Development of biosensors for mycotoxins detection in food and beverages

DISSERTATION ZUR ERLANGUNG DES DOKTORGRADES DER NATURWISSENSCHAFTEN (DR. RER. NAT.) DER FAKULTÄT FÜR CHEMIE UND PHARMAZIE DER UNIVERSITÄT REGENSBURG

vorgelegt von

Aleksandra Karczmarczyk

aus Nowy Dwór Mazowiecki, Polen

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Diese Doktorarbeit entstand in der Zeit von Juli 2014 bis Mai 2017 am Institut für Analytische Chemie, Chemo- und Biosensorik an der Universität Regensburg.
Die Arbeit wurde durchgeführt bei Prof. Dr. Antje J. Bäumner und Prof. Dr. Karl-Heinz Feller (Ernst-Abbe-Hochschule Jena).

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ABSTRACT (in English)

Mycotoxins are secondary metabolites of mould, which are ubiquitous in a large variety of food and feed commodities. Thousands of mycotoxins exist, but only a few present significant damages and poisonous properties. Among them, the aflatoxins and ochratoxins are considered to be the most toxic and widely spread in the world and therefore, represent a real threat for human/animal life. Depending on a number of factors like the intake levels, duration of exposure, mechanisms of action, metabolism and defense mechanisms, mycotoxins elicit a wide spectrum of toxicological effects leading to both acute and chronic disease, liver and kidney damage, skin irritation, cancer, immune suppression, birth defects or even death.

To address the adverse effects of mycotoxin contaminants in food and feed, health authorities in many countries all over the world have become active in establishing regulations to protect their citizens and livestock from the potential damages caused by those compounds. The European Commission, the US Food and Drug Administration (FDA), the World Health Organization and the Food and Agriculture Organization of the United Nations have set up regulations and maximum levels for major mycotoxins in foods and feeds. To fulfill expectations of these regulatory limits, there is an increasing need for the development and validation of new, simple, fast and precise methods for toxins detection.

Therefore, this thesis reveals different strategies for rapid, cost-effective and ultrasensitive bioanalysis of two major mycotoxins: aflatoxin M_1 and ochratoxin A. Inhibition competitive assays with surface plasmon resonance spectroscopy (SPR, optical technique), quartz crystal microbalance (QCM, acoustic device) and electrochemical based readout were developed and compared. Presented biosensors were challenged in a red wine and milk samples with no need for pre-treatment or pre-concentration of the sample extract.

In order to prevent fouling on the sensor surface by the constituents present in milk samples, the gold surface of the sensor chip was modified and different surface architecture and compared (antifouling polymer brushes and self-assembled monolayer - SAM). Complete resistance to the non-specific interactions was observed for coating with p(HEMA) brushes resulting in two times lower LOD compared to that on thiol SAM. The SPR biosensor for AFM_1 allowed for highly sensitive detection in milk with an excellent precision (the average calculated CV was below 4%), limit of detection of 18 pg mL^{-1} for p(HEMA) brushes and 38 pg mL^{-1} for thiol SAM and with the analysis time of 55 min. It is worth highlighting that it is

the first time that an SPR chip modified with such polymer brushes was used for real time detection of a small target antigen opening a new avenue for highly precise analysis.

In the case of wine samples tested for OTA detection, a simple but very effective pre-treatment procedure was successfully applied. It was proved that the addition of the 3% of the binding agent poly(vinylpyrrolidone) (PVP) to red wine completely reduces non-specific interactions by binding polyphenolic compounds (which may be responsible for inactivation of antibody and blocking the sensor surface) through hydrogen bonding, making their elimination easier. Moreover, in this study, the authors evaluated the influence of gold nanoparticles (AuNPs) on signal enhancement and thereby biosensor sensitivity. For this purpose two assays were performed: with and without implementation of NPs. Obtained results allowed for OTA detection at concentrations as low as 0.75 ng mL^{-1} however, its limit of detection was improved by more than one order of magnitude to 0.068 ng mL^{-1} by applying AuNPs as a signal enhancer.

The combination of indirect competitive assay and AuNPs with QCM-D gave a straightforward tool, which can simultaneously measure frequency and dissipation changes resulting in information about the sensitivity but also about the mass attached to the sensor surface as well as viscoelastic properties and the hydration state of the film. A linear detection range of 0.2–40 ng mL^{-1} has been achieved with LOD of 0.16 ng mL^{-1}.

The same assay format was also tested in voltammetric detection of mycotoxins using modified gold screen printed electrodes (AuSPE). An excellent LOD of 15 ng mL^{-1} for OTA and 37 pg mL^{-1} for AFM_1 were obtained. Additionally, AuSPE modified with SAMs based on different types of alkanethiols (long and short chains) were tested and compared in terms of electron transfer resistance.

Proposed biosensors offer vast range of advantages such as high sensitivity (at pg or ng levels), short analysis time (55 min) in comparison to for example, ELISA which require multiple steps that translates to prolonged analysis time, possibility for online monitoring, characterization of binding kinetics, low consumption of primary antibody (cost reduction), excellent antifouling surface and simple pre-treatment procedure.

Combining all most desirable aspects of a good biosensor such as high sensitivity, low costs, short analysis time and simple but effective cleaning-up technique make proposed approaches an important and very promising tools for widespread biosensing applications.

Kurzfassung (in Deutsch)

Mykotoxine sind sekundäre Schimmel-Metaboliten, die allgegenwärtig in einer großen Anzahl von Lebensmittel und Futter-Erzeugnissen enthalten sind. Tausende von Mykotoxinen existieren, aber nur einige wenige haben signifikante Schadens- und Vergiftungseigenschaften. Unter diesen sind die Aflatoxine und die Ochratoxine die am meisten toxischen und auch am weitesten verbreiteten, und stellen deshalb eine reale Gefahr für das menschliche und tierische Leben dar. Abhängig von einer Anzahl von Faktoren wie dem Aufnahme-Niveau, der Dauer der Belastung, dem Wirkungsmechanismus, dem Metabolismus und Schutzmechanismen, Mykotoxine rufen ein weites Spektrum an toxikologischen Effekten hervor, die sowohl zu akuten als auch zu chronischen Krankheiten, Leber und Nieren-Schäden, Hautirritationen, Immunerkrankungen, zu Geburtsschäden und sogar zum Tod führen können.

Um die nachteiligen Effekte der Mykotoxin-Kontamination in Lebensmitteln und Futter zu adressieren, sind Gesundheitsbehörden in vielen Ländern auf der ganzen Welt aktiv, Betimmungen zu erlassen, um ihre Einwohner und den Tierbestand für eine potentiellen Gefährdung durch diese Verbindungen zu schützen. Die europäische Kommission, die US Food and Drug Administration (FDA) als auch die Weltgesundheitsorganisation der Vereinigten Nationen haben Verordnungen erlassen und maximale Niveaus für die Haupt-Mykotoxine in Lebensmitteln und im Futter erlassen. Um die Erwartungen dieser regulatorischen Grenzwerte zu erfüllen, ist es im wachsenden Masse erforderlich, neue, einfache, schnelle und präzise Methoden des Nachweises von Mykotoxinen zu entwickeln.

Aus diesem Grunde werden in der Promotionsarbeit verschiedene Strategien für eine schnelle, kosten-effektive und ultrasensitive Bioanalyse von 2 Haupt-Mykotoxinen: Aflotoxin M_1 und Ochratoxin A vorgestellt. Ein Inhibitions-kompetitiver Assay unter Nutzung der Oberflächenplasmonenresonanz (SPR, optische Technik), der Quarzkristall-Mikrowaage (QCM, akustische Technik) sowie ein elektrochemisch-basierter Ansatz werden entwickelt und verglichen. Die vorgestellten Biosensoren wurden in Rotwein und in Milchproben eingesetzt ohne jegliche Vorbereitung oder Anreicherung des Probenextraktes.

Um einen möglichen Faulprozess auf der Sensoroberfläche durch die Bestandteile, die in der Milch vorhanden sind zu verhindern, wird die Goldoberfläche des Sensorchips modifiziert und verschiedene Oberflächenarchitekturen wurden getestet und verglichen (Antifaul-

Polymerbürsten und selbst-organisierende Monoschichten - SAM). Eine komplette Unterdrückung von nicht-spezifischen Wechselwirkungen wurde beobachtet durch eine Beschichtung mit p(HEMA)-Bürsten, was zu einer um den Faktor zwei verringerten LOD verglichen mit den des Thiol-SAM führt. Der SPR-Biosensor für das AFM_1 ermöglicht einen hoch-sensitiven Nachweis in der Milch mit einer exzellenten Genauigkeit (der mittlere berechnete CV war unter 4 %), einer Nachweisgrenze von 18 pg/ml für p(HEMA) – Bürsten und 38 pg/ml für das Thiol-SAM und mit einer Analysezeit von 55 min. Es sollte darauf hingewiesen werden, dass damit zum ersten Mal ein SPR-Chip benutzt wurde, der mit solchen Polymerbürsten modifiziert wurde für den Echtzeit-Nachweis eines kleinen Ziel-Antigens, was eine völlig neue Richtung für die hochpräzise Analyse eröffnet.

Im Falle der Weinproben, die für die OTA-Detektion getestet wurden, wurde eine simple aber sehr effektive Vorbehandlungsprozedur angewendet. Es konnte gezeigt werden, dass die Zugabe von 3 % einer Bindungssubstanz ((Poly(vinylpyrrolidon), PVP) zum Rotwein die nichtspezifischen Wechselwirkungen total reduziert, indem die polyphenolischen Verbindungen (die für die Inaktivierung des Antikörpers und dem Blockieren der Sensoroberfläche verantwortlich zu sein scheinen) durch Wasserstoffbrückenbindungen gebunden werden. Dieses Verfahren hat wesentliche Vorteile bei der Eliminierung der polyphenolischen Komponenten im Wein. Des weiteren wurde im Rahmen der Dissertation der Einfluss von Gold-Nanopartikeln (AuNPs) auf die Signalverstärkung und somit die Sensorempfindlichkeit untersucht. Für diesen Zweck wurden zwei Assays entwickelt: mit und ohne Benutzung von NPs. The erhaltenen Ergebnisse erlaubten es, OTA bis zu Konzentrationen von 0,75 ng/ml (Nachweisgrenze) zu detektieren, während die Nachweisgrenze durch die Anwendung von NPs als Signalverstärker um eine Größenordnung auf 0,068 ng/ml verringert werden konnte.

Die Kombination von indirektem kompetitiven Assay und NPs mit QCM-D liefert ein ideales Werkzeug, das simultan die Frequenz und Dissipationsänderungen messen kann, was sowohl zu einer Information über die Empfindlichkeit, über die Masse, die an der Sensoroberfläche angelagert ist, als auch über die visko-elastischen Eigenschaften und den Hydrationszustand des Filmes führt. Ein linearer Nachweisbereich von 0,2 – 40 ng/ml mit einem LOD von 0,16 ng/ml wurde erreicht.

Dasselbe Assayformat wurde auch für eine voltammetrische Detektion der Mykotoxine bei Nutzung von modifizierten gedruckten Goldelektroden (AuSPE) getestet. Ein exzellentes

LOD von 15 ng/ml für OTA und 37 pg/ml für AFM_1 wurde erhalten. Zusätzlich wurden AuSPEs modifiziert mit SAMs basierend auf unterschiedlichen Typen von Alkanethiolen (lang- und kurzkettigen) getestet und in Bezug auf den Elektronentransferwiderstand verglichen.

Die vorgeschlagenen Biosensoren bieten sehr vielfältige Vorteile, wie eine sehr hohe Sensitivität (im pg oder ng Bereich), kurze Analysenzeiten (55 Minuten) im Vergleich zu z. B. ELISA, was multiple Schritte benötigt, und dazu führt, das solche Faktoren, wie die Analysenzeit, die Möglichkeit eines on-line-Monitorings, der Charakterisierung von Bindungskinetiken, dem geringen Verbrauch an Antikörpern (Kostenreduktion), der exzellenten Antifaul-Oberfläche und nicht zuletzt mit einer einfachen Vorbereitungsprozedur vorteilhaft sind.

Indem man die wichtigsten Aspekte eines guten Biosensors wie hohe Sensitivität, geringe Kosten, kurze Analysezeit und einfache UND effektive Reinigungstechniken betrachtet, zeigt sich, dass der vorgeschlagene Zugang ein wichtiges und sehr erfolgversprechendes Werkzeug für weitgespannte Biosensor-Anwendungen darstellt.

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LIST OF ABBREVIATIONS

Ab – antibody

Ab_1 – primary antibody

Ab_2 – secondary antibody

ACT - acetate buffer

AFs – aflatoxins

AFB_1 – aflatoxin B_1

AFB_2 - aflatoxin B_2

AFG_1 - aflatoxin G_1

AFG_2 - aflatoxin G_2

AFM_1 - aflatoxin M_1

AFM_2 - aflatoxin M_2

Ag - antigen

AMP - amperometry

AOAC - Association of Official Analytical Chemists

AP - alkaline phosphatase

APCI - atmospheric pressure chemical ionization

ATR - attenuated total reflection

AuNPs - gold nanoparticles

BEN - Balkan Endemic Nephropathy

BSA - bovine serum albumin

CE - capillary electrophoresis

CONTAM - Panel of Contaminants in Food Chain

CV - coefficient of variation

CZE - capillary zone electrophoresis

DAD - diode array

DC - direct competitive assay

DMAP - 4-(dimethylamino) pyridine

DMF - N,N-dimethylformamide

DON - deoxynivalenol

DPV - differential pulse voltammetry

DSC - N,N-disuccinimidyl carbonate

EC - European Commission

EDC - 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrchloride

ELISA - enzyme-linked immunosorbent assay

ESI - ion electrospray ionization

EU - European Union

FAO - Food and Agriculture Organization

FB_1 – fumonisin B_1

FB_2 - fumonisin B_2

FD - fluorescence detection

FDA - US Food and Drug Administration

FUM – fumonisins

GC - gas chromatography

GCE - glassy-carbon electrode

HPLC - high-performance liquid chromatography

HRP - horse radish peroxidase

IAC - immunoaffinity clean-up

IARC - International Agency for Research on Cancer

IC - indirect competitive assay

ISE - ion-selective electrodes

ISFET - ion-sensitive field effect transistors

ITO - indium tin oxide

IUPAC - International Union of Pure and Applied Chemistry

LIF - laser-induced fluorescence

LOD – limit of the detection

LOQ – limit of quantification

LSP - localized surface plasmon

MCC - multifunctional clean-up columns

MECC - micellar electrokinetic capillary chromatography

MHDA - (11-mercaptoundecyl)tetra(ethylene glycol)

MIP - molecularly imprinted polymer

MNPs - magnetic nanoparticles

MUTEG - 16-mercaptohexadecanoic acid

MW - molecular weight

NHS - N-hydroxysuccinimide

NIP - non-imprinted polymer

NPs - nanoparticles

OTs - ochratoxins

OTA – ochratoxin A

OTB – ochratoxin B

OTC – ochratoxin C

PBS - phosphate buffered saline

PEG - polyethylene glycol

p(HEMA) - poly(2-hydroxyethyl methacrylate)

POT - potentiometry

PVP - poly(vinylpyrrolidone)

QCM - quartz crystal microbalance

QCM-D - quartz crystal microbalance with dissipation

QD - quantum dot

RU - resonance units

SAMs - self-assembled monolayers

SP - solid-phase extraction

SPE - screen printed electrode

SPR - surface plasmon resonance

TLC - thin layer chromatography

TWI - tolerable weekly intake

UV - ultra violet

WHO - World health Organization

ZEA - zearalenone

CHAPTER ONE, INTRODUCTION

1.1. Thesis outline and objectives

The main aim of presented thesis concerns the development of biosensors applied to the determination of naturally occurring secondary metabolites: aflatoxin M1 (AFM_1) and ochratoxin A (OTA), chosen for this study as they represent two of the most important mycotoxins classes. The major goal was to create a biosensor exhibiting all most desirable properties: high sensitivity and specificity, rapidity of analysis, low costs and portability. For this purpose different strategies were applied, tested and compared.

Current routine analysis of those compounds in foodstuff is mostly performed by chromatographical methods including thin layer chromatography, high-performance liquid chromatography with fluorescence detection or capillary electrophoresis. Those techniques are generally straightforward and yield reliable results however, they require extensive preparation steps and are time-consuming. Thus, alternative approaches offering high sensitivity and simplicity are urgently needed. To fulfill those expectation and the European Union regulations in the field of food control and safety, the author propose novel strategies of biosensors utilizing indirect competitive immunoassay combined with three different detection techniques:

- surface plasmon resonance spectroscopy (SPR)
- quartz crystal microbalance (QCM)
- electrochemistry.

Those methods, although based on different principles and readouts can provide all desired properties of a good biosensor (such as sensitivity, rapidity etc.) however, a deep knowledge about their functioning is still urgently needed. Due to the increased complexity in the food industry and competition within companies, new, well-described and tested approaches for rapid mycotoxin analysis have become increasingly important.

Here, the question arises as what actually do we mean by "rapid method". This term usually refers to a method which is faster than respective reference methods and has a tendency of promoting the method [2]. Nevertheless, such rapid techniques should have also other common features: should be simple, user-friendly, relatively fast (yielding results within minutes) and able to work in the field [2]. Resented in this thesis approaches (SPR, QCM,

electrochemistry) fulfill all abovementioned expectations. Moreover, a detailed analysis of each technique has been performed, providing a wide range of information, paying attention to challenges and difficulties which can arise during analysis and showing their possible solutions as well as highlighting pros and cons of every used method.

The structure of this work is divided into five main parts, from which independent conclusions are drawn.

The first chapter gives a general overview about classes of mycotoxins (putting special attention to aflatoxins and ochratoxins description which are compounds chosen for investigation in this thesis), their toxic effect on humans and animal health, occurrence in a daily life products as well provides information about international regulations and limitations concerning food and beverages safety. Moreover, brief description supplemented with a large number of examples from the literature of conventional analytical methods for mycotoxins analysis is presented. Important part of this chapter is related to the alternative techniques based on biosensing systems posing the base of the current research. Therefore, methodologies such as SPR, QCM and electrochemistry are described in detail.

The second and the third chapters show results of the rapid and sensitive detection of AFM_1 in milk and OTA in red wine utilizing gold nanoparticles-enhanced surface plasmon resonance spectroscopy. To overcome the matter concerning low molecular weight of the analyte that hampers its detection using SPR, an indirect competitive inhibition assay was performed. To reduce matrix interferences coming from real samples, different strategies were applied: modification of the surface architectures (in case of milk analysis) and simple pre-treatment of sample (red wine) with binding agent. Moreover, the influence of gold nanoparticles on signal enhancement was investigated as well as a detailed analysis of kinetic parameters (association/dissociation constants and association/dissociation rate constants) was provided and compared with available literature.

Chapter four is focused on the OTA determination in wine using quartz crystal microbalance with dissipation (QCM-D) as a detection technique. The combination of indirect competitive assay with QCM-D was shown to give a straightforward device, which can simultaneously measure frequency (Δf) and dissipation (ΔD) changes resulting not only in information about the sensitivity of the assay but also providing a detailed description about the mechanical and viscoelastic properties of the biofilm.

In chapter five the author presents an electrochemical biosensor for AFM_1 and OTA analysis. A competitive immunoassay that uses a secondary antibody conjugated with an enzyme (alkaline phosphatase) as a tag was explored for the voltammetric detection using modified gold screen printed electrodes (AuSPE). Additionally, AuSPE modified with self-assembled monolayers based on different types of alkanethiols (long and short chains) were tested and compared in terms of electron transfer resistance.

Last, sixth chapter summarizes all developed biosensors based on different detection techniques and provides a detailed comparison between them taking into account various aspects which need to be considered when choosing the best methodology for mycotoxins detection.

Summarizing, in the presented thesis, the author proposed three strategies based on combination of biosensors methodology with indirect competitive immunoassay and surface plasmon resonance spectroscopy, quartz crystal microbalance and electrochemistry as a readout. Proposed biosensors offer vast range of advantages such as high sensitivity (at pg or ng levels), short analysis time (55 min) in comparison to for example, ELISA which require multiple steps that translates to prolonged analysis time, possibility for online monitoring, characterization of binding kinetics, low consumption of primary antibody (cost reduction), excellent antifouling surface and simple pre-treatment procedure.

Therefore, all most desirable aspects of a good biosensor - sensitivity, low costs, short analysis time and simple but effective cleaning-up technique are shown and supported with detail characterization.

Thus, this thesis comprises an important and very promising study not only for small molecules determination in food and beverages but is also a valuable development in the field of biosensing and food safety and/or control.

1.2. Mycotoxins

Mycotoxins are low-molecular-weight natural compounds produced as secondary metabolites by certain filamentous fungi (more specifically, the molds) that may occur in almost all food and feed commodities (Table 1.1) [3]. They are known since more than a half century - the first report about mycotoxins existence dates back to 1962, as a consequence of unusual and mysterious veterinary crisis near London (England), which killed over one hundred thousand turkeys (later called turkey 'X' disease) [4]. After an extensive investigation, abstruse deaths were linked to a peanut meal coming from Brazil which had become mouldy during the shipment. Further researches demonstrated that the transported feed was heavily contaminated with secondary metabolites from *Aspergillus flavus* (hence the name Aflatoxin) causing incurable liver cancer in the poultry [5]. Information about carcinogenic properties of aflatoxin gave a concern that other occult mold metabolites might be toxic or even deadly. In later studies, it was shown that the target and metabolite concentration are playing the main role. Thus, although all mycotoxins are of fungal origin, not all toxic compounds produced by fungi are called mycotoxins, e.g. fungal products toxic to bacteria are called antibiotics, the name - phytotoxins refers to compounds imposing a hazard on plants [6].

Fungi are pervasive in nature and part of the microflora of the worldwide food chain. Under suitable conditions (temperature, humidity) they can grow on a large variety of foods and feeds. The most important mycotoxigenic fungi belong to the genera *Aspergillus, Fusarium* and *Penicillium* [7]. Mycotoxins are a structurally diverse group; they vary from teeny, simple molecules like moniliformin, to large complexes such as phomopsins [8, 9]. Hundreds of mycotoxins have been identified till now, although only a few (proven to be carcinogenic and/or toxic) are under scientific attention. The major mycotoxin classes (considering also public health, agro-economic significance and an impact on global agriculture) are aflatoxins, ochratoxins, fumonisins, trichothecenes (most importantly deoxynivelanol), patulin and zearalenone [10].

Table 1.1. Mycotoxins and associated with them commodities, toxic effects and producing fungal species [3].

Mycotoxin	Matrix	Toxic effect	Fungal species
Aflatoxins	Peanuts, maize, tree nuts, cottonseed, milk	Hepatotoxicity, cancer, probable immune suppression and childhood stunting reduced growth	*Aspergillus flavus, A. parasiticus*
Ochratoxins	Cereals, coffee, cocoa, wine, beer, grapes, dried fruits	Nephrotoxicity, hepatotoxicity, neurotoxicity, teratogenicity, immunotoxicity	*Aspergillus ochraceus, A.carbonarius, Penicillium verrucosum*
Fumonisins	Maize	Neurotoxicity, genotoxicity, immunotoxicity, cancer	*Fusarium verticillioides, F. proliferatum*
Trichothecenes	Grains	Inhibition of protein synthesis, human intestinal upsets	*Fusarium graminearum*
Patulin	Apples	Genotoxicity, teratogenicity, cancer	*Penicillium expansum*
Zearalenone	Corn, oats	Hepatotoxicity, genotoxicity, immunotoxicity	*Fusarium graminearum*

Even if mycotoxin-producing fungi differ according to ecological conditions, it is important to emphasize that mycotoxins exist all over the world mainly due to the trade that contributes to their worldwide dispersal [11]. Fig.1.1 shows a very recent and detailed study that depicts the relation between geographical origin and mycotoxins [11]. The number of reports about different mycotoxins strongly depends on the location, climate and conditions in which fungal growth is preferable. Thus, e.g. the mixture of aflatoxins (noted as AFs) and fumonisins (FUM) dominates in Africa, Asia, and South America. Maize harvested in the tropical and subtropical areas of the world with hot and humid climates is the major commodity contaminated with those two toxins. Nevertheless, because of the movement of agricultural goods around the globe, no region of the world is aflatoxin-free [11]. In Europe and North America, considered as relatively colder regions, mixture of trichothecenes (deoxynivalenol,

DON) and zearalenone (ZEA) are the most common, emphasizing the role of the climate conditions on fungal contamination, growth and metabolism.

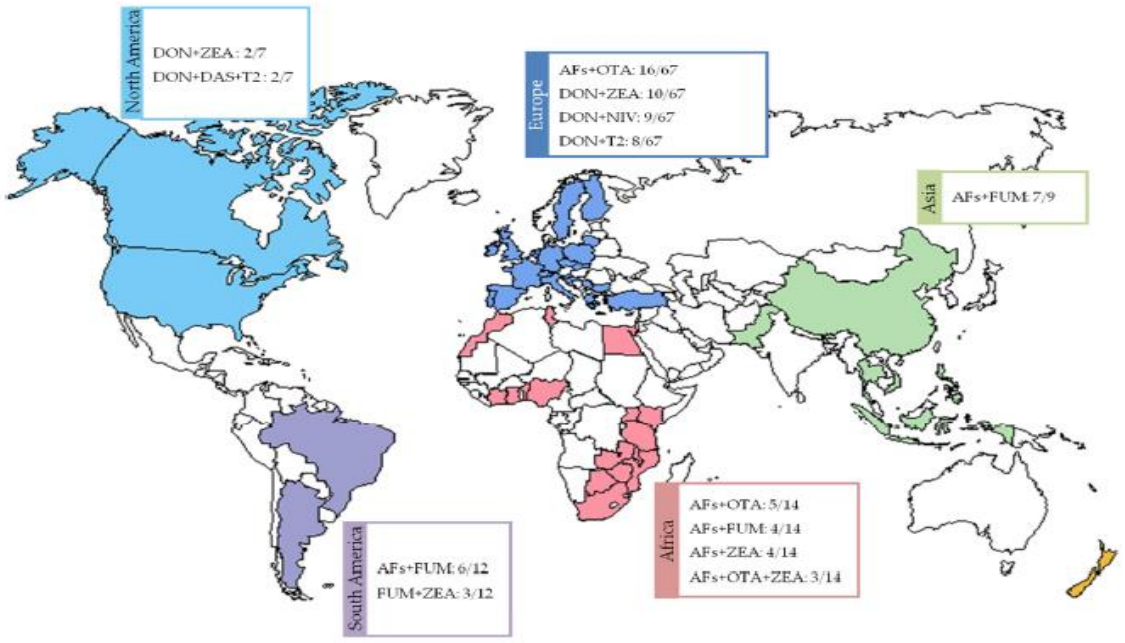

Fig.1.1. Main mycotoxins citied in literature depending on their geographical origin. Reprinted from [11]. Abbreviations: DON - deoxynivalenol, ZEA - zearalenone, AFs - aflatoxins, FUM - fumonisins, OTA - ochratoxins, T2 - toxin belonging to the group of trichothecenes.

The main route of exposure to mycotoxins is through the consumption of contaminated plant-derived foods, although it may also occur via the ingestion of mycotoxins and their metabolites present in animal products such as meat, eggs or milk, which can cause their accumulation in different organs and tissues [12, 13]. It is well established that these compounds elicit a wide spectrum of toxicological effects leading to both acute and chronic disease, liver and kidney damage, cancer or immune suppression (Table 1.1) [3]. Therefore, they impose a hazard on both human and animal health. Due to this fact, in 1993, the International Agency for Research on Cancer (IARC) classified aflatoxins as carcinogenic to humans (Group 1), while ochratoxins, fumonisins and patulin were placed in a Group 2 as a possible carcinogens [14]. Trichothecenes and zearalenone were not classified as human carcinogens (Group 3) [10]. Since then, health authorities in many countries all over the world have become active in establishing regulations to protect their citizens and livestock from the potential damages caused by mycotoxins [15]. Several times in recent decades (1981, 1987, 1995, 2003) international inquiries were held and published about regulations for mycotoxins in food and feed [16]. The most recent one, conducted by the National Institute for Public Health and the Environment, under contract to the FAO (Food and Agriculture Organization),

gathered information from 119 countries about the existence or absence of specific mycotoxin limits and regulations in food and feed (Table 1.2). Therefore, specific, broaden with newer requirements of sampling procedures and analytical methods regulations exist for thirteen mycotoxins [16].

Table 1.2. Maximum limits for mycotoxins in foods in various countries [7].

Mycotoxin	Country	Maximum level [μg kg^{-1}]	Matrix
Patulin	Japan, Moldavia	50	Apple juice
	EU countries	25	
Trichothecenes	USA	1000	Wheat
	Russia	1000	Cereals
	Austria	750	Wheat
Zearalenone	Romania	30	Cereals, vegetable oils
	France	200	
	Russia	1000	
Fumonisins	Bulgaria, France, Switzerland	1000	Maize and processed products
Ochratoxin	Czech Rep.	5	Children's food
	Denmark	25	Pigs
	Sudan, Turkey	15	Wheat, dried raisins
	The Netherlands	0	Cereals
Aflatoxin B1	Finland, Germany	2	All
	Belgium, Spain, Luxembourg, Ireland, Greece	5	Cereals
	Portugal	25	Peanuts
Aflatoxin B1, B2, G1 and G2	Norway, Belgium	5	Peanuts
	Italy	50	
	Germany, England	4-5	All
Aflatoxin M1	Sweden, Austria, Germany, Belgium	0.05	Milk
	USA	0.5	
	Switzerland	0.25	Cheese
	The Netherlands	0.2	Butter

1.2.1. Patulin

Patulin (Fig.1.2) is a toxic fungal metabolite produced by a wide range of fungal species of the genera *Penicillium*, *Aspergillus* and *Byssochlamys*, from which *Penicillium expansum* is the most important due to its existence in damaged fruits [17]. Patulin occurs mostly in apples that have been spoiled by mold growth. Furthermore, pears, peaches and berries can also be affected. It has been also found in vegetables, cereal grains and cheese [18]. Nevertheless, apples and apple products (juices, pies, conserves) are considered to be the main vectors of this mycotoxin. The European Union (EU) maximum permissible level is between 10 and 50 μg kg^{-1} [19]. Patulin has been shown to be mutagenic [20], carcinogenic [21] and teratogenic [22]. *In vitro* studies have demonstrated that patulin inhibits several macrophage functions. Some of these studies conducted on mice revealed bad influence on immune system (e.g. increase in the number of neutrophilsers) [1, 23].

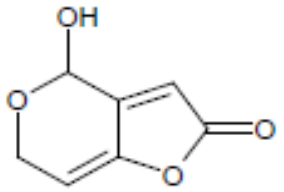

Fig.1.2. Molecular structure of patulin [1].

1.2.2. Trichothecenes

Trichothecenes are a group of over hundred structurally related compounds with the same basic structure, occurring worldwide in grains and other commodities (corn, wheat, barley, oats etc.) [24]. All of them contain an epoxide at the $C_{12,13}$ position, recognized to be a culprit of their toxicological activity [3]. This class of mycotoxins has been divided into four groups according to their molecular structure. Type A is represented by HT-2 and T-2 toxins, while type B includes well-known deoxynivalenol (DON) [1]. Types C and D contain less important compounds, in terms of toxicity. The structures of the mentioned examples of trichothecenes are shown in Fig.1.3. The major effects on human and animal health – related to toxin concentration in the commodity – are reduced feed uptake, vomiting and immune suppression. Moreover, they are in general very stable, both during storage and food processing (e.g. cooking) and do not degrade at high temperature [25].

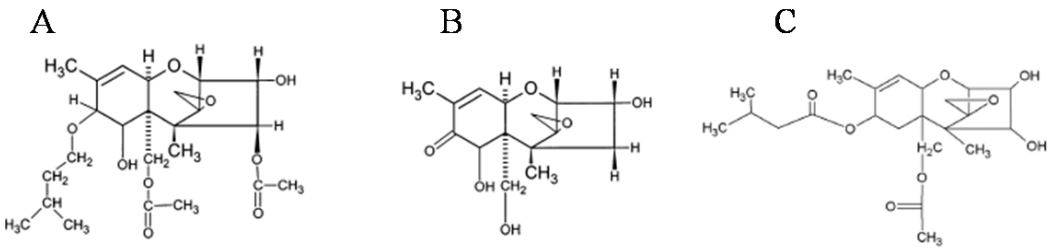

Fig.1.3. Molecular structures of T-2 toxin (A), DON (B) and HT-2 (C) [1].

1.2.3. Zearalenone

Zearalenone (Fig.1.4) is a mycotoxin produced by several *Fusarium* species (mainly *Fusaria graminearum)* using corn, oats and sorghum as substrates [3]. Generally, they grow in moist, cool field conditions during blooming [26]. This toxin exhibits oestrogen-like activity in certain animals such as sheep, pigs or cattle [3]. Zearalenone is stable upon heating (up to 150ºC) and degrade only under alkaline conditions and very high temperatures [27].

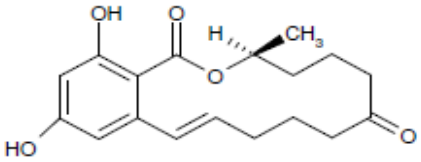

Fig. 1.4. Molecular structure of zearalenone [1].

1.2.4. Fumonisins

Fumonisins (FB_1 and FB_2) are cancer-promoting mycotoxins possessing a long-chain hydrocarbon unit responsible for their toxicity [3]. At least twelve structurally similar compounds are known, although the most important ones are fumonisin B_1 and B_2 (Fig.1.5). From the toxicological point of view, FB_1 gives rise to a real threat for humans and animals health. It can cause leucoencephalomalacia in horses and porcine pulmonary edema, while in humans fumonisins are associated with cancer growth [28]. Moreover, hepatotoxic, nephrotoxic and embryotoxic properties have been also reported [29]. Fumonisins are frequently found in corn and corn-based foods [30]. FB_1 can be also found in beer, rice, sorghum, triticale, cowpea seeds, soybeans and asparagus [28]. They are all heat-stable and their content can be minimized only during processes where the temperature exceeds 150 ºC [31].

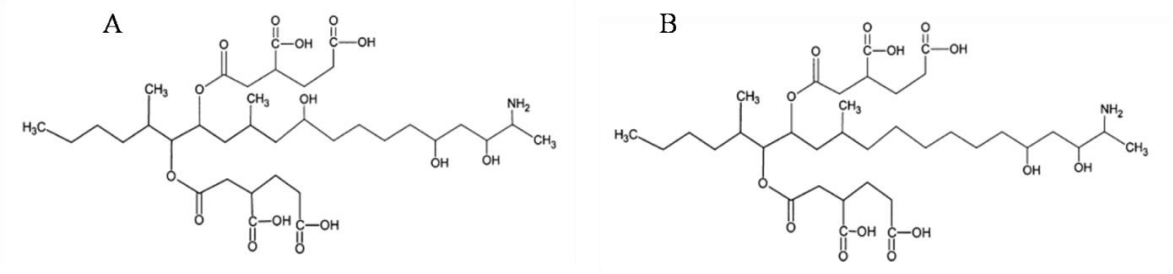

Fig.1.5. Molecular structures of FB_1 (A) and FB_2 (B) [1].

1.2.5. Ochratoxins

Ochratoxins are a group of mycotoxins produced by a variety of fungal species (see Table 1.1) containing in their structure two moieties: a substituted dihydroisocoumarin and L-phenylalanine [32]. The main forms are ochratoxin A (OTA), B (OTB, non-chlorinated form of OTA) and C (OTC, an ethyl ester of OTA) but the most prevalent and relevant member of this family is OTA (Fig.1.6) [33].

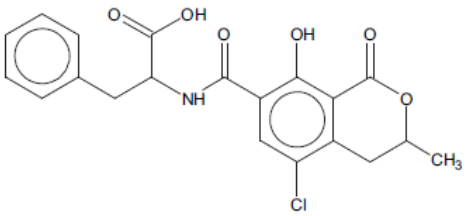

Fig. 1.6. Molecular structure of OTA [1].

OTA is a colorless crystalline compound soluble in organic solvents and in alkaline water, optically active and exhibiting blue fluorescence under UV light, but the ultraviolet spectrum varies with pH and with the solvent polarity [34]. Fluorescence emission is maximum at 467 nm in 96 % ethanol and 428 nm in absolute ethanol [35]. OTA is a very stable mycotoxin in different solvents, possesses a resistance to acidity and high temperatures. Thus, once foodstuffs are contaminated, it is very difficult to totally remove this molecule [34].

Ochratoxin A is a frequent natural contaminant of a daily life foodstuff such as cereals, coffee, cacao, grapes, wine, fish, soy, peanuts, beer and so on [33]. Fig.1.7 shows the contributions to the total human OTA exposure reported by the European Union [36].

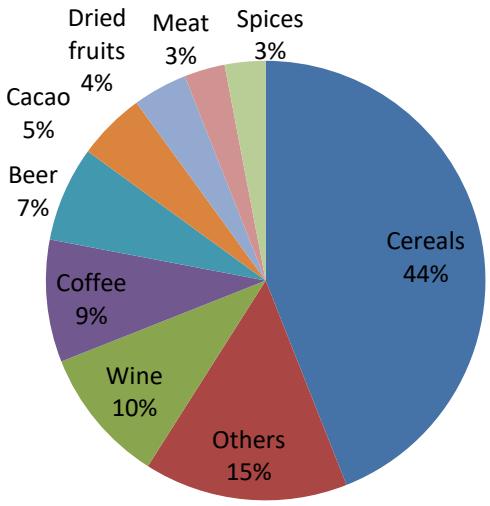

Fig.1.7. Contribution to the total human adult OTA exposure.

As it can be seen, cereals are the most important dietary source of this mycotoxin contributing to 44% of the intake. The reason of such high value is the fact that cereals have a variety of uses as a food which is consumed almost every day by humans e.g. bread, breakfast cereals, cookies or cakes. Another common usage of cereals is in the preparation of alcoholic drinks such as whiskey, beer, vodka or Japanese sake [37]. The second major source of OTA consumption by EU population is wine (10%) which is one of the products taken under investigation in the presented thesis. Wine is an important beverage in the world trade with France, Italy and Spain known as the main exporters [32]. In general, red wines have higher levels of OTA than rose and white wines due to the increased contact time between berry skins and grape juice during the mashing stage [38]. Some results suggest also that wines from the South contain more OTA than those from the North, at least in Europe and North Africa [39]. This difference is attributed to climate, grape cultivation and storage conditions. Examples of OTA occurrences in three types of wine (red, white, rose) produced in different places are shown in Table 1.3 [40].

Table 1.3. Occurrence of OTA in wines produced in different places and estimated exposure to this toxin [40].

Place	Type	Estimated exposure [ng kg^{-1}]
Brazil	Red	< 0.04-11.25
	White	< 0.07
China	Red	< 0.07-14.12
	White	< 0.07-0.17
	Rose	< 0.07-0.55
Spain	Red	0.001-0.23
	Rose	0.017-0.22
Italy	Red	< 0.18-2.35
	White	0.025-2.42
	Rose	< 0.025-2.88
France	Red	< 0.025-0.6
Portugal	Red	2.50-6.00
	White	2.50-3.08
Greece	Rose	0.47-6.3
	Red	< 0.0251.78

This chlorophenolic mycotoxin is widely recognized as a significant threat for human and animal life. OTA has been reported to be teratogenic, genotoxic, carcinogenic and immunotoxic [41]. Therefore, it has been classified by the International Agency on Cancer as a possible human carcinogen (Group 2B) [42]. OTA exhibits unusual toxicokinetics, with a half-life time in blood of 840 h after oral ingestion and its elimination from the body is slower in humans than in all other species, providing more time for damage to occur [43]. It was also found that its toxicity is most acute in the kidney, recognized as potent nephtrotoxin [1]. Therefore, OTA has been linked to the so-called Balkan Endemic Nephtropathy (BEN) disease which causes kidney damage.

Taking into account the global importance of cereals, wine, coffee and other products which might be contaminated with OTA, the Panel of Contaminants in Food Chain (CONTAM) of the European Food Safety Authority derived a Tolerable Weekly Intake (TWI) for OTA of 120 ng kg^{-1} [44]. Recent analysis of the human dietary exposure (mainly via food and beverages) of adult European consumers to OTA, shows that the weekly exposure ranges from 15 to 60 ng OTA kg^{-1}, which is lower than TWI [32]. Moreover, due to the worldwide OTA occurrence and its wide spectrum of toxicological and carcinogenic effects, maximum permitted level of ochratoxin A have been set up by nations all over the world (see Table 1.2). Also, the European Commission has conducted detailed risk assessments and defined a

maximum allowable level for different types of food and feed (e.g. 5 ng mL^{-1} for unprocessed cereals, 3 ng mL^{-1} for products derived from unprocessed cereals, 10 ng mL^{-1} for coffee beans and 2 ng mL^{-1} for all types of wine [45]).

Ochratoxin-producing fungi can contaminate agricultural products in the field (pre-harvest spoilage), during storage (post-harvest spoilage) or during food processing (e.g. sorting, cleaning, brewing, cooking, roasting, frying etc.) and therefore, the deep knowledge about the stability and reactivity of toxins as well as possible methods for their elimination from the food chain is essential [46]. Thus, several strategies, classified into three categories: prevention of mycotoxin contamination, decontamination of affected foods and inhibition of the absorption of consumed toxin, have been proposed to minimize the toxic effect of those molecules in foods and feeds. The best and most common approach for lowering the pre-harvest contamination is field treatment with fungicides. It was demonstrated that organophosphate fungicide, dichlorovos or iprodione can successfully inhibit OTA production of *A. ochraceus*, *A verrucosum* and *A. westerdijkiae* by disrupting cell division through linking to the nuclear spindle, which slow down the fungal growth [47-49]. The effect of such a treatment has been tested (among others) on the OTA content of wines involving Euparen (sulfamide type of fungicide), Mycodifol and captan as an effective solutions against black aspergilli, which colonize grape berries [46, 50].

Nevertheless, pre-harvesting procedures of contamination reduction are usually not sufficient enough and mycotoxins formation is unavoidable under environmental conditions. Thus, the main goal of post-harvest strategies is to lower fungal contamination of agricultural products during further stages - storage, handling, processing and transport. Those approaches are based on the improvement of storage conditions together with the use of chemical and natural agents as well as irradiation [46]. The major factors influencing the mycotoxins presence in food and feed, which affect the physiology of fungal producer, are temperature, moisture content and insect activity [51]. Moulds grow over a temperature range of 10-40 ºC, a pH range of 4 to 8 and above 14.5% moisture content [52]. Therefore, those parameters must be regularly controlled and kept under a safe storage conditions. Since mycotoxin-producing moulds are aerobic, the modification of atmospheric gases (such as CO_2, N_2, O_2 and SO_2) in storage silos may reduce theirs formation. It has been demonstrated (on example of *P. verrucosum* and *A. ochraceus*) that at least 50% CO_2 is needed to inhibit growth and OTA production showing also that the spore germination is not markedly affected, although germ tube extension and thus colonization is significantly reduced [46, 53]. Another possibility for

fungi growth inhibition is the use of chemical preservatives (e.g. potassium sorbate, calcium propionate), antioxidants (e.g. vanillic acid), essential oils extracts, cinnamon and clove leaf which affect mould evolution and OTA synthesis [54-57]. The mechanisms of phenolic antioxidant activity may be directly or indirectly related to primary metabolism, as evidenced by effects on fungal growth, or involved in secondary metabolism, or a combination of the two [55].

Unfortunately, the prevention methods for mycotoxins elimination during pre- and post-harvesting are usually not able to their complete removal from food and feed. The processes which may have an influence on mycotoxins include sorting, cleaning, brewing, cooking, baking etc. Therefore, various detoxification approaches (physical, chemical and/or biological) have to be employed to assure toxin-free commodities. During the segregation and sorting of damaged, discolored crops with visible mould growth, the clean product is separated from the contaminated grains mechanically however, those operation do not destroy mycotoxins itself. Similar results might be obtained during milling, where the toxins contamination can be redistributed and concentrated in certain mill fractions, but without mycotoxins destruction [58]. Cleaning steps eliminate dust, hair and shallow particles while washing procedures which involve the use of water or sodium carbonate can significantly reduce the amount of *Fusarium* mycotoxins [59]. Most mycotoxins, including OTA are relatively stable upon heating within typical food processing temperatures (80-121ºC) therefore, may survive normal cooking conditions such as boiling or frying [52]. However, the degradation level strongly depends on the type of mycotoxin, its concentration, the degree of heat penetration as well as heating temperature and/or time. Several examples of OTA content reduction during roasting using varies conditions (time, temperature) are shown in Table 1.4. The differences between obtained results may be caused by different spiking techniques, initial concentration of OTA in the sample or inhomogeneous toxin distribution [46].

Tabel 1.4. OTA reduction during heat-treatment[46] .

Heating conditions	OTA content reduction [%]	References
180 ºC, 10 min	31.1	[60]
200 ºC, 20 min	77-87	[61]
250 ºC, 150 sec	14-62	[62]
223 ºC, 4 min	84	[63]
175-204 ºC, 7-9 min	>90	[64]

There is no exact explanation of the mechanism of observed OTA reduction during heating however, in several studies it was shown that the physical removal of OTA with the silverskins (chaff) may be one the reason [63]. Another possible explanation given by Studer-Rohr et al. is related to the isomerization of the C-3 position into a less toxic diastereomer [62]. Moreover, the thermal degradation with the possible involvement of moisture can also play an important role in decrease of OTA contamination [65]. In turn of the opposite process - freezing (-20 ºC), the reduction of toxins was also observed what could be explained by lesions induces by ice crystals in the spores [66]. Another largely used practice improving toxicological safety of wine making process is microfiltration through a 0.45 µm membrane which can reduce OTA contamination by even 80%. This reduction was likely a result of retaining the toxin by the filtration bed formed on a 0.45-µm membrane by wine macromolecules during treatment [67].

Detoxification of mycotoxins can be also performed by implementation of adsorbent materials which have the ability to tightly bind and immobilize toxins. Minerals (e.g. aluminosilicates), biological adsorbents (e.g. yeast, bacterial cells) and synthetic resins (e.g. polyvinylpyrrolidone, cholestyramine) are examples of fining agents mainly used in lowering OTA contamination on wine and must [46]. However, they may also influence on the reduction of some important wine constituents such as aroma compounds and polyphenols responsible for the quality, color, bitterness, oxidative level as well as health beneficial effects of wine. In general, adsorption is based on the accumulation of molecules from a solvent onto the exterior and interior (i.e. pore) surfaces of an adsorbent and therefore it is curtail that the interaction between e.g. OTA and adsorbent are stronger than the one between OTA and solvent [68]. The efficiency of the binding is strongly dependent on the molecular size and physico-chemical properties of toxin. OTA is a weak acid with a pK_a value for the carboxyl group of the phenylalanine moiety of 4.4, suggesting partial dissociation of OTA at the wine pH (ca.3.5) what result in negatively charged molecule that can interact with positively charged surface [68, 69]. Nevertheless, adsorption may also occur onto a negatively charged surface via hydrogen bonding and/or charge transfer when phenol moiety and carboxylic groups are involved [70]. Thus, fining agents like activated carbon, egg albumin and potassium caseinate have been shown to be the most effective solutions for reduction of OTA (even up to 90%) content in wine [67, 68].

Recently, a biological approaches have gained a lot of interest in the field of detoxification as a very promising alternative to physical and chemical methods for toxins elimination form

food (restricted due to the safety issues, possible losses in the quality of treated commodities combined with the limited efficiency [52]). OTA can be biodegraded through the hydrolysis of the amide bond that links the L-β-phenylalanine molecule to the OTα moiety (Fig. 1.8). Since OTα and L-β-phenylalanine are non-toxic this mechanism can be considered to be a detoxification pathway [71].

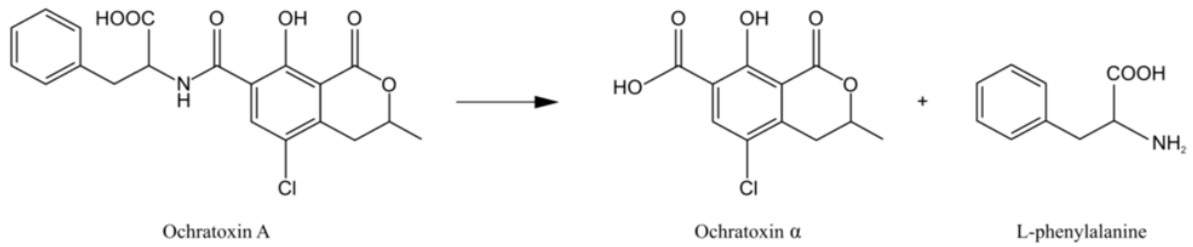

Figure 1.8. Degradation of Ochratoxin A [71].

A number of different fungi, bacteria, yeasts and protozoa have been shown to detoxify OTA. Moreover, some enzymes, lipases and commercial proteases have been identified to carry out the reaction of OTA degradation. Examples of aforementioned compounds and references are summarized in Table 1.5.

It is clear that mycotoxins can contaminate a wide range of agricultural products in the field, during storage and processing. Pre- and post-harvest prevention strategies nowadays are commonly used as most effective methodologies for the reduction of toxins occurrence. However, it is impossible to entirely eliminate production of harmful molecules and therefore, additional decontamination and detoxification approaches are necessary to minimize toxicity of commodities.

Table 1.5. Microbes and enzymes with the ability of OTA degradation [71, 72].

	Microbes or enzyme	References
Bacteria	*Acinetobacter calcoaceticus*	[73]
	Phenylobacterium immobile	[74]
Protozoa		[75, 76]
Fungi	*Aspergillus niger, A. fumigatus*	[77]
	Saccharomyces cerevisiae	[78]
Enzymes	Carboxypeptidase A	[79]
	Commercial proteases (Protease A and Prolyve PAC)	[72]
	Commercial hydrolases (Amano A)	[80]

1.2.6. Aflatoxins

Aflatoxins (AFs) are a group of toxic metabolites produced by certain fungi in/on foods and feeds and probably the most studied mycotoxins in the world (>5000 publications) since their discovery in 1962 as the cause of the Turkey disease (see section 1.1). There are four major aflatoxins B_1, B_2, G_1, G_2 (the nomenclature is based on their fluorescence under UV light – **B**lue or **G**reen) plus two additional metabolic products, M_1 (derivative of aflatoxin B_1) and M_2 (derivative of aflatoxin G_2) occurring in **M**ilk and milk products, that are of significance as direct contaminants (Fig.1.9) [3]. These mycotoxins, produced by at least three *Aspergillus* species, are able to colonize a wide range of crops both in the field as non-destructive plant pathogens and in storage, and can grow and produce aflatoxins at quite low moisture levels over a broad temperature range (13-41 ^{o}C) [81]. However, the level of contamination strongly depends on different parameters such as temperature, humidity, water activity and other storage conditions [1].

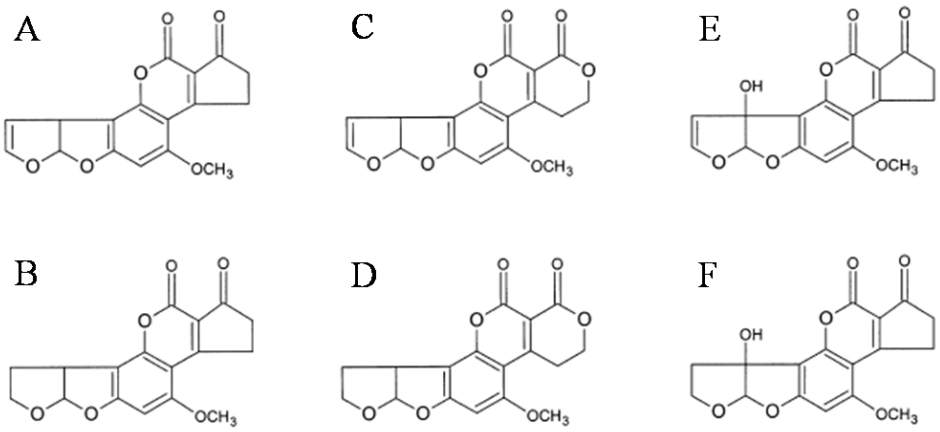

Fig. 1.9 Molecular structures of AFB_1 (A), AFB_2 (B), AFG_1 (C), AFG_2 (D), AFM_1 (E) and AFM_2 (F) [1].

From the physico-chemical and biochemical point of view (important in case of detoxification), the characteristics of the AFB_1 reveals two sites for toxicological activity [82]:

- Double bond in position 8,9 of the furo-furan ring. The aflatoxin-DNA and -protein interactions which occur at this site can change the normal biochemical functions of these macromolecules, leading to deleterious effects at the cellular level [83].
- The lactone ring in the coumarin moiety - can be easily hydrolyzed and therefore a vulnerable site for aflatoxin degradation [82].

Therefore, all actions undertaken to prevent aflatoxins occurrence should be aimed at removing the double bond of the terminal furan ring or in opening the lactone ring [82]. General methods for minimization mycotoxins existence in food during pre- and post-harvesting were described in section 1.2.5. However, the application of physical and chemical approaches is dependent on the type of mycotoxin, its structure, properties and can differ one from each other.

Aflatoxins are quite stable compounds and survive relatively high temperatures (decomposition temperatures ranging from 237 ºC to 306 ºC [52]) with little degradation but their heat stability is influenced also by other factors, such as moisture level and pH. Rustom et.al. suggested that a high level of humidity may amplify degradation via hydrolysis of the lactone ring and formation of a terminal carboxylic acid which undergo a heat-driven decarboxylation, but on the other hand AFs can also be "protected" in food by their ability to bind with proteins [84]. It has been shown that the minimum temperature required for (at least) partial detoxification should be above 100 ºC. The varying degree of AFs degradation during different heat-treatment procedures are shown in Table 1.6.

Aflatoxins are also sensitivity to UV radiation at 222, 265, and 362 nm, with the greatest absorption occurring at 362 nm which activates AFB_1 and increases it possibility of affecting the structure of the terminal furan ring and thus eliminating the active binding sites [82]. For example, 56.2% of AFM_1 in milk was destroyed by UV radiation at 365 nm for 20 min [85].

Table 1.6. Aflatoxins reduction during different heat-treatment procedures [82].

Heating conditions	AFs degradation [%]	Matrix	References
Boiling	28	Corn	[86]
Baking at 120 ºC, 30 min	80	Wheat flour	[87]
Heat up 120 ºC, 10 min	50	Peanut oil	[88]
Heat up to 250 ºC	65	Olive oil	[89]
Frying	33-53	Corn	[86]
Roasting at 190 ºC, 15 min	80	Pecans	[90]

In case of chemical detoxification, a wide range of substances such as sodium hypochlorite, chlorine dioxide, hydrogen peroxide, ozone, sodium disulphide and the hydrolytic agents (acids and alkalis) have been already tested and described with very effective way for aflatoxins elimination. Those reagents can either oxidize the double bond of the terminal furan ring or hydrolyze and oxidize the lactone ring of AFB_1 [82]. Hypochlorite anion which is a strong oxidizing agent, under acidic conditions, is able to convert ABM_1 into unstable

8,9-dichloro-AFB (exhibiting carcinogenic properties) which further easily hydrolyze to 8,9-dihydroxy-AFB (non-toxic compound) [91]. Hydrolysis of the lactone ring followed by decarboxylation to two non-toxic molecules as a result of treatment with ammonia has been shown to reduce AFB_1 concentration [92]. Another example - very powerful oxidizing agent, ozone, is promoting the reaction across the 8,9- double bond of the furan ring through electrophilic attack (at a room temperature, within few minutes) and therefore, can affect on AFs existence [82]. However, it is worth to mention that aflatoxins which do not have a double C=C bond in the furan ring (AFG and AFM) are resistant to oxidation by ozone [52]. Besides chemical and physical methods for AFs detoxification, biological or enzymatic approaches (Table 1.7.) can also affect and modify the structure of toxic compounds resulting in less toxic or even non-toxic derivatives. Generally, they are based on two pathways: modification of the difuran ring or modification of the coumarin structure [93].

Table 1.7. Biological degradation of aflatoxins [93].

Microorganism		References
Fungi	*Phanetochaete sordida*	[94]
	Pleurotus ostreatus	[95]
	Pseudomonas putida	[96]
Bacteria	*Rhodococcus erythropolis*	[97]
	Flavobacterium aurantiacum	[98]
Enzyme	Laccases	[99]

Degradation of AFB_1 into AFB_1-8,9-dihydrodiol was performed by manganese peroxidase from the white rot fungi *Phanerochaete sordida* - the authors suggested that aflatoxin degradation initially involves formation of AFB_1-8,9-epoxide, after which a hydrolysis resulted in a non-toxic dihydrodiol-derivate [94]. Another studies involving microorganism showed that 91.76% of AFB_1 was converted into a component which could be a hydrolyte of AFB_1, named dihydrohydroxy aflatoxin B1 (AFB2a) which also has a reduced mutagenicity [95].

Aflatoxins may be present particularly in cereals, oilseeds, spices and tree nuts but also maize, groundnuts (peanuts), pistachios, brazils, chilies, black pepper, dried fruit and figs are known to be high-risk foods for AFs contamination [100].

Aflatoxins are associated with both toxicity and carcinogenicity in humans and animals with a high risk of death or immune suppression [3]. Nevertheless, both aflatoxin B_1 (AFB_1) and

aflatoxin G_1 (AFG_1) have been shown to cause various types of cancer in different animal species, only AFB_1 has been identified by the IARC as carcinogenic to humans (Group 1). Therefore, AFB_1 is considered as the most toxic aflatoxin and the biggest threat for humans. It has been demonstrated that liver is the principal organ affected, followed by lungs, kidneys and brain [101]. Low levels of AFs ingestion was often times linked to primary liver cancer, chronic hepatitis, jaundice, childhood stunting growth reduction and Reye's syndrome [101]. Considering the hazard impact on people health and the wide occurrence of aflatoxins, the EU sets limits for AFB_1 and total aflatoxins (B_1, B_2, G_1 and G_2) in nuts, dried fruits, cereals and spices. Limits vary according to the commodity, but range from 2 to 12 $\mu g\ kg^{-1}$ for B_1 and 4-15 $\mu g\ kg^{-1}$ for total AFs. In US, food safety regulations include a limit of 20 $\mu g\ kg^{-1}$ for total aflatoxins in all foods except milk [102].

More recent studies show that the most threatening aspect of AFB_1 contamination is now related to AFM_1 – the main monohydroxylated metabolite of AFB_1 [1, 7]. About 0.3-6.2% of AFB_1 feed is transformed into AFM_1 through enzymatic hydroxylation process [103]. Mammals who ingest AFB_1-contaminated diets eliminate into milk amounts of metabolite known as "milk toxin" or AFM_1 [104]. Its occurrence was confirmed in human and animal milk, infant formula, dried milk, cheese, yoghurt, butter and eggs.

Even though AFM_1 is less toxic than its parent compound, IARC has recently reconsidered its carcinogenicity categorization, initially classified as a Group 2B human carcinogen, and changed it to Group 1. Besides carcinogenic properties, it is also hepatotoxic and mutagenic [105].

Since milk can be proceeded in numerous ways, its storage (temperature, time) and preparation methods are of great concern. It has been shown that the amount of AFM_1 decreases by 25% after 3 days at 5ºC, 40% after 4 days at 0ºC and even 80% after 6 days at 0ºC [104]. Moreover, milk stored at -18ºC for one month reduces AFM_1 content by 14% and 85% after two months [106]. Some authors have also linked AFM_1 with the year season, indicating that during winters its concentration in milk samples is higher than during other warm months [104]. Nevertheless, AFM_1 has been demonstrated to be stable during the pasteurization, storage and processing, and therefore, implies a significant threat to human health [105]. Due to this fact, more than one hundred countries have implemented regulations in order to control the content of AFM_1 in daily products. In US, the maximum permissible

level in milk is 500 pg mL^{-1} whereas the one set up by the EU is much more restrictive - 50 pg mL^{-1} [107].

The consumption of milk and milk products by the human population is very high, especially in infants and children, who are the major group exposed to aflatoxin M_1. Due to its persistence in the food chain, high stability, resistance during food processing and most importantly evidence of hazard on both human and animal health, it is essential to provide rapid, sensitive and selective methods for their early detection. Thus, in this thesis we took this challenge and proposed different ways for AFM_1 analysis.

1.3. Conventional analysis techniques

The common occurrence of mycotoxins in food and feed creates a real threat all over the world due to their wide spectrum of toxicological properties affecting humans and animals health. National and international institutions/organizations, such as the European Commission (EC), the US Food and Drug Administration (FDA), the World Health Organization (WHO) and the Food and Agriculture Organization have recognized the potential harmful impact of mycotoxins and set up regulatory limits for major classes and selected individual molecules [108]. Ochratoxins and aflatoxins are the most spread mycotoxins and hence, in order to protect population and minimize economic losses, their control in daily products has become a main objective for researchers worldwide. In the last century there was a great development of analytical methods for toxins detection. However, the diversity of chemical structures and varying concentration ranges in different types of commodities make it impossible to use a single standard technique for all mycotoxins analysis and/or detection. Therefore, analytical methods used nowadays typically require additional steps prior to detection, including extraction, clean-up and separation. Those steps are crucial (though time consuming) for a successful protocol and directly affect the final choice for the detection procedure. On the one hand they may result in the partial loss of some compounds, increase labor and costs, but on the other hand insufficient pre-cleaning can cause unfavorable effects like masking of residue by matrix components, occurrence of false positives and/or inaccurate quantification [109]. In the extraction step, the presence of co-extractives can mask the analytical signal of the target analyte and thus, increase the limit of detection (LOD). Generally, liquid-liquid partitioning, solid-phase extraction (SP), supercritical fluid extraction, gel permeation chromatography, immunoaffinity clean-up (IAC) and multifunctional clean-up columns (MCC) are used for the purification of extracts [110].

The most common quantitative methods for almost all kinds of mycotoxins use immunoaffinity clean-up combined with high-performance liquid chromatography (HPLC) with UV or fluorescence detection [111]. Another frequently used technology is thin layer chromatography (TLC), which provides qualitative or semi-quantitative results. Recently, capillary electrophoresis (CE) has gained a great interest, especially in ochratoxins and aflatoxins detection. Moreover, the discovery of antibodies for the most abundant mycotoxins in 1970's led to an increasing use of enzyme-linked immunosorbent assays (ELISAs). Over the years, this technology has been significantly improved, validated, commercialized, and become a useful, rapid and sensitive tool for screening.

Due to the emerging need for newer, faster and more sensitive technologies for food control and safety, a significant increase in development of alternative solutions in the field of mycotoxins detection has been recently noted. Nevertheless, up to now, none of them has elicited such popularity as the separation methods aforementioned, which have been already validated by the Association of Official Analytical Chemists (AOAC) – an international organization in which scientists worldwide contribute with their expertise to standard and method development and the systematic evaluation and review of already-in-use methods. A compilation of methods for the detection of major mycotoxins in different types of food is presented in Table 1.8 [112].

Table 1.8. Examples of validated and official methods for mycotoxins detection in food [112].

Mycotoxin	Matrix	Method	Reference
DON	Grain	TLC	[113]
FB_1	Grain	HPLC	[114]
FB_1	Corn, rice	ELISA	[115]
FB_2	Cornflakes	HPLC	[116]
OTA	Beer	HPLC	[112]
OTA	Roasted coffee	HPLC	[117]
OTA	Wine, white	HPLC	[112]
OTA	Wine, red	HPLC	[118]
ZEA	Cereal	TLC	[119]
Patulin	Apple products	HPLC	[120]
AFs	Peanut butter	ELISA	[121]
AFs	Nuts	HPLC	[122]
AFs	-	TLC	[123]

1.3.1. High performance liquid chromatography (HPLC)

As mentioned above, HPLC is one of the most frequently used technique for aflatoxins and ochratoxins quantification in food. In essence, all HPLC protocols are similar and used jointly with detection techniques such as UV absorption, fluorescence (which rely on the presence of a fluorophore in the molecule) or mass spectrometry. To minimize matrix interferences, sample pretreatment with immunoaffinity clean-up [124, 125] or conventional SP [118, 126] is typically required. Among all detection methods, fluorescence (FD) ranks the first place, due to its high specificity and sensitivity. Although a number of toxins exhibit natural florescence activity, there is a small group of them that require a previous derivatization step in order to be detected after the chromatographic column.

HPLC-FD is most often used for OTA detection and thus, a well-defined protocol has been set for its determination in various food stuff and beverages such as dried fruits [127], green or roasted coffee [128], blue cheese [129] or wine [130]. The latter product has gained a lot of interest of researchers around the world resulting in a large number of published reports. For instance, the presence of OTA in wine has been determined using commercial IAC and separation with reverse-phase C_{18} column [131]. The implementation of FD allowed for OTA detection in 0.01 ng mL^{-1} concentration. In a similar study, HPLC-FD was used with dispersive liquid-liquid microextraction with ionic liquid as a solvent which enabled to achieve LOD of 0.005 ng mL^{-1} [132]. Obtained LOD levels are comparable to the protocols' utilizing fluorescence (e.g. [130, 133, 134]).

Aflatoxins can also be detected using HPLC-FD. However, in this case the analysis becomes more complicated due to the quenching effect of their native fluorescence emission by aqueous mixtures used for reversed-phase chromatography [135]. Fluorescence amplification can be achieved by pre- or post-column addition of cyclodextrins to the HPLC eluent [136] or by pre-column derivatization of the hemiacetal [135, 137]. The usual chromatographic conditions are reverse-phase (C_{18}) column and isocratic mobile phase regime, consisting of a mixture of methanol, acetonitrile and water [138]. Nevertheless, the usage of HPLC-FD is less popular for AFs detection then for OTs.

The determination of other mycotoxins (trichothecenes, FUM, ZEA) is usually based on HPLC combined with mass spectrometry detection (HPLC-MS) due to the absence of natural fluorescence. Recently, a great development has been noticed in this field, allowing for highly accurate and specific analysis. Accordingly, fumonisins were detected using positive ion

electrospray ionization (ESI) mode as they elute from a C_{18} reverse-phase column during a methanol-water gradient containing acetic acid to facilitate the elution [139]. DON detection involved positive or negative ions in the atmospheric pressure chemical ionization (APCI) mode resulting in LOD of 1 μg g^{-1} [139].

Compared to fluorescence and mass spectrometry, alternative detections such as UV absorption are rarely used mostly because of higher LOD unable to trace the amount of the investigated substances and lack of specificity. Moreover, some mycotoxins do not absorb in the UV part of spectra (trichothecenes, FUM), or absorb only at rather non-specific wavelengths in a range of 200-225 nm (DON, OTs, ZEA) [138].

HPLC clearly has a useful place in mycotoxins analysis. As an analytical tool it offers the advantages of high resolution, sensitivity and the possibility to combine multiple detection systems allowing for simultaneous detections of compounds from one sample. On the other hand, chromatographic assays are expensive, time-consuming and require expensive equipment and clean-up procedures [140].

1.3.3. Thin layer chromatography (TLC)

One of the most effective, simple, widely used and also the first chromatographic screening method for mycotoxins detection is thin layer chromatography (TLC). In general, it is non-destructive, cheap and rapid analytical technique, yielding qualitative or semi-quantitative information [138]. Moreover, the ease of identification of targets using UV-Vis spectral analysis, a wide choice of stationary and mobile phases as well as an array of spraying agents used for the detection make this technology a powerful tool in the field of food safety [138, 141]. Nevertheless, it requires intrinsic need for sample preparation and clean-up methods dependent on the type of studied toxin. Besides the most common silica gel columns used for purification there are also reports describing the use of ELISA for AFs in corn and peanuts [142], C_2, C_8 and C_{18} pH-bonded phases for AFs in maize [143], SP [144] or IAC [145]. TLC is widely used for AFs and OTs detection in food samples since they are naturally fluorescent compounds. For instance, one-dimensional TLC involving IAC clean-up procedure was used for the determination of AFB_1, AFB_2, AFG_1 and AFG_2 in different food matrixes. The limit of quantification was found to be significantly lower than current regulatory limits for AFs [146]. The same protocol was used for OTA determination in green coffee achieving LOD of 0.5 μg kg^{-1} [147]. There are also some studies showing even greater accuracy than TLC when compared with comprehensive HPLC on the example of OTA detection [148]. TLC

methodology is cost effective, user friendly and fast methods for mycotoxin analysis however, further development of more sensitive systems and improved automation could make this technique a more popular tool in the future [110]. However, nowadays TLC is still the method of choice when HPLC is not available and the precise determination of aflatoxins is not required [146].

1.3.4. Capillary electrophoresis (CE)

Capillary electrophoresis (CE) leads to a fast separation of components based on charge- and mass-dependent migration in electric fields [140]. It is described as a rapid analytical technique with high column efficiency and fast separation accomplished in aqueous buffer solutions (minimal use of organic solvents) [149]. CE systems are available with laser-induced fluorescence (LIF) and diode array (DAD) detectors that extend the range of compounds that can be analyzed and allows the detection of mycotoxins at trace levels [150].

Capillary zone electrophoresis with diode array detection (CZE-DAD) and micellar electrokinetic capillary chromatography with diode array detection (MECC-DAD) have been developed for quantitation of AFs achieving LOD of 15 pg AFB_1 in 30 nL of buffer [150]. However, in further studies, sensitivity has been significantly improved by the implementation of a LIF system. This way, FB_1 was detected in corn within the range of 0.25 to 5.0 μg g^{-1}. Other studies present OTA analysis utilizing CE-LIF in serum [151, 152], beer [153] and in wine [154] with satisfying results. In contrast to CE-LIF methods, UV detection of OTA wine has given too high quantification limits and therefore, this technique is rarely used nowadays [155].

Nevertheless, despite a wide range of advantages, CE has never gained such popularity as HPLC, mostly due to lower sensitivity [138].

1.3.5. Enzyme-Linked Immunosorbent Assay (ELISA)

A widely used application based on interactions between antibody (Ab) and antigen (Ag), and therefore, estimation of the amount of target molecules in the sample, is the enzyme-linked immunosorbent assay, introduced in 1971 at Stockholm University in Sweden [156, 157]. Since that time, ELISA has been significantly improved and become an extraordinary useful tool for the screening and quantification of a wide range of analytes. This methodology is a plate based (typically performed in 96-well polystyrene plates) assay in which the antigen (in fluid phase) is immobilized to a solid surface [158].

ELISAs involve the stepwise addition and reaction of reagents to a solid phase-bound substance through incubation and separation of bound and free reagents using washing steps [159]. The antigen is allowed to bind to a specific antibody, which is itself further detected by a secondary, enzyme-coupled antibody [158]. The final stage in all ELISA systems is the detection of bounded Ab or Ag. An enzymatic reaction is utilized to yield color and to quantify the reaction through the use of enzyme labeled reactant [159]. The intensity of recorded signal should be directly proportional to the amount of antigen immobilized on the microtitre plate and bound by the detection reagents. The most popular and widespread enzymes used in ELISA are horse radish peroxidase (HRP) and alkaline phosphatase (AP) due to their flexibility and accessibility of a variety of substrates for chromogenic, chemifluorescent and chemiluminescent imaging [160]. The basic protocol of ELISA can provide a wealth of information. However, this technique can be also performed in more complex versions providing signal amplification and more precise results. Therefore, there are three major types of ELISAs: direct, sandwich and indirect presented in Fig. 1.10 [161].

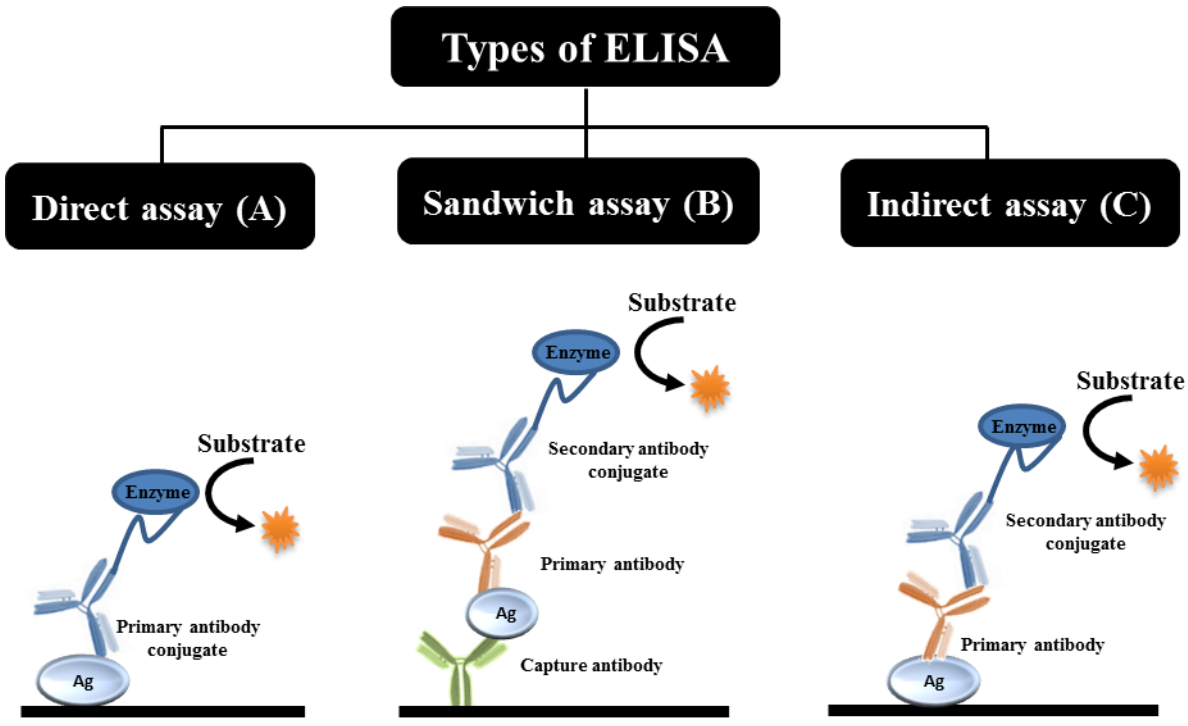

Fig. 1.10. Types of ELISA formats: direct (a), sandwich (B) and Indirect (C). Abbreviation: Ag - antigen.

A. Direct assay

A direct assay is considered to be the simplest type of ELISA which relies on the antigen immobilization onto the plate followed by blocking the remaining binding sites by the addition of another protein (usually bovine serum albumin, BSA) [162]. Afterwards, an antibody conjugated to an enzyme is applied to recognize the Ag. The aim is to allow development of a color reaction through enzymatic catalysis (for a defined period, after which the reaction is stopped by altering the pH of the system, or adding an inhibiting reactant [159]. Afterwards, the color is quantified by the use of a spectrophotometer reading at the appropriate wavelength for the color produced. This format is fast, since only few steps are required and eliminates cross-reactivity between other Ab. On the other hand, the primary Ab must be labeled individually, which significantly extends a time and also obtained signal is not as high as compare to other formats, resulting in a lower sensitivity.

B. Sandwich assay

In a sandwich format of ELISA, the Ag to be measured must contain at least two antigenic epitope capable of binding to Ab, since in this assay at least two Ab are involved. The first Ab (called capture Ab) is attached to the microtiter well. Next, the analyte or sample solution is added, followed by the injection of detection Ab. If the latter Ab is labeled with an enzyme, the assay is called a direct sandwich ELISA which involves the passive attachment of antibodies to the solid phase that subsequently bind antigen [159]. After incubation and washing, the captured Ag is detected by the addition of enzyme-labeled specific antibodies. At the last step, the bound enzyme is developed by the addition of substrate/chromogen, stopped and finally read using a plate reader. However, if injected antibody is not conjugated, then a second detection Ab is required resulting in so-called indirect sandwich assay. Sandwich format provides a high specificity and sensitivity. It is suitable for complex samples due to the fact that the Ag does not require purification prior the measurement and offers a big flexibility, since direct and indirect methods can be used [158].

C. Indirect assay

Another commonly used type of ELISA format is indirect assay. In this technique, the target is immobilized on the platform surface followed by the addition of the sample containing primary antibodies (unlabeled, specific for the Ag) [162]. Next, the secondary Ab (Ab_2, which

has the specificity for the primary Ab) labeled with an enzyme are added to bind with the primary Ab. A substrate for the enzyme is introduced to quantify the primary antibody through a color change [158]. The concentration of primary antibody present in the serum directly correlates with the intensity of the color. An indirect ELISA combines a lot of advantages such as high sensitivity, flexibility or cost savings. Moreover, a wide variety of labeled secondary Ab is commercially available. However, the use of Ab_2 might result in high cross-reactivity and therefore, non-specific signals may occur [162].

Beside three main ELISA formats described above, it is worth to mention about one other existing type – **Competitive ELISA**, used especially for the detection of small molecules (low molecular weight compounds) with only one epitope. This strategy is based on antigen immobilization on the surface followed by the injection of the mixture of primary antibody and sample containing free antigen [163]. Therefore, the higher the Ag concentration in the sample, the lower the amount of antibodies available for binding the antigen linked to the surface. After washing, labeled Ab_2 are added and the enzymatic reaction is measured.

Due to the high sensitivity, strong specificity, flexibility in the choice of detection methods, and time effectivity, ELISA has become a useful and powerful technique with a large variety of applications, either in scientific research or clinical diagnosis of diseases. Recently, a lot of commercially available ELISA kits have been developed offering portable, rapid and user friendly method for the detection of different target molecules. In the field of food control, ELISA is considered to be a suitable alternative for all chromatographic technologies. In the last years there have been a lot of reports about the use of ELISA as an analytical tool for mycotoxins determination in food and beverages. Some examples of protocols which employ different formats of ELISA for ochratoxins and aflatoxins are shown in Table 1.9.

Table 1.9. Examples of different ELISA formats used for the detection of most common mycotoxins

Mycotoxin	Protocol	Matrix	Reference
AFs	Indirect	Peanuts	[164]
AFs	Indirect competitive	Maize	[165]
AFB_1	Direct competitive	Grain	[2]
AFB_1	Direct competitive	Corn, soybeans,	[166]
AFM_1	Indirect competitive	Milk	[167]
AFM_1	Competitive (Ridascreen AFM_1)	Cheese	[168]
AFs, OTA	Direct competitive	Cereals, feed	[119]
OTA	Direct competitive (AgraQuant)	Corn, wheat, soybeans, green coffee	[169]
OTA	Direct competitive	French wine	[170]
OTA	Indirect competitive	Wine, beer	[171]

As it can be observed, both direct and indirect ELISA formats are widely used for the detection of mycotoxins. All of those techniques have advantages and some limitations described above. The choice of a proper format belongs only to the researcher who considers pros and cons of each methodology. However, worth to notice is the fact that in all publications regarding mycotoxins detection the authors combine a direct/indirect style with competition step due to the small size of analyzed toxins which preclude the use of ELISA in easier way.

1.4. Biosensors

The availability of fast, sensitive, simple, portable and cheap methods for rapid determination of food contaminants is an increasing need for human safety. Official techniques such as HPLC, TLC or CE offer high sensitivity and selectivity at the expense of time, cost and a need for sample pre-treatment or pre-concentration. Therefore, the use of analytical procedures based on affinity biosensors has recently gained a lot of interest, mainly due to their capability to resolve a potentially large number of analytical problems and challenges in very diverse areas such as defense, homeland security, agriculture and food safety, environmental monitoring, medicine, pharmacology, industry, etc. [172].

The term biosensor (schematically depicted in Fig. 1.11) can be, in general, described as a device containing a biological recognition component (e.g. enzymes, antibodies, nucleic acids or artificial receptors) combined with a sensor element (transducer/detector).

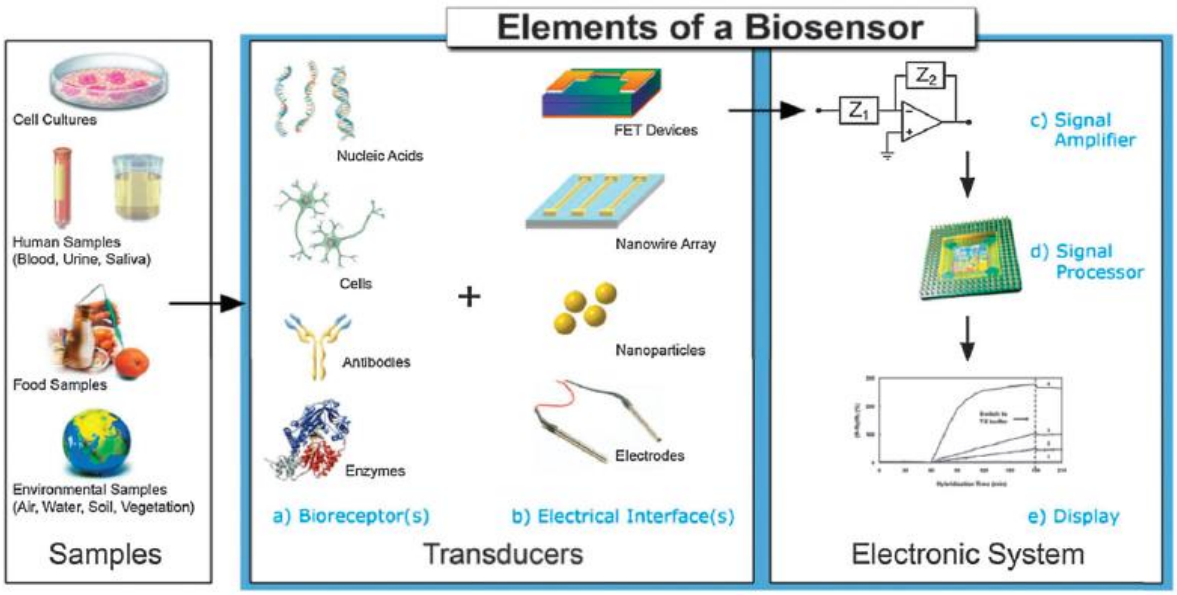

Fig. 1.11. Elements and selected components of a typical biosensor. Reprinted from [172]

The official IUPAC (International Union of Pure and Applied Chemistry) definition states *"A biosensor is a self-contained integrated device, which is capable of providing specific quantitative or semi-quantitative analytical information using a biological recognition element (biochemical receptor) which is retained in direct spatial contact with an transduction element."* [173].

Related to the physicochemical properties of mycotoxins and/or the type of transduction, biosensors technology can be divided into three groups [174]:

- Optical sensors (e.g. SPR)
- Piezoelectric sensors (e.g. QCM)
- Electrochemical sensors.

The main advantages of biosensors in comparison with traditional analytical methods are summarized in rapid detection, high sensitivity, easy preparation, reusability and low costs [175]. In the last 30 years there has been a significant increasing interest in the field of biosensors development. Such a fast growth is driven by several factors including medical and health problems like growing population with a high risk of diabetes and obesity, the rising incidence of chronic diseases such as heart disease, stroke, cancer, chronic respiratory diseases, tuberculosis, significant problems with environmental monitoring; and of course serious challenges in security and military applications and agriculture/food safety [172, 176]. In the latter one, the biosensor technology is currently the most active area of mycotoxins

analytical research. Examples of major mycotoxins determination in food and beverages utilizing biosensors with optical, acoustic and electrochemical detection are shown in Table 1.10.

Table 1.10. Examples of mycotoxins detection utilizing various types of biosensors

Mycotoxin	Matrix	Detection	Reference
AFM_1	Milk	SPR + fluorescence	[177]
AFM_1	Milk	Electrochemical	[178, 179]
AFB_1	Groundnut	Piezoelectric	[180]
OTA	Wheat	Electrochemical	[181]
OTA	Coffee	Piezoelectric	[182]
OTA	Cereals	SPR	[45]
DON	Wheat	SPR	[183]
T-2, HT-2 toxins	Cereals, maize-based baby food	SPR	[184]
ZEA	Milk, wheat	Electrochemical	[185]

1.4.1. Optical biosensors (Surface Plasmon Resonance Spectroscopy - SPR)

A very promising technology for rapid and sensitive detection of chemical and biological analytes is surface plasmon resonance spectroscopy (SPR). This technique has become increasingly popular with the commercialization of biosensors by the company Biacore in the 90's offering a novel and powerful approach for the determination of kinetic parameters (association and dissociation rate constants) but also providing thermodynamic information (e.g. affinity constants) [186, 187].

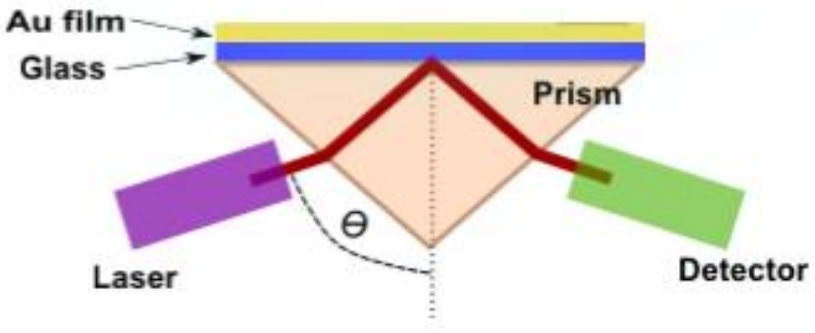

Fig. 1.12. SPR biosensor principle, surface plasmons are excited by polarized laser beam at certain angle Θ and the intensity of reflected light is measured.

SPR is an opto-electronic phenomenon utilizing a thin metal (preferably gold or silver) film between two transparent medias of different refractive index (e.g. a glass prism and sample solution) [186]. When a polarized light beam passes through the higher refractive index medium (e.g. glass prism) it can undergo a total internal reflection above a critical angle of incidence, generating an evanescence wave penetrating the metal layer [186, 188]. This evanescence wave propagates along the interface with a propagation constant which can be adjusted to match that of surface plasmons, by controlling the angle of incidence (the so called attenuated total reflection (ATR) method) [188]. When the wavelength of the photon equals the resonance wavelength of the metal, the photon couples with the surface and induces the electrons in the metal film to oscillate as a single electrical entity (called plasmon) [163]. This movement creates an electromagnetic field that decays exponentially out from the metal surface, with significant electric field strength occurring within 300 nm of the surface [163]. If the molecule binds to the surface within this range, the plasmon might be disturbed, causing a change in the resonance angle of incoming photons. Thus, the SPR system is sensitive for changes in the refractive index of the surface layer of a solution in contact with the sensor chip (Fig. 1.12) [186].

A change in the refractive index of the dielectric material gives rise to a change in the propagation constant of the surface plasmon, which alerts the characteristics of the light wave coupled to the surface plasmon (e.g. coupling angle, wavelength, intensity, phase) [189]. Therefore, SPR sensors with angular modulation use monochromatic light wave for plasmon excitation which is observed as a dip in the angular spectrum of reflected light (at a fixed wavelength, Fig. 1.13 A, upper plot). The angle of incidence providing the strongest coupling is measured and used as a sensor output [189]. In SPR with wavelength modulation a polychromatic light source is utilized, where the plasmon excitation is seen as a dip in the wavelength spectrum of reflected light (at a fixed angle of incidence, Fig.1.13 B). SPR with intensity modulation rely on measurements of coupling strength between the light wave and the surface plasmons at a single angle of incidence and wavelength (Fig.1.13 B) [190]. Sensors with phase modulation measure the shift in phase of the light wave coupled to the surface plasmon at a single angle of incidence and wavelength of the light wave (Fig.1.13 A, lower plot) [189, 191].

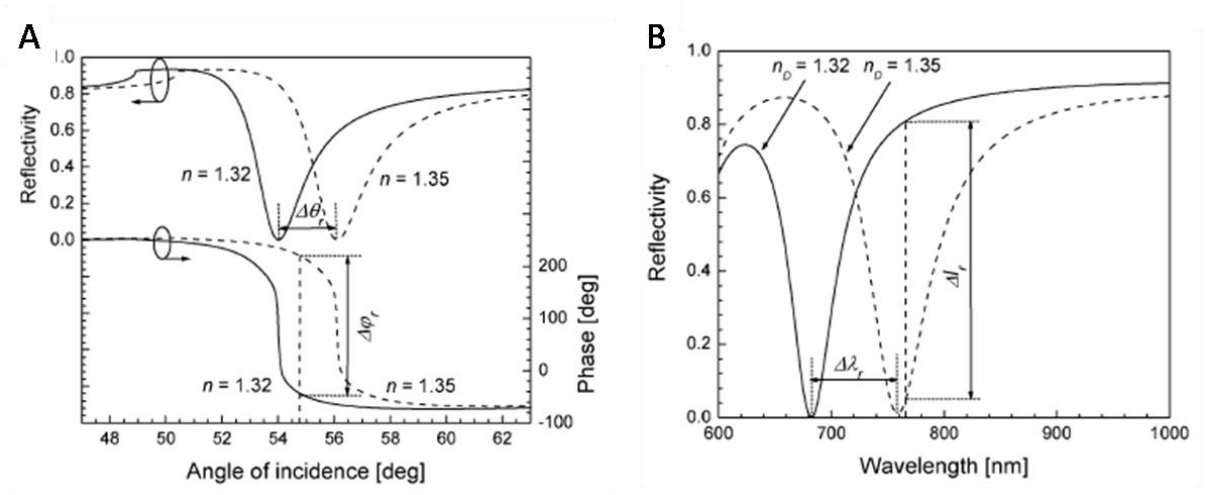

Fig. 1.13. Reflectivity and phase for light wave exciting surface plasma wave vs. (A) the angle of incidence for two different refractive indices of the dielectric and (B)wavelength for two different refractive indices of the dielectric. Reprinted from [188].

In general, SPR biosensors use resonance units (RU) to quantify changes in the refractive index. The signal is proportional to the amount of bounded molecules and therefore, e.g. for proteins 1000 RU corresponds to the surface coverage of 1 ng mm^{-2}. Due to the fact that this technique is sensitive to mass changes occurring at the noble metal surface interface, it has found an application mostly in bioassays of large molecules (with molecular weight > 2 kDa, such as antibody - approximately 150 kDa) utilizing sandwich immunoassay format. Nevertheless, there has recently been an increasing number of reports focusing on strategies involving other formats for the detection of various chemical and biological analytes. Small compounds (such as mycotoxins) usually do not generate a sufficient change in the reflective index and thus are more challenging for the determination using SPR, suffering from low signal and poor sensitivity [163]. However, in combination with competitive or inhibition detection formats and the utilization of additional high mass labels, a clear enhancement of sensitivity can be achieved. In competitive methodologies, the sensor surface is coated with antibody interacting with analyte; when a conjugated antigen is added to the sample, it competes with the analyte for a limited number of biding sites on the surface. Therefore, the recorded signal is inversely proportional to the analyte concentration. The inhibition assay rely on the mixture of a fixed concentration of antibody with a sample containing unknown concentration of antigen which is subsequently injected into the flow cell and passed through a sensor surface, to which antigen is immobilized. Then, the amount of bounded antigen to the modified surface antibody is measured and the obtained signal is proportional to the concentration of analyte [189]. In those cases, the mass is provided by the use of primary

antibody which let obtain the optimum assay sensitivity. However, the signal can be further amplified by the use of secondary antibodies either with or without conjugation to large particles. Other approaches involve the use of fluorescence for LOD improvement or modification of the sensor chip with metallic nanoparticles. The latter ones are widely used in the construction of biosensors due to their unique physical and chemical properties, good biocompatibility and high catalytic activity for many chemical reactions.

Among a large variety of nanomaterials available nowadays, the implementation of gold nanoparticles (AuNPs) has gained the highest interest.

AuNPs - signal amplification

The outstanding optical properties of AuNPs result from participation of their free electrons in the collective oscillation of electrons, called localized surface plasmon (LSP) [187]. When metal nanostructures interact with a light beam, part of the incident photons are absorbed and part are scattered in different directions. Both absorption and scattering are greatly enhanced when the LSPR is excited [192]. The general principle behind LSPR involves the shift in wavelength and/or the change in absorption intensity of the LSPR band upon analyte detection [187]. Therefore, this type of sensors are recently used as an alternative to simple SPR sensors due to the highly localized electromagnetic fields on NPs surfaces which can significantly improve detection of nanoscale biological analytes [193]. Moreover, AuNPs have another advantage - they concentrate a high mass into a small volume, followed by the simple formation of coordinate bonds with thiol functional groups on their surface what results in an easy conjugation of AuNPs to the biomolecules as signal enhancement labels [163].

In case of AuNPs-based biosensors, signal amplification can be achieved by implementation of antigen/antibody-labeled AuNPs to bind with the ligand immobilized on the SPR sensor. The main idea standing behind such tremendous enhancement is linked to the artificial increased mass of the analyte due to the linked AuNPs which results in higher refractive index changes causing a larger SPR shift [187]. However, besides this fact, it is expected that the dominant role belongs to the electromagnetic field coupling between LSP field of NPs and the surface plasmon field of the gold sensor surface [187].

In SPR biosensors, the recognition element (e.g. antigen, antibody/aptamer) is immobilized on a solid surface on a sensor chip. This step is crucial for obtaining a high sensitivity, selectivity

and low LOD. Therefore, the design of surface chemistry must enable immobilization of a sufficient number of biomolecules while keeping their biological activity and minimizing non-specific bindings which may occur during this process [189]. The most widely used immobilization on the sensing (gold) surface is via self-assembled monolayers (SAMs) of alkanethiolates or disulfides.

Self-assembled monolayer

Since the 1980's, with the discovery of spontaneous assembling of alkanethiols on noble metals, a new avenue offering a simple way of creating surfaces of virtually any desired chemistry has been opened. SAMs are ordered molecular assemblies formed by the simple immersion of a substrate and adsorption of an active surfactant solution onto a solid surface (Fig 1.14 A) [194].

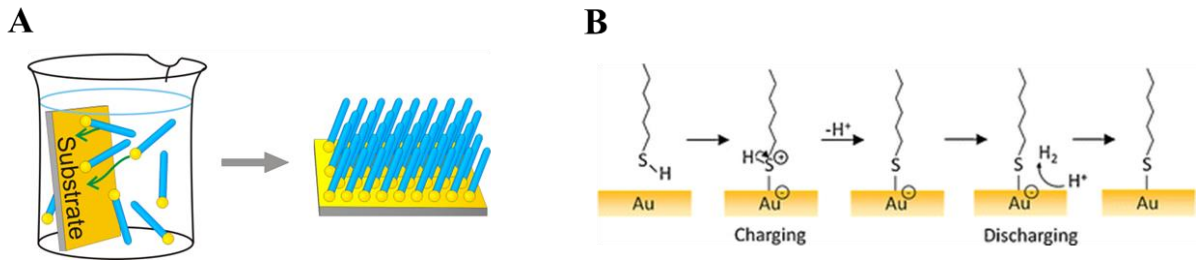

Fig. 1.14. Self-assembled monolayer formations (A) and schematics of the adsorption of thiols on gold (B) Reprinted from [195].

Despite of the existence of different SAMs systems (e.g. silanes on hydroxylated surfaces, fatty acids, organosilicon derivatives) the majority of papers in recent years deal with thiols on gold. Why is gold so popular? There are five main reasons:

- It is easy to obtain, both as a thin film (utilizing e.g. physical vapor deposition, sputtering or electrodeposition) and as a colloid;
- It is simple to pattern by photolithography, micromachining or chemical etchants;
- Au is considered as an inert metal allowing for handling samples under atmospheric conditions;
- Thin films of gold are common substrates used in a number of analytical methods (SPR, QCM, ellipsometry etc.);
- Gold is compatible with cells [196].

Spontaneous adsorption of thiols on gold leading to the formation of exceptionally strong bond makes this system the most commonly used. There are several driving forces responsible for this assembly: the affinity of sulfur for the gold surface (45 kcal mol^{-1} – creation of a stable, semi-covalent bond [197]) and hydrophobic, van der Waals interactions between the methylene carbons on the alkane chains [198]. The longer the chain is, the more ordered SAMs with higher integrity and thermal stability is created (it has been reported that well-ordered monolayer is formed from an alkane chain of at least ten carbons [199]). It has been demonstrated that the mechanism of covalent bond creation consists of charging and discharging steps while releasing H_2 (Fig.1.14 B). The first step of layer formation – attachment of –SH groups to Au atoms is very fast whereas the process of organization of thiols to maximize van der Waals interactions takes place much slower [195].

The most common and the simplest protocol for thiol monolayer preparation providing maximum density of molecules and minimizing defects in the SAM is based on immersion of substrate into ethanolic solution of thiols (usually at the concentration ranging from 1 to 10 mM) for 12-18 h at room temperature. Nevertheless, the structure of the formed layer is also dependent on a number of experimental factors (solvent, temperature, concentration, immersion time, purity of reagents, concentration of oxygen in solution, cleanliness of the substrate, and chain length) which have to be taken into account during gold surface functionalization.

As mentioned before, long alkyl chain thiols are the best solution for formation of a well-organized and stable monolayer. This strategy is successfully used in such techniques as SPR or QCM. However, the limitation of using SAMs of thiols for electroanalytical applications derives from the necessity of a conducting interface. For this reason, usually short chains of alkanethiols are used, which not only enable electron transfer across the layer, but also reduce the stability of the interface. Nevertheless, it was intensively investigated and demonstrated that also those constraints can be overcome by implementation of a proper compounds [195, 200].

Design flexibility, simplicity, dense and stable structures – all those properties makes SAMs important components for many researches. They have found an application in a large variety of areas e.g. corrosion prevention, wear protection, surface wetting, non-fouling property, electro-optic devices or chemical and biochemical sensing systems (protein binding, DNA assembly, biological arrays, and cell interactions).

Coming back to SPR, this technology offers a number of benefits over other methodologies such as label-free detection, real-time monitoring, ability to handle complex samples and replicate measurements, possibility to reuse a sensor chip (regeneration of the surface) which significantly reduces cost. Therefore, it has found an application e.g. in medical diagnostics, environmental monitoring and food safety and security. The acceptance of SPR biosensors in food analysis caused an interest among researches all over the world resulting in an increasing number of publications which appeared recently. Several assays employing SPR for measuring mycotoxins concentration were described - Table 1.11 shows examples of major toxins determination and obtained LOD.

Table 1.11. Mycotoxin detection utilizing SPR spectroscopy

Mycotoxin	Type of detection	LOD	Reference
DON	Indirect	0.05 mg kg^{-1}	[201]
FB_1	Direct	10 ng mL^{-1}	[202]
FB_1	Direct	50 ng mL^{-1}	[203]
ZEA	Direct	30 ng g^{-1}	[204]
ZEA	Indirect	0.01 ng g^{-1}	[186]
T-2 toxin	Direct	0.05 pg mL^{-1}	[205]
OTA	Indirect	0.05 ng mL^{-1}	[206]
OTA	Indirect	0.042 ng mL^{-1}	[45]
AFM_1	Indirect	0.6 pg mL^{-1}	[177]
AFB_1	Indirect	0.2 ng g^{-1}	[207]
AFB_1	Indirect	3 ng mL^{-1}	[208]

1.4.2. Acoustic biosensors (Quartz Crystal Microbalance - QCM)

Another example of exceptional technique which has gained importance in the field of biosensors is an acoustic device - quartz crystal microbalance (QCM), introduced in late 1950s' by Sauerbrey, who demonstrated the dependence of quartz oscillation frequency on the change in surface mass. This phenomenon led to the use of quartz plate resonators as sensitive microbalances for thin films [209]. A QCM is a shear mode device which includes a quartz crystal wafer (cut to a specific orientation with respect to the crystal axes – AT or BT where the acoustic wave propagates perpendicularly to the crystal surface [209]) sandwiched between two metal electrodes [210].

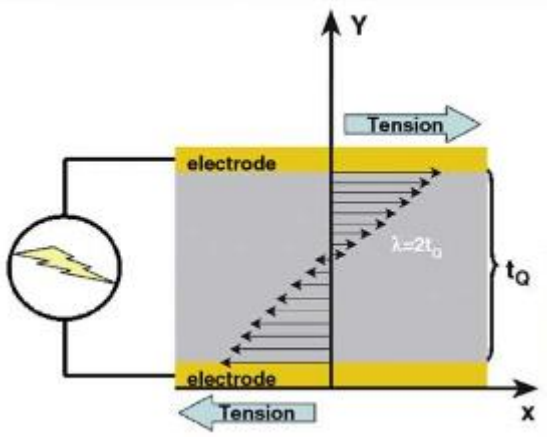

Fig. 1.15. QCM principles - the application of the electric field produces deformation that results in an acoustic wave which propagates across the crustal material. Reprinted from [211].

When electric field is applied, it induces oscillations of the crystal at a specific frequency by producing a shear deformation where both surfaces move in parallel but opposite direction and thereby generating acoustic waves which propagate through the bulk of the material across the crystal, in a direction that is perpendicular to the surface (Fig. 1.15) [211]. The change in mass on the quartz surface is directly related to the change in frequency of the oscillating crystal, as shown in the Sauerbrey equation [212]:

$$\frac{\Delta f}{\Delta m} = -\frac{2f_n^2}{\rho v n}$$

where Δf is the frequency shift, Δm is the surface mass density change on the active sensor's surface, ρ is the quartz density, v the propagation velocity of the wave in the AT cut crystal, f_n is the frequency of the selected harmonic resonant mode and n is the harmonic number (n=1 for the fundamental mode). However, this equation is valid only for coatings exhibiting elastic properties which do not dissipate any energy during oscillation [209]. In case of inelastic subjects (e.g. cells, polymers) this formula cannot be applied due to the energy loss caused by damping during oscillation. When the change in mass is greater than 2% of the crystal mass, the Sauerbrey equation becomes inaccurate - there is no linear relationship between Δf and Δm [209]. Therefore, the so-called QCM with dissipation (QCM-D) device was developed to enable simultaneous monitoring of the changes in frequency and dissipation, and thus provides an unique information about the effective layer thickness, conformational changes, viscoelastic properties and the hydration state of the film [213]. Thus, acoustic sensor technology has become a powerful methodology providing a detailed description of analyzed subjects. Due to this fact QCM technology has found a broad range of applications in the field

of biochemistry, biotechnology, environmental monitoring or food control as an excellent tool for detection of a variety of analytes; from interfacial chemistries and lipid membranes to small molecules, whole cells, disease biomarkers and pathogens [214]. In the case of mycotoxins determination utilizing QCM technology only few examples have been found in the literature: OTA [215], AFB_1 [216, 217], patulin [218] and T-2 toxin [219].

Nowadays, these biosensors are widely used for the direct, marker-free measurements of a specific interactions between immobilized molecules and analytes in solution [220]. The sensitivity and specificity highly depend on the immobilization process of recognition layer. The most common strategy is based on generation of strongly bonded carboxyl groups (SAMs) on the QCM gold surface by treatment with appropriate thiols followed by the activation of modified surface and generation of active moieties able to bind antibody. When a target proteins are captured by the immobilized receptor, the effective mass of the oscillator increases, resulting in the decrease in the resonance frequency of the oscillator [220]. Subsequently, an injection of washing solution causes dissociation of the target and recovery of the resonance frequency (Fig. 1.16).

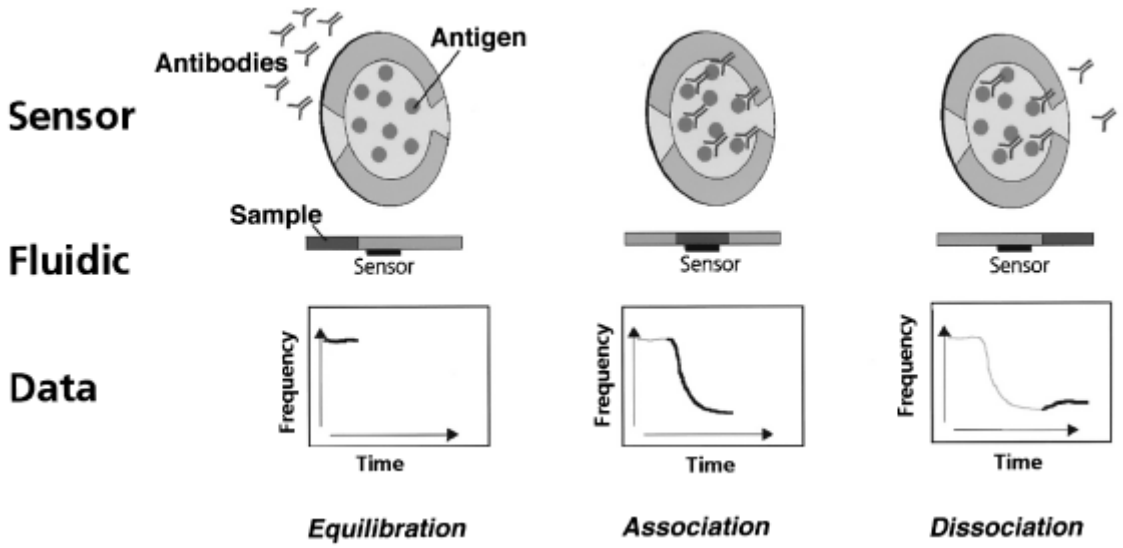

Fig. 1.16. Scheme of the antibody–antigen on sensor surface (sensor) and resulting frequency versus time curve changes (data) [220].

Therefore, the possibility of a real-time monitoring of association and dissociation reactions between molecules provides quantitative information about their binding affinity.

In the quartz crystal microbalance, similarly to the SPR technology, the bioreaction generates a change in the mass, and thus, gives a rise to a change in the resonant frequency of the

microbalance [214]. Hence, the use of AuNPs labels in immunoassays is widely applied to increase the mass of the immune complex, what allows for the sensitivity improvement (lower LOD). Studies about implementation of metallic nanoparticles in different strategies (Fig.1.12) were already reported e.g. in the detection of human IgG [221], bacteria (e.g. *Escherichia coli*) [222] and mycotoxins (e.g.AFB_1) [223].

Concluding, utilization of QCM-based biosensors which offer high sensitivity and stability, fast response, portability and non-hazardous label-free real-time monitoring of various molecules is an excellent alternative (or complementary) to conventional methods.

1.4.3. Electrochemical biosensors

Electrochemical biosensors have been the subject of research in a large range of applications, including food analysis. For more than sixty years due to their unique properties which combine simplicity and rapidity of the measurement, portability, low-cost of the equipment and integration in automated devices thereby offering high sensitivity and selectivity [174]. Therefore, they have become a novel and very promising alternative tool, which do not require sophisticated instrumentation and well-trained personnel.

Typically, electrochemical biosensors utilize the presence of electroactive analytes that are oxidized or reduced on the working electrode surface and further, generating an electrochemical signal, which is measured by the detector [224]. The choice of a proper working electrode is a key for successful measurements. Recently, due to the fast development in the field of photolithography, microcontact printing etc., commonly used solid electrodes of gold, platinum, silver, nickel or copper have been more often replaced by microelectrodes (2 mm dimension) which have found an application in *in vivo* and *in vitro* studies offering a significant reduction of analyte and reagents volumes. Moreover, the possibility to use inexpensive, highly reproducible and disposable sensors offered by screen-printed technology is currently undergoing widespread growth [225]. The great versatility, commercial availability and easy modification (addition to the printing ink or deposition on the surface different substances such as metals, enzymes, polymers) of screen-printed electrodes (SPEs) makes this approach an interesting solution in the field of electrochemical biosensing.

Most of electrochemical biosensors for mycotoxins are based on the use of specific antibodies, aptamers or artificial receptors as affinity ligands which allows binding the analyte

to the sensor for the measurement with minimum interference from other components that can occur in the sample [224]. Such affinity based sensors that comprise an electrode with the bioreceptors – e.g. antibody (or antigen) labelled with an enzyme (usually horseradish peroxidase or alkaline phosphatase) which generate an electroactive signal, offer great selectivity and sensitivity [226]. Based on their operating principles, a variety of electrochemical techniques have been used to convert the chemical information into a measurable analytical response [227]:

- **Potentiometric**: based on ion-selective electrodes (ISE) and ion-sensitive field effect transistors (ISFET). The primary outputting signal is possibly due to ions accumulated at the ion-selective membrane interface [227]. The signal is measured as the potential difference between the working and the reference electrodes.
- **Amperometric**: based on the measurement of the current resulting from the oxidation or reduction of an electroactive biological element providing specific quantitative analytical information [228].
- **Impedimetric**: combines the analysis of both the resistive and capacitive properties of materials. Measures the resistance of the generated electric current at certain applied voltage.

Electrochemical detection strategy which assures simplicity, frugality, high sensitivity and selectivity has gained recently a lot of attention if the field of food control. These kinds of biosensors have been the most popular solution used for detection of various analytes including mycotoxins due to a numerous advances leading to their well-understood biointeraction and detection process [224]. Table 1.12 shows some examples of electrochemical affinity sensors for major mycotoxins detection found in the literature.

Table 1.12. Electrochemical immunosensors for mycotoxins

Mycotoxin	Technique	LOD [ng mL^{-1}]	Matrix	Reference
AFB_1	IC/AMP on SPE	0.09	Barley	[229]
AFB_1	IC/DPV on SPE	0.03	Barley	[230]
AFB_1	IC/DPV on ITO electrodes	0.006	Red paprika	[231]
AFM_1	EIS	1	Milk	[232]
AFM_1	DC/AMP on SPE	0.039	Milk	[179]
AFM_1	DC/POT	0.04	Milk	[233]
OTA	DC/DPV	0.18	-	[234]
OTA	IC/AMP	0.3	Wine	[235]
OTA	IC with AuNPs/DPV	0.2	Wheat	[236]
OTA	DC/EIS	0.0008	Coffee	[237]
ZEA	DC/AMP	0.01	Maize, cereal, baby food	[238]
ZEA	DC/AMP	0.41	Foodstuff	[174]
FB_1+FB_2	DC/AMP on SPE	5	Corn	[239]
DON	DC/EIS	0.0003	Food samples	[174]
T-2 toxin	IC/AMP	0.3	Corn	[240]

Abbreviations: *IC - indirect competitive assay; DC - direct competitive assay; AMP - amperometry, DPV - differential pulse voltammetry; POT - potentiometry; SPE - screen printed electrodes; GCE - glassy-carbon electrode; ITO - indium tin oxide electrode.*

Still, there are many avenues to be opened in the field of electrochemical sensors aiming at amplification of electron transfer in order to improve sensitivity. One of them may combine the recent development in nanotechnology and discovery of newer and better nanomaterials. For example, the versatility and high applicability of nanoparticles and carbon nanotubes makes them clear candidates to be further used in electrochemical nanosensors for food analysis utilizing their unique properties like good conductivity and high electrocatalytic activity [224]. Undoubtedly, the integration of novel nanobiotechnological concepts in electrochemical biosensors for the analysis of toxins require further investigation to fulfill the demand for more robust systems capable of detecting genetically modified ingredients and allergens [224].

CHAPTER TWO, SENSITIVE AND RAPID DETECTION OF AFLATOXIN M1 IN MILK UTILIZING ENHANCED SPR AND p(HEMA) BRUSHES

The content of this chapter has been already published in **Biosensors and Bioelectronics** Journal.

Authors and their contribution:

- **A. Karczmarczyk** - study conception and design, acquisition of data, analysis and interpretation of data, drafting of manuscript,
- M. Dubiak-Szepietowska - critical revision of the article,
- M. Vorobii - p(HEMA) brushes provider,
- C. Rodriguez-Emmenegger - scientific advisor, critical revision of the article,
- J. Dostalek - conception and design, scientific advisor, critical revision of the article,
- K-H. Feller - scientific advisor, critical revision of the article, final approval of the version to be published.

Sensitive and rapid detection of aflatoxin M1 in milk utilizing enhanced SPR and p (HEMA) brushes. Biosensors and Bioelectronics, 2016. 81: p. 159-165

2.1. Introduction

Aflatoxins are a family of extremely toxic and carcinogenic secondary metabolites (mycotoxins) secreted by certain species of *Aspergillus*. In particular, *Aspergillus flavus*, *A. parasiticus* and *A. nominus* contaminate a large variety of food and feed commodities [7]. Aflatoxin M_1 (AFM_1) is the hydroxylated derivative of aflatoxin B_1 (AFB_1) originating from the activity of cytochrome P450-associated enzyme in liver. It is excreted into the milk of both human and animals that have been fed with AFB_1 polluted diet [241, 242]. About 0.3–6.2% of AFB_1 in animal feed is transformed to AFM_1 in milk [243]. This compound elicits a wide spectrum of toxicological and carcinogenic effects causing liver cirrhosis, tumors or liver damage of human as well as animals [244-246]. The International Agency for Research on Cancer (IARC) recently reconsidered its carcinogenicity categorization, initially classified as a Group 2B human carcinogen, and changed it to Group 1 [247]. Despite the fact that milk and dairy products, such as cheese and yoghurt are an important source of many essential nutrients like proteins, magnesium, calcium or vitamins B12 and A, unfortunately, they are also the most potent providers of AFM_1 among foods. Due to the relative stability during heat treatments (*e.g.* pasteurization) and significant threat to human health, especially to children

who are the major consumers of milk, many countries have implemented regulations to control the content of AFM_1 in daily products [104]. The European Commission stipulates a maximum permissible level of 50 ng L^{-1} for AFM_1 in milk and dried or processed milk products [248]. In China and United States the regulations mandate AFM_1 levels below 500 ng L^{-1} [249].

Sampling and analysis of mycotoxins are regulated by the European Commission Directives. The stipulated methods include high performance liquid chromatography with fluorimetric detection (HPLC-FD) coupled with the pre-cleaning by immunoaffinity columns [250]. This procedure relies on extensive sample preparation and the analysis requires couple of hours. Other, currently performed, techniques such as thin-layer chromatography (TLC) [251, 252] or enzyme-linked immunosorbent assay (ELISA) [167] are also time consuming and require highly trained personnel, expensive equipment deployed in specialized laboratories. In order to simplify the analysis of mycotoxins, research is carried out to provide faster and sensitive techniques suitable for routine assay of milk and other dairy products. Over the last years we witnessed efforts to expedite AFM_1 detection based on immunoassays combined with fluorescence [253], electrochemistry [178], colorimetry [254] or chemiluminescence [255] readout.

A promising alternative to fluorescence assays are label-free biosensors based on surface plasmon resonance (SPR) [189]. This technology exploits the measurement of changes in the reflective index occurring upon the affinity binding of molecules in the proximity of a metallic surface. As the response of SPR sensor is proportional to the mass of target molecule, direct detection of small molecules, such as mycotoxins, and/or analytes at very low concentration is challenging. Therefore, alternative assay formats are required for mycotoxin detection by using SPR. In order to enhance the sensor response, it was utilized a competition for binding to the surface between an antigen conjugated with a high molecular weight label and the un-labeled sample antigen. An alternative way is to immobilized the same molecule —antigen— which will be measured to the sensor surface, followed by injection of the primary antibodies and sample containing free antigen mixture. In this latter case, the signal can be further amplified by using the secondary antibodies labeled with metallic nanoparticles (NPs), magnetic nanoparticles (MNPs), fluorophores or quantum dots (QDs) [256-259]. Recently, several studies have reported the signal amplification by the implementation of gold nanoparticles (AuNPs). It can be designed to harness several effects including enhanced surface area, refractive index changes by the particles mass, and electromagnetic field

coupling between the plasmonic properties of the particles and propagating plasmons [187, 260].

Another challenging aspect in SPR analysis of complex samples such as milk and dairy products is the non-specific interaction at the surface that is associated with the deposition of non-targeted molecules or entities. SPR biosensors require an interface design for anchoring specific bioreceptors that is at the same time resistant to non-specific sorption [261]. To overcome the problem with fouling, different strategies of surface modification were proposed: grafting of carboxymethyl dextran [262], passivation with albumin [263] or a relatively new, however, very promising modification with various types of non-fouling polymer brushes. Such surface architecture has been shown to provide significantly higher fouling suppression [264-267], demonstrating their applications in a real-world biosensing [258, 265, 268, 269]. Considering milk analysis, it has been reported remarkably ultra-low fouling properties of antibody functionalized poly(2-hydroxyethyl methacrylate) p(HEMA) for the direct detection of *Cronobacter* in milk [261].

In this work, we describe an SPR biosensing for rapid, sensitive and specific detection of low molecular weight analyte AFM_1 in complex milk samples. The resistance to non-specific adsorption from milk was assessed by using the p(HEMA) brushes and the assay performance was compared to recent state-of-the-art antifouling polyethylene glycol (PEG) moieties. An indirect competitive immunoassay was developed for the analysis of low molecular analyte and the amplification of the sensor response by using secondary antibodies with metallic nanoparticles labels.

2.2. Materials and methods

2.2.1. Reagents

All reagents were used as received without further purification. Dithiol PEG6-COOH and dithiol PEG3-OH were purchased from SensoPath Technologies. 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and *N*-hydroxysuccinimide (NHS) were obtained from Pierce (USA). *N*,*N*-dimethylformamide (DMF, 99.8%), 4-(dimethylamino) pyridine (DMAP), *N*,*N*'-disuccinimidyl carbonate (DSC), aflatoxin M_1 (AFM_1), the conjugate of AFM_1 with bovine serum albumin (BSA-AFM_1), PBS buffer tablets and Tween 20 were from Sigma-Aldrich. The primary rabbit antibody against AFM_1 (Ab_1) was from AntiProt and gold nanoparticles (AuNPs, 20 nm)-labeled goat anti-rabbit secondary

antibody (Ab_2-AuNPs) from Abcam. The experiments were performed in PBS-Tween buffer (PBS-T) (pH 7.4) prepared by adding Tween 20 (0.05%) in PBS buffer solution. 20 mM acetate buffer (ACT, pH 4) was prepared from sodium acetate trihydrate and acetic acid (both from Sigma-Aldrich) and the pH was adjusted by HCl and NaOH. Glycine buffer with pH 1.5 and ethanolamine were purchased from Biocore (Germany). The ERM (European Reference Material) BD282 (zero level of AFM_1) was obtained from the Institute for Reference Materials and Measurements (Belgium).

2.2.2. Optical setup

An optical SPR biosensor setup utilizing angular spectroscopy of surface plasmons (SPs) was used. A transverse magnetically (TM) polarized beam with a wavelength of $\lambda = 632.8$ nm emitted from a HeNe laser (2 mW) was coupled to a right angle LASFN9 glass prism. To the prism base, a sensor chip was optically matched by using immersion oil. The sensor chip was prepared on the top of a BK7 glass substrate that was coated by sputtering (UNIVEX 450C form Leybold, Germany) with 41 nm thick gold layer. Then, the gold surface was either modified by bicomponent SAM of thiols or polymer brushes for subsequent covalent immobilization of BSA-AFM_1 conjugates. A transparent flow-cell with a volume of approximately 10 μL was pressed against the surface of the sensor chip in order to flow liquid samples over the sensor surface by using a peristaltic pump at a flow rate of 500 μL min^{-1}. The intensity of the laser beam reflected from the sensor surface was measured using a photodiode detector. The resonant coupling to the SP is manifested as a resonance dip in the angular reflectivity spectrum *R(Θ)*. The binding of molecules to the gold layer was observed as a shift in the reflectivity dip, $\Delta\Theta$, and evaluated by fitting with a transfer matrix-based model implemented in the software Winspall (developed at the Max Planck Institute for Polymer Research in Mainz, Germany). The whole sensor system and the supporting electronics were controlled by using the customized software Wasplas.

Since the refractive index is a linear function of concentration over a wide range of concentrations, the absolute amount of biomolecules bound at the surface (Γ) can be calculated using Feijter's formula [270]:

$$\Gamma = \frac{(n_h - n_b)d_h}{\partial n/\partial c} \quad (1)$$

where n_h and n_b are the refractive indices of a protein monolayer (n_h=1.5) and a buffer, respectively. The factor of $\partial n/\partial c$=0.18 cm^3 g^{-1} relates to the refractive index changes with

the concentration of molecules and it was obtained from the literature [271]. The thickness of a layer d_h where the biomolecular binding occurs was determined by fitting the respective SPR spectrum using the following parameters: refractive index of the prism n_p=1.845, complex refractive index for the gold film n_m=0.22+3.67i, refractive index of the PBS-T buffer n_b=1.3337.

2.2.3. Preparation of the chip

As Fig. 2.1 shows, two surface architectures were used for the attachment of AFM_1-BSA conjugate. The first (A) was based on mixed thiols SAM with PEG moieties and the second (B) utilized p(HEMA) polymer brushes. The thiol SAM was formed by overnight incubation of a gold surface at room temperature in a mixture of carboxyl-terminated and PEG-terminated dithiols (molar ratio 1:9) dissolved in ethanol at a total concentration of 1 mM. Subsequently, the sensor surface was rinsed with ethanol and dried in a stream of nitrogen. Afterwards, BSA-AFM_1 conjugate was *in situ* immobilized by using standard amine coupling chemistry. First, the carboxylic terminal groups were activated with a solution of EDC and NHS (concentrations in deionized water of 37.5 and 10.5 mg mL^{-1}, respectively) for 7 min. Afterward, a 40 μg mL^{-1} solution of BSA-AFM_1 conjugate in ACT buffer was circulated through the flow cell for 15 min to react via their amine groups with the sensor surface. The unreacted active ester groups were then blocked by 10 min incubation in 1 M ethanolamine at pH 8.5.

Polymer brushes of 2-hydroxyethyl methacrylate p(HEMA) were polymerized from a SAM of ω-mercaptoundecyl bromoisobutyrate on gold as described elsewhere [261]. Briefly, p(HEMA) brushes were grown using a solution of $CuBr_2$, 2,2´dipyridyl, HEMA and CuCl in a mixture of water:ethanol 1:1 as a solvent (for the advancing and receding water contact angles measurement as well as FTIR-GASR spectra of p(HEMA) brushes. Brushes with thickness of 23.0±0.3 nm were used for further experiments. Hydroxyl groups in p(HEMA) brushes were activated with a solution of DSC and DMAP (26 and 12 mg mL^{-1}, respectively, in anhydrous DMF) overnight at room temperature. Afterwards, the chips were rinsed with dry DMF, deionized water, blow dried with nitrogen and plugged to the SPR flow cell. BSA-AFM_1 conjugate was pumped through the flow cell and subsequently, unreacted groups reacted with ethanolamine.

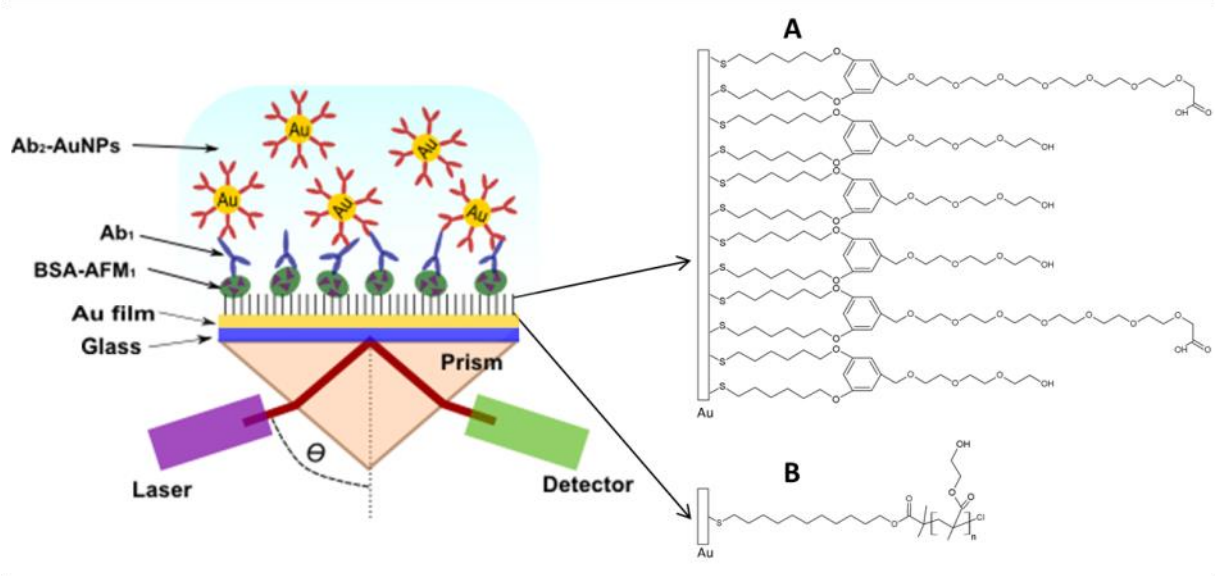

Fig. 2.1. The scheme of the optical setup and sensor chip with different surface architecture (A) mixed SAM and p(HEMA) brushes (B).

2.2.4. Immunoassav procedure

For the detection of AFM_1, an inhibition immunoassay was carried out. PBS-T buffer or milk was spiked with AFM_1 at concentrations ranging from 10^{-1} to 10^{3} ng mL^{-1}. These samples were prepared by sequential diluting of AFM_1 stock solution (concentration of 10 µg mL^{-1} in PBS buffer). The ERM-BD282 milk powder was dissolved in deionized water at the concentration of 0.1 g mL^{-1}. Then, samples containing AFM_1 were centrifuged at 5000 rpm for 20 min at the temperature of 4°C. The upper fat layer was completely removed, and the obtained aqueous phase was directly used for the further analysis.

The analyzed sample was pre-incubated with Ab_1 antibody (concentration of 70 ng mL^{-1}) for 30 min. The mixture of sample and Ab_1 was flowed through the sensor for 10 min in order to allow the unreacted free Ab_1 to bind the BSA-AFM_1 conjugate immobilized on the surface. Subsequently, the sensor surface was washed with PBS-T buffer for 2 min to remove weakly bound Ab_1 molecules. Afterward, the Ab_2-AuNPs antibody (concentration of 0.1 µg mL^{-1}) was circulated through the sensor for 10 min, followed by 2 min rinsing with PBS-T. After each detection cycle, the sensor surface was regenerated by 5 min incubation in glycine buffer (pH 1.5, 20 mM) followed by rinsing with sodium hydroxide (20 mM).

2.3. Results and discussion

2.3.1. Affinity binding at thiol SAM and p(HEMA) brush architectures

SPR was used for the *in situ* observation of the covalent immobilization of BSA-AFM_1 and the subsequent affinity binding of Ab_1 and Ab_2-AuNPs. SPR reflectivity curves $R(\Theta)$ were recorded for gold surface modified with thiol SAM and p(HEMA)-based brush, surfaces with immobilized BSA-AFM_1 conjugate, and after its affinity reaction with primary antibody Ab_1 and secondary antibody Ab_2 conjugated with AuNPs. On the chip modified by a mixed thiol SAM with PEG moieties, the resonant excitation of SPs occurs at Θ=57.1° which is manifested as a dip in the reflectivity spectrum $R(\Theta)$, see Fig.2.2A. In case of chips functionalized with p(HEMA) brushes, this resonance is observed at higher angle of Θ=58° (Fig.2.2B) as a consequence of the higher thickness. Upon the binding of biomolecules to the sensor surface, the surface mass density Γ increases which leads to a shift in SPR angle Θ. As seen in Fig.2.2, after the covalent functionalization with BSA-AFM_1 conjugate the resonant angle shift $\Delta\Theta$=0.11° and $\Delta\Theta$=0.14° for the PEG-SAM and p(HEMA) brush, respectively. By using Eq.1, the corresponding surface mass density of BSA-AFM_1 was estimated to be Γ=1.6 ng mm^{-2} on the mixed SAM. The analysis of the reflectivity curves in Fig.2.2B showed that a bare p(HEMA) brushes exhibit the thickness of 23 nm and refractive index of $n_h = 1.4714$. These data correspond to a surface mass density of Γ=17.5 ng mm^{-2}. After the immobilization of BSA-AFM_1 in p(HEMA) brushes the surface mass density increases to 20.3 ng mm^{-2} indicating that the net surface mass density of coupled BSA-AFM_1 is 2.8 ng mm^{-2}. Afterward, the affinity binding of Ab_1 was tested for both tested architectures. Obtained results reveal a small shift of $\Delta\Theta \approx 0.02°$ corresponding to $\Gamma \approx 0.16$ ng mm^{-2}. Although, the molecular weight of IgG antibody is about 2.2 times higher than BSA-AMF_1, the surface mass density is significantly lower. The low concentration of the primary antibody Ab_1, short incubation time for which the binding may not reached the saturation and lack of antigen exposed for binding are among the feasible reasons for the low Ab_1 binding. Nevertheless, a very strong amplification of the sensor response $\Delta\Theta$ was obtained by the subsequent reaction of the captured Ab_1 with Ab_2-AuNPs. An order of magnitude higher shift of $\Delta\Theta$=0.23° was measured for the mixed SAM and lower shift $\Delta\Theta$=0.13° was recorded for p(HEMA) architectures. This indicates that the affinity binding of large objects such as AuNPs decorated with Ab_2 to BSA-AFM_1 conjugate is partially hindered by the surrounding brush polymer chains. In order to check for the specificity of the interaction of Ab_1 and Ab_2 conjugated with

AuNPs, the previous experiment was repeated for a sensor chip with immobilized BSA that was not conjugated with AFM_1. Not measurable response was observed after the flow of either Ab_1or Ab_2-AuNPs antibodies (data not shown).

The obtained results are in a good agreement with previous studies which showed that the immobilization density strongly depends on the surface density of hydroxyl groups [261]. In a mixed SAM only carboxylic end groups can participate in the binding process, while in the case of p(HEMA) brushes, only the external layer of brushes with high density of functional groups provided by the polymer chain takes part in immobilization resulting in a similar range of functionalization [272].

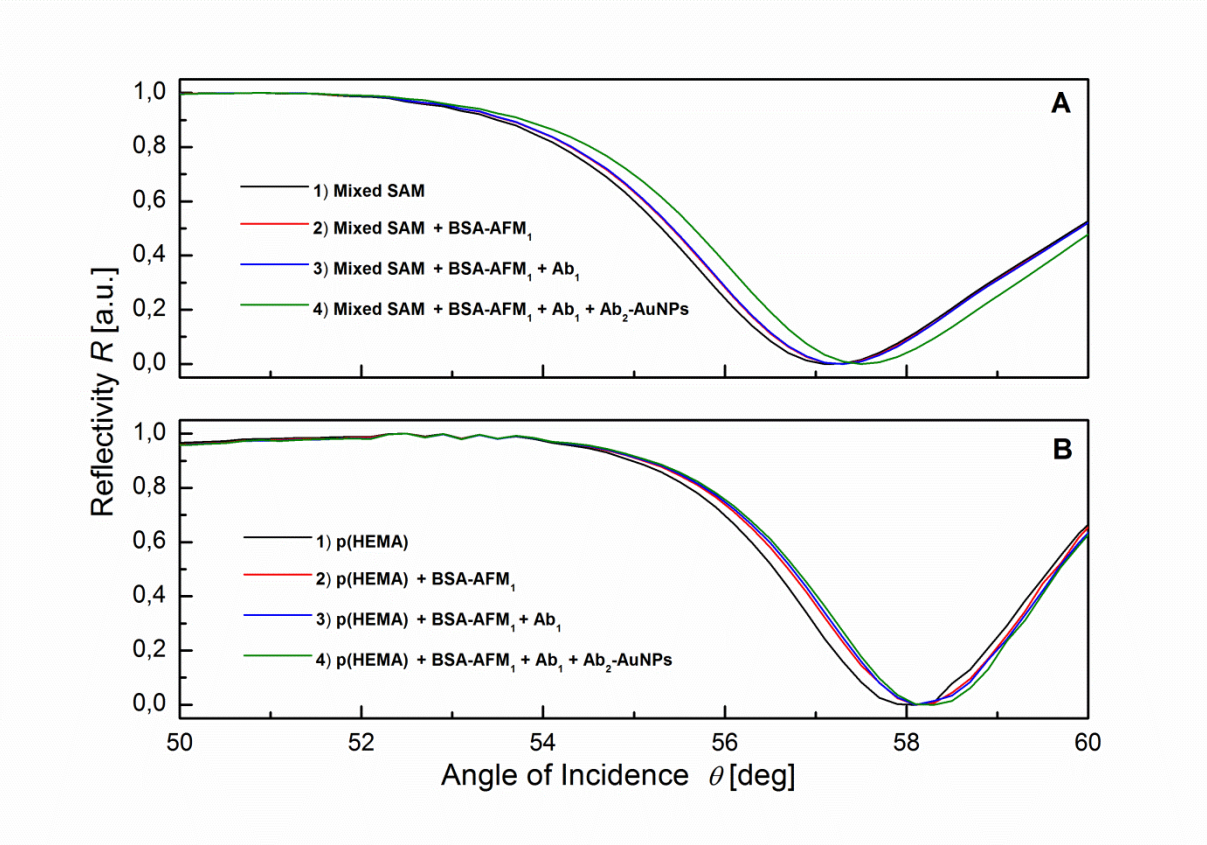

Fig. 2.2. Angular reflectivity spectra measured from a sensor chip for mixed SAM (A) and p(HEMA) brushes (B) prior the modification (1, only thiols SAM), after the immobilization of BSA-AFM_1 conjugate (2), and after affinity binding of Ab_1 (3) and Ab_2-AuNPs (4). The spectra were measured for the surface brought in contact with buffer.

2.3.2. Resistance to fouling from milk

Milk is a complex biological fluid composed of constituents including whey proteins (particularly β-lactoglobulin), lipids and calcium phosphate, which are involved in the fouling

process through interacting mechanisms (denaturation, aggregation, local supersaturation) [273]. The used surface architectures were exposed to the prepared standard milk samples and compared. As seen in the measured data in Fig.2.3, these observations were made in real time and SPR changes were measured for an angle of incidence Θ fixed at the resonance edge where the highest slope $\Delta R/\Delta\Theta$ is occurs. The angle of incidence was set to Θ=56.7° and Θ=57.3° for mixed SAM and p(HEMA), respectively.

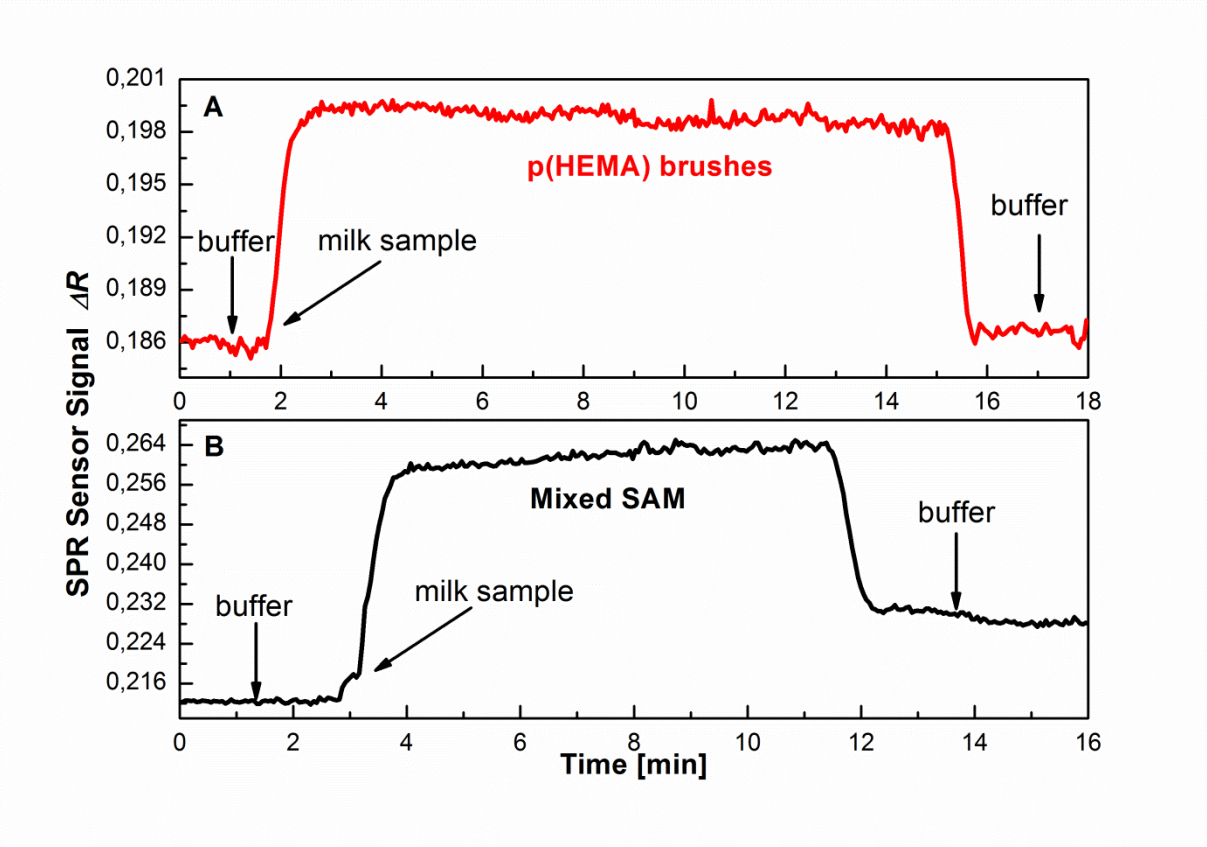

Fig. 2.3. (A) Resistance to the non-specific interactions on the sensor coated with p(HEMA) brushes. (B) Adsorption of milk components on mixed SAM.

After obtaining a stable baseline in PBS-T, a milk sample was circulated over the sensor for *ca* 10 min, followed by 2 min washing with buffer. The amount of deposited material, fouling, was quantified as the difference in the resonance signal measured before and after the injection. As shown in Fig.2.3, non-specific adsorption of components from milk is clearly visible on commonly used thiol SAMs with PEG moieties (Fig.2.3B). Contrary to these results, excellent resistance to the non-specific interactions is observed for the sensor coated with p(HEMA) brush (Fig.2.3A). After the washing step the reflectivity change is negligible. The sensitivity of SPR to fouling is more than 1 order of magnitude smaller than the fouling

recorded in SAMs. According to previous studies, this phenomenon might be due to a water barrier resulting in minimization of hydrophobic effect with the lipids components form milk as well as to entropic barrier resulting from the brush architecture [261].

2.3.3. Aflatoxin M1 detection

In order to push the limit of detection of low molecular weight analyte AFM_1 to concentrations stipulated by regulatory bodies, a competitive assay format was applied and the mass provided by the binding of primary antibodies Ab_1 was amplified by secondary antibodies Ab_2 labeled with gold nanoparticles AuNPs. The sensor response was evaluated as the difference in the reflectivity ΔR before and after the assay cycle for series AFM_1 concentrations. Fig.2.4 shows the obtained calibration curves normalized with the sensor response ΔR_0 measured in the absence of AFM_1 for the above-mentioned assay format. For each concentration, the sensor response ΔR was measured in triplicate and the standard deviation (SD) is indicated further as an error bar. The calibration curves were fitted with a sigmoidal function and the limit of detection (LOD) was determined as the concentration of AFM_1 which correspond to a sensor output equal to three standard deviation of the sensor output for the non-spiked liquids (blank samples). As seen in Fig.2.4, the reflectivity change ΔR decreases with the increasing AFM_1 concentration due to the blocking of Ab_1 binding sites resulting in its lower surface mass density at the sensor surface with AFM_1 conjugate.

The LOD for a sensor with thiol mixed SAM with PEG moieties was determined as 26 pg mL^{-1} and 38 pg mL^{-1} for the buffer and milk samples, respectively. The difference between LOD can be ascribed to the non-specific adsorption of milk constituents to the surface resulting in higher background response and deteriorated reproducibility.

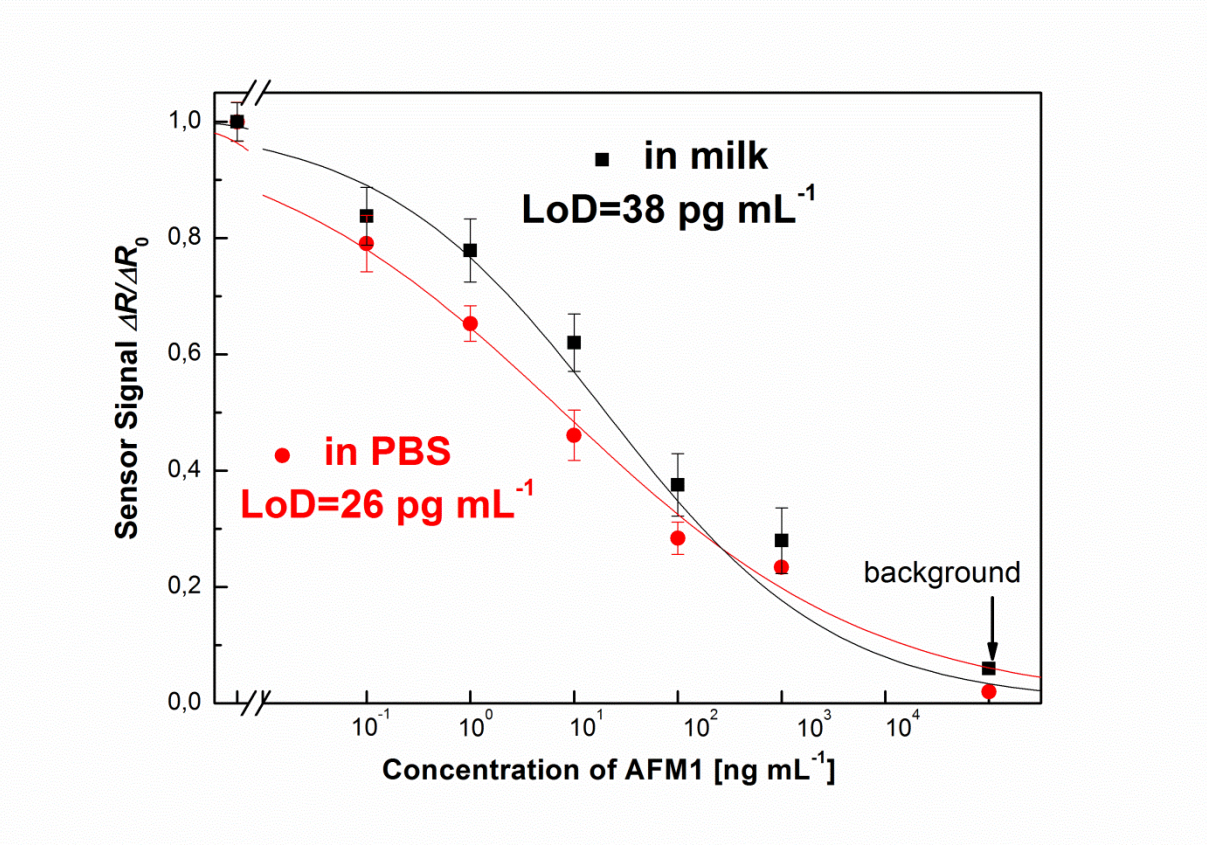

Fig. 2.4. Normalized calibration curves for the detection of AFM_1 using inhibition immunoassay on mixed SAM performed in a buffer (red circles) and milk (black squares).

In order to reduce fouling from the milk samples, the assay was performed on the surface modified with p(HEMA) brush moieties. Fig.2.5 shows a comparison of calibration curves obtained for two studied surface architectures. LOD of the sensor with p(HEMA) was determined as 18 pg mL^{-1} which is more than two-times lower compared to that on thiol SAM with PEG groups. The improvement of the LOD can be attributed to the elimination of the non-specific adsorption to the surface. This concomitantly, leaded to better reproducibility represented by error bars and lower background signal.

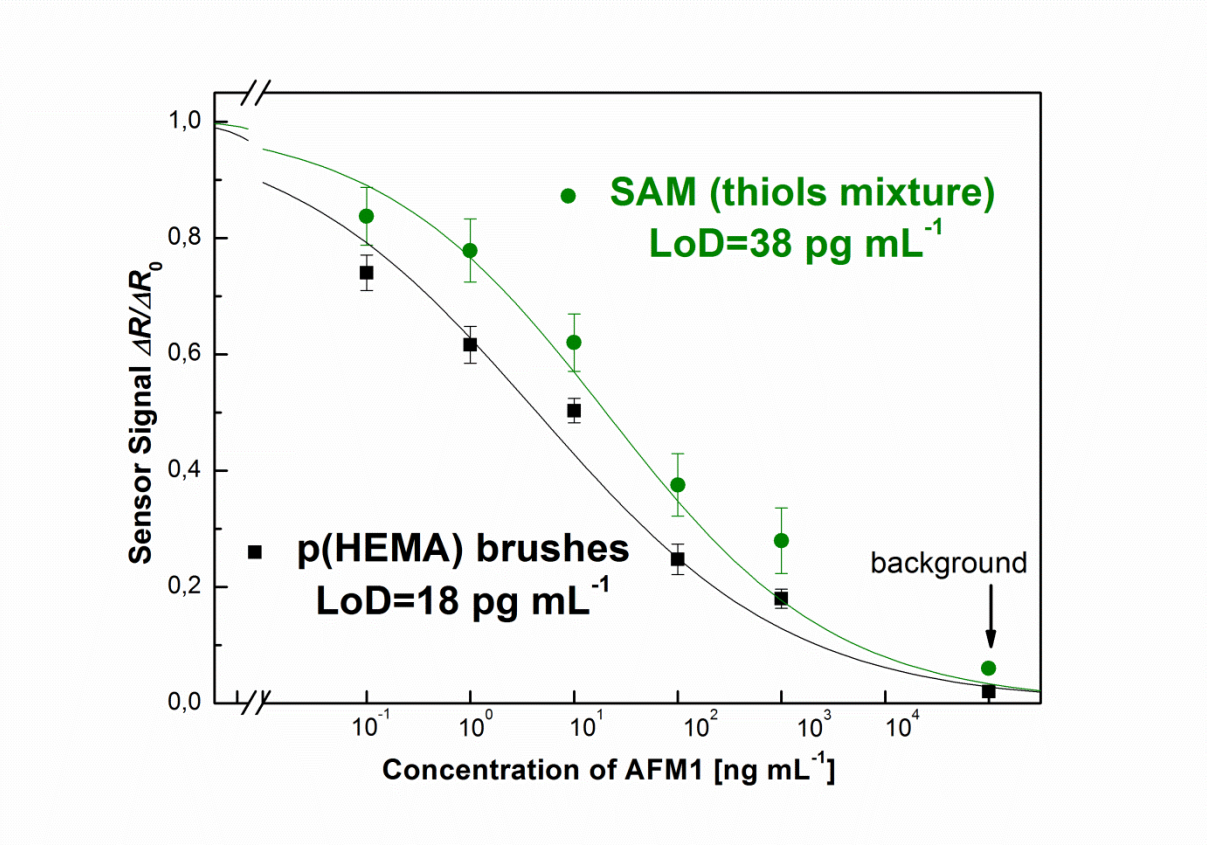

Fig. 2.5. Comparison of normalized calibration curves for the detection of AFM_1 in milk on the sensor surface coated with p(HEMA) brushes (black squares) and mixed SAM (green circles).

Compared to the performance of the other reported methods for the detection of aflatoxin M_1 in milk and milk products including electrochemistry [178, 232] or indirect and direct ELISA [250, 274, 275] the presented biosensor provides about one order of magnitude higher sensitivity. Regarding the chemiluminescent technique reported by Magliulo et al. (2005) or LSPR detection described by Wang et al. (2009) where the obtained LOD was lower, the presently developed sensor offers shorter analysis time (ca. 55 min) and permits the detection of AFM_1 without compensation for the fouling. Nevertheless, the LOD of the developed biosensor can be further reduced by increasing the number of AFM_1 per BSA molecules, increasing the size of the gold nanoparticles and the time could be decreased by implementation of microfluidics, where smaller volume of the samples would reduce the time as well as improve the efficiency of binding.

2.4. Conclusions

A novel and highly sensitive SPR biosensor for AFM_1 analysis in milk was developed using inhibition assay format that allowed for the detection of toxin of interest at pg mL^{-1} levels.

The limit of the detection was one order of magnitude lower than the maximum permissible level required by the European Commission. Modification of a gold chip surface with p(HEMA) showed excellent specificity and complete resistance to non-specific interaction, fouling. This is the first time that an SPR chip modified with such polymer brushes was used for real time detection of a small target antigen (AFM_1) utilizing indirect competitive immunoassay with AuNPs in milk samples. SPR-based sensors for small molecules suffer from high LOD due to high concentration of Ab_1 being needed to generate a sufficient signal. The current study shows that a combination of SPR and AuNPs enables signal enhancement and sensitivity improvement. Furthermore, such a system uses cheap reagents (Ab_2 and AuNPs) and significantly reduces the concentration of valuable Ab_1.

CHAPTER THREE, FAST AND SENSITIVE DETECTION OF OCHRATOXIN A IN RED WINE BY NANOPARTICLE-ENHANCED SPR

The content of this chapter has been already published in **Analytica Chimica Acta** Journal.

Authors and their contribution:

- **A. Karczmarczyk** - study conception and design, acquisition of data, analysis and interpretation of data, drafting of manuscript,
- M. Dubiak-Szepietowska - critical revision of the article,
- S. Hageneder - critical revision of the article,
- C. Reiner-Rozman - affinity constants calculations,
- J. Dostalek - conception and design, scientific advisor, critical revision of the article,
- K-H. Feller - scientific advisor, critical revision of the article, final approval of the version to be published.

Fast and sensitive detection of ochratoxin A in red wine by nanoparticle-enhanced SPR. Analytica chimica acta, 2016. **937**: p. 143-150.

3.1. Introduction

Ochratoxin A (OTA), a highly toxic fungal secondary metabolite of *Aspergillus ochraceus* and *Penicillium verrucosum* is one of the most widely spread mycotoxin that contaminates a large variety of agricultural commodities [276]. It exhibits multiple toxicities in animals and mankind, including nephrotoxic, hepatotoxic, immunotoxic, teratogenic and carcinogenic effects, which represent serious health risks to livestock and the general population [277]. It has been linked to the Balkan Endemic Nephropathy (BEN), a kidney disease occurring in some regions in south-eastern Europe (Croatia, Bosnia, Serbia, Croatia, Bulgaria and Romania) and development of tumors in the urinary tract in humans [278, 279]. As a result, the International Agency for Research on Cancer (IARC) has classified OTA as a potential carcinogen (group 2B) for humans [280].

The worldwide occurrence of OTA pollution has been already precisely reported. It contaminates a various foodstuffs and beverages including cereal grains, oil seeds, dried fruits, coffee, cocoa beans, grape juice, beer and wine [281-283]. It has been established that wine is the second major source of OTA daily intake in EU, following cereals. After the first report on the occurrence of OTA in wine [284] several studies were performed to assess the pertinence of this toxin [285-287]. Ottender and Majerus noticed the fact that higher level of

OTA occurs in a red wine then in a white and rose which may be due to differences in winemaking process [38].

Due to the persistence of OTA in the food chain, high stability and resistance during food processing (*e.g.* cooking, roasting or fermenting), this mycotoxin represents a serious threat for human health. Therefore, the European Commission has established maximum permissible level of OTA in food, feed products, raw materials and beverages (*e.g.* 5 ng mL^{-1} for unprocessed cereals, 3 ng mL^{-1} for products derived from unprocessed cereals, 10 ng mL^{-1} for coffee beans and 2 ng mL^{-1} for all types of wine) (EC No. 123/2005). Such low allowable levels require very sensitive and precise methods of detection. Analysis of OTA is nowadays performed by established analytical techniques including thin-layer chromatography (TLC) [281], gas chromatography (GC) [288] and high-performance liquid chromatography (HPLC) [289] with immunoaffinity columns and fluorescence detection. These technologies provide sufficient detection limit but relay on multiple steps prior to the detection, sophisticated equipment and trained personnel, which cannot meet the requirements of on-site and rapid detection. Therefore, alternative methods such as capillary electrophoresis with diode array detection [155], immunochemical techniques like enzyme-linked immunosorbent assays (ELISA) [290], electrochemical immunosensors [234] or optical techniques either using the optical waveguide light mode spectroscopy or surface plasmon resonance (SPR) has been successfully developed. These methods present good sensitivity and selectivity with the potential for high-throughput screening. In particular, SPR spectroscopy is a powerful, label-free technique enabling monitoring of affinity molecular interactions in a real time and in a noninvasive manner. This opto-electronic phenomenon utilizes refractive index changes to detect mass changes occurring at noble metal surface interfaces [291]. Moreover, the kinetics information on the affinity binding between native biomolecules can be also provided. Nevertheless, SPR biosensors were shown to be suitable for the direct analysis of medium and large molecular weight analytes which induce measurable refractive index changes upon their binding on the surface from samples with the analyte concentration above ng mL^{-1} [292]. Regrettably, mycotoxins are small chemical compounds that possess inadequate mass to cause significant changes in the refractive index. In order to overcome this limitation, sandwich or indirect competitive inhibition assays are developed to detect such molecules. In addition, signal amplification by gold nanoparticles (AuNPs) offers efficient means to increase the SPR response in order to detect binding of minute amounts of target molecules on the surface. It has been already demonstrated that, electronic coupling between the localized surface

plasmons of AuNPs and the surface plasmons wave associated with the SPR gold chip can significantly enhance the SPR response [187]. AuNPs exhibit several distinct physical and chemical attributes that make them an excellent scaffold for novel biochemical and chemical sensors. Relatively easy and inexpensive synthesis, stability, unique optoelectronic properties, high surface-to-volume ratio with excellent biocompatibility, safety for humans and small amounts of AuNPs needed in the test allow researchers to develop sensing strategies with higher sensitivity, stability and selectivity [293]. AuNPs have been successfully applied in SPR detection of DNA, proteins and drug molecules. Despite of broad use of AuNPs in SPR biosensors, the role of the size of NPs in the interactions with SPR surface is not fully understood. Studies performed by Uludag and Tothill [294] show higher sensor response with increasing size of AuNPs. On the other hand, Mitchell et al. [260] observed no significant differences in signal amplification for AuNPs with diameter ranging from 25 to 50 nm. The competing effect of enhanced SPR signal, steric hindrance and diffusion mass transfer rate depending on the size of AuNP was investigated by Springer et al. [295].

In this work, we report on the development of fast and sensitive SPR assay for ochratoxin A detection in a red wine. To overcome the matter concerning low molecular weight of the analyte that hampers its detection using SPR, an indirect competitive inhibition assay was performed. Moreover, the signal amplification and sensitivity improvement was achieved by using secondary antibodies conjugated with gold nanoparticles labels. In this study, we also investigate the ability of functionalized AuNPs to enhance the response of an SPR biosensor in a biomolecular detection assay with special attention given to the study of the effect of the size of AuNPs. Furthermore, a detailed analysis of kinetic parameters (association/dissociation constants and association/dissociation rate constants) was made and compared with available literature. To reduce matrix interferences in untreated wine (*e.g.* ethanol, polyphenols) that imposes unspecific sorption and potential inactivation of used antibodies, a very simple pre-treatment of samples with binding agent poly(vinylpyrrolidone) (PVP) was successfully applied.

3.2. Materials and methods

3.2.1. Reagents

All reagents were used as received without further purification. Dithiol PEG6-COOH and dithiol PEG3-OH were purchased from SensoPath Technologies (USA). 1-ethyl-3-(3-

dimethylaminopropyl) carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) were obtained from Pierce (USA). Ochratoxin A (OTA), the conjugate of OTA with bovine serum albumin (BSA-OTA), poly(vinylpyrrolidinone) (PVP), PBS buffer tablets and Tween 20 were from Sigma-Aldrich (Austria). The primary rabbit antibody against OTA (Ab_1) was from AntiProt. Goat anti-rabbit secondary antibody (Ab_2) and gold nanoparticles (AuNPs, 10, 20 and 40 nm)-labeled goat anti-rabbit secondary antibody (Ab_2-AuNPs) were from Abcam (UK). The experiments were performed in PBS-Tween buffer (PBS-T) (pH 7.4) prepared by adding Tween 20 (0.05%) in PBS buffer solution. 20 mM acetate buffer (ACT, pH 4) was prepared from sodium acetate trihydrate and acetic acid (both from Sigma-Aldrich) and the pH was adjusted by HCl and NaOH. Glycine buffer with pH of 1.5 and ethanolamine were purchased from Biocore (Germany). The ERM (European Reference Material) BD476 (OTA in red wine) was obtained from the Institute for Reference Materials and Measurements (Belgium). This material was prepared from commercial wine sources intended for human consumption and it was characterized by mass spectrometry and HPLC. The concentration of OTA was determined as 0.52 ±0.11 ng mL^{-1}.

3.2.2. Optical setup

Surface plasmon (SP) resonance measurements were carried out by using the Kretschmann-type of attenuated total reflection configuration, as described previously [103]. Briefly, a monochromatic light beam at a wavelength of λ=632.8 nm emitted from a HeNe laser (2mW) passed through a chopper and a polarizer selecting transversal magnetic (TM) polarization. Then it was made incident at a surface of a sensor chip with gold film that was optically matched using immersion oil to an optical prism. The sensor chip was made of BK7 glass substrate which was coated by sputtering (UNIVEX 450C form Leybold, Germany) with 37 nm thick gold film. To the sensor chip, a transparent flow-cell with a volume of approximately 10 μL was attached in order to flow aqueous samples by using a peristaltic pump at the a flow of 0.5 mL min^{-1}. The assembly of the sensor chip and prim was mounted on a rotation stage in order to control the angle of incidence of the laser beam θ. The resonant coupling to SP is manifested as a narrow dip in the reflectivity spectrum $R(\Theta)$. The binding of molecules to the gold layer was observed as a shift of the angular position of the reflectivity dip, $\Delta\Theta$, and evaluated by fitting with a transfer matrix-based model implemented in the software Winspall (developed at the Max Planck Institute for Polymer Research in Mainz, Germany). The whole sensor system and the supporting electronics were controlled by using

the customized software Wasplas (developed at the Max Planck Institute for Polymer Research in Mainz, Germany).

Surface mass density Γ of biomolecules adsorbed to the surface was calculated using Feijter's formula [270]:

$$\Gamma = \frac{(n - n_b) d_h}{\partial n / \partial c} \quad (1)$$

where the refractive index of the protein sublayer and a buffer is denoted by n=1.465 and n_b=1.3337, respectively. The ratio $\partial n/\partial c = 0.182\ \mathrm{mm^3\ mg^{-1}}$ at a wavelength of 632.8 nm was taken from literature [270]. The thickness d_h was determined by fitting the respective SPR spectrum using following parameters: refractive index of the prism n_p=1.845, complex refractive index for the gold film n_m=0.22+3.67i.

For the SPR measurements of kinetics of surface reactions, the angle of incidence was fixed close to the angle Θ=55.6° where resonance edge with the highest slope $\Delta R/\Delta\Theta$ occurs. At this angle, the time dependent reflectivity signal was measured. The reflectivity changes were converted to variations in refractive index by calibrating the sensor with series of standard aqueous samples. These standards were prepared with known refractive index in refractive index units (RIU).

3.2.3. Sensor chip functionalization and detection format

The gold surface of the sensor chip was modified with a mixed thiol self-assembled monolayer (SAM). First, the gold chip was incubated overnight at room temperature in a molar ratio 1:9 mixture of dithiols: PEG6-COOH and PEG3-OH (dissolved in ethanol at the total concentration of 1 mM). Subsequently, the sensor surface was rinsed with ethanol and dried in a stream of nitrogen. The covalent *in situ* immobilization of BSA-OTA was carried by using amine coupling. By using EDC/NHS (concentrations in deionized water of 37.5 and 10.5 mg mL^{-1}, respectively) carboxylic acid groups at the gold surface were activated. Afterward, BSA-OTA conjugate dissolved in ACT buffer at a concentration of 30 μg mL^{-1} was circulated through the flow cell for 15 min in order to react via their amine groups with the activated carboxyl groups at the sensor surface. Finally, the unreacted active ester moities were deactivated by 10 min incubation in ethanolamine (1M and pH 8.5).

For the detection of OTA, an indirect competitive immunoassay was applied. Analyzed samples were prepared by spiking the PBS-T buffer or certified red wine standard with

purified OTA (at concentrations between10^{-1} and 10^{3} ng mL^{-1}). To minimize the matrix effect that may interfere with the OTA analysis in wine, samples were spiked with 3% of PVP and subsequently shaken for 5 min at room temperature, filtrated, and the pH of each aliquot was adjusted to 7.4 with NaOH prior to analysis. The standards were then mixed and incubated (for 30 min) with an equal volume of Ab_1 (concentration of 100 ng mL^{-1}), followed by the detection of unreacted Ab_1. Subsequently, the mixture was passed over the chip for 10 min to allow binding the free Ab_1 to the BSA-OTA conjugate. To remove unbounded molecules, the sensor surface was then washed for 2 min with PBS-T buffer. Afterward, the Ab_2-AuNPs antibody was flowed through the sensor for 10 min, followed by 2 min rinsing with PBS-T. After each detection cycle, the substrate surface was regenerated by 5 min incubation in glycine buffer (pH 1.5, 20 mM) followed by rinsing with NaOH (20 mM).

3.3. Results and discussion

3.3.1. Sensor chip and assay characterization

Firstly, the surface mass density of *in situ* immobilized BSA-OTA and its ability to bind Ab_1 was evaluated. As shown in Fig.3.1, SPR reflectivity curves $R(\Theta)$ were recorded upon the subsequent modification of the sensor surface with mixed thiol SAM, BSA-OTA, and after the affinity binding of Ab_1 and Ab_2. Prior to the surface modification by protein molecules, the resonant excitation of SPs occurs at Θ=57.04° on a gold surface with mixed thiol SAM. This resonance shifts to higher angles by $\Delta\Theta$=0.27° after the covalent binding of BSA-OTA conjugate to the sensor surface that is associated with increased thickness of an adlayer (d_h=2.05 nm was determined by fitting the reflectivity curve). Based on Eq.1, the surface mass density of covalently bounded BSA-OTA was estimated to be Γ=1.48 ng mm^{-2}. Additional shift of $\Delta\Theta$=0.06° was observed after the affinity binding of Ab_1 which corresponds to Γ=0.32 ng mm^{-2}. These values are much lower than those for the BSA-OTA due to the very low concentration of Ab_1 and incubation time not long enough to reach saturation. Nevertheless, the application of an additional high mass provided by secondary antibodies Ab_2 labeled with gold nanoparticles (Ab_2-AuNPs, here example with 20 nm AuNPs) caused significant enhancement of the SPR shift $\Delta\Theta$ of 0.2º.

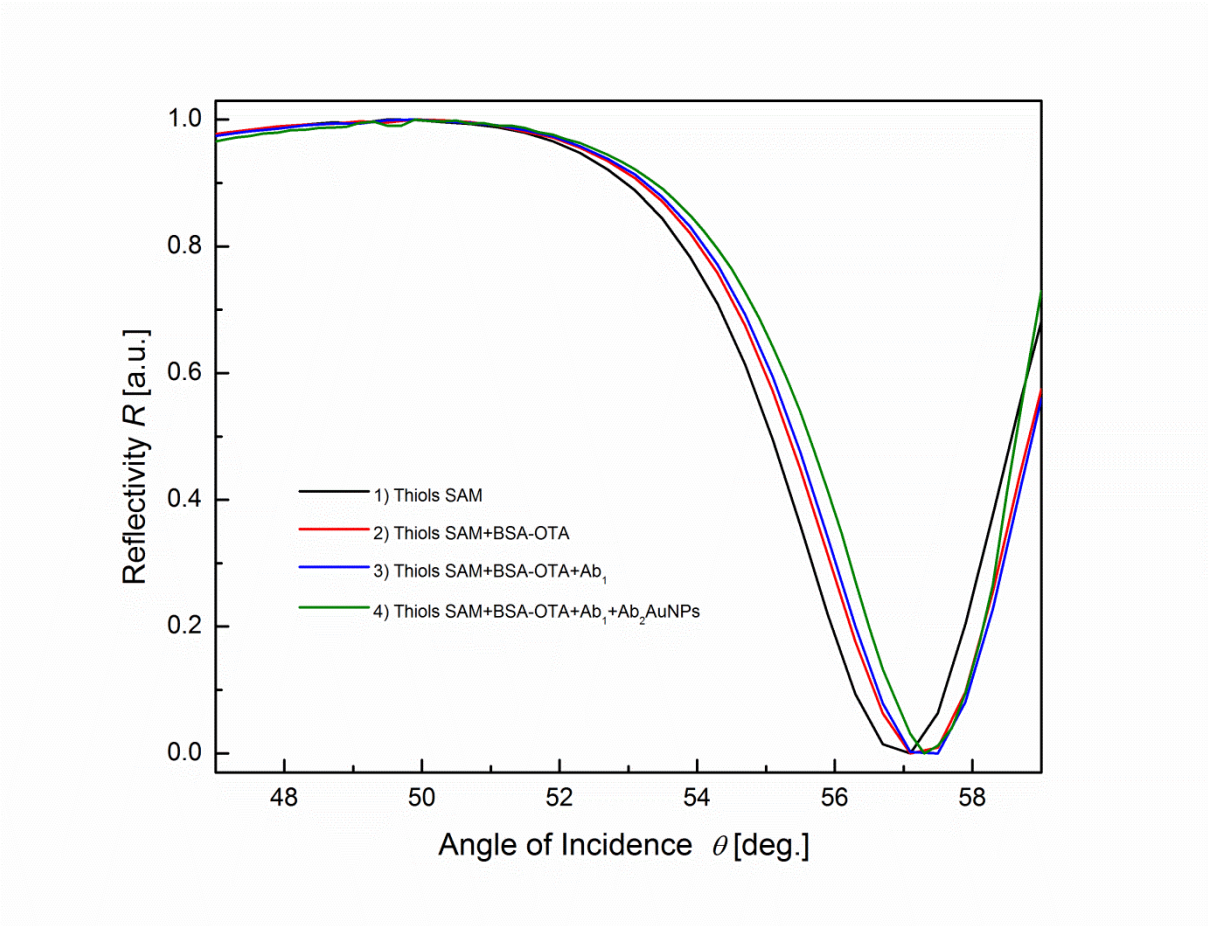

Fig.3.1. Angular reflectivity spectra measured for a sensor chip prior to the modification (1, thiol SAM on Au), after the immobilization of OTA-BSA conjugate (2), after affinity binding of Ab_1 (3) and after reaction with Ab_2-AuNPs (4). The spectra were measured for the surface in contact with PBS-T.

The non-specific binding of species in a sample at the sensor surface with tethered ligands [296] can lead to false positive results in screening assays and incorrect determination of analyte concentration. In biomolecular interaction analysis, non-specific binding generally gives results that do not fit to an interaction model and the binding constants are way off the results from the literature. Therefore, the specificity of primary Ab_1 and secondary Ab_2 antibodies used in the detection format was tested. For this purpose, BSA (not conjugated with OTA) was immobilized on a sensor chip, followed by the injection of Ab_1 and Ab_2-AuNPs. The assay showed an excellent specificity as no significant SPR response was recorded for antibodies (data not shown).

Wine is a complex alcoholic beverage and it contains constituents that may have a strong influence on the sensitivity of the detection. Wine matrix is composed of two main fractions, the non-volatile fraction containing ethanol, polyphenols, variety of proteins, and the volatile

fraction, that include flavor and aroma compounds [297]. The presence of mentioned polyphenolic compounds may cause inactivation of antibodies and unspecific sorption that blocks the sensor surface. To overcome this matter, a simple pre-treatment of the wine sample with the binding agent poly(vinylpyrrolidone) (PVP) was applied.

In order to evaluate the unspecific sorption of constituents in wine to the surface and its reducing by PVP, SPR observation of surface mass density change was carried out. First, a stable baseline was established upon the flow of PBS-T. Then, 3% PVP was injected for 10 min followed by rinsing with PBS-T. The sensorgram in Fig.3.2 shows no measurable change in the SPR response which returned back to the baseline. Afterwards, the surface was regenerated and wine sample spiked with 3% PVP was injected. After the subsequent rinsing with PBS-T, a small increase in the SPR response of 4×10^{-5} RU was observed which can be attributed by a weak non-specific interactions coming from the wine. After the regeneration step, the SPR response returns to the original baseline indicating fully reversible assay cycle. Finally, un-treated wine was injected and after the rinsing with PBS-T a huge SPR sensor signal change of 1.2×10^{-3} RU was measured. The regeneration allowed to removing most of the deposit but substantial fraction of the adsorbed constituents corresponding to 1.7×10^{-4} RU fouled the surface irreversibly. These data reveal the excellent ability of PVP to bind polyphenols through hydrogen bonding making it easier to eliminate them from the solution [298].

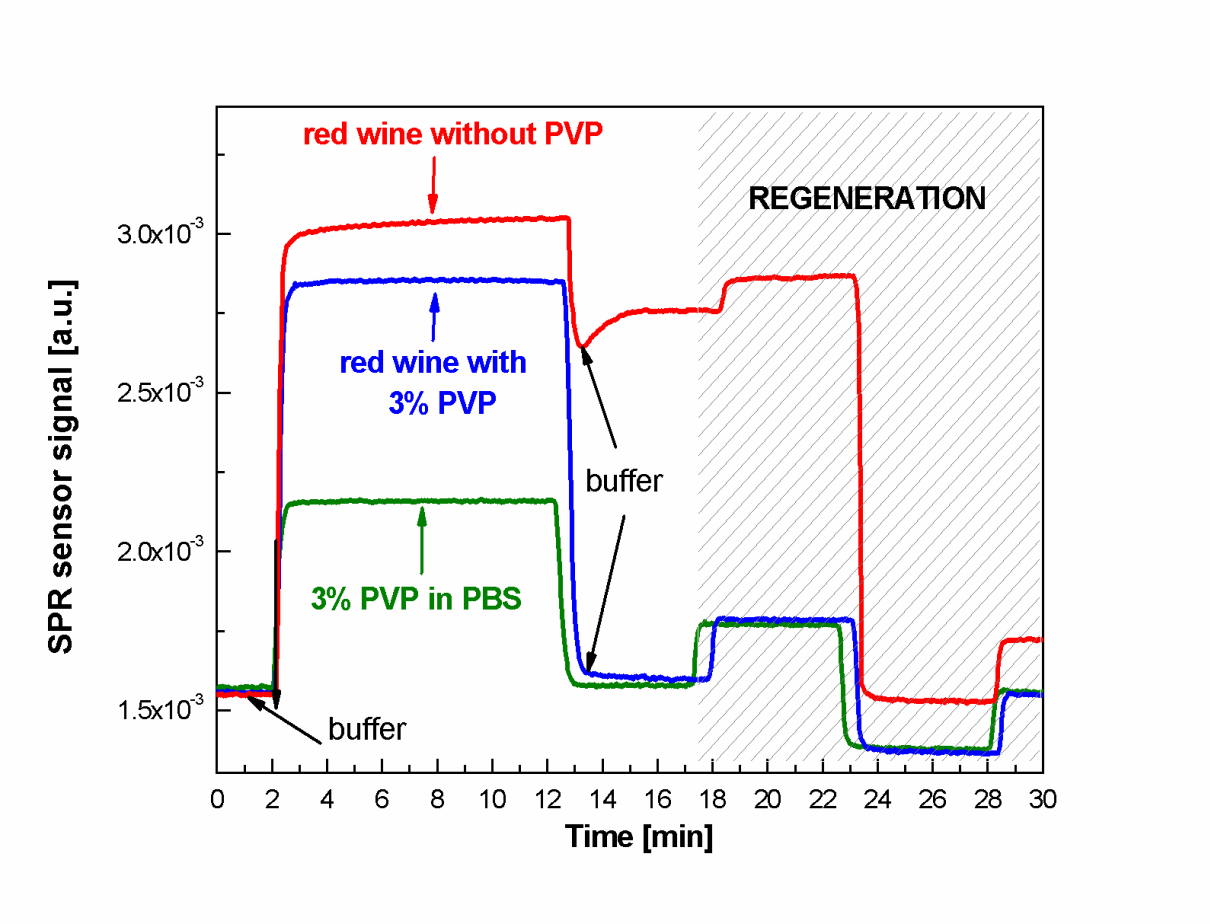

Fig.3.2. Sensorgrams showing SPR signal recorded upon injections before and after injection of 3% PVP in PBS-T (green line), wine spiked with 3% PVP (blue line) and pure wine sample (red line) followed by regeneration step.

The SPR was used for the measurement of affinity binding constants of interaction between Ab_1 and the OTA moieties. The equilibrium association and dissociation constants K_A and K_D, respectively, were calculated by the Langmuir binding theory [299] (Fig.3.3). K_D is related to the rate of complex formation (described by association rate constant k_{on}) and the rate of breakdown (described by dissociation rate constant k_{off}) such that $K_D = \frac{k_{off}}{k_{on}}$. Association constant ($K_A$) can be then calculated as K_D^{-1}.

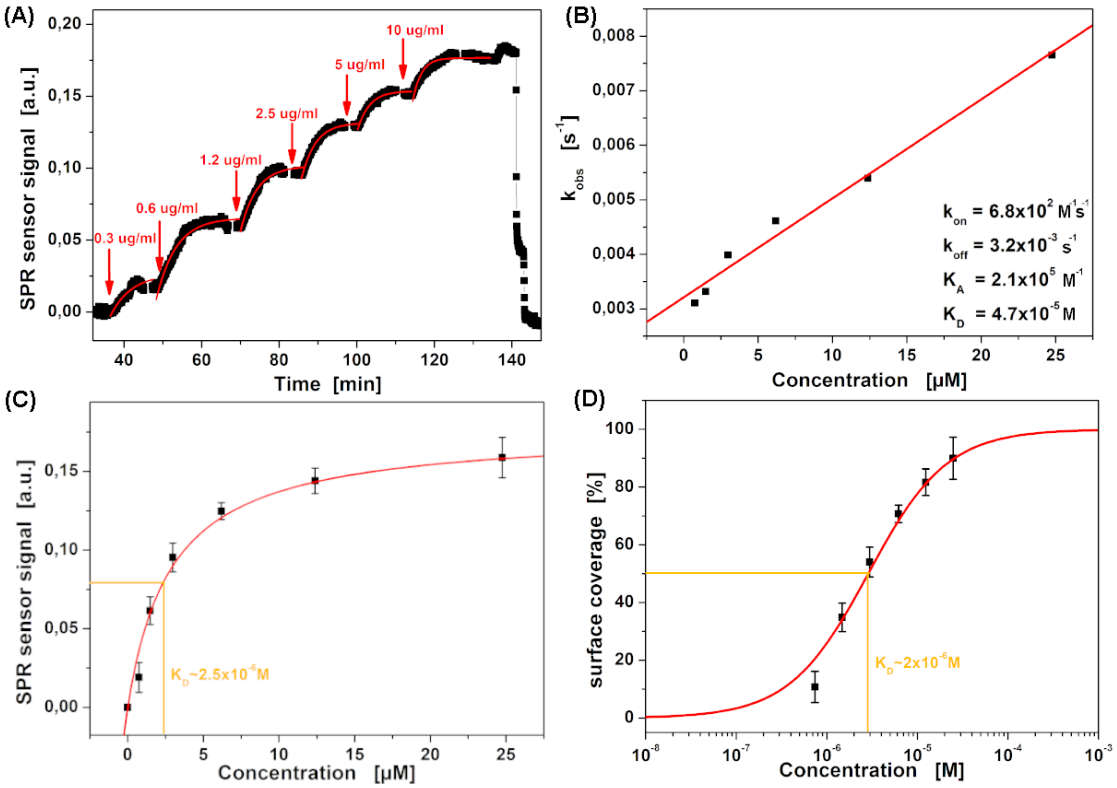

Fig.3.3.(A) Binding kinetics of the titration of OTA antibody. The arrows mark the injection of the spiked concentrations. The binding kinetics for each injection were fitted with simple exponential fits, obtaining the observed binding parameters k. (B) Plot of the observed binding parameters k for each measured concentration against the respective concentrations. The linear fit gives a direct relation to $k=conc[c]+k_{off}$, therefore association and dissociation rate constants are obtained from the slope and intercept of the linear fit. The calculated binding affinity K_A is shown in the insert. (C) Injected concentration plotted against the sensor response signal. The red line is the best fitted Langmuir isotherm, showing a half saturation concentration of around $2x10^{-6}$M, which corresponds to the K_D value. (D) Surface coverage of the biosensor, calculated for the response signals. The estimated K_D from this fitting routine - the IC50 concentration - gives very comparable results to the values obtained from the analysis of the kinetic reaction.

The limit of detection (LOD) was found below 300 ng mL^{-1} and the measured binding affinity of 2.7×10^{-6} M (806 ng mL^{-1}) was determined for the interaction of Ab_1 to surface immobilized BSA-OTA. This values indicates weaker affinity in comparison to other works reported in literature. Houwlingen et al. [300] obtained affinity values for the binding of ochratoxin A to several antibody fragments ranging between K_D=12 ng mL^{-1} up to 476 ng mL^{-1}. Bodarenko et al. [301] obtained affinity values between K_D=14 to < 500 ng mL^{-1} when detecting OTA using a fluorescence polarization immunoassay with various tracers. The deviation from the results obtained in this work can be ascribed to the different antibodies used, and/or the inhibition of affinity by the used wine matrix. In the work of Heusser et al. [302] a monoclonal antibody was used for the detection of ochratoxin A using ELISA. This experiment is most comparable to our results, since authors have obtained an IC50 value of 5.7×10^{-6} M and a LOD of 320

nM, which is slightly higher than the demonstrated LOD in this work, but very comparable since the measured LOD in this work still has a good signal strength and could be even lower if the corresponding concentrations were tested.

3.3.2. AuNPs enhancement

SPR analysis of small molecules (MW<1kDa) or analytes at extremely low concentration is hindered by weak refractive index changes occurring upon their capture on the sensor surface [303]. To overcome this major impediment of SPR biosensor technology, gold nanoparticles have been demonstrated as efficient signal enhancers [258].

In order to maximize the enhancement of SPR biosensor response for the OTA immounoassay, Ab_2-AuNPs conjugates with diameters of 10, 20 and 40 nm were tested. As shown in Fig.3.4A and 3.4B, the binding of non-labeled Ab_2 dissolved at concentration of 2 μg mL^{-1} to affinity captured Ab_1 induced a change in SPR signal of δR=0.01 and resonance shift to higher angles θ. This value is approximately two times higher than that recorded for the primary antibodies Ab_1. When the Ab_2 was conjugated with AuNPs and diluted at one order of magnitude lower concentration (0.2 μg mL^{-1}), the sensor response increased to δR=0.018 for 10 nm diameter and to δR=0.026 for 20 nm diameter which corresponds to the enhancement factors of 3.2 and 5, respectively. Even when the AuNP-Ab_2 conjugate with the diameter of 40 nm was diluted at the two orders of magnitude lower concentration (0.02 μg mL^{-1}), recorded signal δR=0.061 was 10-fold amplified compared to that for non-labeled Ab_2. Obtained results are in a good agreement with previously reported studies on the dependence of the optical enhancement on the size of AuNPs [295, 304]. The magnitude of the reflectivity shift depends on the particle size and the bigger particles lead to larger shifts. When an object with a high mass (like AuNPs) is applied when the effect of diffusion-limited mass transfer is weak, higher mass is allowed to bind to the surface which leads to the stronger SPR shift. Nevertheless, SPR response is influenced not only by size of NPs but also by the electromagnetic field coupling between localized surface plasmons of the nanoparticle and propagating plasmon field of the surface [187].

Furthermore the enhancement factor strongly depends on the distance between the NPs and the gold surface. This effect is dominant especially at certain sizes of the NPs. From Leveque and Martin it is known that for distances d < 50 nm most of the energy is concentrated between the particle and the film leading to enhancement factors between 10^2 and 10^3 [305]. Due to the fact that a distribution of distances between NP and gold surface is to be expected

the presented results are in good agreement with the theoretical calculations by various authors [187, 305]. Work is in progress to verify the distance dependency by measurements of the signal enhancement at various fixed distances between the NPs and the gold surface.

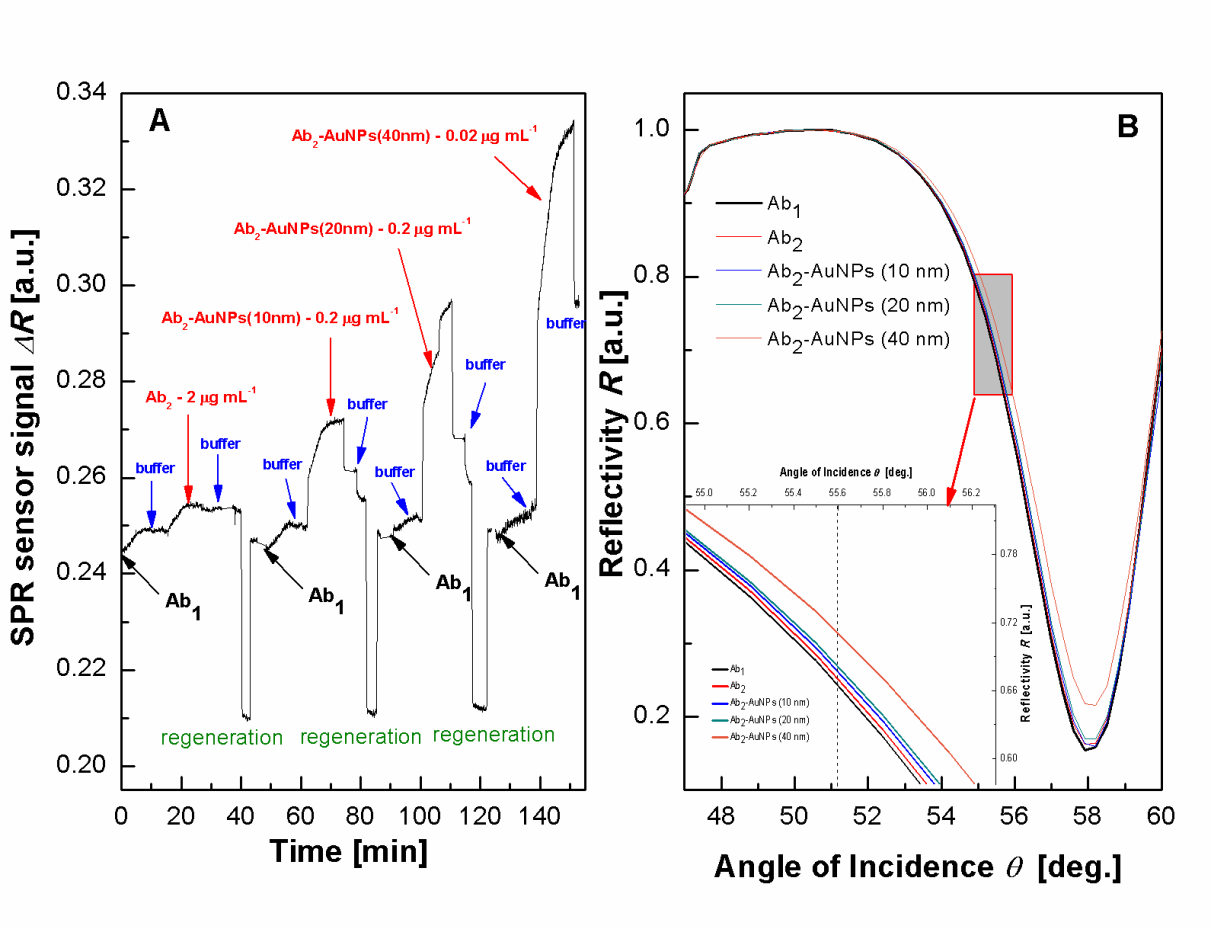

Fig.3.4. (A) Sensogram showing affinity binding ofAb_1 and subsequent reaction with non-labeled Ab_2 and Ab_2-AuNPs conjugates (size 10, 20 and 40 nm).(B) Angular reflectivity spectra measured from a sensor chip after affinity binding of Ab_1 (black line) and non-labeled Ab_2 (red line) and Ab_2-AuNPs (10 nm - blue line, 20 nm – green line and 40 nm – orange line). The spectra were measured for the surface brought in contact with PBS-T.

3.3.3. Ochratoxin A detection

Ochratoxin A was detected using indirect competitive format described in section 2.3. For calibration of the OTA biosensor, Ab_2-AuNPs conjugates with the diameter of 40 nm were chosen due to the highest recorded enhancement of the signal from all tested Ab_2-AuNPs (see section 3.3). To evaluate the effect of the SPR sensor signal enhancement by AuNPs, two types of assays were performed. In the first one, only the binding of Ab_1 antibody mixed with analyzed samples was measured. In second assay, the binding of Ab_1 was followed by the reaction with secondary antibodies conjugated with AuNPs. Fig.3.5 shows obtained

calibration curves normalized with the sensor response measured for the blank sample (not spiked with OTA). The sensor response ΔR was defined as a difference in the SPR sensor signal R before the reaction of the surface with spiked sample and after the rinsing with PBS-T. The sensor response ΔR was measured for series of buffer and red wine samples spiked with OTA at concentrations ranging from 1.5×10^{-1} to 10^{3} ng mL^{-1}. The calibration curves were fitted with a sigmoidal function. The limit of the detection (LOD) and the limit of quantification (LOQ) were defined as the concentration of OTA equivalent to three times (for LOD) and ten times (for LOQ) the value of the standard deviation (SD), measured in the absence of OTA (no competition point). The sensitivity of the AuNPs enhanced format was significantly better comparing to the one non-enhanced. For those two assays (performed in PBS-T buffer) LOD was found to be 0.068 ng mL^{-1} and 0.75 ng mL^{-1}, respectively (Fig.3.5). The higher signal has clearly reduced the errors in the determination of LOD, and in the case of low antibody concentration this reduction in coefficient of variation (CV, estimated to be 10,2% and 35% for assay with and without AuNPs, respectively) has been so significant that it made the LOD of the enhanced format significantly lower than without enhancement. When such detection format (utilizing AuNPs) was performed in red wine samples, LOD and LOQ of 0.19 ng mL^{-1} and 0.68 ng mL^{-1}, respectively, were calculated. Higher LOD in comparison to the assay established in PBS indicate some non-specific adsorption of wine components to the surface resulting in higher background response (Fig.3.5B). Nevertheless, both values are one order of magnitude lower than maximum allowed level of OTA in red wine established by European Commission. Thereby, presented biosensor could be considered as a new, sensitive and fast tool for mycotoxins detection in food and beverages.

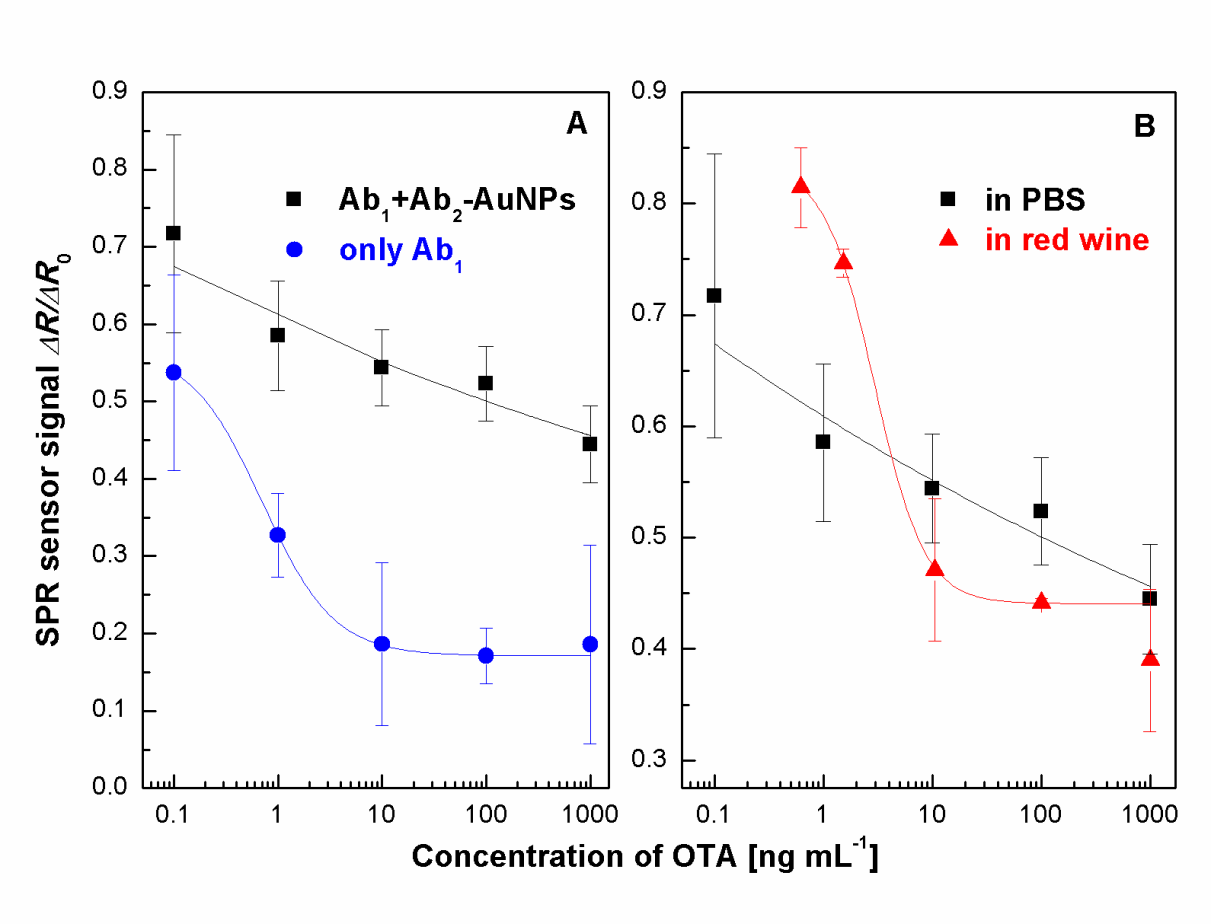

Fig.3.5. (A) Normalized calibration curves for the detection of OTA using inhibition immunoassay with Ab_2-AuNPs (40nm, black squares) and without secondary antibodies measured in buffer solution (blue circles). (B) Comparison of calibration plots performed in PBS-T and red wine (red triangles).

Comparing to the conventional methods for OTA detection in foodstuff including TLC [306], HPLC and GC-MS [307] that offer a very good sensitivity at the expense of complicated and time consuming pre-cleaning techniques. Most methods used for determination of a mycotoxin must rely on the correct extraction and clean-up procedures like liquid-liquid extraction (LLE) [131], supercritical fluid extraction (SFE) [308] or solid phase extraction (SP) [309]. An interesting study reported by Maier [310] shows a two-dimensional extraction procedure employed SP and MIP (molecularly imprinted polymers) for the extraction of OTA. Prior removal of the interfering acidic matrix compounds by C-18 SPE was shown to be successful and direct sample loading onto the MIP resulted in low recoveries. The extracts after the combined SPE protocol enabled OTA quantification by HPLC–FD. However, in this study, a similar result was observed in control experiments in which the MIP was replaced by the corresponding non-imprinted polymer (NIP). In yet another study, detection of OTA using SPME–LC–MS/MS has been reported [311]. High-throughput was achieved by simultaneous

preparation of up to 96 samples using multi-fibre SPME (Solid phase micro extraction) device and multi-well plates. A carbon-tape coating was chosen for extracting phase. The SPME technique was reported to be successful as clean-up procedures for OTA extraction from urine with LOD and LOQ of 0.3 and 0.7 ng mL^{-1} in urine, respectively. Pelegri et al. [312] presented a sensitive protocol for OTA detection and quantification using SAX (strong anion exchange) columns in clean up resulting with LOD of 0.02 ng mL^{-1} with HPLC–FD readout. There are several types of very sensitive chromatographic methods (TLC, HPLC, GC) available for mycotoxin analysis however, in all cases sample pre-treatment plays a major part in the analysis. Presented biosensor combine both aspects – detection of toxin of interest at ng mL^{-1} level as well as a very simple and fast treatment utilizing addition of 3% PVP to analyzed sample.

Among other separation techniques, ELISA became very popular recently due to its relatively low cost and easy application [313]. Commercially available ELISA kits offer highly specific as well as simple-to-use tool for mycotoxins detection. The disadvantage of these kits lies in the fact that they are for single use, which can increase costs of bulk screening, require multiple steps that translates to prolonged analysis time, and in the lower sensitivity compared to chromatographic methods [314]. Flajs et al. [130] reported a comparison of ELISA kit for OTA detection in red wine with HPLC and showed that the method of OTA-extraction recommended by the ELISA manufacturer is not appropriate for red wines due to the interference of chromogenes. The introduction additional clean-up with bicarbonate eliminate this interference, and the latter method gives results that correlate well with the results obtained by HPLC. In contrast to HPLC, ELISA could not detect very low OTA concentrations. In other study, Barthelmebs [315] modified typical ELISA by using aptamer instead of antibody. The limit of detection attained (1 ng mL^{-1}), the midpoint value obtained (5 ng mL^{-1}) and the analysis time needed (125 min) for the real sample.

Nowadays, also other alternative methods for OTA detection eg. electrochemistry or chemiluminescent has been intensively investigated. Barthelmebs [316] proposed an aptasensor, based on disposable screen-printed electrodes with electrochemical detection using differential pulse voltammetry. The aptasensor obtained using this approach allowed detection limit of 0.11 ng mL^{-1}, and was also validated for real sample analysis. Another very interesting work done by Novo et al. [279] demonstrates an integrated analytical system that conjugates an indirect competitive enzyme-linked immunosorbent assay strategy developed in PDMS microfluidics with integrated microfabricated hydrogenated amorphous silicon photodiodes for chemiluminescence detection. A limit of detection of 0.85 ng mL^{-1} was

obtained for OTA detection in a PBS solution using a straight-channel configuration. Comparable limits of detection were obtained for beer extracts but for red wine extracts a higher limit of detection of OTA of 28 ng mL^{-1} was obtained.

Presented in this work sensor offers shorter analysis time (*ca.* 55 min) with sensitivity reaching the most sensitive techniques. Indeed, the LOD of the developed biosensor can be further improved by increasing the binding capacity of the sensor surface and by using higher affinity antibodies. The analysis time can be decreased by the implementation of more sophisticated microfluidic devices with smaller volume of *e.g.* additional means for sample mixing.

3.4. Conclusions

Fast indirect competitive-based biosensor with application of AuNPs as a signal amplifier for OTA detection in red wine has been successfully developed. The analysis relies on the SPR readout that offers a real-time, label-free aspect of measurements. Estimated LOD of nanogold-enhanced assay of 68 pg mL^{-1} is 10 times more sensitive than non-enhanced one and one order of magnitude lower than the maximum level of OTA in wine specified by the European Commission. The LOD performed in red wine is slightly higher than the LOD of the pure assay indicating a certain amount of unspecific adsorption of red wine components to the surface resulting in higher background signal response. Moreover, to reduce interference of polyphenols which impede the analysis, a simple pre-treatment of the wine samples with the binging agent PVP was applied. It was shown that the addition of 3 % PVP to red wine completely reduces non-specific interactions. This is due to the excellent ability of PVP to bind polyphenols through hydrogen bonding and the possibility to eliminate them from the solution with a simple washing step. To overcome a matter of the small size (low molecular weight) of antigen that hampers its detection via SPR, secondary antibodies with metallic nanoparticle (NPs) labels with different sizes of AuNPs have been used as a signal enhancer. The highest signal amplification was obtained for 40 nm AuNPs and for distances $d > 50$ nm between the nanoparticles and the gold surface leading to enhancement factors of more than 100. The precision of the agreement between the theoretical prediction [21, 38] and the experimental results with a distribution of the distances is so excellent that the enhancement factor becomes a good measure of the averaged distance.

The low limit of detection, the superior signal response time of less than one hour and low consumption of primary antibodies (reduction of costs) make the developed assay an excellent alternative to conventional methods for the detection of OTA in red wine and other beverages.

CHAPTER FOUR, DEVELOPMENT OF A QCM-D BIOSENSOR FOR OCHRATOXIN A DETECTION IN RED WINE

The content of this chapter has been already published in **Talanta** Journal.

Authors and their contribution:

- **A. Karczmarczyk** - study conception and design, acquisition of data, analysis and interpretation of data, drafting of manuscript,
- K. Haupt - scientific advisor, critical revision of the article,
- K-H. Feller - scientific advisor, critical revision of the article, final approval of the version to be published.

Development of a QCM-D biosensor for Ochratoxin A detection in red wine. Talanta, 2017.

4.1. Introduction

Ochratoxis are a group of highly toxic fungal secondary metabolites produced mainly by *Aspergillus* (chiefly *A. ochraceus* and *A. niger*) and some *Penicillium* species (*P. verrucosum* and *P. carbonarius*) [317]. The most ubiquitous and toxic ochratoxin occurring in agricultural products is Ochratoxin A (OTA). Other structurally related ochratoxins (Ochratoxin B, C, α and β) are much less harmful or even do not exist in food [34]. The worldwide OTA occurrence in a broad range of raw and processed food commodities e.g. cereals, wine, coffee, dried fruits, beer, cocoa, nuts, beans, peas, bread and rice has already been amply described [45, 281, 283, 318].

Studies show that this molecule can have several toxicological effects such as nephrotoxicity, hepatotoxicity, neurotoxicity, teratogenicity and immunotoxicity [34]. It is also suspected of being the main etiological factor responsible for Balkan endemic nephropathy and its association to urinary tract tumors has also been proven [319]. Due to the general concern about the OTA present in the food chain, its high stability and potentially negative effects on human and animal health, a number of countries have set up regulations including maximum permitted, or recommended levels for specific commodities. The European Union (E.U.) has established limits for OTA depending on the food product: 5 μg kg^{-1} for unprocessed cereals, 10 μg kg^{-1} for dried fruits, 15 μg kg^{-1} for spices, including chili powder, paprika, pepper or nutmeg, and 2 mg mL^{-1} for all types of wines [45].

Currently, routine analysis of OTA in foodstuff is mostly performed by chromatographical methods including thin layer chromatography (TLC) [320], liquid chromatography (LC) [276, 321], gas chromatography (GC) [322] and high-performance liquid chromatography with fluorescence detection (HPLC) [131, 323]. Those techniques are generally straightforward and yield reliable results, however, they require extensive preparation steps and are time-consuming. Thus, alternative approaches offering high sensitivity and simplicity such as enzyme-linked immunosorbent assay (ELISA) [130, 324], electrochemistry [325], fluorescence [133] or chemiluminescence [326] have been developed. Moreover, label-free immunosensors for real-time toxins detection based on optical (e.g., surface plasmon resonance spectroscopy, SPR) [318] and piezoelectric or acoustic devices (e.g., quartz crystal microbalance, QCM) [182] have recently been investigated.

Biosensors with QCM-based readout are gaining increasing popularity in the detection of chemicals and biomolecules. This technique is a powerful and well-established noninvasive tool for online monitoring and quantification of molecular interactions on a solid surfaces [327]. The transducer in a QCM sensor is an oscillating quartz crystal whose resonance frequency (Δf) changes with the change in the mass (Δm) according to the Sauerbrey equation [212]. In addition to adsorbed mass (ng cm^{-2} sensitivity), the damping behavior of the crystal related to the conformational or structural properties of the viscous layer can be defined by measuring the energy dissipation loss (ΔD) of the freely oscillating crystal [327-329]. The so-called QCM-D device enables simultaneous monitoring of the changes in frequency and dissipation, and thus provides additional information about the effective layer thickness, conformational changes, viscoelastic properties and the hydration state of the film [330].

Despite a wide variety of approaches, these sensors do not always fulfil the requirements of high sensitivity, especially regarding detection of small molecules. Mycotoxins are low molecular weight compounds and therefore, cannot generate sufficient QCM-D signal (frequency change). In order to obtain optimal assay sensitivity, that is, increase the signal, reduce the background, while keeping the detection time short, the competitive inhibition immunoassay format was applied in the present study. This format is based on antigen immobilization on the sensor surface followed by the injection of the mixture of primary antibody and sample containing free antigen [163]. To reach a lower limit of detection, the signal can be amplified by the use of additional high mass compounds such as a secondary antibody with or without conjugation to gold nanoparticles (AuNPs).

In this work, we present the development of a fast and sensitive QCM-D biosensor for the detection of ochratoxin A in red wine. We have reached good QCM-D sensitivity with the

specific detection format of indirect competitive assay. To increase sensitivity, the signal was further amplified by the implementation of a secondary antibody labeled with gold nanoparticles. With this system we were able to reach a detection limit at the ng mL^{-1} level. Additionally, by combining simultaneous monitoring of the frequency and dissipation changes, the mechanical and viscoelastic properties of the biofilm were characterized. Moreover, to minimize the matrix effect and non-specific adsorption of wine commodities such as polyphenols (resulting in antibodies inactivation), a very simple procedure (addition of 3% of binding agent poly(vinylpyrrolidone) (PVP)) was employed with no need for cleanup or preconcentration of the sample extract [318].

4.2. Materials and methods

4.2.1. Reagents

All reagents were used as received without further purification. 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) were obtained from ThermoFisher Scientific (Germany). Thiols: (11-mercaptoundecyl)tetra(ethylene glycol) (MUTEG) and 16-mercaptohexadecanoic acid (MHDA), ochratoxin A (OTA), the conjugate of OTA with bovine serum albumin (BSA-OTA), poly(vinylpyrrolidinone) (PVP), Tween 20, hydrogen peroxide (30%), ethanolamine and glycine were purchased from Sigma-Aldrich (Germany). The primary rabbit antibody against OTA (Ab_1) was from AntiProt. Gold nanoparticles (AuNPs, 20 nm)-labeled goat anti-rabbit secondary antibody (Ab_2-AuNPs) were from Abcam (UK). 20 mM acetate buffer (ACT, pH 4) was prepared from sodium acetate trihydrate and acetic acid (both from Sigma-Aldrich) and the pH was adjusted by HCl and NaOH. Phosphate-buffered saline pH 7.4 (PBS) (0.12 g KH_2PO_4, 0.72 g Na_2HPO_4, 4g NaCl and 0.1 g KCl in 0.5 L distilled water) or PBS containing 0.1% Tween 20 (PBS-T) were used as running buffers. The ERM (European Reference Material) BD476 (OTA in red wine) was obtained from the Institute for Reference Materials and Measurements (Geel, Belgium). This material was prepared from commercial wine sources intended for human consumption and it was characterized by mass spectrometry and HPLC with optical detection. The concentration of OTA was determined as 0.52 ±0.11 ng mL^{-1}.

4.2.2. Apparatus

Piezoelectric measurements were performed with AT-cut gold-coated quartz crystals (Ø 14 mm, 100 nm thickness) with a resonance frequency of 5 MHz (LOT-Quantum Design GmbH, Germany) in flow-through mode (flow rate = 50 µL min^{-1}) with a quartz crystal microbalance with dissipation monitoring QCM-D (E1 model, Q-Sense AB, Sweden) at 22°C. In a QCM-D measurements, the fundamental resonance frequency of the crystal is excited and the variations in the resonance frequency, Δf, and energy dissipation ΔD, are recorded simultaneously for several overtones [331]. The oscillating frequency of the piezoelectric crystal decreases with the adsorption of a foreign substances on the surface. To calculate the mass uptakes (Δm) the simplified relation between the shift in frequency (Δf) and the mass of the adsorbed layer described by the Sauerbrey equation was used [212]:

$$\Delta m = \frac{-C\,\Delta f_n}{n} \qquad (1)$$

where C is the mass sensitivity constant (C = 17.7 ng $cm^{-2}Hz^{-1}$ at f = 5 MHz), n = 1 for the fundamental frequency and n > 1 is the overtone number (n = 3,5, …). In the QCM-D measurements, Δf is not only related to the mass uptake but may also be caused by the hydration of proteins and water trapped in the pores of the layer. In very dissipative systems it is required to utilize a more complex model for surface mass density estimation, e.g. the Voigt model [332]. However, in the current paper the frequency shift of the 5^{th} overtone was chosen (due to appropriate sensitivity and reproducibility as compared to other overtones) and the Sauerbrey equation was employed in the data analysis.

4.2.3. Sensor chip functionalization and detection format

As shown in Fig.4.1, the gold surface of the sensor chip was modified with a mixed thiol self-assembled monolayer (SAM) to which the BSA-OTA conjugate was attached. Prior the functionalization, the QCM resonator wafers were cleaned with a 3:1 (v/v) piranha solution (concentrated H_2SO_4 and 30% H_2O_2) for 10 min, followed by rinsing with water. Afterwards, the sensor was dried in a nitrogen stream and treated with a UV/ozon cleaner for 30 min. The thiol SAM was formed on the gold surface upon overnight incubation in a mixture of MUTEG and MHDA (molar ratio 9:1) dissolved in ethanol at the total concentration of 1 mM. Afterwards, the sensor surface was rinsed with ethanol and dried in a stream of nitrogen. The carboxylic terminal groups were activated by immersing the chip in EDC/NHS solution (concentrations in deionized water of 37.5 and 10.5 mg mL^{-1}, respectively) for 45 min. Then,

the sensor was rinsed with water, dried in a nitrogen stream and inserted into the QCM-D cell. Subsequently, the immobilization of BSA-OTA (dissolved in ACT buffer at a concentration of 40 μg mL^{-1}) was done by 20 min circulation of the conjugate solution over the flow cell, followed by deactivation of unreacted active ester moieties using 10 min incubation in ethanolamine (1M and pH 8.5).

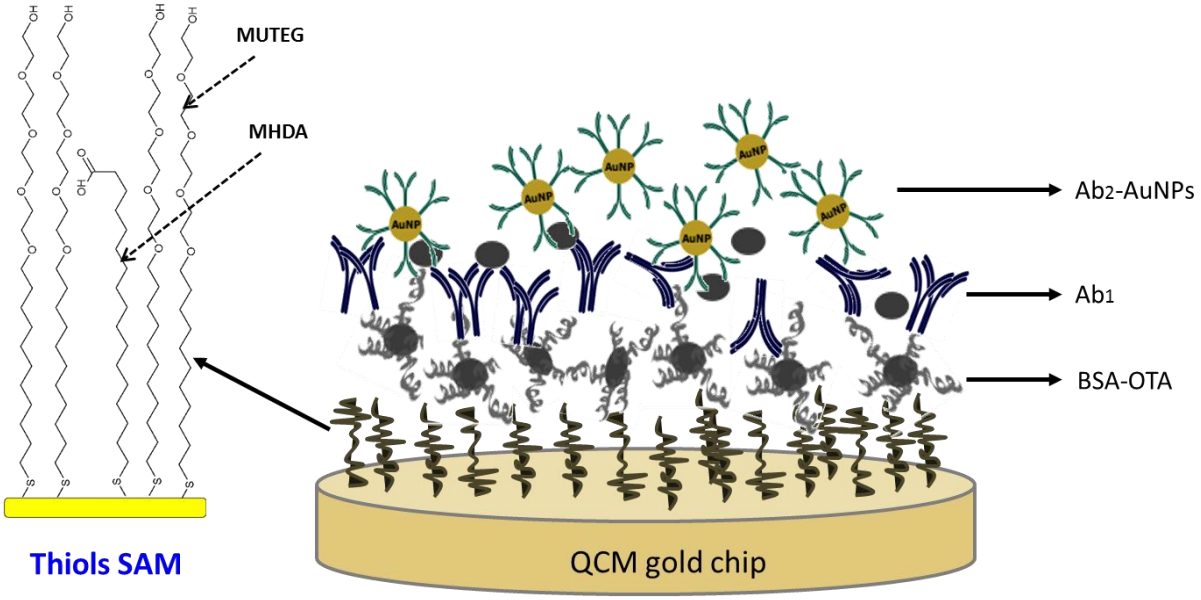

Fig. 4.1. Scheme of the interfacial molecular architecture for the detection of OTA by a competitive immunoassay utilizing QCM.

For the detection of OTA, an inhibition immunoassay was applied. In this assay, the samples were pre-incubated with Ab_1 for 15 min (with an equal volume) followed by detection of the amount of unreacted Ab_1 antibody. PBS-T buffer or certified red wine standard were spiked with OTA at concentrations between 10^{-2} and 10^{4} ng mL^{-1}. In order to decrease the influence of polyphenolic compound (matrix effect) that may interfere with the OTA analysis (inactivation of antibodies or unspecific sorption) in wine, samples were mixed with 3% of PVP and subsequently shaken for 5 min at room temperature, filtrated, and the pH of each aliquot was adjusted to 7.4 with NaOH prior to analysis. The mixture of Ab_1 with sample was pumped through the sensor for 10 min followed by washing with PBS-T buffer for 2 min to remove weakly bounded Ab_1 molecules. Afterward, the Ab_2–AuNPs antibody (concentration of 0.1 μg mL^{-1}) was flowed through the sensor for 10 min. Finally, the sensor surface was washed for 3 min with PBS-T buffer and the changes in the frequency owing to the binding of Ab_2-AuNPs to the captured Ab_1 were recorded. After each detection cycle, the sensor surface was regenerated by 10 min incubation in glycine buffer (pH 2.0, 20 mM).

4.3. Results and discussion

4.3.1. Sensor chip and assay characterization

The QCM-D is an acoustic wave device measuring the resonance of a piezoelectric quartz crystal upon electrical excitation (bulk excitation). The resonance frequency decreases linearly with addition of mass on the sensor surface. BSA-OTA immobilization showed a slight increase in dissipation energy ($\Delta D = 10.77 \times 10^{-6}$) therefore, the frequency measurement was directly converted to mass by using the Sauerbrey equation (Eq.1) and the recorded frequency shift (Δf = 57.31 Hz). The surface coverage of the covalently bound antigen conjugate was calculated to be 202.84 ng cm^{-1}. A typical QCM-D plot, with real-time changes in the frequency and dissipation of adsorption sequence is shown in the Figure 4.2. Due to the very low concentration of the primary antibody, the recorded signal for both the frequency and dissipation is weak (Δf = 17.97 Hz and $\Delta D = 2.02 \times 10^{-6}$). Hence, an additional high mass provided by Ab_2-AuNPs injection was applied resulting in a signal enhancement (Δf = 52.53 Hz) and consequently an improvement of immunoassay sensitivity.

The combined Δf and ΔD data analysis provide also information about the viscoelastic and mechanical properties of the protein layer. Moreover, according to the general understanding of the dissipative behavior of the biofilm, water is known to be trapped and sensed as additional mass [333]. A larger dissipation or $\Delta D/\Delta f$ ratio are usually correlated with high water content, loose bindings between interacting molecules or creation of a soft and dissipative film. On the other hand, the low dissipation per mass unit could be attributed to the formation of a well-structured complex with a high affinity binding and low degree of hydration [327]. Estimated $\Delta D/\Delta f = 1.7 \times 10^{-7}$ for BSA-OTA layer indicates formation of a homogeneous and fairly rigid film.

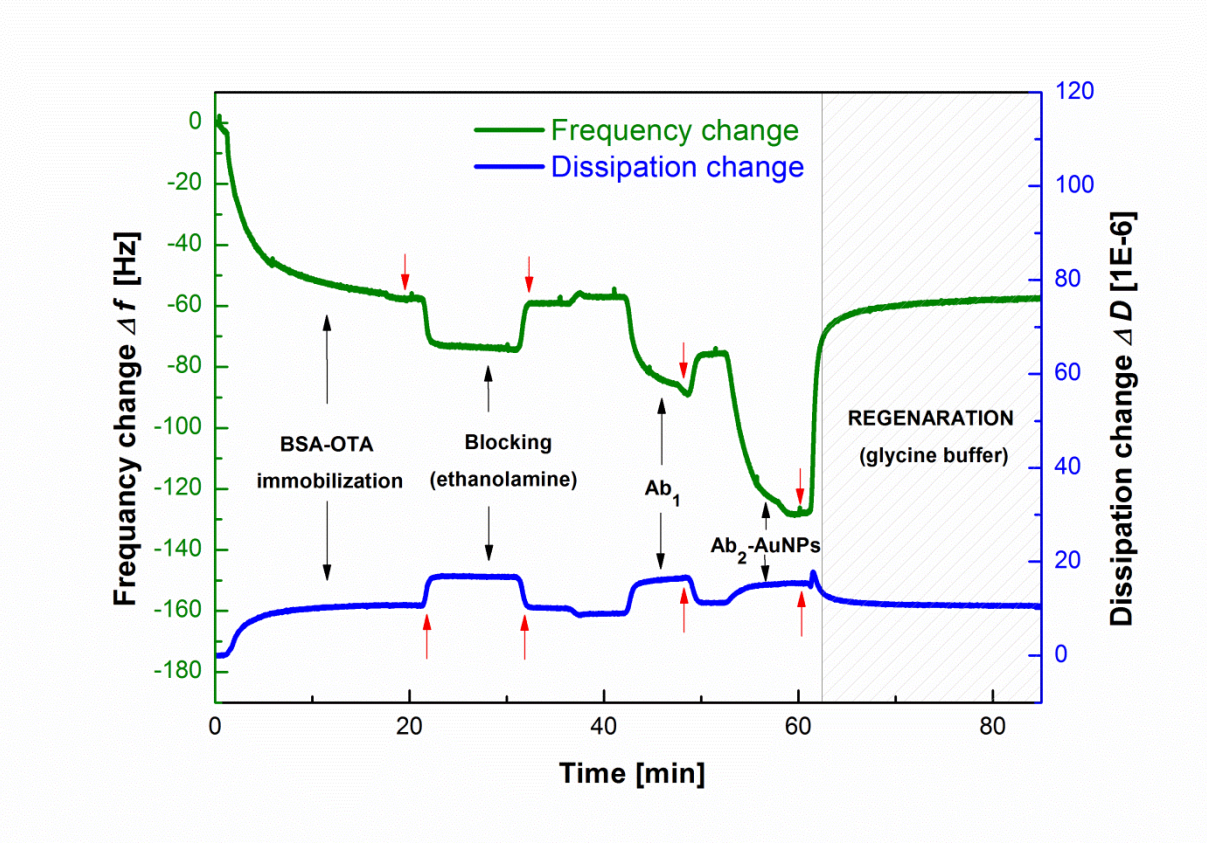

Fig. 4.2. QCM-D curves showing the frequency (Δf, green curve) and dissipation (ΔD, blue curve) changes during the adsorption sequence of the indirect immunoassay. Red arrows represent the washing steps.

Surface regeneration for cyclic use of the same chip is an important part of QCM-D immunoassays. In order to reuse the sensor chip surface, a simple regeneration protocol based on breaking the strong non-covalent interaction between toxin and antibody was applied [334]. This was accomplished by injection of glycine buffer at pH 2.0 over the sensor surface. As shown in Figure 4.2, after the regeneration step the frequency and dissipation signals return almost to the original baseline level (negligible increase of Δf and $\Delta D \approx 0.\ 1\%$) indicating complete breakdown of the immuno-complex interaction and revealing fully reversible assay cycle. In this case, the immobilized antigen was still present at the sensor surface and hence, the assay procedure for the next cycle started with the injection of the primary antibody.

After six cycles, the used quartz crystals were cleaned with piranha solution to remove all adsorbed on the gold surface materials including antigen (BSA-OTA) as well as SAM. SAMs are mechanically fragile surfaces, and thus, techniques for polishing or roughening surfaces of metals can remove the SAM and expose a clean surface on bulk metal substrates [196]. There

are a number of different techniques for removing SAMs from gold substrates such as: thermal desorption [335], ion sputtering [336] or UV/ozonolysis [337]. Chemical oxidants or reductants such as concentrated acids or bases or piranha solutions also are effective for cleaning substrates [338]. In our work piranha solution was used both for cleaning the surface before SAM functionalization as well was for removing thiols from the sensor surface. Moreover, a gold surface exposed to UV light (also used in this study) can be purged of sulfur impurities via oxidation of chemisorbed sulfur to sulfonates which can then be removed by washing with water [339]. The UV/ozone method allows one to remove old thiol monolayers and to provide a fresh surface for additional experiments. After such a treatment, the gold surface could be reused – a new SAM could be created followed by a fresh antigen solution immobilization.

The detection scheme is based on an indirect competitive format where Ab_2-AuNPs are used as a signal enhancement tag. The antigen attached to the surface (BSA-OTA conjugate) competes with toxins from the sample to bind with the added primary antibodies. Subsequently, captured Ab_1 bind with secondary antibodies labeled with AuNPs resulting in signal amplification and improvement of the assay sensitivity. In order to check the specificity of antibodies a simple experiment was performed. Briefly, BSA was immobilized on a chip, followed by Ab_1 injection and incubation for 10 minutes. After washing with PBS-T, only a negligible increase of the signal was recorded, indicating no adsorption of Ab_1. Naturally, in the absence of Ab_1, no non-specific binding of the secondary antibodies was observed (data not shown).

Due to the fact that wine is a very complex beverage containing a large variety of macromolecules which can interfere with the affinity binding on the surface, it is required to implement a pre-treatment of the wine samples prior the analysis. It might also seem that the percentage of alcohol (≈ 12%) in wine can influence the proper functionality of the assay. However, Ngundi et al. [340] demonstrated that the amount of ethanol is not a major factor which hampers the analysis. On the other hand, Ogunjimi and Choudary [341] have shown antibody inactivation due to the presence of polyphenols in fruit juice, wines and vegetables. Thus, a simple pre-treatment of wine samples involving the use of 3% PVP, a binding agent commonly used to remove polyphenols from plant extracts, was applied. We have previously proved an excellent ability of PVP to eliminate unwanted compounds from the solution and therefore preserve antibody activity [318].

4.3.2. Ochratoxin A detection

The sensor response was measured for series of buffer and wine samples spiked with OTA at concentrations ranging from 10^{-2} to 10^{4} ng mL^{-1}. Fig.4.3 shows the calibration curves normalized with the sensor response for the blank sample (not spiked with OTA). The calibration curves were fitted with a sigmoidal function. The limit of the detection (LOD) and the limit of quantification (LOQ) were defined as the concentration of OTA equivalent to three times (for LOD) and ten times (for LOQ) the value of the standard deviation (SD), measured in the absence of OTA.

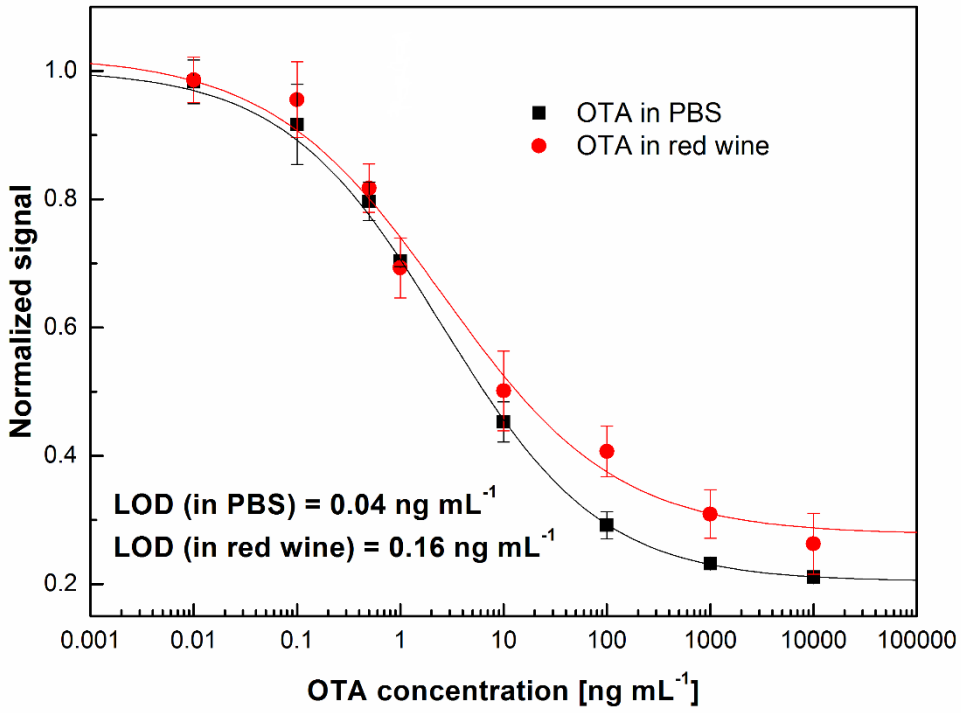

Fig. 4.3. Normalized calibration curves for the detection of OTA using inhibition immunoassay performed in PBS-T buffer (black squares) and red wine (red circles). Each point is an average of three replicates.

Fig.4.3 clearly shows that the recorded signal is proportional to the amount of bound antibody (gradual decrease when increasing the concentration of OTA in the sample assigned to the blocking of Ab_1 binding sites). The LOD and LOQ for the assay established in a PBS-T buffer were 0.04 and 0.13 ng mL^{-1}, respectively. The same parameters estimated for experiments performed in red wine samples were 0.16 and 0.55 ng mL^{-1} with the working linear range (IC20-IC80) between 0.2-40 ng mL^{-1}. The coefficient of variation (CV) of the

standard points of the calibration curves were 8.77% and 3.79% for the assay carried out in red wine and PBS-T, respectively, showing a good repeatability of the detection method. A midpoint value (IC50) of 2.53 and 2.58 ng mL^{-1} were obtained during competition assays performed in PBS and red wine, respectively. The higher LOD value obtained from wine measurements indicates adsorption of wine components on the sensor surface causing a somewhat higher background. However, this value is still one order of magnitude lower than the maximum residue level of OTA required by the European Commission.

The presented QCM-D biosensor offers higher sensitivity, shorter analysis time and avoids complicated and time consuming pre-cleaning methods with respect to other reports on the detection of this toxin [37, 39, 110, 135, 342, 343]. High-performance liquid chromatography (HPLC) [307], gas chromatography-mass spectrometry, thin-layer chromatography (TLC) [306], enzyme linked immunosorbent assay (ELISA) [313] and immunochromatographic assays as lateral flow strips [344] show good performance for OTA detection however, they all require multiple steps prior to the detection (including extraction and clean-up procedures), what extend the analysis time and causing extra costs. Compared to other alternative methods such as capillary electrophoresis with diode array detection [155], electrochemical or optical techniques like surface plasmon resonance (SPR) [45, 318], our QCM sensor offers a similar detection limit but shorter detection time.

4.4. Conclusions

An indirect competitive bioassay was successfully established for the detection of Ochratoxin A in red wine utilizing gold nanoparticles for QCM-D signal enhancement. Small molecules (such as OTA) suffer from poor LOD due to the high concentration of Ab_1 being needed to generate adequate signal. To overcome this problem, in the present study we have reported implementation of a secondary antibody with gold nanoparticle label resulting in the enhancement of the sensitivity of the low molecular weight compound assay. The decrease of the frequency and dissipation changes (Δf and ΔD) are proportional to the mass of molecules adsorbed on the chip surface and therefore, an additional mass provided by AuNPs results in a great signal amplification in comparison to the assay only with Ab_1. A linear range 0.2-40 ng mL^{-1} has been achieved with excellent LOD of 0.16 ng mL^{-1}, which is one order of magnitude lower then LOD specified by E.U. legislation concerning limit of exposure in food. Additionally, the biosensor can be easily regenerated with 10 min incubation in glycine buffer. Moreover, a real-world application of the system was tested with the determination of

OTA in spiked red wine samples. Finally, a matrix effect (caused by the occurrence of polyphenol in wine) and associated non-specific interactions with the sensor surface was minimized by a simple pre-treatment of the wine with the addition of 3% of the binding agent PVP.

The method described here is a rapid (less than one hour detection time), sensitive and cost effective (inexpensive reagents, reduction of the concentration of the valuable primary antibody) alternative for the detection of various mycotoxins in food and beverages and therefore, an important tool in the field of food security and thus, human health.

CHAPTER FIVE, INHIBITION-BASED ASSAY FOR THE ELECTROCHEMICAL DETECTION OF AFLATOXIN M1 AND OCHRATOXIN A IN MILK AND RED WINE

The content of this chapter has been already submitted to **Electrochmica Acta** Journal.

Authors and their contribution:

- **A. Karczmarczyk** - study conception and design, acquisition of data, analysis and interpretation of data, drafting of manuscript,
- A. Beaumner - conception and design, scientific advisor, critical revision of the article,
- K-H. Feller - scientific advisor, critical revision of the article, final approval of the version to be published.

Rapid and sensitive inhibition-based assay for the electrochemical detection of mycotoxins in food, Analytica chimica acta, 2017

5.1. Introduction

Mycotoxins are secondary metabolites of fungi (the major mycotoxigenic fungi belonging to the genera *Aspergillus*, *Fusarium* and *Penicillium*) that may appear in almost all food and feed commodities [343]. Examples of mycotoxins of greatest public health and agroeconomic significance include aflatoxins, ochratoxins, trichothecenes, zearalenone, fumonisins and ergot alkaloids [10]. Due to high exposure to mycotoxins through a large variety of daily life products, they are widely recognized as a serious hazard for both human and animal health [345]. When present in a foodstuff at significantly high levels, they can cause adverse and toxic effects leading to liver and kidney damage, cancer or even immune suppression [12].

Among all mycotoxins, the most toxic/carcinogenic are aflatoxins (secondary metabolites produced by *Aspergillus flavus* and *Aspergillus parasiticus* [346]). Aflatoxin M1 (AFM_1) is a major derivative of aflatoxin B1, found in milk of animals that have ingested feed contaminated with AFB_1 (about 0.3–6.2% of AFB_1 in animal feed is transformed to AFM_1 [243]). Because of the relatively high stability during milk pasteurization or other thermal treatments, AFM_1 can also occur in milk-derived dairy products, such as cheese and yogurt [347]. The toxic and carcinogenic effects of AFM_1 have been convincingly demonstrated [348] and therefore, WHO–International Agency for Research on Cancer (IARC) has classified AFM_1 as Group 1 carcinogen [247]. Because of a high human consumption of milk

products (especially children), AFM_1 contamination poses a significant threat to human health. Hence, the European Commission stipulates a maximum permissible level of 50 ng L^{-1} for AFM_1 in milk and dried or processed milk products [103].

Ochratoxins belong to a family of structurally related secondary fungal metabolites, constitute another example of highly toxic mycotoxins which can be found mainly in cereals, coffee, cacao, grapes, wine, soy, nuts, beer and so on [45, 281, 283, 318]. The main forms are ochratoxin A, B, and C, which differ in that ochratoxin B (OTB) is a non-chlorinated form of ochratoxin A (OTA) and ochratoxin C (OTC) is an ethyl ester of OTA [33]. However, among all these forms, the most harmful and dominant is OTA, which has demonstrated to be nephrotoxic, carcinogenic, teratogenic and immunotoxic [349]. The main source of OTA intake by the European Union population are cereals, followed by wine and peanuts [206]. Due to its existence in the food chain, relatively high stability during food processing and therefore hazard imposed on both human and animal life, maximum permitted levels of OTA have been established by nations all over the word. For example, the European Commission has conducted detailed risk assessments and set up maximum allowable levels for different types of food and feed (e.g. 5 ng mL^{-1} for unprocessed cereals, 3 ng mL^{-1} for products derived from unprocessed cereals, 10 ng mL^{-1} for coffee beans and 2 ng mL^{-1} for all types of wine [45]).

To minimize the occurrence of mycotoxins, it is essential to trace the sources of contamination by using fast, sensitive and cost-effective techniques. Most of the established conventional analytical methods for their detection involve enzyme-linked immunosorbent assay (ELISA) [350], thin layer chromatography (TLC) [351], high performance liquid chromatography (HPLC) in combination with the appropriate detection methods [352] and liquid chromatography/electrospray – tandem mass spectrometry [276, 321, 353]. However, these techniques require extensive sample preparation, highly trained personnel and thereby are time and cost consuming. Thus, the development of rapid, simple and sensitive methodologies for analysis of mycotoxins in our dairy products is necessary. Recently, some new technologies have emerged for the detection of small molecules utilizing e.g. surface plasmon resonance spectroscopy (SPR) [45, 263, 318] or quartz crystal microbalance (QCM) [213, 354]. The main advantages of those methods rely on real-time, label-free and non-invasive measurements at the expense of high-priced equipment (SPR, QCM devices, sensor chips). However, the majority of novel approaches are based on immunoassays with colorimetric [355], fluorescence [253] or electrochemical [105, 235] detection. The latter,

electrochemistry, has been reported as a sensitive (in the ng cm^{-2} range), practical and fast tool used for various biosensor applications therefore, opening a new path for their development [356-358].

In this regard, the use of screen-printed electrodes (SPE) has spread widely over the last years as a simple, fast and inexpensive approach for the development and production of disposable biosensors. The ease of surface modification and broad variety of transducer compositions allow the design of on-demand working sensors, suitable to multiple analytes [359]. Furthermore, the miniaturization of these electrochemical sensing platforms results in significantly lower sample consumption, facilitating the integration to microfluidic and point-of-care devices, as well as portable diagnostic systems [360].

The immobilization of biomolecules (e.g., biotin, DNAs, saccharides, peptides, and proteins) on transducers is an important requirement for the fabrication of biosensors. Self-assembled monolayers (SAMs) can facilitate this process. The spontaneous formation of a monolayer containing functional molecules [361] is most often employed with gold substrates taking advantage of the strong bond between thiol groups and gold. Biomolecules are in turn anchored to the SAM by covalent attachment to the functional molecules available on the SAM surface [362, 363].

In this article, the development of an electrochemical biosensor for rapid and sensitive mycotoxins (OTA and AFM_1) determination is reported. Detection is achieved by using a competitive immunoassay format based on the immobilization of the antigen onto the modified gold surface, followed by the injection of the mixture of primary antibody and sample containing free antigen [213]. Detection is accomplished via a secondary antibody labeled with alkaline phosphatase. The voltammetric signal associated to the enzymatically generated product was measured in gold screen-printed electrodes. The biosensor was tested in red wine and milk samples with no need for pre-treatment or preconcentration of the sample extract. Moreover, AuSPE modified with SAMs based on different types of alkanethiols (long (MHDA) and short (MPA) chains) were investigated and compared in terms of electron transfer resistance and thus, better substrate for assay performance.

5.2. Materials and methods

5.2.1. Reagents

All reagents were used as received without further purification. 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) were obtained from ThermoFisher Scientific (Germany). Thiols: (11-mercaptoundecyl)tetra(ethylene glycol) (MUTEG), 16-mercaptohexadecanoic acid (MHDA), and 3-mercaptopropionic acid (MPA), ochratoxin A (OTA), aflatoxin M1 (AFM_1), the conjugates of OTA and AFM_1 with bovine serum albumin (BSA-OTA, BSA-AFM_1, respectively), poly(vinylpyrrolidinone) (PVP), potassium ferricyanide ($K_3Fe(CN)_6$) and ferrocyanide ($K_4Fe(CN)_6$), Tween 20, ethanolamine were purchased from Sigma-Aldrich (Germany). The primary rabbit antibody against OTA and AFM_1 (Ab_1-OTA, Ab_1-AFM_1, respectively) were from AntiProt. Alkaline phosphatase-labeled (ALP) goat anti-rabbit secondary antibody (Ab_2) was from Abcam (UK). 1-naphtyl phosphate disodium salt (α-NP) was from VWR (Germany). The ERM (European Reference Material) BD476 (OTA in red wine) was obtained from the Institute for Reference Materials and Measurements (Geel, Belgium). This material was prepared from commercial wine sources intended for human consumption, and was characterized by mass spectrometry and HPLC with optical detection. The concentration of OTA was determined as 0.52 ±0.11 ng mL^{-1}. The ERM BD282 (zero level of AFM_1) was also obtained from the Institute for reference Materials and Measurements.

The compositions of the buffers used for the experiments were as follows:

- Immobilization solution: 20 mM acetate buffer (ACT, pH 4) was prepared from sodium acetate trihydrate and acetic acid (both from Sigma-Aldrich) and the pH was adjusted by HCl and NaOH (Buffer A)
- Affinity solution: Phosphate-buffered saline pH 7.4 (PBS) (0.12 g KH_2PO_4, 0.72 g Na_2HPO_4, 4g NaCl and 0.1 g KCl in 0.5 L distilled water) (Buffer B)
- Washing solution: Buffer B+ 0.1% Tween 20 (PBS-T, Buffer C)
- Detection solution: 0.2 M Tris-HCl pH 9.8 (Buffer D)

5.2.2. Electrochemical measurements

Electrochemical measurements were carried out with a computer-controlled potentiostat with CV50W software at room temperature. Screen printed gold electrodes (AuSPE) were obtained

from DropSens (Spain, ref. DRP-C220AT). The electrodes incorporate a conventional three-electrode configuration, which comprises a disk-shaped Au working (0.4 mm diameter), Au counter and silver pseudo-reference electrodes. Cyclic voltammetry (CV) was performed in 0.1 M buffer B in the presence of 10 mM $Fe(CN)_6^{3-/4-}$as a redox probe from -0.2 V to +0.6 V at a scan rate of 100 mV s^{-1}. Differential pulse voltammetry (DPV) was carried out from -0.1 V to 0.55 V at a scan rate of 100 mV s^{-1} [364].

5.2.3. Analysis of samples

The determination of AFM_1 in milk was carried out by spiking a milk sample (before centrifugation). ERM-BD282 milk powder was dissolved in deionized water at a concentration of 0.1g mL^{-1}. Then, samples containing AFM_1 were centrifuged for 20 min. The upper fat layer was removed completely, and the obtained aqueous phase was directly used for further analysis.

In order to decrease the influence of polyphenolic compound (matrix effect) that may interfere with the OTA analysis in wine, samples were mixed with 3% of PVP and subsequently shaken for 5 min at room temperature, filtrated, and the pH of each aliquot was adjusted to 7.4 with NaOH prior to analysis [318].

5.2.4. Competitive assay procedure

Immobilization of BSA-toxin conjugates (BSA-OTA or BSA-AFM_1) was based on the covalent attachment to a self-assembled monolayer of MPA (3-mercaptopropionic acid) on the gold surface of screen-printed electrode (Fig. 5.1). Briefly, AuSPE were first modified overnight by incubation with a 1 mM ethanolic solution of MPA at room temperature. Subsequently, the gold surface was rinsed with ethanol followed by deionized water to remove all unattached species. The MPA-modified Au electrode was then treated with EDC/NHS solution (concentrations in deionized water of 37.5 and 10.5 mg mL^{-1}, respectively) for 45 min to convert the terminal carboxylic groups into an active NHS ester. After rinsing with water and drying, the AuSPE surface was covered with a droplet (40 µL) of BSA-toxin conjugate (BSA-OTA or BSA-AFM_1, both dissolved in a buffer A at a concentration of 40 µg mL^{-1}) for 40 min. Afterwards, the unbound molecules were removed from the electrode by slow dipping into buffer A, followed by deactivation of unreacted active ester moieties using 10 min incubation with 1M ethanolamine.

For the detection of toxins – OTA or AFM_1, competitive immunoassay was used. Samples were pre-incubated with Ab_1 (concentration of 100 ng mL^{-1}) for 20 min (equal volume) followed by the detection of the amount of unreacted Ab_1 antibody. Buffer B, certified red wine (for OTA detection) and milk (for AFM_1 detection) standards were spiked with toxins (OTA or AMF_1) at concentrations between 10^{-2} and 10^4 ng mL^{-1}. The droplet (40 μL) of the mixture of Ab_1 containing the sample was placed on the electrode surface for 30 min followed by a washing step with buffer C to remove weakly bounded Ab_1 molecules. Afterwards, a droplet with Ab_2 (labeled with ALP enzyme) was added to the surface and incubated for 30 min. Finally, the sensor was washed with buffer C. Once the enzymatic conjugate was attached, both the enzymatic reaction and the electrochemical measurements were carried out. For this purpose, a droplet (40 μL) of the enzymatic substrate (α-NP) prepared in buffer D (at a concentration of 4 mM) was placed on the modified AuSPE. After 10 min of enzymatic reaction, the product, α-naphthol (α-N) was detected by DPV.

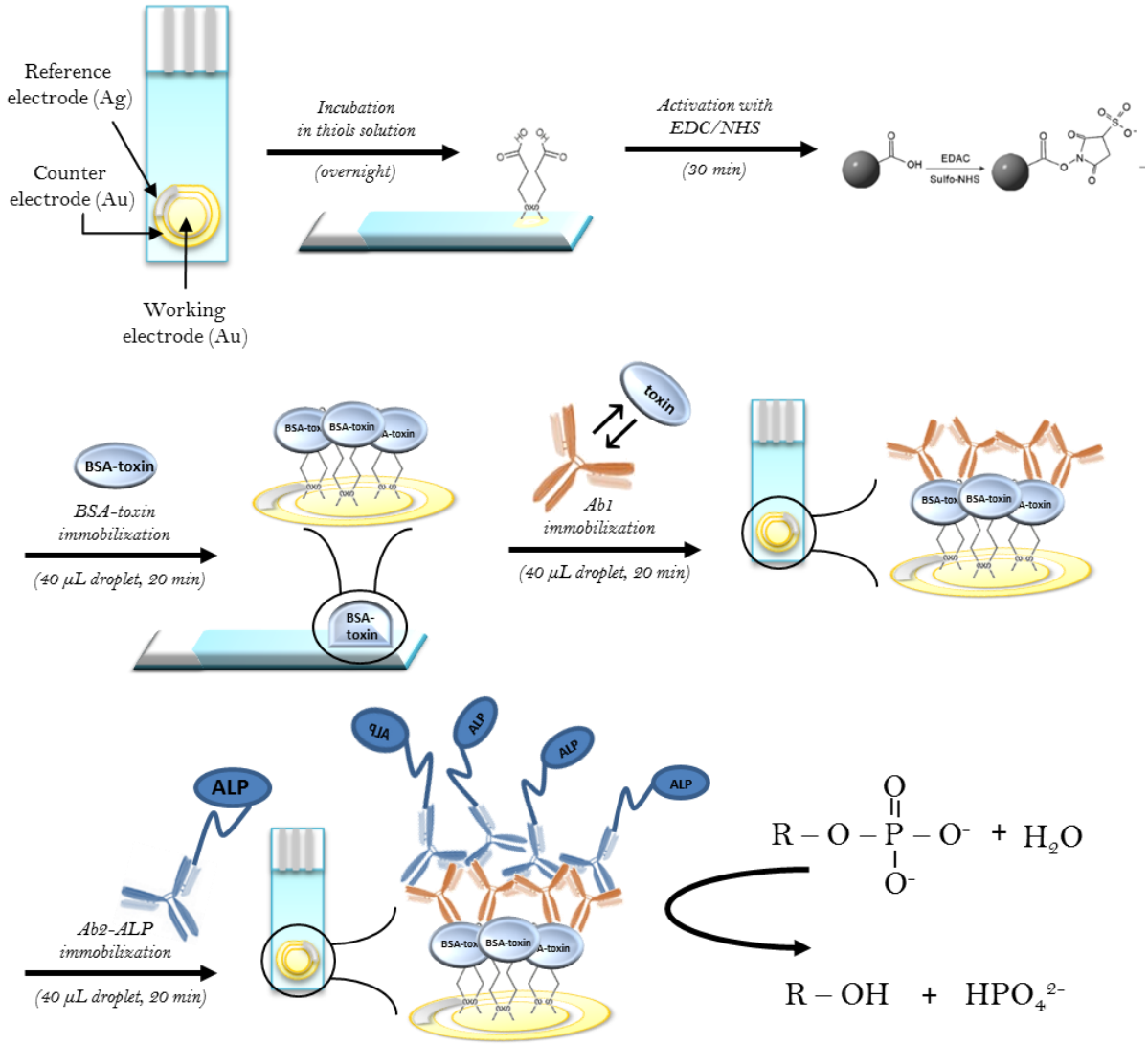

Fig.5.1. Schematic illustration of the preparation of the biosensor for toxin detection utilizing a competitive immunoassay format.

5.3. Results and discussion

5.3.1. Cyclic voltammetry studies

Cyclic voltammetry is an efficient analytical technique commonly used to monitor surface modifications, since it provides useful and rapid information on the changes of the electrode behavior after the assembly step. In our previous study (utilizing SPR and QCM for toxin detection) we used mixed thiols solutions (e.g. MHDA+MUTEG) for surface modification [103, 213, 318]. In this case, long aliphatic chains led to strong van der Waals interactions and hence, produced well-ordered SAMs with high integrity and thermal stability [195]. However, SAMs generated from such a long thiols typically block the electrode surface and render it

less reactive. For this reason, in electrochemical approaches usually short chains of alkanethiols are used enabling electron transfer across the layer, but unfortunately typically also present a reduced stability of the interface in comparison to longer chain alkanethiols [195]. The short-chain thiol (MPA) was tested in the present work and the voltammetric response compared to two other thiol-SAMs (MHDA and MHDA+MUTEG) and bare AuSPE as shown in Fig.5.2. The reversible redox couple, $Fe(CN)_6^{3-/4-}$ was selected as a redox probe.

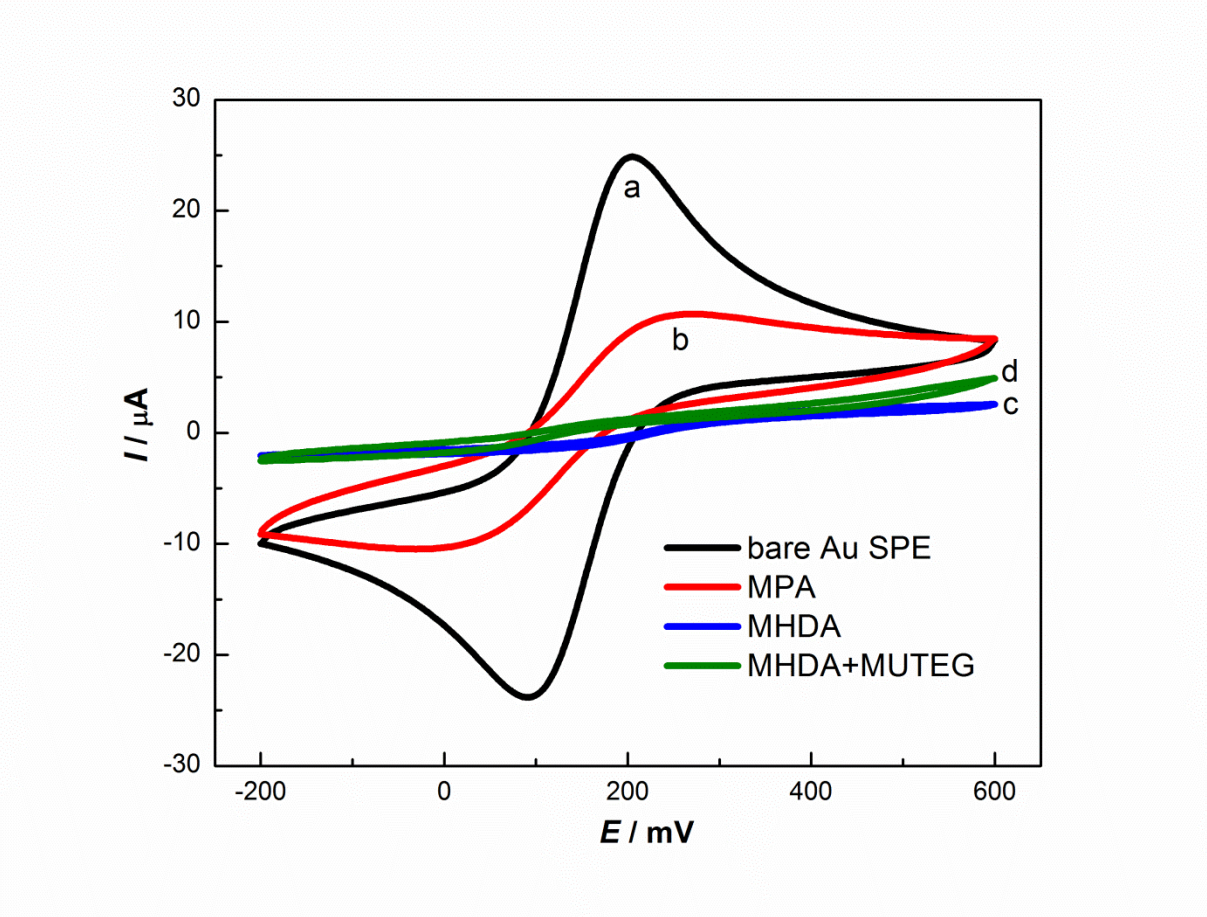

Fig.5.2. Cyclic voltammogram of 10 mM $Fe(CN)_6^{3-}$/ $Fe(CN)_6^{4-}$ (scan rate 100 mV s^{-1}) on a bare AuSPE (a, black curve), modified with MPA (b, red curve), MHDA (c, blue curve) and MHDA+MUTEG (d, green curve).

As expected, $Fe(CN)_6^{3-/4-}$ showed a reversible behavior on the bare AuSPE with a peak-to-peak separation (ΔE_p) of 113 mV (Fig.5.2a, black curve). However, the reduction and oxidation reactions were blocked after the modification of the Au surface with MHDA (Fig.5.2c, blue curve) and MHDA+MUTEG (Fig.5.2d, green curve) thiols solutions. This was most likely caused by densely packed MHDA+MUTEG molecules. In the case of the short chain thiol – MPA (Fig.5.2b, red curve) the redox reactions can still occur, however with slower electron transfer kinetics as observed through lower peak current and bigger peak-to-

peak separation (ΔE_p approximately 240 mV) when compared to a bare AuSPE. The carboxylic groups of the SAM can experience ionization and become negatively charged and hence hinder the electron transfer due to electrostatic repulsion [200]. Nevertheless, the electron transfer can occur partially through bare spots on the electrode and by tunneling across the SAM [365].Thus, the MPA based SAM was used for further immobilization steps.

5.3.2. Assay characterization

The gold surface modification with an MPA SAM provided a film containing –COOH groups on the surface that could be used for covalent bonding to the antigen. After activation with a mixture of EDC/NHS, the carboxyl terminal groups of MPA reacted with NH_2-groups of the BSA-toxin covalently attaching the antigen to the electrode surface. This immobilized antigen competes with the free toxin molecules for binding to Ab_1. Thus, the immobilization of BSA-toxin conjugate is a crucial step since the competitive immunoreaction is dependent on the amount and functionality of BSA-toxin on the surface. In our previous study with SPR and QCM methods for toxins detection [213, 318], the incubation times for antigen and antibody immobilization were monitored online. The recorded signal reached a plateau after 10 min (for BSA-toxin conjugate) and 15 min (for Ab_1) indicating surface saturation. However, in both techniques the analyte was transported by a flow system to the surface limiting the effect of the diffusion-limited mass transport. As in the electrochemical set-up a droplet format was chosen in which no active mixing or mass-transport occurs, the optimum incubation times were investigated (Fig. 5.3) testing five different times. As expected, the amount of BSA-toxin conjugate increased with longer incubation times, reaching a plateau after 40 minutes which was used for further experiments. Also, a similar experiment was performed for the determination of the optimal antibody incubation time (data not shown). A comparable response curve was obtained indicating that 30 min incubation was sufficient to obtain maximum signals.

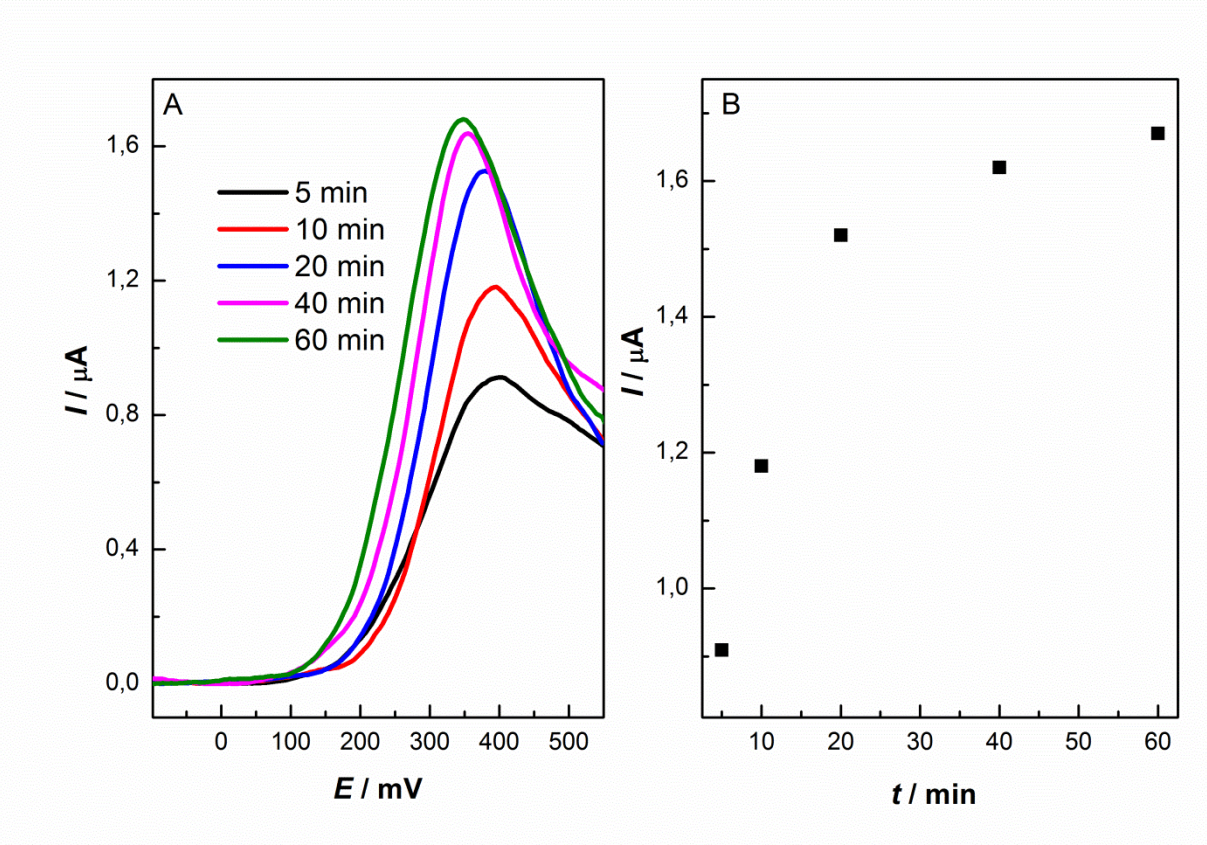

Fig.5.32. Determination of the assay optimal BSA-toxin conjugate immobilization time.

5.3.3. Toxins detection

Once the experimental conditions were optimized, the competitive assay was performed as explained above, testing OTA and AFM_1 solutions at concentrations ranging from 10^{-2} to 10^{3} ng mL^{-1}. Fig.5.4 shows the calibration curves normalized with the sensor response for the blank sample (not spiked with OTA - Fig.5.4A or AFM_1 – Fig.5.4B). The calibration curves were fitted to a sigmoidal function. The limit of detection (LOD) and the limit of quantification (LOQ) were defined as the concentration of toxin equivalent to three times (for LOD) and ten times (for LOQ) the value of the standard deviation (SD), measured in the absence of toxins. The working range of the assay is defined as the most linear part of calibration curve, usually between 15-20% and 80-85% inhibition and is represented by IC20 and IC80 values [366].

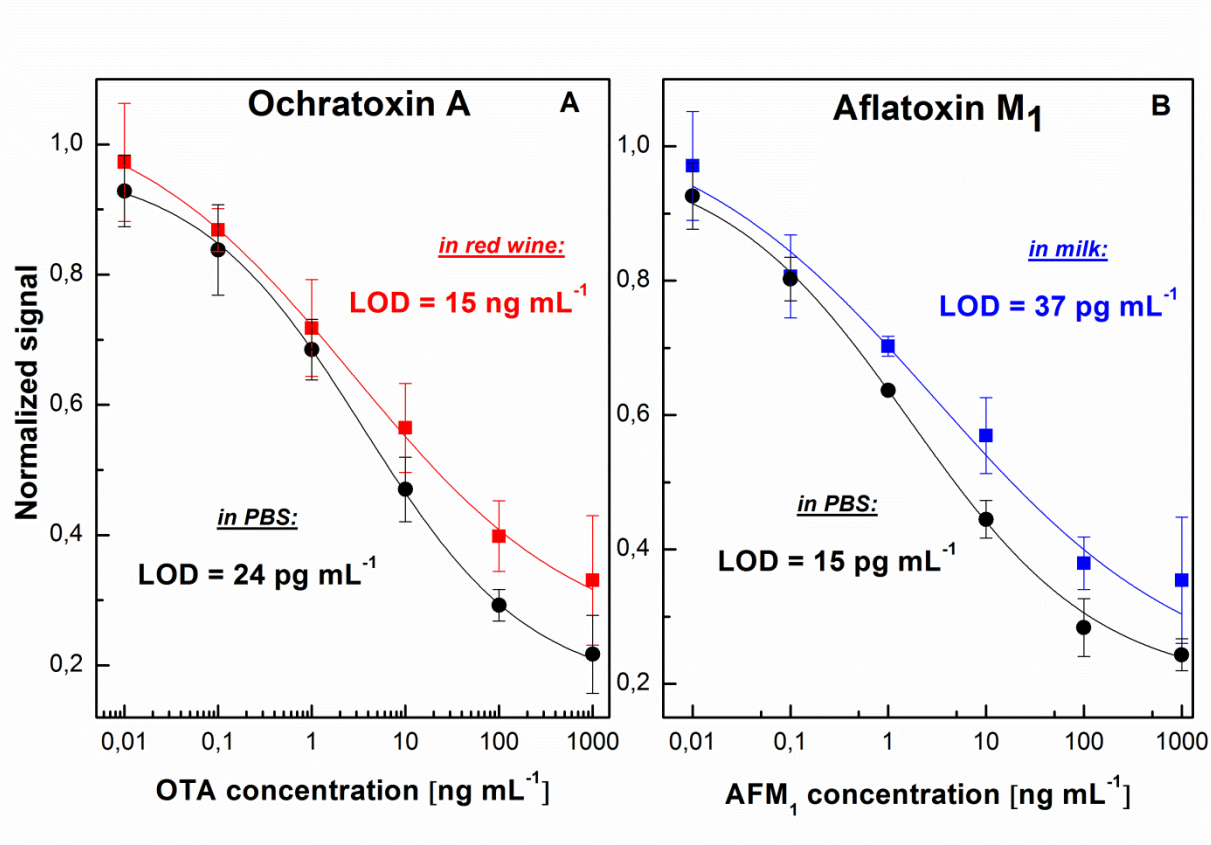

Fig.5.4. Normalized calibration curves for the detection of (A) OTA performed in PBS buffer (black circles) and red wine (red squares) and (B) AFM_1 in PBS buffer (black circles) and milk (blue squares). Each point is an average of three replicates.

As a competitive immunoassay was used, signals decreased with higher toxin concentrations in the sample. One can notice that the calibration curves for measurements performed in real samples (red wine – Fig.5.4A and milk – Fig.5.4B) are shifted towards higher concentrations with respect to the curves obtained from experiments carried out in PBS buffer. This difference can be ascribed to the non-specific adsorption of milk constituents or wine components to the surface resulting in higher background response. Nevertheless, the developed assay shows excellent values of relevant analytical parameters summarized in Table 5.1. As it can be observed, the current electrochemical approach with the LOD and IC50 of 15 and 2.75 ng mL^{-1} for OTA and 37 pg mL^{-1} and 3.04 ng $mL^{-1}$for AFM_1 detection, can be a good method for the analysis of various mycotoxins in food and beverages. Estimated LODs values are well below of the most restricted limits set by the European Union (for both toxins) indicating the suitability of the immunosensor as a novel tool for small molecules analysis.

Table 5.1. Analytical characterization of OTA and AFM_1 biosensors utilizing competitive immunoassay preformed in red wine and milk samples, respectively.

	LOD [ng mL^{-1}]	LOQ [ng mL^{-1}]	IC50 [ng mL^{-1}]	IC20-IC50 [ng mL^{-1}]	CV [%]
OTA (in red wine)	15	50	2.75	0.05-136	9.65
AFM_1 (in milk)	0.037	0.123	3.04	0.04-205	11.61

The developed biosensors for the two toxins combine the most desirable aspects for mycotoxins detection such as high sensitivity, low cost and short analysis time making it a competitive method in comparison to conventional analytical methods including thin-layer chromatography [320], high-performance liquid chromatography [131, 323] and gas chromatography [322]. These techniques offer good sensitivity at the expense of long analysis time and extra costs because they employ solid phase column clean-up of extracts procedures to remove interferences occurring during real samples analysis. Though, known for more than a decade, mycotoxin ELISAs typically require multiple steps and sample pre-treatment with those described for red wine not always being sufficient enough to remove interference of chromogenes [130]. Our biosensor merges mycotoxins detection at ng mL^{-1} level and simple pre-treatment of the sample. Recently developed, alternative approaches based on optical (e.g. SPR [103]) or acoustic (e.g. QCM [213]) technologies show comparable LODs and shorter analysis time however, require highly trained personnel and very expensive equipment. Gold screen printed electrodes used in this study have the advantages to be miniaturized (thus, the volume of reagents is reduced to the size of a droplet - ca.15-50 µL), mass produced and cost-effective.

5.4. Conclusions

An electrochemical immunosensor for the detection of mycotoxins (Aflatoxin M_1 and Ochratoxin A) utilizing a competitive assay format was carried out on a MPA-modified gold screen-printed electrodes (SPEs). Indeed, the use of SPEs has made it possible to design a low-cost, disposable and sensitive biosensor for the analysis of the aforementioned toxins, operating in a wide working linear range with a limit of detection at the low ng mL^{-1} level. It becomes worth highlighting that this level remains one order of magnitude below (for both analyzed compounds) the maximum residue level required by the European Commission. In fact, utilization of SPEs implies remarkable advantages when it comes to the development of

biosensors, such as smaller needed volumes of sample [225] and reagents, as well as the inherent miniaturization of the biosensor itself [367]. We have harnessed these benefits coming forward with a fully-miniaturized, fast electrochemical biosensor, in comparison with other methodologies described for these particular analytes in literature [178, 368] . Moreover, the use of short-chain alkanethiol SAMs enabled simple immobilization of the antigens and helped protect the electrode surface from unwanted matrix effects from red wine and milk samples. The high sensitivity, low costs (use of inexpensive and disposable SPE and the reagents volume reduction to the size of a droplet), short analysis time and simple but effective cleaning-up technique makes this approach an important and very promising tool for widespread biosensing applications of small molecules in food and beverage samples.

CHAPTER SIX, FINAL DISCUSSION AND CONCLUSIONS

In the presented thesis, the author proposed three strategies based on combination of biosensors methodology (utilizing biological recognition component - antibody) with indirect competitive immunoassay and surface plasmon resonance spectroscopy, quartz crystal microbalance and electrochemistry as a readout. Biosensors for aflatoxin M1 and ochratoxin A detection (chosen for this study as they represent two of the most important mycotoxins classes) were challenged in red wine and milk to proof the usability of offered approaches for real samples analysis.

The work of this thesis can be clustered into three main parts as follows:

(I) Biosensors for mycotoxins detection utilizing surface plasmon resonance spectroscopy (based on [103, 318]).

This study revealed a novel and highly sensitive biosensors for the detection of aflatoxin M_1 in milk and ochratoxin A in red wine by using SPR spectroscopy. Taking into account that SPR response is proportional to the mass of analyzed molecule and the fact that mycotoxins are small chemical compounds that possess inadequate mass to cause significant changes in the refractive index, an indirect competitive immunoassay was applied to overcome those limitations. For further signal amplification and sensitivity improvement secondary antibodies conjugated with gold nanoparticle labels were used and the interplay of size of AuNPs and affinity of recognition elements affecting the efficiency of the signal enhancement were examined. Obtained results showed that with increasing size of AuNPs the magnitude of the reflectivity shift is larger, what is in a good agreement with the theoretical divagations (weaker effect of diffusion-limited mass transfer allow for higher mass binding to the surface). Moreover, in order to prevent fouling on the sensor surface by the constituents present in analyzed milk samples, the gold surface of the sensor chip was modified and different surface architecture were tested and compared (antifouling polymer brushes and self-assembled monolayer (SAM) using a mixture of thiols). Complete resistance to the non-specific interactions was observed for coating with p(HEMA) brushes resulting in two times lower LOD compared to that on thiol SAM with PEG groups. The biosensor for AFM_1 determination allowed for highly sensitive detection in milk with an excellent precision (the average calculated CV was below 4%), limit of detection of 18 $pg\,mL^{-1}$ for p(HEMA)

brushes and 38 pg mL^{-1} for thiol SAM and with the analysis time of 55 min. It is worth highlighting that it is the first time that an SPR chip modified with such polymer brushes was used for real time detection of a small target antigen opening a new avenue for highly precise analysis.

In the case of wine samples tested for OTA detection, a simple but very effective pre-treatment procedure was successfully applied. It was proved that the addition of the 3% of the binding agent poly(vinylpyrrolidone) (PVP) to red wine completely reduces non-specific interactions by binding polyphenolic compounds (which may be responsible for inactivation of antibody and blocking the sensor surface) through hydrogen bonding, making their elimination easier. Moreover, in this study, the authors evaluated the influence of AuNPs on signal enhancement and thereby biosensor sensitivity. For this purpose two assays were performed: with and without implementation of NPs. Obtained results allowed for OTA detection at concentrations as low as 0.75 ng mL^{-1} however, its limit of detection was improved by more than one order of magnitude to 0.068 ng mL^{-1} by applying AuNPs as a signal enhancer.

Proposed biosensors offer vast range of advantages such as high sensitivity (at pg or ng levels), short analysis time (55 min) in comparison to for example, ELISA which require multiple steps that translates to prolonged analysis time, possibility for online monitoring, characterization of binding kinetics, low consumption of primary antibody (cost reduction), excellent antifouling surface and simple pre-treatment procedure.

(II) Biosensor for ochratoxin A detection utilizing quartz crystal microbalance (based on [213].

Successful and detailed studies on mycotoxins detection with mentioned above SPR spectroscopy became a base for further research and development in this field. The author used previously optimized and well characterized parameters of the assay (with AuNPs implementation) for OTA detection in wine, to create another novel biosensor however, this time utilizing the quartz crystal microbalance with dissipation as a readout device. The combination of indirect competitive assay and AuNPs for signal amplification with QCM-D gave a straightforward tool, which can simultaneously measure frequency (Δf) and dissipation (ΔD) changes resulting in information not only about the sensitivity but also about the mass attached to the sensor surface as well as viscoelastic properties and the hydration state of the film. Therefore, obtained results indicated the formation of a homogeneous and fairly rigid

film and let for sensitivity parameters estimation. A linear detection range of 0.2–40 ng mL^{-1} has been achieved with an excellent LOD of 0.16 ng mL^{-1}, which is one order of magnitude lower than LOD specified by European Union legislation concerning the limit of OTA in food.

(III) Electrochemical biosensors for mycotoxins detection.

SPR and QCM are two very powerful techniques receiving an increasing attention in the field of biosensing systems. However, beside a number of advantages, as every other method, they exhibit some drawbacks and limitations (e.g. high-priced equipment, lack of sensitivity when monitoring low molecular weight molecules, problems with mass transport etc.). Among different approaches used for analysis of toxins, electrochemical detection seems especially promising due to high sensitivity, feasibility of low cost, compatibility with portability and miniaturization. Therefore, this part of thesis is based on the on a competitive immunoassay that uses a secondary antibody conjugated with an enzyme (alkaline phosphatase) as a tag for the voltammetric detection of mycotoxins (OTA and AFM_1) using modified gold screen printed electrodes (AuSPE). The analytical signal of presented biosensor was proportional to the toxin concentration in a wide working linear range, showing an excellent limit of detection of 15 ng mL^{-1} for OTA in red wine and 37 pg mL^{-1} for AFM_1 in milk. Additionally, AuSPE modified with self-assembled monolayers based on different types of alkanethiols (long and short chains) were tested and compared in terms of electron transfer resistance. As expected, the reduction and oxidation reactions were blocked after the modification of the Au surface with long chain thiols and therefore, MPA (3-mercaptopropionic acid) was chosen for SAM formation allowing for electron transfer occurrence.

Table 6.1. Characterization of presented in this thesis OTA and AFM_1 biosensors utilizing different detection techniques: SPR, QCM and electrochemistry.

	SPR		QCM	Electrochemistry	
	AFM_1	**OTA**	**OTA**	**AFM_1**	**OTA**
LOD [ng mL^{-1}]	0.04	0.07	0.16	0.04	15
Time	≈55		≈55	≈80	
Cost	$$$		$$	$	
Volume	> 1 mL		> 1 mL	40 µL	
Regeneration	+++		++	-	
Online monitoring	yes		yes	no	
Limitations	Small analytes, non-specific adsorption		Temperature, non-specific adsorption	Need of a label	
Sample consumption	++		++	+	

Table 6.1 summarizes the most important properties of presented biosensors utilizing three different detection techniques: SPR, QCM and electrochemistry. As it can be seen, every methodology has its strong and weak points what will be further discussed in detail. All proposed approaches exhibit a very high sensitivity with LOD at least one order of magnitude lower than the one specified by European Union. This parameter describes the smallest amount of target molecules adsorbed onto the sensor that can be detected however, is also highly dependent on the matrix effect and interferences resulting in some non-specific adsorption of food components to the surface. This can lead to the higher background response, influence on the accuracy, reproducibility, linearity and sensitivity and therefore, causing erroneous quantitation. Consequently, is it necessary to study those effects and employ efficient methods for their minimization, prior the development and validation of any new method in order to obtain reliable and satisfying results. One of the limitations of QCM and SPR devices is non-specific adsorption of molecules present in real matrices, since they are both mass sensitive, any molecule able to bind or to be adsorbed on the surface is a potential interference. Due to the fact that wine is a complex alcoholic beverage and it contains constituents such as polyphenols, which can bind with proteins by hydrophobic interactions, hydrogen bonds and covalent bonds as well as may cause inactivation of

antibodies what results in unspecific sorption that blocks the sensor surface and strongly influence on the sensitivity of the detection. In order to evaluate the unspecific sorption of constituents in wine to the surface, in the presented work, a simple pre-treatment with a binding agent poly(vinylpyrrolidone) (PVP) was applied and SPR observation of surface mass density change was carried out. Obtained results revealed the excellent ability of PVP to bind polyphenols through hydrogen bonding making it easier to eliminate them from the solution and therefore minimize the possibility of their influence on sensitivity. In case of milk, which is also a complex biological fluid composed of constituents including whey proteins (particularly β-lactoglobulin), lipids and calcium phosphate, which are involved in the fouling process through interacting mechanisms (denaturation, aggregation, local supersaturation), the use of surfaces coated with p(HEMA) showed excellent resistance to the non-specific interactions. This phenomenon might be due to a water barrier resulting in minimization of hydrophobic effect with the lipids components form milk as well as to entropic barrier resulting from the brush architecture. Due to the large variety of matrices and to the unpredictable effect that they might have on the final results, it is impossible to propose a uniform protocol for ME elimination however, it must be considered, tested and minimized/eliminated to ensure acceptable and credible quantitative results. It is also important to remember about the methods which can prevent mycotoxins occurrence (see. Section 1.2.5) during pre- and post-harvesting. This can significantly reduce/eliminate the content of toxic molecules, simplify further detection, save time dedicated for additional pre-cleaning, lower general costs and therefore, simplify the whole procedure of analysis.

Considering SPR and QCM - techniques based on different principles however, both being mass sensitive devices, estimated LOD diverge from each other. LOD is determined not only by the mass sensitivity but also how precisely the sensor signal can be measured (frequency in QCM and angular shift in SPR). For QCM generally LOD is about 2 ng cm^{-1} when in case of SPR, this parameter is usually at the level of 0.1 ng cm^{-2}, which is significantly better than that of QCM. Moreover, the sensing area of QCM is typically around 1 cm^2 whereas for SPR is determined by the size of illumination spot and usually is smaller than 10^{-5} cm^2. Therefore, the LOD defined as a total detectable mass will be always on much lower level for SPR. Moreover, any external pressure applied to the QCM may reduce the oscillation of the crystal and destroy the sensing performance. This problem can be overcome by increasing the cell volume at the expense of sample consumption. The level of sensitivity of SPR is comparable

to the one obtained from electrochemical measurements, which are able to detect every single electron behavior involved in electrochemical reaction what can affect on recorded signal.

Moreover, SPR measures the changes in the refractive index of material binding to the sensor surface but is also dependent on the optical properties of the used materials and therefore, small chemical compounds (with low molecular weight, such as mycotoxins) that possess inadequate mass to cause significant changes in the refractive index, are a limiting factor for this methodology. This problem can be overcome by the use of very high concentration of active immobilized ligand however, at such high concentration accurate kinetic analysis is not possible because of mass-transport limitations. On the contrary, the most applied principle of detection in acoustic sensing for biochemical applications is based on mass (gravimetric) properties and it is, therefore, independent of the optical properties of the materials, allowing to perform studies over a great variety of surfaces (4). On the other hand, QCM is sensitive for temperature changes, as it is easily disrupted by internal stresses caused by temperature gradients (low and high with respect to room temperature, ~25 °C) what can cause undesirable frequency shifts in the crystal, decreasing its accuracy.

Another aspect that has an influence on good biosensing system creation is time. In presented study, the analysis based on SPR and QCM offered desirable rapid readout within 55 min. In the both techniques the analyte was transported by a flow system to the surface limiting the effect of the diffusion-limited mass transport. As in the electrochemical set-up a droplet format was chosen in which no active mixing or mass-transport occurs, the detection time was extended to 80 min. Conventional analytical methods for mycotoxins (TLC, HPLC, GC etc.) employ solid phase column cleanup of extracts and immunoaffinity techniques to remove interferences to improve the measurement and therefore, yield results within hours or days. Rapid methods presented in this thesis are less expensive, easier to use and can be moved to an on-site environment. Nevertheless, the strategy involving the droplet formation, beside aforementioned drawback, offers also an improvement in the field of samples volume and thus, overtops two other approaches. Unlike SPR and QCM, where more than 1 mL of reagents was necessary for the assays performance, electrochemical detection based on a droplet, dramatically reduces this amount - it required only 40 µL of the solution. For biosensing applications, sensor chips have to be modified with an appropriate layer of ligand or a layer of matrix to reduce non-specific adsorption. The methodology for both QCM and SPR are similar due to the fact that the most commonly used platforms utilize gold-coated surfaces. However, for in-situ or real-time pre-functionalization, QCM requires more reagents

because of its larger surface area and cell volume. In a typical QCM, the flow-cell volume is 40 µL and the flow rate vary from 50 to 200 µL min^{-1}, while in SPR measurements are carried out usually with a flow rate of 5-50 µL min^{-1} in a 1 µL flow-cell. Therefore, when for example the sample consumption in SPR for a single injection of 5 min at 50 µL min^{-1} is 250 µL, the QCM has to run at 350 µL min^{-1}, which means 2.2 mL for one injection.

Next issue worth consideration pertain costs. SPR and QCM are relatively expensive methodologies requiring very sophisticated equipment. The main distributors of SPR technology - Biacore Company and Q-Sense - Biolin Technologies, in case of QCM, offer highly sensitive tools for various applications supported with detailed information about their capabilities. Nevertheless, beside the high price of the instruments itself, all additional items like sensing chips, fitting to the desirable device which design, project and properties are usually encompassed with a trade secret, increase the total costs of those approaches. The Biocore instrument is priced at 112.000$, the chip costs about 200$, with one chip capable of performing 100 analyses. A QCM system, although much cheaper (approximately 13.000$) offers sensing elements (e.g. crystals) for 25$ but allowing up to 25 analyses per surface [369]. However, in this study, SPR measurements were performed in the home-build system with the home-made sensors what significantly reduced the expenses and offered more flexibility. Regarding experiments carried out in QCM, the commercial instrument was used, what resulted in additional costs due to the need of purchasing chips which would meet all expectations (size, design, properties) of the provided QCM device to assure reliable results. As opposed to SPR and QCM, electrochemical analysis, in general, belongs to inexpensive approaches which do not require complicated instrumentation and offer easy and cheap possibility of creation home-build systems. When talking about costs, it is also worth to mention about the regeneration of the sensor chip which has a significant influence on the total expenses (lower consumption of reagents, lower amount of sensor chips). This process is based on the removal of bound analyte from the surface after an analysis without destroying the ligand. The successful regeneration after each detection cycle was obtain for SPR and QCM measurements allowing for the reuse of the sensor for further experiments. After the regeneration step the SPR and QCM signals return almost to the original baseline level (negligible increase of 0. 1%) indicating complete breakdown of the immuno-complex interaction and revealing fully reversible assay cycle. The immobilized antigen was still present at the sensor surface and hence, the assay procedure for the next cycle started with the injection of the primary antibody. In the case of SPR the sensors could have been regenerated

more than 20 times without a change in its performance. It means that once the antigen is immobilized on the surface, 20 analysis per day could be done. For the QCM measurements, after six cycles of regeneration, crystals did not show the reproducible results and therefore, were cleaned with piranha solution to remove all adsorbed on the gold surface materials including SAM. After such a treatment, the gold surface could be reused – a new SAM could be created followed by a fresh antigen solution immobilization. The variations between the number of possible regenerations in QCM and SPR can be assigned to the quality of gold (differences in a crystallographic structure) used for chip formation. The same protocol was applied to screen printed electrodes however, the final result was not satisfactory, as expected. SPEs are meant to be disposable and for only one use. There is no concrete methodology on the reusability of the electrodes and for better results achievement; the provider (DropSens) recommends their utilization only for one measurement. Probably due to this reason, at least the price of commercially available SPEs is relatively low and affordable for everyone.

The use of SPR and QCM technologies has another advantage - provides a simple but very useful way to observe how different biomolecules interact in real time allowing also for binding kinetics determination. Moreover, it enables the control of every step of performed assay, giving online information about bindings at exactly the same time when the sample is injected. Thanks to this fact, researchers know which parts of the tested protocols are working perfectly well and which could be improved. In electrochemical measurements, only the final step is recorded. Therefore, the determination of any problem/deviation that may occur and influence on the assay sensitivity is much more complicated and thus, is time-consuming.

To sum up, in all presented in this thesis approaches a number of advantages significantly exceeded drawbacks. Nevertheless, the choice of the best detection system depends on varied factors (e.g. purpose, budget etc.) and the final decision must be carefully made after a detailed analysis of every single advantage and disadvantage of considered methodology. However, there are still open ways for further improvement and elimination of limitations in offered biosensors. For example, the LOD could be reduced by increasing the number of toxin per BSA molecules or by increasing the size of the gold nanoparticles, in case of SPR and QCM techniques. Another aspect worth consideration concerns surface modification and the development of new antifouling materials in order to reduce/eliminate matrix effects and non-specific adsorption of constituents present in real samples. Moreover, the performance of combined techniques (e.g. SPR with fluorescence detection) may result in higher sensitivity as well as the replacement of antibodies with very promising technologies based on aptamers

or molecularly imprinted polymers can have a significant influence on this parameter. Screen printed electrodes could be exchanged with microelectrodes where the signal to noise ratio is enhanced due to lowered capacitive current and increased rates of mass transfer to the electrode surface. The time and reagents consumption could be decreased by implementation of microfluidics, where smaller volume of the samples are required what further would reduce the time as well as improve the efficiency of binding.

Those are only a couple of examples which can be used for further development in the field of food safety and control. Nevertheless, all most desirable aspects of a good biosensor such as high sensitivity, low costs, short analysis time and simple but effective cleaning-up technique were obtained in this thesis and supported with detail characterization, making proposed approaches an important and very promising tools for widespread biosensing applications.

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Résumé

07.2014 – 06.2017	**Ph.D. Studies** – University of Regensburg / University of Applied Sciences Jena Department: Analytical Chemistry, Bio- and Chemosensors Supervisors: Prof. Antje Bäumner, prof. Karl-Heinz Feller Subject: "Biosensors for toxins detection in food"
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03.2015 – 09.2015	**Visiting Scientist** – Austrian Institute of Technology, Vienna, Austria. "Plasmonic biosensors for detection of Aflatoxin M1 and Ochratoxin A"
08.2012 – 06.2014	**Laboratory assistant -** Institute of Physical Chemistry PAS, Warsaw, Poland. "Electrocatalysis – from single nanoparticle to their films."
09.2013 – 12.2013	**Internship** – National Institute for Material Sciences, Tsukuba, Japan. "Study of the thin films of phosphorylcholine-modified chitosan and

hyaluronic acid at various pH values, SPR spectroscopy and QCM."

09.2011 – 06.2012 – **Internship** – Institute of Physical Chemistry / Laboratory of High Pressure PAS, Warsaw, Poland.
"SERS platforms for molecular diagnostics."

03.2011 – 08.2011 – **Internship** – Warsaw University of Technology, Warsaw, Poland.
"Physico-chemical analysis of highly energetic materials."

09.2010 – 10.2010 – **Internship** – BaKaChem Sp. z.o.o, Warsaw, Poland.
"Preparation of explosives, blasting and demolition works."

09.2009 – 10.2009 – **Internship** – Central Institute of Labor Protection – National Research Institute, Department of Chemical and Dust, Warsaw, Poland.
"Environmental monitoring, analysis of harmful airborne chemical substances at the work place utilizing gas chromatography and high-efficiency liquid chromatography methods."

Publications:

- **Karczmarczyk A.**, Haupt K., Feller K-H., *Development of a QCM-D biosensor for Ochratoxin A detection in red wine*, Talanta 2017, 193-197.
- Dubiak Szepietowska M.**, Karczmarczyk A.**, Feller K-H., Winckler T., *A cell-based biosensor for nanomaterials cytotoxicity assessment in three dimensional cell culture*, Toxicology 2016, 370, 60-69.
- **Karczmarczyk A.**, Reiner-Rozman C., hageneder S., Dubiak Szepietowska M., Dostalek J., Feller K-H*., Fast and sensitive detection of Ochratoxin A in red wine by nanoparticle-enhanced SPR*, Analytica Chimica Acta 2016, 937, 143-150.
- **Karczmarczyk A.**, Dubiak Szepietowska M., Vorobii M., Rodriguez-Emmenegger C., Dostalek J., Feller K-H., *Sensitive and rapid detection of aflatoxin M1 in milk utilizing enhanced SPR and p(HEMA) brushes*, Biosensors and Bioelectronics 2016, 81, 159-165.
- Dubiak Szepietowska M.**, Karczmarczyk A.**, Jönsson-Niedziółka M., Feller K-H., Winckler T., *Development of complex-shaped liver multicellular spheroids as a human-based model for nanoparticle toxicity assessment in vitro*, Toxicology and Applied Pharmacology 2016, 294, 78-85.
- **Karczmarczyk A.**, Celebanska A., Nogala W., Sashuk V., Chernyaeva O., Opallo M., *Electrocatalytic glucose oxidation at gold and gold-carbon nanoparticulate film prepared from oppositely charged nanoparticles*, Electrochimica Acta 2014, 117, 211-216.

Conferences:

- **BIOSENSORS 2016,** Gothenburg (Sweden), poster contribution: "Ultrasensitive biosensors based on enhanced SPR for detection of AFM1 in milk and OTA in red wine"
- **EUROPT[R]ODE XII – Optical, Chemical Sensors and Biosensors 2016,** Graz (Austria), poster contribution: "Ultrasensitive biosensors based on enhanced SPR for detection of AFM1 in milk and OTA in red wine"

- **11th Workshop on Biosensors & Bioanalytical Microtechniques in Environmental, Food & Clinical Analysis 2015**, Regensburg (Germany), poster contribution: "Ultrasensitive biosensors based on enhanced SPR and nano-ELISA for toxins detection"
- **65th Lindau Nobel Laureate Meeting Interdisciplinary: Physiology/Medicine, Physics, Chemistry 2015**, Linadu (Germany)
- **ElecNano6 – "Electrochemistry at the nanoscale from basic aspect to applications" 2014,** Paris (France), poster contribution: "The effect of functionalities on electrocatalysis at gold nanoparticulate film electrode prepared from oppositely charged particles"
- **9th ECHEMS Meeting – "Electrochemistry in Particles, Droplets, and Bubbles" 2013,** Łochów (Poland), poster contribution: "Electrocatalytic activity of gold nanoparticles in films or suspensions toward direct glucose electrooxidation"
- **Faraday Discussion 164 – "Electroanalysis at the Nanoscale" 2013**, Durham (UK), poster contribution: "Electrocatalytic activity of gold or carbon nanoparticulate films towards direct electrooxidation of glucose or ascorbic acid"
- **ElecNano5 – "The nanoscale and electroanalysis: surface nanostructuration, nanobiological system, coupled techniques, microsystems" 2013**, Bordeaux (France), poster contribution: "Electrocatalytic glucose oxidation at gold nanoparticles: films vs suspensions"
- **SAMOSS ITN Project Meetings** (oral presentations):
    - Madrid, Spain **2014** – "Development of multi-receptor assay/array for food analytics"
    - Groningen, The Netherlands **2015** – "Biosensors based on enhanced SPR and nano-ELISA for toxins detection in food"
    - Beer-Sheva, Israel **2015** – "Biosensors for toxins detection in food"
    - Vienna, Austria **2016** – "*French style* milk analysis"

Workshops:

- "Biosensors – application workshop", **Biosensor srl**, Rome, Italy (10.2016)
- "Optical biosensors and biochips", **Austrian Institute of Technology**, Vienna, Austria (06.2016)
- "Light microscopy and Fluorescence techniques", **Ben-Gurion University of the Negev**, Beer-Sheva, Israel (10.2015)
- "Microfluidics", **University of Groningen**, Groningen, The Netherlands (05.2015)
- "Synthesis and characterization of new sensing materials", **Universidad Complutense de Madrid**, Madrid, Spain (07.2014)
- "Materials Phenomena at Small Scale", **National Institute for Material Sciences**, Tsukuba, Japan (11.2013)

Languages:

- Polish (mother tongue)
- English (full professional proficiency)
- German (elementary proficiency)

Other:

- Certificate of Internal Auditor Quality management systems PN-EN ISO 9001:2009
- Driver's license cat. B
- Computer skills: MS Office , Origin, Inkscape, Chemsketch

Eidesstattliche Erklärung

Ich erkläre hiermit an Eides statt, dass ich die vorliegende Arbeit ohne unzulässige Hilfe Dritter und ohne Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe; die aus anderen Quellen direkt oder indirekt übernommenen Daten und Konzepte sind unter Angabe des Literaturzitats gekennzeichnet.

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