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Innovative Ochratoxin A (OTA) extraction platforms using OTA-binding proteins

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ABSTRACT

Wine is a widely consumed product that is often associated with contaminations of toxic metabolites called mycotoxins. The most important mycotoxin associated to wine is ochratoxin A (OTA), and its detection usually involves the clean-up of samples with immunoaffinity columns (IAC) and quantification by HPLC coupled with fluorescence detection (FL). However, the several drawbacks associated with the use of the IAC led to the search for alternative clean-up methods. Thus, in this work, new platforms for OTA clean-up from wine were developed, based on proteins whose affinity towards OTA makes them suitable candidates to mimic the binding properties of the antibodies used in the IAC method.

In a first approach, a protein with high affinity towards OTA, was used to develop a new solid phase extraction (SPE) method for the extraction of the mycotoxin from wine and subsequent quantification by HPLC-FL. The capture of OTA by the columns constructed with agarose-immobilized OTA binding protein was optimized to allow the full recovery of OTA in wine, and the method was further validated by the evaluation of various parameters such as recovery rates, selectivity and limits of detection (LOD) and quantification (LOQ). The developed method was selective enough for a reliable determination of OTA in wine, presenting recovery rates superior to 98% and LOD and LOQ of 0.02 and 0.05 μg L^{-1}, respectively. Furthermore, the performance of the developed method revealed no significant differences in relation to the IAC method in concentrations up to 2 μg L^{-1} of OTA. In comparison with other conventional SPE methods reported in the literature, the developed method has proved to be suitable to be employed in the determination of OTA in wine.

In a second approach, the domain where lies the primary binding site of OTA in the OTA-binding protein used in the first approach was evaluated as OTA ligand for developing OTA extraction platforms based on this domain. For that, the domain was recombinantly produced, fused to a 6xHis tag, with and without the thioredoxin (TrxA) solubility partner, in two *E. coli* strains, BL21 (DE3) and Origami 2 (DE3). Soluble proteins, with and without TrxA, were produced by both strains, but the Origami strain provided higher yields of production (18.7 and 23.4 mg per litre of culture, respectively). In addition, differences observed in the affinity of the proteins for the nickel resin in the purification suggested that the structures acquired by the recombinant proteins produced in each strain were different. Furthermore, the fact that fusion proteins were less prone to degradation suggested that TrxA contributed for their stability. Studies of fluorescence spectroscopy revealed that only the recombinant proteins produced by the Origami strain were capable of interacting with OTA, thus indicating that these proteins were functional. The ability of the proteins produced from this strain, with and without TrxA, immobilized in nickel via the 6xHis tag, to capture the mycotoxin in buffer solutions was evaluated. SPE columns constructed with the nickel-immobilized recombinant proteins did not show the ability to effectively capture OTA. On the other hand, incubation assays performed in eppendorfs allowed decreasing OTA in solution up to 54 and 63%, respectively for immobilized proteins, with and without TrxA. These results open perspectives for the development of OTA extraction platforms based on the recombinant domain of this OTA-binding protein with matrixes less expensive than the agarose used in the first approach by means of specific purification tags.

RESUMO

O vinho é um produto largamente consumido que está frequentemente associado a contaminações com metabolitos tóxicos denominados de micotoxinas. A micotoxina mais importante associada ao vinho é a ocratoxina A (OTA), e a sua deteção envolve normalmente um passo de concentração das amostras com colunas de imunoafinidade (IAC) e deteção por HPLC acoplada com deteção por fluorescência (FL). Contudo, as diversas desvantagens associadas ao uso das IAC levaram à procura de métodos de concentração alternativos. Assim, neste trabalho, foram desenvolvidas novas plataformas de concentração de OTA do vinho, baseadas no uso de proteínas cuja afinidade para a OTA as torna candidatos adequados para mimetizar as propriedades de ligação dos anticorpos usados no método IAC.

Numa primeira abordagem, uma proteína com alta afinidade para a OTA foi usada para desenvolver um novo método de extração em fase solida (SPE) para extrair esta micotoxina do vinho e subsequente quantificação por HPLC-FL. A captura de OTA por colunas construídas com a proteína em estudo imobilizada em agarose foi otimizada de forma a permitir uma recuperação total da micotoxina presente no vinho, e o método foi posteriormente validado através da avaliação de parâmetros como taxas de recuperação, seletividade e limites de deteção (LOD) e quantificação (LOQ). O método desenvolvido foi suficientemente seletivo para permitir uma quantificação confiável de OTA no vinho, apresentando taxas de recuperação superiores a 98% e um LOD e LOQ de 0.02 e 0.05 μg L^{-1}, respetivamente. Em adição, o desempenho do método desenvolvido não apresentou diferenças significativas em relação ao método das IAC em concentrações até 2 μg L^{-1} de OTA. Em comparação com outros métodos convencionais de SPE reportados na literatura, o método desenvolvido revelou ser adequado para aplicação na determinação de OTA no vinho.

Numa segunda abordagem, o domínio da proteína usada na primeira abordagem que contém o principal local de ligação da micotoxina, foi avaliado como ligando da OTA para o desenvolvimento de plataformas de extração de OTA baseadas nesse domínio. Para isso, este foi produzido de forma recombinante, em fusão com um 6xHis tag, com e sem tiorredoxina (TrxA) como parceiro de solubilidade, em duas estirpes de *E. coli,* BL21 (DE3) e Origami 2 (DE3). O domínio proteico solúvel, com e sem TrxA, foi produzido por ambas as estirpes, mas a estirpe Origami obteve maiores rendimentos de produção (18.7 e 23.4 mg por litro de cultura, respetivamente). Em adição, as diferenças observadas na afinidade das proteínas para a resina de níquel na purificação sugeriram que as estruturas adquiridas pelas proteínas produzidas em cada estirpe eram diferentes. Além disso, o facto de que que as proteínas de fusão se mostraram menos suscetíveis a degradação sugere que a TrxA contribuiu para a sua estabilidade. Estudos de espectroscopia de fluorescência revelaram que apenas as proteínas recombinantes produzidas pela estirpe Origami foram capazes de interagir com a OTA, indicando assim que estas se encontravam funcionais. A capacidade das proteínas produzidas por esta estirpe, com e sem TrxA, imobilizadas em níquel a partir do 6xHis tag, para capturarem a micotoxina em soluções tampão foi avaliada. Colunas SPE construídas com estas proteínas recombinantes imobilizadas em níquel não mostraram a capacidade de capturar efetivamente a OTA. Por outro lado, os ensaios realizados em "eppendorfs" permitiram reduções de OTA em solução até 54 e 63%, respetivamente para as proteínas imobilizadas com e sem TrxA. Estes resultados abrem perspetivas para o desenvolvimento de plataformas de extração de OTA baseadas neste domínio da proteína estudada com matrizes menos caras que a agarose usada na primeira abordagem por meio de tags de purificação específicos.

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LIST OF ABBREVIATIONS

6xHis tag	Tag of six histidines
a_w	Water activity
BAC	BSA-agarose column
BDII	Domain II of BSA
bl-BDII	Domain II of BSA produced from *E. coli* BL21 (DE3)
bl-TrxBDII	Domain II of BSA fused to the thioredoxin protein produced from *E. coli* BL21 (DE3)
cBDII	Cleaved domain II of BSA
BSA	Bovine serum albumin
dH_2O	Distilled water
ddH_2O	Distilled deionized water
EDTA	Ethylenediamine tetraacetic acid
FL	Fluorescence detection
gor	Glutathione reductase gene
HPLC	High performance liquid chromatography
HSA	Human serum albumin
IAC	Immunoaffinity columns
IARC	International Agency for Research on Cancer
IMAC-Ni	Immobilized nickel affinity chromatography
IPTG	Isopropyl β-D-1-thiogalactopyranoside
kDa	Kilodaltons
LB	Luria-Bertani broth medium
LOD	Limit of detection
LOQ	Limit of quantification
MIP	Molecularly imprinted polymer
Mw	Molecular weight
OIV	Organization of Vine and Wine
OD_{600}	Optical density at 600 nm
ori-BDII	Domain II of BSA produced from *E. coli* Origami 2 (DE3)
ori-TrxBDII	Domain II of BSA fused to the thioredoxin protein produced from *E. coli* Origami 2 (DE3)
OTA	Ochratoxin A

OTA^{-1}	Mono-anion form of OTA
OTA^{-2}	Di-anion form of OTA
PES	Polyethersulfone
pI	Isoelectric point
PMSF	Phenylmethylsulfonyl fluoride
RSD	Relative standard deviation
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate–polyacrylamide gel electrophoresis
SELEX	Systematic evolution of ligands by exponential enrichment
SOC	Super optimal broth with catabolite repression
SPE	Solid-phase extraction
SPME	Solid-phase microextraction
SPPS	Solid-phase peptide synthesis
TEV	Tobacco Etch Virus
TLC	Thin layer chromatography
TrxA	Thioredoxin protein
trxB	Thioredoxin reductase gene
TrxBDII	Domain II of BSA fused with the thioredoxin protein
uH2O	Ultrapure water
WHO	World Health Organization

CHAPTER I.

INTRODUCTION

1.1 Fungi

Fungi are a widely spread and diverse group of organisms composed of more than 100 000 species that have been already described, although it is estimated that, in total, there may exist as many as 1.5 million species [1]. They present a wide variety of forms, ranging from multicellular moulds and mushrooms to unicellular yeasts. Moulds body, also called *thallus*, consists of long filaments called *hyphae* that are joined together in a loosely organized mass designated by *mycelium*, which is embedded in either decaying organic matter, soil or tissues of living organisms [2]. Fungi are chemoheterotroph organisms that are fed by secreting extracellular enzymes into their environment. Those enzymes digest polymeric materials, such as polysaccharides or proteins into monomers that are further assimilated as sources of carbon and energy. They play a very important role in the food chain since they can live as decomposers, digesting dead animal and plant materials, but can also be parasites of plants or animals, using the nutrients from their living cells [3]. In fact, although often not noticed, fungi are involved in our daily life for centuries in processes such as the fermentation of, for example, beer, wine and bread. More recently, as a result of the biotechnological development, fungi have been used as producers of antibiotics and other pharmaceuticals, enzymes, alcohols and organic acids [4]. However, despite their importance, fungi can also be very harmful and cause big impacts in economics, food industry and in human and animal health.

1.1.1 Fungal diseases: Mycoses and Mycotoxicosis

Of the great number of fungal species described, only about 400 species have shown to be pathogenic to humans or domestic animals [5]. However, despite the fact of being less frequent than bacterial and viral diseases, fungal diseases are still a worldwide health problem. They can be classified as mycoses or mycotoxicosis [6]. Mycosis are caused by a fungal growth in the host's tissues [5]. On the other hand, mycotoxicosis occurs after exposure to fungal toxic metabolites, called mycotoxins, through ingestion, inhalation or absorption through skin [7,8]. Mycotoxicosis symptoms are dependent on many factors, such as the type of mycotoxin, the concentration and time of exposure, the synergic effects when there is a combination of different mycotoxins and also on the age, health status and gender of the affected person [6,9].

1.1.2 Fungal spoilage of food

Despite the development of new and advanced technologies, a large portion of the food supply of our society is still lost due to spoilage or wasted. Food losses occur from various causes, such as from insect or rodent damage, chemical and physical spoilage and also microbial spoilage. The World Health Organization (WHO) estimated that, in developing countries, microbial spoilage ranges from more than 10% in cereal grains to as much as 50% for vegetables and fruits. In fact, it is estimated that, about one-fourth of the world's food spoilage is caused by microbial activity [10,11]. In the specific case of fungi, mostly moulds, due to their ability to use a wide variety of substrates and to their tolerance to low pH values, low water activities and low temperatures, they can be found in a wide range of environments. Since foodstuff have the essential nutrients for fungal growth, fungi often appear and spoil different types of food and feed leading to major economic losses [12,13]. An additional problem caused by food contamination with fungi, is the fact that some species have the ability to produce toxins, designated by mycotoxins, that can be hazardous to human and animal health. These fungi are named mycotoxigenic fungi and belong mainly to four genera: *Aspergillus, Penicillium*, *Fusarium* and *Claviceps* [14].

It is important to emphasize that food contamination with mycotoxins is an additive process, which begins in the field and increases during harvest, drying and storage. During each stage, different fungi can grow and contaminate foodstuff: *Aspergillus* and *Penicillium* can grow at environments with high temperatures and low water activity (a_w) and, due to that ability, they are usually found during the stages of drying and storage of food and feed. On the other hand, fungi belonging to the genus *Fusarium* are most commonly found before or immediately after harvesting [15]. Also typical, is the association of particular fungi with specific agricultural crops or ecological environments. For example, fungi belonging to the genus *Aspergillus* are most commonly found in regions with subtropical and warm temperate climates while fungi belonging to the genus *Penicillium* are very abundant in regions with temperate climates [16].

1.2 Mycotoxins

Mycotoxins can be defined as a group of chemical compounds that are produced by filamentous fungi. These compounds are low molecular weight secondary metabolites, which means, they are not directly involved in fungal growth, development or reproduction [17]. The fact that these toxins occur naturally and affect nearly 25% of the global food and feed crop output, make them a great concern to food and feed safety [18]. Every day, humans are directly and indirectly exposed to mycotoxins since,

besides entering the food chain by contaminating foodstuffs, mycotoxins can also enter the feed chain by contaminating feeds for animals that are destined for human consumption [19].

It is important to note that the conditions for optimum toxin production do not necessarily correspond to those of optimum fungal growth. These conditions are dependent on, for example, fungal strain variation, genetic susceptibility of the host plant or commodity, commodity composition, moisture content, temperature, aeration, stress factors and microbial population [20].

Mycotoxins became known as a potential danger to human and animal health shortly after an outbreak in England of a disease characterized by internal bleeding and liver necrosis, which culminated in the death of over 100 000 turkeys in 1960 [21]. Later in 1969, it was revealed that the disease was caused by the ingestion of peanut meal contaminated with *Aspergillus flavus* and, consecutively, with aflatoxins [22]. Since their discovery, mycotoxins have been found in numerous food matrixes such as corn, wheat, rice, barley, sorghum, nuts and various fruits, quickly becoming a target of intensive research. Currently, more than 300 mycotoxins are already known, however, only some of them are considered a real threat to food safety, such as aflatoxins, ochratoxin A (OTA), fumonisins, zearalenone, trichothecenes, patulin, and ergot alkaloids [23].

As mentioned before, mycotoxins can often appear in food and feed, causing harm to both humans and animals. Upon its ingestion, some of these mycotoxins have shown to be carcinogenic, mutagenic, teratogenic, cytotoxic, neurotoxic, nephrotoxic, immunosuppressive and estrogenic. The severity of these effects is largely dependent on factors such as the quantity ingested, duration of exposure and also on toxic synergies resulting from the simultaneous ingestion of different mycotoxins. The ingestion of high quantities of mycotoxins results in acute toxicity which sometimes leads to death, while the ingestion of very low levels of mycotoxins during long periods of time can lead to the suppression of immune functions and the development of chronic diseases and tumours [24].

1.2.1 Ochratoxin A

Ochratoxins are considered one of the most relevant groups of mycotoxins in food safety. They are secondary metabolites produced by various fungi belonging to the *Aspergillus* and *Penicillium* genera, such as *A. niger*, *A. carbonarius* and *P. verrucosum* [25]. This family of mycotoxins includes three main compounds (ochratoxins A, B, and C) whose chemical structures differ only slightly from each other but possess different toxic potentials. Structurally they consist of a polyketide-derived dihydroisocoumarin moiety linked to a L-β-phenylalanine by an amide bond [26].

Ochratoxin A (OTA) (Figure 1.1) is the most abundant and toxic of the three. This mycotoxin has a crystalline structure with a molar mass of 403.8 g mol^{-1} that can range from colourless to white. Under UV light, it presents different fluorescence behaviours according to various pH conditions: green fluorescence in acid medium and blue fluorescence in alkaline medium [27]. Its pKa values range from 4.2-4.4 and 7.0-7.3 for the carboxyl group of the phenylalanine and the phenolic hydroxyl group of the isocoumarin, respectively [28].

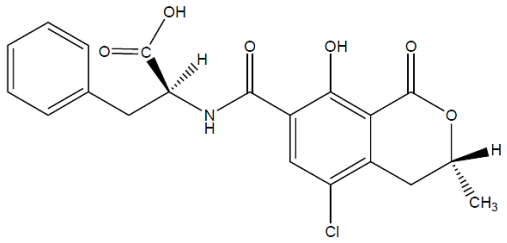

Figure 1.1. Chemical structure of Ochratoxin A (Adapted from Khoury et al. [27]).

It was isolated for the first time in 1965 after discovering that the ingestion of corn contaminated with the fungus *Aspergillus ochraceus* caused the death of several experimental animals [29]. Since then, OTA has been found in several food matrixes such as cereals, corn, wheat, oats, nuts, peanuts, rice, barley, sorghum, cottonseed, coffee beans and some beverages like beer and wine [30,31]. Also, OTA was found in different animal tissues and in human blood and breast milk [32,33].

The problem of food contamination with OTA arises from its high stability. Since it is highly resistant to acid mediums and high temperatures, it is difficult to remove it from contaminated food [27]. Contamination of food with OTA is considered a severe problem since it can deeply affect human health not only after a single heavy exposure but also, and most commonly, after continuous exposure to low doses [34]. Its toxicity is due to its similarity to phenylalanine which makes it a competitive inhibitor to amino acid phenylalanine in reactions catalysed by phenylalanine-t-RNA [35]. This mycotoxin is absorbed in the gastrointestinal tract, mainly in the small intestine and, according to some studies of OTA's toxicokinetics in pigs, rats, chickens and goats, OTA binds to serum proteins and is distributed through the blood, to the kidneys, liver, muscle and fat [36].

According to the International Agency for Research on Cancer (IARC), OTA is classified as Group 2B (possibly carcinogenic agent to humans) [37]. In addition to its carcinogenic properties, it is known to have nephrotoxic, immunosuppressive, genotoxic and teratogenic effects, also acting as an endocrine disruptor [38]. In addition, it is thought to be the cause of the human Balkan Endemic Nephropathy [39].

1.3 Wine

Wine is a widely consumed natural product that possesses proven health benefits and is very important for the world's economy [40]. In fact, according to the International Organisation of Vine and Wine (OIV), in 2017, the worldwide production of wine rounded a value as high as 247 million of hectolitres [41]. It presents a very high complexity and its composition depends on both the grapes used as raw material and on the production procedures [42]. Although its chemical composition varies from wine to wine, it is essentially composed of water, ethanol, glycerol, polysaccharides, different types of acids and phenolic compounds. The phenolic compounds have high antioxidant capacity and are important in terms of wine colour, aroma and acidity. They can be divided into flavonoids (such as flavonols, flavones and anthocyanindins) and non-flavonoids (such as hydroxybenzoic acids, resveratrol and hydrolysable tannins) [43,44].

During the whole winemaking process, each stage can be associated with the occurrence of contaminations, either chemical or biological, that compromise not only the quality of the product but also the consumer's health [45]. Mycotoxins are an example of natural toxic compounds that can contaminate wine and, therefore, require to be controlled and monitored in the product [46].

1.3.1 Wine contamination by ochratoxin A

The most important mycotoxin associated to wine is ochratoxin A [46]. In fact, wine is the second biggest source of OTA intake nowadays, representing about 10% to 15% of OTA daily intake, while cereals are the main source representing 50% of human daily intake of this mycotoxin [27].

The European Commission fixed the regulatory limit of OTA in wine, grape juices and grape beverages at 2 μg L^{-1}, therefore, there has been a growing need to monitor OTA concentration in these beverages [47,48].

In the past decades, studies revealed the occurrence of OTA in wine samples from several countries. OTA's presence in wine is tightly related to the existence of fungal contamination in the vineyards, mainly by fungi belonging to the genus *Aspergillus* [49]. Normally, *A. carbonarius* is considered the most important ochratoxigenic species in grapes since it occurs very commonly and has the ability to produce high amounts of this toxin. Species like *A. niger*, *A. welwitschiae* and *A. tubingensis* are also found in grapes but usually possess lower production levels. Although infrequent, fungi belonging to the genus *Penicillium* have also been found in grapes but their contribution to OTA contamination is still not well established [50].

The growth of ochratoxigenic fungi in vineyards and consecutively grape's contamination with OTA is critical between early veraison and harvest because abiotic and/or biotic damaged grapes allow the easier access of the fungi. Berry damage, which can be caused by insects, fungal pathogens, excessive irrigation and rain damage, increases rot and OTA production in grapes [51].

The spoilage and growth of ochratoxigenic fungi is also dependent of the climate conditions such as temperature and humidity. Different fungal species have different temperature ranges in which production of high OTA levels in grapes occurs. Humidity is also a key point for the contamination [52]. Usually, higher relative humidity leads to maximum amounts of OTA. For instance, rainfall results in increasing humidity, more damaged grapes and, consecutively in higher OTA production [53]. Studies suggest that the geographical region has also an influence on which species appear to contaminate grapes [50]. It has been shown that there is an increase of OTA concentration in wines originating from southern Europe countries such as France, Italy, Greece and Portugal. These countries have warmer climates that favour fungal growth and OTA contamination [54].

In the specific case of Portugal, the climatic conditions of the several winemaking regions are quite diverse, as they may be influenced by the Atlantic and the Mediterranean climate. These different weather conditions determine the variety of grapes cultivated and influence its properties. Surveys show that the highest prevalence of ochratoxigenic fungi was observed in Alentejo region, which has the highest sun exposure during the summer, followed by Douro and Ribatejo regions [55]. In 2010, a study with 60 Portuguese wines showed that OTA was found in 12 samples (9 of red wine and 3 of white wine). One of the white wine samples presented a level of OTA of 2.4 μg L^{-1}, which is above the established limit of 2 μg L^{-1} [56].

At last, the winemaking procedure appears to also be a critical factor when it comes to OTA's contamination in wines. OTA appears in lower concentrations in white wines, followed by rosé wines and finally red wines, that normally have higher OTA contents. This order may reflect a relationship between maceration and the dissolution of OTA in the grape must during the winemaking process of red wines, since the production of white wines usually lacks or has a very limited maceration process [57]. In red wines, the production of ethanol during the initial stages can increase the solubility of OTA contributing to the higher levels of the mycotoxin [58].

In sum, as seen before, OTA has been found in wines produced all over the world and, due to the harmful effects that the ingestion of this mycotoxin can exert in humans, it is necessary to create sensitive and fast analytical methods for the determination of OTA in wine to protect the consumer's health.

1.4 Determination of ochratoxin A in wine

The monitorization of OTA levels in wine is essential to prove that the wines are in agreement with the legal regulations and to guarantee their safety. The analytical methods of OTA detection are usually based on thin-layer chromatography (TLC) or high-performance liquid chromatography (HPLC) coupled with fluorescence detection (FL). The TLC technique is based on the mycotoxin blue fluorescence under UV radiation but its low sensitivity and quantification difficulties render this method obsolete [59]. On the other hand, HPLC-FL is a technique that provides a sensitive, fast and reliable detection of OTA because of the natural fluorescence properties of this mycotoxin [60,61]. Regardless the analytical technique used, the low detection limits and the protection of the HPLC column requires a previous step of sample clean-up. The protocol for OTA determination in wines recommended by the European Commission includes a solid-phase extraction (SPE) clean-up method based on immunoaffinity columns (IAC), and subsequent determination by HPLC-FL [62,63].

1.4.1 Sample clean-up with immunoaffinity columns

The use of immunoaffinity columns in the samples clean-up is based in the use of specific binding agents, named antibodies, that establish a highly selective and reversible interaction with the mycotoxins present in wine, other food matrixes and even biological fluids. These antibodies are usually immobilized in a solid phase, which is further packed in a column. Thus, when the samples are loaded in the columns, the mycotoxin in the food matrix binds to the antibody while the impurities are further removed with water or aqueous solutions, being finally eluted with an organic solvent such as methanol (Figure 1.2) [64,65].

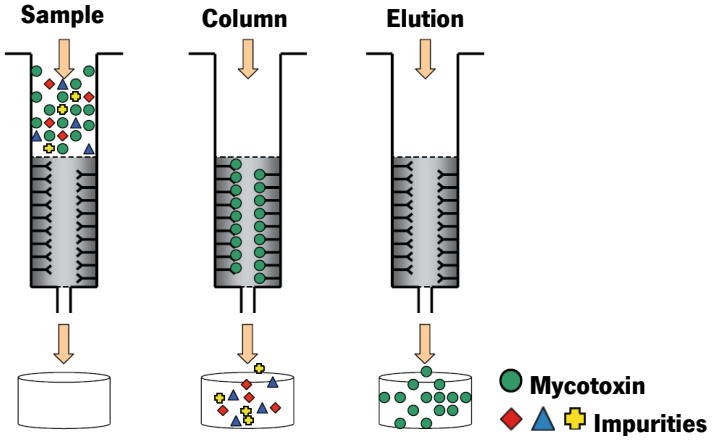

Figure 1.2. Sample clean-up principle of the immunoaffinity columns (Adapted from Zheng e*t al.* [66]).

The high specificity of the antibodies towards the mycotoxin (or group of mycotoxins) allows to obtain clean extracts that can be directly injected into the HPLC column [30]. The celerity and simplicity of this technique also allows its automation in order to overcome problems associated with the loading of samples into the columns and the low recoveries caused by irregular flow-rates [67].

Despite all of the advantages mentioned before, this technique presents several drawbacks, such as the expensive cost associated to the antibodies. Also, the denaturation of the antibodies due to chaotropic salts, acid or basic pH values and organic solvents causes the shortening of the column's life [30].

1.4.2 Alternative sample clean-up systems

All the disadvantages associated with the IAC methods led to the search of alternative methods that could be more suitable, resistant and cost-effective for the clean-up of samples. Aptamers, molecularly imprinted polymers, and binding peptides have been studied as suitable candidates for this purpose due to their potential to mimic the binding properties of the antibodies [34].

1.4.2.1 Aptamers

Aptamers are artificial single strand oligonucleotides (DNA or RNA) with sequences of 20-100 base pairs that can be synthetized against a wide variety of analytes and that possess high binding affinity and selectivity. Their binding properties result from the sequence folding in complex three-dimensional structures, which includes stems, loops, bulges, hairpins, and others that are complementary to the target molecule [68].

In contrast to antibodies, aptamers offer multiple advantages including reduced cost, ease of chemical modification, stability at storage, and others [69]. Despite these advantages, aptamers also present several drawbacks. Their negative charge makes them hydrophilic and easily degraded by nucleases. Furthermore, with some exceptions of modified aptamers, their structural diversity is very scarce due to the existence of only four nucleic acid building blocks. Although this last drawback can be overcome by modifications of the aptamer's backbone and the addition of functional groups to increase their stability, these approaches come with additional costs that are not desirable [70].

Aptamers are produced by a complex procedure called SELEX (Systematic Evolution of Ligands by Exponential Enrichment) which is based in an *in vitro* selection and amplification. Since their discovery, a wide variety of aptamers has been developed and integrated into the development of aptasensors for the detection of mycotoxins, bacterial toxins and cyanotoxins [68,69].

1.4.2.2 Molecularly imprinted polymers

Molecularly imprinted polymers (MIP) consist of another approach designed to mimic antibodies properties towards mycotoxins. MIP are synthetic materials with binding sites that allow the selective recognition of a target molecule [34].

They can be prepared using semi-covalent and non-covalent approaches [69]. MIP synthesis is based on the polymerization of cross-linkers and functional monomers around a template, by interactions with its functional groups through, most commonly, non-covalent interactions. After the removal of the template, which can be the target molecule itself or another related molecule mimicking the target, the result is a highly cross-linked three-dimensional network polymer whose binding sites have complementary shape, size and functionalities towards the template molecule (Figure 1.3) [71]. Just like antibodies, these artificial binding sites present binding reversibility and high selectivity and affinity constants. The mycotoxin's extraction with MIPs usually includes the packaging of the imprinted polymer in an open column or closed HPLC cartridge through which the toxin is loaded and further trapped in the polymer matrix [68].

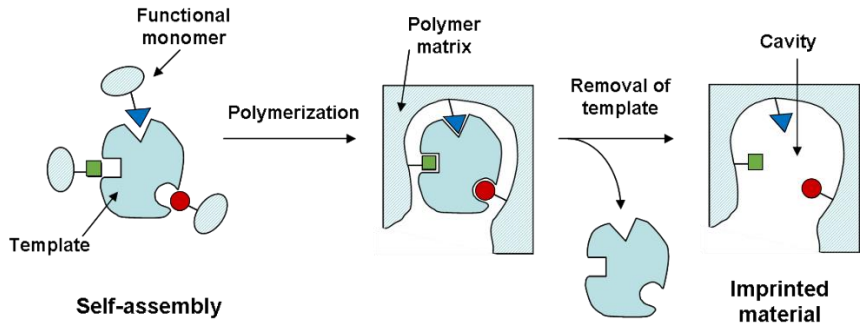

Figure 1.3. The molecular imprinting process. The self-assembly of functional monomers around the template molecule is followed by the polymerization process that culminates in a polymer matrix that is complementary to the template. The template molecule is then removed leaving the polymer ready to be used on recognition processes towards the molecule it was designed for (Adapted from Tadeo [72]).

During these last years, MIPs have proliferated in the scientific community as an affordable, accessible and efficient tool in different areas, including separation and purification processes, biosensors, catalysis and drug delivery [71]. The application of MIPs as sorbents allows the pre-concentration and clean-up of the sample and also the selective extraction of the target analyte, something essential when the sample matrix is complex and quantification can be affected by the impurities [73].

OTA was the first reported mycotoxin to be successfully extracted from wine with a SPE method based on MIPs. Jodlbauer and co-authors developed in 2002 MIPs with specific recognition properties towards OTA using rationally designed analytes as templates mimicking the phenol functionality, the

amide group and the phenylalanine amino acid of OTA's side chain with a correct stereochemistry and arrangement [74]. Later, Maier *et. al.* [75], using those same MIPs, developed a protocol for sample clean-up of red wine that resulted in recoveries of more than 90% of OTA in spiked red wines.

The use of MIPs offers multiple advantages such as their excellent stability under a wide range of temperatures and pH values, low costs and ease of preparation. However, their specificity and affinity are often lower than those for antibodies [69]. Also, sometimes the residual template is not completely removed from the newly formed polymeric matrix and slowly starts leaking during the loading, washing and elution of the sample and is detected at trace levels, representing a significant source of interferences and systematic errors in the target molecule detection [34]. Still, ever since their discovery, MIPs are slowly gaining more popularity and are seen as one of the most promising separation techniques, being considered a suitable alternative method for the extraction of mycotoxins in the food field.

1.4.2.3 Synthetic binding peptides

The use of peptides with the capacity to mimic the recognition and binding properties of antibodies is another possible approach to extract mycotoxins from food. In fact, thanks to their chemical nature and functional groups, it has been already shown that peptides have great potential as capturing agents [30]. They are essential in a wide variety of biochemical processes such as signal transduction, metabolism, cell growth and immune defence. Their molecular recognition is based on non-covalent interactions such as hydrogen bonds, salt bridges, hydrophobic and Van der Waals interactions [76]. Artificial peptides are usually obtained in large libraries of well-characterized peptides that can be synthesized by chemical methods, such a solid-phase peptide synthesis (SPPS), and biological methods such as the phase display technique [77].

In short, binding peptides are a valuable choice to replace the use of antibodies in the sample clean-up steps. Some of the advantages they present include the low costs of amino acids and the fact that their small weight allows them to be more stable in a wider range of buffer solutions with fewer probabilities of losing their activity [30,78]. Also, when these peptides are packed in a column to perform the extraction of mycotoxins, it has been shown that, after their use, they do not lose their molecular recognition properties allowing the recycling of the column [79].

There are many examples of well-succeeded peptides obtained by this technique. Giraudi *et al.* [30], for example, synthesized the hexapeptide Ser-Asn-Leu-His-Pro-Lys that was further used in the clean-up of OTA from wine samples and originated recovery rates up to 98%. However, none of the reported techniques have been able to achieve the affinity and selectivity obtained using the immunoaffinity columns [80].

1.5 Serum proteins

Serum albumins are the most abundant proteins present in the blood plasma of various species. Some of their major roles include the conservation of the plasma's pH value and osmotic pressure, as well as bring substances such as hormones, fatty acids and medicinal drugs in the bloodstream to their target organs [81]. Since very early on, serum proteins and, particularly human serum albumin (HSA) and bovine serum albumin (BSA), have been the target of extensive studies due to the high importance of its physiological functions and to their wide applications and therapeutic potential [82]. Their polypeptide chain is composed of, respectively, 585 and 607 amino acids and possess very similar sequences (76% of identity). Their crystallographic structures are also very similar and consist of three homologous helical domains (I, II and III) that assemble a heart-shaped molecule and are connected by random coil. Each one of these domains is divided in two subdomains (A and B) (Figure 1.4). Studies regarding interactions between various drugs and HSA revealed the existence of two important drug-binding sites named Sudlow's site I and Sudlow's site II which are located, respectively, in the hydrophobic cavities of the subdomains IIA and IIIA [83].

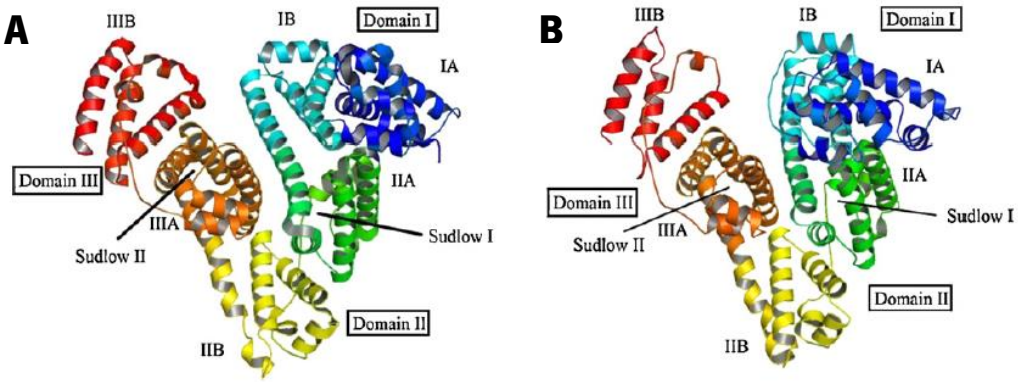

Figure 1.4. Ribbon representations of HSA (A) and BSA (B) with the locations of their subdomains IA (dark blue), IB (light blue), IIA (green), IIB (yellow), IIIA (orange) and IIIB (red) and the indication of their binding sites, Sudlow I and Sudlow II. (Adapted from Khodarahmi, *et al.* [83]).

As mentioned before, studies have shown that OTA binds to albumins with very high affinities immediately after its absorption in the gastrointestinal tract. The strength of this interaction is responsible for long biological half-lifes (from a few days to a month, depending on the species) and, for aggravate the mycotoxin toxicity [26]. The higher binding constants for that interaction were identified for HSA (5.2×10^{6} M^{-1}) [84] and BSA (3.2×10^{5} M^{-1}) [85]. Furthermore, studies regarding the interaction between HSA and OTA revealed that the primary binding site of this mycotoxin is located in the Sudlow's Site I. A secondary OTA binding site is located in the Sudlow's Site II but with much less affinity [86,87]. These

properties make of HSA and BSA a source of OTA binding sites that can be used to develop novel selective clean-up methods for the determination of OTA in food matrixes such as wine.

HSA and its domains have been recombinantly produced in *Escherichia coli* and *Pichia pastoris*, respectively [88,89], but not BSA. Thus, the current work is reporting for the first time the recombinant production of the domain II of BSA in *E. coli* and the study of its interaction with OTA.

CHAPTER II.

AIMS OF THE WORK

Wine is a widely consumed product that is often associated with contaminations of mycotoxins such as ochratoxin A (OTA). The severe health hazards caused by the ingestion of this mycotoxin and the frequency of its occurrence in wines resulted in the imposition of a regulatory limit of 2 μg L^{-1} by the European entities. Ever since, there has been a growing need to monitor OTA concentration in this beverage.

The analytical method most used for the determination of OTA in wine involves the clean-up of samples with immunoaffinity columns (IAC) and detection by HPLC-FL. However, the several drawbacks associated with the use of the IAC method, such as the high cost and short columns life, demands for alternative sample clean-up methods.

Thus, the main objective of this work was to develop novel OTA-extraction platforms based on the BSA protein, which has a remarkable ability to bind OTA, for OTA determination in wine samples.

The first conducted approach was based on the use of the entire BSA protein, obtained from a commercial supplier, and had as specific goals:

(1) Construction of BSA-agarose SPE columns;

(2) Optimization of OTA capture by BSA-agarose SPE in wine;

(3) Validation of the BSA-agarose SPE for OTA determination in wine;

(4) Application of BSA-agarose SPE in the determination of OTA in real wine samples.

The second approach was based on the use of the domain II of BSA (BDII), obtained in this work from a recombinant bacterial host, and emerged as an attempt to build OTA extraction platforms using resins less expensive than the activated agarose used in the first approach. With the long term aim of fusing BDII with tags that allow its binding to more affordable matrixes (e.g. fusion with a cellulose-binding module allows a direct printing of the recombinant protein in cellulose), the current work sought the validation of the capacity of this domain to bind OTA, having a small purification tag (6xHis tag), and had as specific goals:

(1) Cloning, expression and purification of BDII in *Escherichia coli*, with and without the protein thioredoxin (TrxA) as solubility fusion partner, having the 6xHis tag for purification and immobilization;

(2) Study of the interaction of OTA with the obtained recombinant versions of BDII by fluorescence spectroscopy;

(3) Evaluation of the capacity of recombinant BDII proteins immobilized in nickel resin to capture OTA from aqueous solutions;

CHAPTER III.

DEVELOPMENT OF A BSA-AGAROSE SPE METHOD FOR OTA DETERMINATION IN WINE

3.1 Materials and methods

3.1.1 Construction of BSA-agarose SPE columns

In order to study the capacity of BSA to capture OTA, BSA was immobilized in Cyanogen bromide-activated agarose (C9210, Sigma-Aldrich) according to the protocol provided by Sigma and packed in empty solid phase extraction (SPE) cartridges.

Briefly, 1 g of resin was swollen and lactose was removed by performing five incubations of 10 minutes with 40 mL of cold 0.001 M HCl. Between each incubation the resin was gently filtered in 10 mL columns equipped with polyethylene disks of 15 mm of diameter and 30 µm pore size (29925, Thermo Scientific) to remove the supernatant. The resin was then washed with 10 mL of distilled water (dH_2O) and once again, filtered to remove the supernatant. Next, 5 mL of coupling buffer (0.1 M $NaHCO_3$/0.5 M NaCl at pH 8.3) were added to the resin before immediately adding a 10 mL solution of 2.5 mg mL^{-1} BSA in coupling buffer. This solution was incubated with the resin overnight at 4 °C under gentle shaking, filtered and the flow-through was saved to quantify unbound protein (Section 3.1.1.1). The resin was further washed with 5 mL of coupling buffer by incubating it during 30 minutes at room temperature under gentle shaking and, after filtration, the flow-through was also saved. The unreacted groups were blocked by incubating the resin with 0.2 M glycine at pH 8.0 during 2 hours at room temperature. Once again, the resin was filtered, and the supernatant was discarded before being washed with 10 mL of coupling buffer and then with 10 mL of 0.1 M acetate buffer/0.5 M NaCl at pH 4. These sequence of washing steps with coupling buffer followed by acetate buffer were repeated 4 times. Finally, the resin was equilibrated in 0.01 M Tris buffer at pH 7.0 and different resin volumes (0.5, 1 and 1.5 mL) were packed in empty gravity flow SPE columns of 3 mL when necessary. Negative control resins were also prepared following the described protocol but without the addition of the solution of 2.5 mg L^{-1} BSA to the resin.

3.1.1.1 BSA monotorization

The quantity of BSA bound to the resin was determined using a Bradford assay-based method following the microtiter plate protocol from Bio-Rad Protein Assay (N° 500-0002). Briefly, 10 µL of each sample and 200 µL of diluted dye reagent (1 part dye reagent concentrate and 4 parts dH_2O) were pipetted into separate wells of a 96-well microplate. After an incubation of 5 minutes at room temperature, the plates were read in a microplate reader (Citation™ 3 from Biotek) at 595 nm. A calibration curve with concentrations between 0.1 and 0.8 mg mL^{-1} of BSA was prepared (Annex I) and used to convert the

absorbance values into concentrations. Finally, the quantity of BSA immobilized in the resin was calculated using the following formula:

(1) Immobilized BSA = Initial BSA - (BSA after incubation + BSA after washing step)

With "Initial BSA" being the milligrams of protein dissolved in coupling buffer before the incubation with the resin, "BSA after incubation" being the milligrams of protein remaining in solution after the overnight incubation with the resin and finally, with "BSA after the washing step" being the milligrams of protein in solution after the incubation of 30 minutes in coupling buffer.

3.1.2 OTA capture by BSA-agarose SPE in buffer solutions

Before proceeding to the target matrix (wine), the optimum conditions for OTA capture by BSA-agarose SPE were determined. For that, OTA capture was studied in buffer solutions at different pH values (3.5, 7.0 and 8.0) and ionic strength (0.1 and 0.01 M).

Initially, the columns previously packed with 0.5 mL of BSA-agarose were washed under gravity-flow with 5 mL of the buffer under study. Next, 1 mL of buffer containing OTA at a concentration of 10 or 100 μg L^{-1} was applied to the columns. The influence of ethanol in OTA capture by BSA-agarose was also studied by loading into a column 1 mL of 0.01 M Tris at pH 8.0 containing 15% (v/v) of ethanol with 10 μg L^{-1} of OTA, to mimic the quantities of this alcohol existing in real wine samples. After loading the samples containing the mycotoxin, columns were washed with 5 mL of the same buffer, and the mycotoxin was eluted with 3 mL of methanol 100% or acetonitrile/dH_2O/acetic acid (99/99/2, v/v/v). A negative control was also performed with a cartridge in which the resin packed had no BSA immobilized.

Throughout the protocol, each millilitre was saved separately and kept at 4 °C until OTA quantification via HPLC-FL (Section 3.1.7). BSA was also quantified in each collected fraction (Section 3.1.1.1) to see if there was any leak of protein from the resin during the assay.

3.1.3 Optimization of OTA capture by BSA-agarose SPE in wine

The procedure used to study OTA capture from wine samples was similar to the one used in the assays with buffer solutions. Different volumes of wine samples were supplemented with different concentrations of OTA, diluted or not with different solutions to reach pH values close to 7.0 or 8.0, and loaded into columns packed with 0.5, 1 or 1.5 mL of resin. The collected 1 mL fractions were preserved at 4 °C until analysis by HPLC-FL to quantify OTA (Section 3.1.7).

The first optimizations are presented in Table 3.1. They were done with 1 mL of wine spiked with OTA and SPE columns packed with 0.5 mL of BSA-agarose. In the first trial the wine pH was not adjusted; in the second trial, wine pH was adjusted with some drops of 10 M NaOH to reach a final pH value of

8.0; in the third trial, wine was diluted with dH_2O; in the fourth trial, wine was diluted with a solution of 5% (w/v) $NaHCO_3$ pH 8.3 to reach a pH close to 7.0; and in the following trials wine was diluted with Tris buffer with two different ionic strengths.

Table 3.1. Conditions tested for the optimization of OTA capture with 1 mL wine spiked with OTA and using SPE columns packed with 0.5 mL of BSA-agarose.

Wine adjustment	OTA (µg L^{-1})	Final pH	Washing steps buffer
None	200	3.5	0.5 M Tris pH 8.0
	20		
	10		0.01 M Tris pH 8.0
NaOH	10	8.0	0.01 M Tris pH 8.0
Dilution 1:2 in dH_2O	200	3.5	0.5 M Tris pH 8.0
	20		
	10		0.01 M Tris pH 8.0
Dilution 1:2 in 5% (w/v) $NaHCO_3$ pH 8.3	10	7.3	0.01 M Tris pH 8.0
Dilution 1:2 in 0.5 M Tris pH 8.0	200	7.8	0.5 M Tris pH 8.0
	20		
	10		0.01 M Tris pH 8.0
Dilution 1:2 in 0.3 M Tris pH 8.0	10	7.2	0.01 M Tris pH 8.0

The second optimizations (Table 3.2) were done with larger volumes (5 mL) of wine spiked with OTA, which were diluted with 0.1 M Tris pH 8.0 or 0.3 M Tris pH 8.0 in different proportion. In this phase, higher volumes of BSA-agarose were also tested (1 or 1.5 mL). In addition, the volume used in the elution steps was increased from three to four mL.

Table 3.2. Evaluation of different dilution factors and BSA-Agarose volumes to optimize OTA capture from 5 mL of wine by SPE columns packed with BSA-agarose.

Resin volume (mL)	Wine dilution buffer	OTA (µg L^{-1})	Dilution Factor	Final volume (mL)	Final pH
0.5	0.3 M Tris pH 8.0	1	1:2	10	7.2
		10	1:4	20	7.9
0.5	0.1 M Tris pH 8.0	10	1:4	20	7.2
1.0	0.3 M Tris pH 8.0	10	1:2	10	7.2
1.5	0.3 M Tris pH 8.0	10	1:2	10	7.2
1.5	0.1 M Tris pH 8.0	10	1:4	20	7.2

The percentages of OTA captured by the BSA-agarose, OTA that was still retained after the elution steps and the final balance were calculated using the following formulas:

(2) % OTA captured = % OTA loaded in the column – (% OTA in the flow-through + % OTA washed out)

(3) % OTA retained after elution = % OTA captured - % OTA eluted

(4) Final balance = % OTA in the flow-through + % OTA washed out + % OTA eluted

Where "% OTA in the flow-through", "% OTA washed out" and "% OTA eluted" are, respectively, the percentages of OTA present in the saved fractions of the flow-through, washing and elution steps.

The last optimization trials, included minor changes to the optimized protocol. Namely, it was performed a trial where the diluted wine sample was filtered through a syringe (Nylon, 0.45 µm) prior to its passage through the SPE columns to guarantee that no clogging occurred due to wine particles. In addition, a last assay was performed with the final optimized conditions: 5 mL of wine spiked with 10 µg L^{-1} of OTA and diluted by a factor of four in 0.1 M Tris at pH 7.0 were loaded into a column. This assay was performed in a vacuum chamber to reach a flow rate of 1 drop per second and the solution used to elute OTA from the column was changed to 1% (v/v) of acetic acid in methanol. Finally, the final extracts were evaporated at 40 °C with a slight nitrogen flow and resuspended in 0.5 mL of the HPLC mobile phase to allow a 10-fold concentration of final extract.

3.1.4 Validation of BSA-agarose SPE for OTA determination in wine

After the whole optimization process, we proceeded to the validation of the BSA-agarose column (BAC) method. Thus, starting from an initial stock of 10 000 µg L^{-1} of OTA, five red wine samples supplemented with five different concentrations of this mycotoxin were prepared: 0.05, 0.5, 1, 2 and 3 µg L^{-1}. The extraction of OTA from each sample was performed in duplicate using both BAC and immunoaffinity columns (Section 3.1.6) in order compare the performance of the developed method with a method that is already validated [62]. The conditions used in the BAC assays were the final optimized conditions described in Section 3.1.3. Since both methods result in extracts that have a 10-fold concentration of OTA in comparison with the original wine sample, the real concentration of OTA in wine was calculated using the following formula:

$$\textbf{(5)}\ [OTA]\ in\ wine = \frac{[OTA]\ determined\ by\ HPLC}{10}$$

Where "[OTA] determined by the HPLC" is the concentration of OTA in the sample extract determined by the HPLC instrument (µg L^{-1}).

After OTA determination in those extracts by HPLC-FL, a series of parameters was evaluated: linearity, recovery rates, precision, selectivity and the limits of detection (LOD) and quantification (LOQ).

3.1.4.1 Linearity

The linearity of the developed method in the range of 0.05-3.0 µg L^{-1} was initially evaluated by plotting OTA concentration measured in each wine sample against the amount of OTA added to the wine sample. A linear regression in the form of $Y = mx + b$ was obtained, with m as the slope and b as the

y intercept. The closeness of the correlation coefficient (R^2) to unity was used to express the linearity [90]. Also, the respective residuals plot was traced and the absence of tendencies and random distribution of the residual values were used to support the linear assumption [91].

3.1.4.2 Recovery rates

The recovery rates represent the fraction of the analyte in the initial sample that is present in the final extract submitted to analysis [92]. They were calculated using the following formula:

$$\textbf{(6)}\ Recovery\ rate\ (\%) = \frac{[OTA]\ determined\ in\ wine}{[OTA]\ added\ to\ wine} \times 100$$

Where "[OTA] determined in wine" is the measured concentration of OTA in wine (µg L^{-1}) and "[OTA] added to wine" is the concentration of OTA that was effectively added to the wine samples (µg L^{-1}). The recovery rate values are expressed in percentage.

3.1.4.3 Precision

To evaluate the precision of the BAC method we analysed the repeatability of the data obtained in terms of OTA quantified in the duplicates of each one of the red wine samples supplemented with the mycotoxin. In order to do that, we determined the relative standard deviations (RSD) for each concentration of OTA studied following the following formula:

$$\textbf{(7)}\ RSD\ (\%) = \frac{SD}{X} \times 100$$

Where "SD" is the standard deviation and "X" is the respective average of OTA quantified in the two replicates of each wine sample. The RSD values are expressed in percentage [93].

3.1.4.4 Selectivity

The selectivity of the developed method was evaluated through the visual inspection of the chromatograms obtained by HPLC-FL. These chromatograms were compared to those obtained with the IAC method.

3.1.4.5 Limits of detection and quantification

For both BAC and IAC method, the OTA peak areas were plotted against the concentrations of OTA determined by the HPLC instrument. The analytical limits of detection and quantification for OTA were determined for each method based on the standard deviation (Sy/x) and slope (m) of the data linear regression. The LOD and LOQ were calculated using the following formulas:

$$\textbf{(8)}\ LOD = 3.3 \times \frac{S_{y/x}}{m} \qquad \textbf{(9)}\ LOQ = 10 \times \frac{S_{y/x}}{m}$$

Since OTA in the extracts is 10x concentrated in comparison with the initial wine samples, the values obtained using these formulas were then divided by 10 to obtain the real method LOD and LOQ.

3.1.4.6 Comparison of BAC and IAC methods

A direct comparison of the results obtained by both methods performed was made by linear regression analysis. The average concentrations of OTA in the wine samples determined by the IAC method were plotted against the concentrations of OTA determined by the BAC method. The proximity of the R^2 of the linear regression to the unit was used as a measure of agreement between the results obtained by both methods. Also, the respective residuals plot was traced and the absence of tendencies and random distribution of the residual values were used to support the linear assumption [91]. Finally, a two-way ANOVA was performed (α=0.05) to analyse the differences between the concentrations of OTA determined by the BAC and IAC methods.

3.1.5 OTA determination in naturally contaminated wines

Six red wines of different Portuguese wine regions that were produced in different years were selected. Both the IAC (Section 3.1.6) and BAC methods were used to extract and determine OTA in each wine.

3.1.6 OTA determination in wine samples by immunoaffinity SPE

Wine samples naturally contaminated and spiked with different concentrations of OTA (0.05, 0.5, 1, 2 and 3 μg L^{-1}) were analysed in duplicate by the immunoaffinity method using OchraTest WB columns (Vicam) and following the protocol reported by Visconti *et al.* [62]. In resume, wine samples were two-fold diluted in 1% PEG 8000/5% $NaHCO_3$ solution (w/v) at pH 8.3 and filtered with a 1 μm Whatman glass microfiber syringe filter. The immunoaffinity column was loaded with 10 mL of this solution and then washed with 5 mL of 0.5% NaHCO3/ 2.5% NaCl (w/v), followed by 5 mL of dH_2O. OTA was eluted with 2 mL of methanol 100% which were further evaporated at 40 °C with a slight nitrogen flow and resuspended in 0.5 mL of HPLC mobile phase. Solutions were passed through the column at a flow rate of 1 drop per second using a vacuum chamber. Samples were preserved at 4 °C until analysed by HPLC-FL (Section 3.1.7).

3.1.7 Quantification of OTA by HPLC-FL

OTA was quantified as described by Abrunhosa *et al.* [94] using HPLC-FL with a system consisting of a Varian Prostar 210 pump, a Varian Prostar 410 autosampler, a Jasco FP-920 fluorescence detector and a Jones Chromatography 7971 column heater that was maintained at room temperature. This system was managed by a Varian 850-MIB data system interface and operated with the Galaxie chromatography software. The chromatographic separation was performed on a C18 reversed-phase YMC-Pack ODS-AQ analytical column (250 x 4.6 mm I.D., 5 µm) connected to a pre-column with the same stationary phase. Samples elution was performed at a flow rate of 1.0 mL min^{-1} with a mobile phase consisting of acetonitrile/dH_2O/acetic acid (99:99:2, v/v/v) previously filtered and degassed with a 0.2 µm membrane filter (GHP, Gelman). The injection volume was 50 µL and the parameters used for OTA detection were: excitation = 333 nm, emission = 460 nm, gain = 1000. Calibration curves were prepared with a certified OTA commercial standard (Sigma-Aldrich) at concentrations between 0.05 µg L^{-1} and 100.0 µg L^{-1} (Annex II). OTA identification in chromatograms was done by comparing the peak retention times with OTA standards. OTA concentration in samples was determined by comparing the obtained peak areas with the calibration curve.

3.2 Results and discussion

3.2.1 Construction of BSA-agarose SPE columns

BSA is an easily obtainable and affordable protein that has high affinity towards OTA [85]. In order to take advantage of this strong interaction, the capacity of this protein to be used specifically for OTA extraction from wine was evaluated. The process started by immobilizing the protein in Cyanogen bromide-activated agarose. During the immobilization protocol, BSA was monitored in the supernatants by taking samples that were further quantified by a Bradford protein assay method. Results from the 21 immobilizations performed showed that up to 4 mg of BSA per mL of resin were immobilized. Then, different resin volumes (0.5, 1.0 and 1.5 mL) were packed in empty gravity flow SPE columns of 3 mL and used to perform OTA capturing assays.

3.2.2 OTA capture by BSA-agarose SPE in buffer solutions

Initially, OTA capture from 1 mL buffer solutions was studied at pH 3.5, 7.0 and 8.0 using the constructed BSA-agarose columns (BAC). In order to do that, OTA extraction was studied at pH 8.0 and 7.0 using Tris buffer with different ionic strengths (0.1 and 0.01 M) and at pH 3.5 using 0.1 M sodium citrate buffer following the extraction protocol described in Section 3.1.2. A negative control for the pH of 8.0 and 0.1 M was performed using agarose columns in which BSA was not immobilized.

As shown in Table 3.3, in the negative control the majority of the OTA loaded was washed out in the flow-through (30.8%) and in the following washing steps (64.6%), leaving only a residual amount (2.2%) that was further washed in the first elution step. On the other hand, in the BAC assay with pH 8.0 at 0.1 M only about 4% of OTA was washed out from the column while the rest was captured by BSA since about 94% of total OTA was successfully eluted with methanol.

The reduction of the ionic strength of the Tris buffer from 0.1 to 0.01 M resulted in the elimination of the small OTA losses in the washing steps and, consecutively, allowed the total retention of OTA by the column at pH 8.0. This indicates that the reduction of the ionic strength of the buffer results in an increased interaction between OTA and immobilized BSA. Nonetheless, the reduction of the ionic strength had a negative effect on the following elution steps since OTA was not totally eluted using pure methanol. Thus, in the assays using buffer with lower ionic strength, instead of methanol, the mobile phase used in OTA determination by HPLC-FL was used in the experiments and, as seen in Table 3.3, allowed total recovery of OTA in the elution steps. The effect of the ionic strength on OTA binding to BSA was reported by Uchiyama *et al.* (1985), and their studies also suggested that the increase of the ionic strength results in a lower affinity between the mycotoxin and BSA [95]. Also, the reduction of the pH from 8.0 to 7.0

does not appear to interfere with the affinity of OTA to BSA since similar results were obtained at both pH values (Table 3.3).

Table 3.3. Amount of OTA (%) recovered in each step of the extraction protocol performed in agarose-columns with and without immobilized BSA.

Extraction step	Agarose column (negative control) *	BSA-agarose column			
		0.1 M Citrate pH 3.5 *	0.1 M Tris pH 8.0 *	0.01 M Tris pH 8.0 *	0.01 M Tris pH 7.0
Flow-through	30.8 ± 20.6	0.0 ± 0.0	0.3 ± 0.6	0.0 ± 0.0	0.0
1st wash	51.6 ± 19.5	1.8 ± 0.0	0.2 ± 0.3	0.0 ± 0.0	0.0
2nd wash	10.7 ± 3.9	12.7 ± 1.0	0.1 ± 0.2	0.0 ± 0.0	0.0
3rd to 5th wash	2.3 ± 1.7	63.5 ± 0.2	3.2 ± 2.9	0.0 ± 0.0	0.0
1st elution	2.2 ± 3.8	20.0 ± 3.1	82.2 ± 10.3	107.1 ± 6.4	124.7
2nd elution	0.0 ± 0.0	0.4 ± 0.3	10.7 ± 6.9	7.3 ± 5.2	9.2
3rd elution	0.0 ± 0.0	0.0 ± 0.0	1.2 ± 0.7	0.3 ± 0.1	0.0
Final balance	97.5 ± 2.2	98.3 ± 1.7	97.9 ± 2.5	114.7 ± 2.7	133.9

*The values represent the average ± standard deviations (SD) of at least two independent experiments.

On the other hand, at pH 3.5 (Table 3.3), although an enhanced delay of OTA from the column was clearly observed in comparison to the negative control, about 80% of total OTA was lost in the washing steps, which means that the interaction between OTA and BSA was not stable at this pH value. These results are in agreement with the literature, since in 1971, studies regarding the interaction between this mycotoxin and BSA revealed that the complex formation is pH dependent. Namely, spectrophotometric and spectrophotofluorometric studies showed that at pH 7.0 the complex is essentially complete and that the interaction remains stable until pH 10.4, above which the complex may dissociate [96]. In addition, it is important to emphasize that throughout these assays, columns were reused many times and still allowed full retention of OTA when the optimum conditions were used. This means that BSA recognition properties towards OTA are maintained intact from assay to assay and, therefore, that BACs can be reused many times to extract the mycotoxin from buffer solutions without losing its efficacy.

Furthermore, in order to see if ethanol could influence OTA capture by BSA, 15% (v/v) ethanol was added to a solution of 1 mL of 0.01 M Tris pH 7.0 containing 10 ng of OTA to mimic the alcohol content of wine. As seen in Table 3.4, the loading of this solution in the columns resulted in total OTA capture and its full recovery in the elution steps which indicates that ethanol at the concentration of 15% (v/v) does not interfere with the BSA-OTA complex formation.

Table 3.4. Amount of OTA (%) recovered from 10 ng of OTA in buffer containing 15% (v/v) ethanol in each step of the extraction protocol performed on agarose-columns with immobilized BSA.

Extraction step	0.01 M Tris pH 7.0 + 15% (v/v) ethanol
Flow-through	0.0
1st wash	0.0
2nd wash	0.0
3rd to 5th wash	0.0
1st elution	102.0
2nd elution	1.8
3rd elution	0.0
Final balance	103.8

*The values are a result of a single experiment.

In summary, these results indicate that the use of the constructed BSA-agarose system at the pH values of 7.0 and 8.0, and under low ionic strengths, allows a stable and total capture of OTA from aqueous solutions.

It is also important to note that all samples collected from each step of the extraction protocol of OTA from pure solutions were quantified by a Bradford protein assay method (Section 3.1.1.1) and the results confirmed that no protein was leaked from the resin, which indicates that a stable interaction was established between the protein and the selected resin under the buffering conditions studied.

3.2.3 Optimization of OTA capture by BSA-agarose SPE in wine

Proven the feasibility of the constructed BSA-agarose system to capture OTA from aqueous solutions, a similar protocol was used to test the capture of the mycotoxin in red wine. In order to do that, initially, the cartridges packed with 0.5 mL of resin were loaded with 1 mL of red wine samples (not diluted) spiked with known amounts of OTA. The percentages of OTA in the flow-through, washing steps and elution steps are presented in Figure 3.1. Initial tests performed with wine samples without any pH adjustment resulted in very low capture percentages regardless the concentration of OTA (10-200 μg L^{-1}) (Figure 3.1A). Next, considering the results previously obtained in the experiments conducted in buffer solutions, wine pH was adjusted in order to obtain a final pH between 7.0 and 8.0. In a first attempt, wine pH was adjusted using a few drops of 10 M NaOH to obtain a final pH of 8.0 (Figure 3.1B). This sample of wine was loaded in the BAC system and 71.2% of OTA was lost in the flow-through and washing steps allowing only a recovery of 25.9% of OTA in the elution steps.

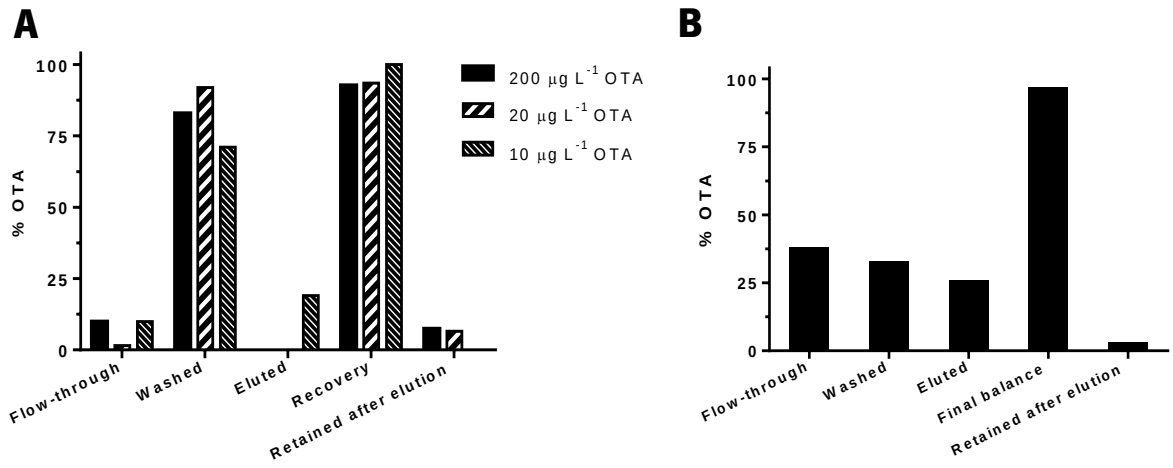

Figure 3.1. Percentages of OTA collected in each step of the SPE method. Experiment conducted with a sample of 1 mL of wine (A) with no pH adjusted, and (B) with pH adjusted to 8.0 with drops of 10 M NaOH. In the assay (A), three OTA concentrations were tested (200, 20 and 10 µg L^{-1}) and in the assay (B) the concentration of 10 µg L^{-1} OTA was tested. The columns used were packed with 0.5 mL of BSA-agarose.

On the other hand, the loading of wine samples submitted to a two-fold dilution in dH_2O resulted in up to 29% of OTA washed out and captures up to 87% (Figure 3.2).

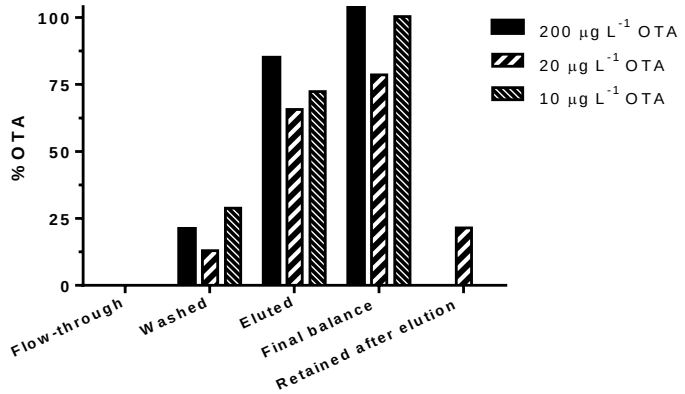

Figure 3.2. Percentages of OTA collected in each step of the SPE method. Experiments conducted with a sample of 0.5 mL of wine submitted to a two-fold dilution in dH_2O. Three OTA concentrations were tested (200, 20 and 10 µg L^{-2}) and the columns used were packed with 0.5 mL of BSA-agarose.

This result is not in accordance with the assays previously conducted with the buffer at pH 3.5 (Section 3.2.2), since in that case about 80% of loaded OTA was lost in the flow-through and washing steps. This may be due to the resin clogging because the columns used in these experiments had already been used in other wine assays.

On the next experiment, 5% $NaHCO_3$ (w/v) at pH 8.3 was used to dilute wine in a 1:2 factor to give a final pH of 7.3 (Figure 3.3). This solution was tested because it is also used in AOAC Official Method 2001.01 to dilute wine prior its clean-up by immunoaffinity [62].This procedure resulted in losses of 39%

of OTA in the flow-through and washing steps, and in a percentage of capture of 61%. Thus, the $NaHCO_3$ solution did not favour OTA capture.

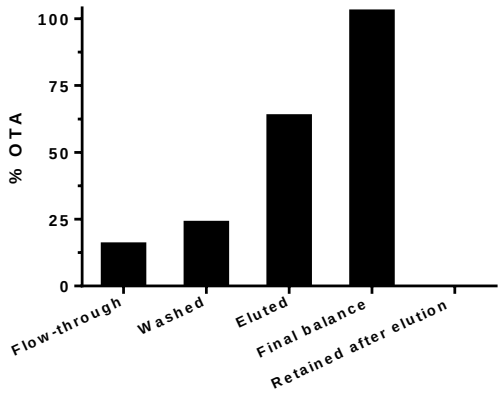

Figure 3.3. Percentages of OTA collected in each step of the SPE method. Experiment conducted with a sample of 1 mL of wine two-fold diluted with 5% $NaHCO_3$ at pH 8.3. Wine was spiked with 10 μg L^{-1} OTA and the column used was packed with 0.5 mL of BSA-agarose.

On the subsequent experiments, wine samples were submitted to a two-fold dilution in Tris buffer with different molarities. Tris buffers with different molarities were also used in the washing steps (Figure 3.4).

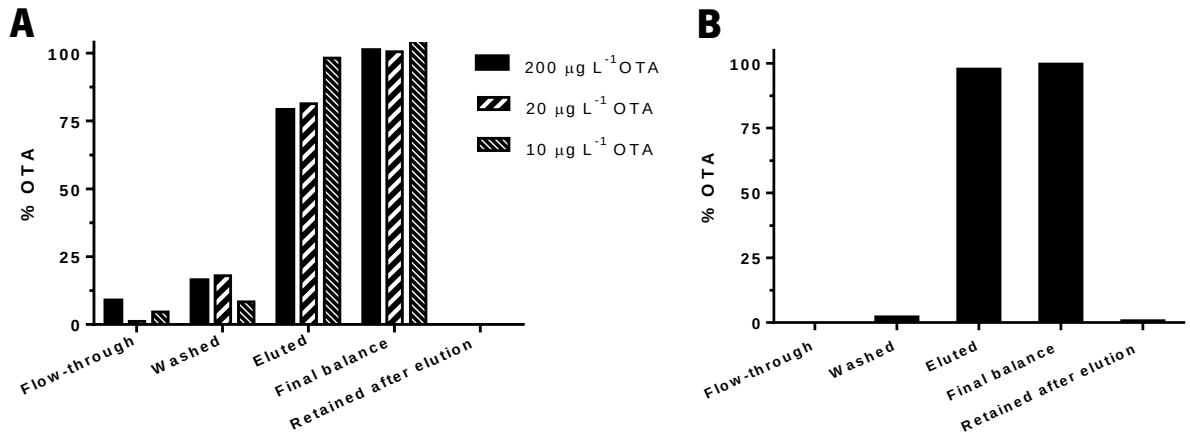

Figure 3.4. Percentages of OTA collected in each step of the SPE method. Experiments conducted with samples of 1 mL of wine two-fold diluted with (A) 0.5 M Tris pH 8.0 and (B) 0.3 M Tris pH 8.0. In the assays (A), three OTA concentrations were tested (200, 20 and 10 μg L^{-1}). In the assay (B) the concentration of 10 μg L^{-1} OTA was tested. The columns used were packed with 0.5 mL of BSA-agarose.

As shown in Figure 3.4A, a two-fold dilution of wine in 0.5 M Tris pH 8.0 (giving a final pH of 7.8) resulted in OTA losses around 13% and 25% in the flow-through and washing steps, and in percentages of capture superior to 75%. In those experiments, it was also observed a slight increase of the capture percentage with the decrease of the washing buffer ionic strength (assay with 10 μg L^{-1}). Following, wine

dilution with a Tris buffer of lower ionic strength (0.3 M) (Figure 3.4B) increased the percentage of OTA capture to 98.0%, showing, once again, the positive effect of the low ionic strength on BSA-OTA interaction.

Nonetheless, despite the 98% capture of OTA obtained with 0.3 M Tris pH 8.0, the method needed further improvement because the 0.5 mL of wine that were passed through the columns were inadequate to obtain a protocol with sufficiently low detection and quantification limits to determine OTA in naturally contaminated wines. Therefore, in order to improve the method sensibility, experiments with 5 mL of wine were conducted. Despite this, the use of higher wine volumes resulted in a drastic reduction of OTA capture percentages when columns packed with 0.5 mL of BSA-agarose were used (Figures 3.5 and 3.6).

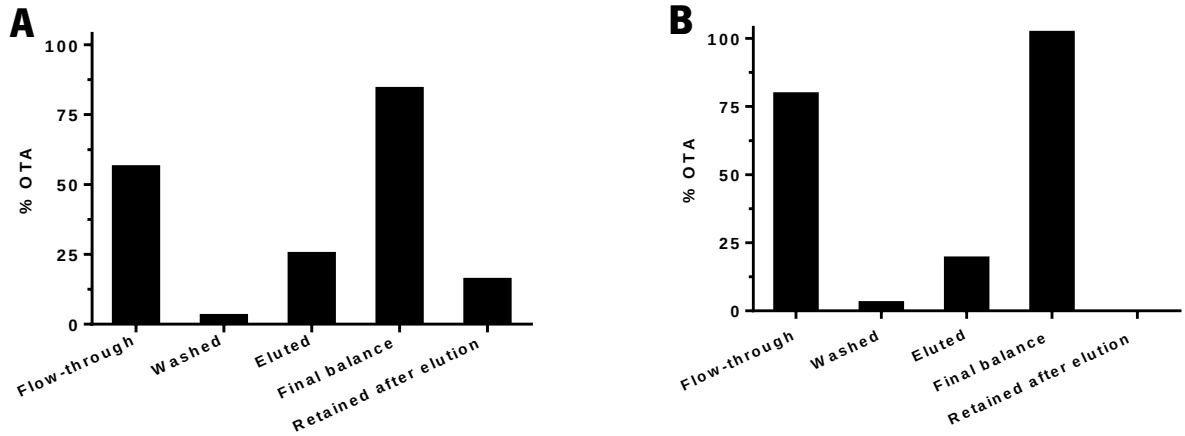

Figure 3.5. Percentages of OTA collected in each step of the SPE method. Experiments conducted with samples of 5 mL of wine (A) two-fold diluted with 0.3 M Tris pH 8.0 and (B) four-fold diluted with 0.3 M Tris pH 8.0. The OTA concentrations were, respectively 1 and 10 μg L^{-1}. The columns used were packed with 0.5 mL of BSA-agarose.

In the first assay conducted, the best conditions previously defined with 0.5 mL of wine were mimicked. Thus, 5 mL of wine were submitted to a two-fold dilution with 0.3 M Tris pH 8.0 and the resulting 10 mL were passed through the cartridges packed with 0.5 mL of BSA-agarose (Figure 3.5A). This increase of wine volume led to the decrease of OTA capture to 41%.

Following, the dilution factor of wine was increased to four (Figure 3.5B) to increase the buffering capacity of the resulting solutions. The resulting 20 mL were loaded in the column but this still originated very low percentages of capture (17%).

Thus, it was tried, once again, to reduce the molarity of buffer solution to see if it could improve the capture of OTA. Tris buffer molarity was decreased from 0.3 M to 0.1 M, and the dilution factor of four was maintained. With this approach, the percentage of OTA in the flow-through and washing steps decreased to 38% and the capture percentage increased to 62% (Figure 3.6). Despite this, it was still not satisfactory for analytical purposes.

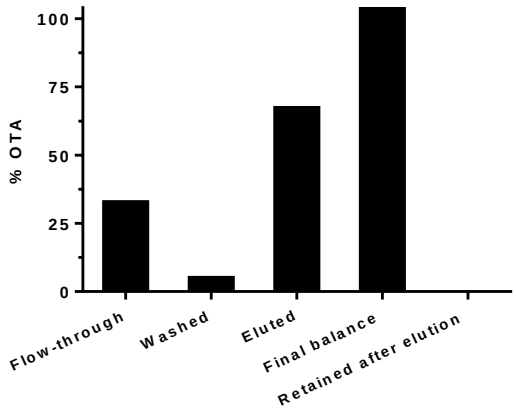

Figure 3.6. Percentages of OTA collected in each step of the SPE method. Experiments conducted with samples of 5 mL of wine spiked with 10 µg L^{-1} OTA submitted to a four-fold dilution in 0.1 M Tris pH 8.0 to reach a final pH of 7.2. The column used was packed with 0.5 mL of BSA-agarose.

Wine is a very complex matrix whose composition includes water, ethanol, glycerol, polysaccharides, various types of acids and phenolic compounds. Thus, it can be hypothesised that other wine components may bind to BSA and therefore, compete with OTA for the binding sites. In fact, the interactions of certain compounds present in wine (such as resveratrol, tannins and anthocyanins) with BSA or HSA have already been described [97–99]. The use of higher volumes of wine increases the quantity of wine compounds that are competing with OTA for the binding sites. In order to overcome that, the volume of resin packed in the cartridges was increased to 1 and 1.5 mL, to increase the amount of BSA available to bind OTA (Figure 3.7).

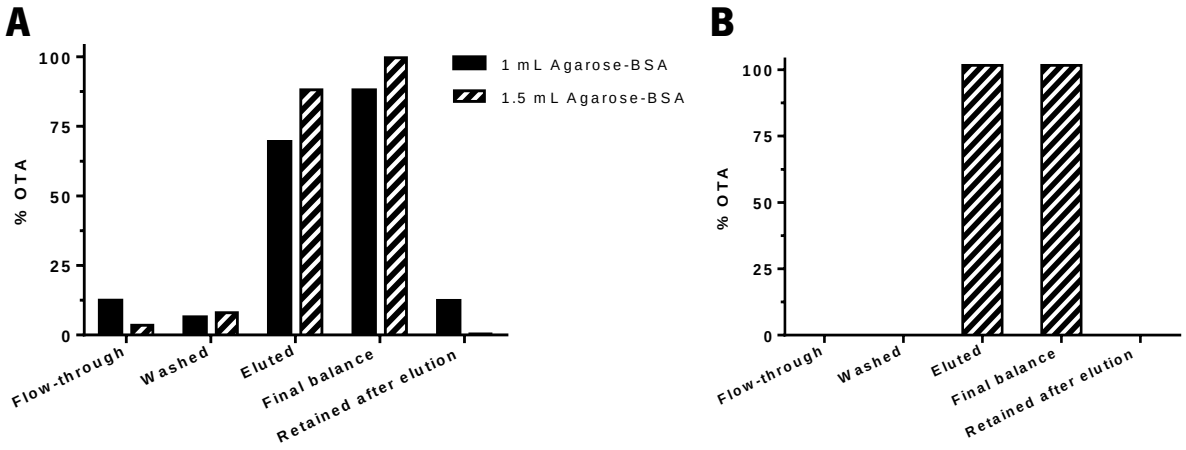

Figure 3.7. Percentages of OTA collected in each step of the SPE method. Experiments conducted with wine samples of 5 mL submitted (A) to a two-fold dilution in 0.3 M Tris pH 8.0 and (B) to a four-fold dilution 0.1 M Tris pH 8.0. In the three assays, wine was spiked with 10 µg L^{-1} OTA. The columns used in the assays (A) packed with 1 and 1.5 mL of BSA-agarose, while in the assay (B) it was packed with 1.5 mL.

When 5 mL of wine diluted in a two-fold factor in 0.3 M Tris pH 8.0 were loaded in cartridges with 1 and 1.5 mL of resin, captures of 81.5 and 88.5% of total OTA were achieved (Figure 3.7A), which is a considerable improvement in comparison to the captures obtained with 0.5 mL of resin. Finally, to further improve these values, the ionic strength of the dilution buffer was reduced to 0.1 M and the dilution factor set in 1:4 in order to obtain a final pH of 7.2 (Figure 3.7B). These final conditions led to total capture and recover of OTA loaded into the column.

It is important to note that with the increase of the resin volume, the total recover of OTA did not occur (assay with 1 mL of BSA-agarose in Figure 3.7A). Thus, it was necessary to increase the volume of solution used in the elution step from three to four millilitres. This increase allowed total OTA recovery.

Nonetheless, OTA was too diluted in the final extract, which compromised the quantification and sensitivity of the final HPLC-FL method. Thus, the elution solvent used so far (acetonitrile/H_2O/acetic acid (99:99:2, v/v/v)) was replaced by methanol containing 1% (v/v) of acetic acid to facilitate its evaporation at 40°C with nitrogen flow and thus its concentration. This change did not affect the recovery of the method. At the end, the dried extracts were resuspended in 0.5 mL of mobile phase (acetonitrile/H_2O/acetic acid (99:99:2, v/v/v)) achieving, thus, a 10-fold concentration. This concentration factor was sufficient to determine OTA in the naturally contaminated wines with very low concentrations of the mycotoxin by the HPLC instrument at a detection limit of 0.01 μg L^{-1}. At last, the columns were set up in a vacuum chamber to allow a flow-rate of 1 drop per second and an additional filtration step was introduced in order to improve the stability of samples: the diluted wine solution was filtered through a syringe nylon filter with a pore size of 0.45 μm prior to its passage through the BSA-agarose columns.

3.2.4 Validation of BSA-agarose SPE for OTA determination in wine

Analytical measurements seek the determination of the value of a compound or parameter using methods and equipment that must be tested to ensure that they are suitable for their purpose. To use a certain analytical method there must be a confirmation, through objective evidences, that the requirements to its application are fulfilled. This confirmation is usually obtained through what is called the method validation, which is highly necessary to ensure the reliability of analytical work obtained [92].

In order to validate the novel clean-up method described above for OTA determination in wine, a series of parameters such as linearity, recovery rates, selectivity and detection and quantification limits were evaluated. Thus, extraction of OTA from red wine samples supplemented with five different concentrations of this mycotoxin in the range of 0.05-3.0 μg L^{-1} was performed in duplicate using the

BACs (Annex III) and the obtained extracts were quantified by HPLC-FL (Section 3.1.7). Table 3.5 summarizes the experimental conditions optimized in Section 3.2.3 to extract OTA from the spiked wine samples. These assays were also performed with IACs (Section 3.1.6) in order to compare the performance of the BAC method with a method that is already validated [62].

Table 3.5. Optimized operating conditions for OTA extraction from wine using the BSA-agarose columns.

1. Column construction	1.5 mL of BSA-agarose resin
2. Sample preparation	5 mL of Red Wine + 15 mL of 0.1 M Tris pH 8.0 Filtration with a syringe nylon filter (0.45 µm)
3. Column equilibration	5 mL of 0.01 M Tris pH 7.0
4. Sample loading	20 mL of diluted wine (final pH 7.2)
5. Column washing	5 mL of 0.01 M Tris pH 7.0
6. Elution	4 mL of 1% (v/v) acetic acid in methanol
7. Post-elution	Evaporation at 40 °C with N2 flow
8. Reconstitution	Resuspension in 0.5 mL of HPLC mobile phase

3.2.4.1 Linearity

Linearity is defined as the ability to obtain results that are linearly proportional to the analyte's concentration in the sample within a defined range. The method's linearity can be represented through the study of a linear regression in the form of $Y = mx + b$, with m as the slope, b as the y intercept and R^2 as the correlation coefficient. The closeness of the correlation coefficient to unity is often used to express the linearity, however, on its own, it does not necessarily mean that there is indeed a linear relationship [90]. An additional way to verify the linearity is by analysing the residuals of the linear regression. The residuals are the distances of the experimental points from the fitted regression line measured in a parallel direction to the horizontal axis. Residuals plot with absence of tendencies and values randomly distributed support the linear assumption [91]. Thus, initially the linearity of the developed method was evaluated in the range of 0.05-3.0 µg L^{-1} by plotting the measured OTA concentration in the wine samples against OTA concentration added (Figure 3.8A). The equation of the linear regression was $Y = (0.9891 \pm 0.0061)x - (0.0006 \pm 0.0103)$ and the correlation coefficient (R^2= 0.9999) obtained was close to the unit. Following, the respective residuals plot was traced (Figure 3.8B), and a random distribution of the values was obtained. Together, these data reflect the excellent linearity of the BAC method. Thus, the developed method can be considered linear within the range of 0.05-3.0 µg L^{-1} of OTA.

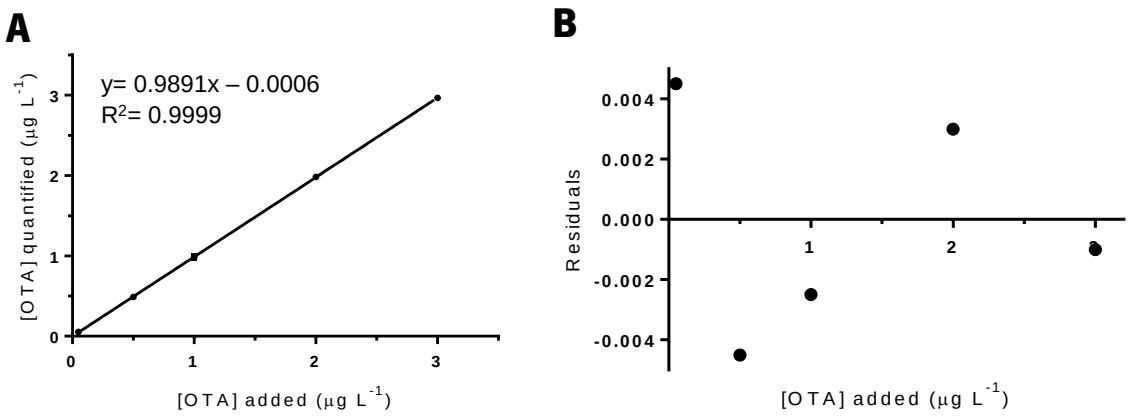

Figure 3.8. Relation between OTA added to wine at different concentrations and OTA in wine determined by HPLC-FL upon extraction by the BSA-agarose method (A) and (B) the respective residuals plot. The error bars in the linear regression represent the standard deviations (SD) of two independent experiments.

3.2.4.2 Recovery rates

Recovery rates are a form to measure the yield of the method, that means, the fraction of the analyte in the initial sample that is present in the final extract submitted to analysis. They were calculated as the percentage of OTA measured in each wine sample relatively to the concentration of OTA added in each sample [92]. The recovery rates obtained are between 98% and 107% (Table 3.6), which is in accordance with the guidelines imposed by the European Commission for the official control of mycotoxin levels in foodstuffs (Commission Regulation (EC) No 401/2006). According to this regulation, the recovery rates must be in the ranges of 50-120% and 70-110% for the concentrations below and above 1 µg L^{-1}, respectively [100].

Table 3.6. Recovery rates obtained using the BAC method.

OTA added (µg L^{-1})	OTA quantified (µg L^{-1}) *	Recovery rate (%) *
0.05	0.05 ± 0.01	107.0 ± 9.3
0.5	0.49 ± 0.02	97.9 ± 3.6
1.0	0.99 ± 0.04	98.6 ± 3.8
2.0	1.98 ± 0.03	99.1 ± 1.3
3.0	2.97 ± 0.01	98.9 ± 0.2

*The values of OTA quantified and recovery rates represent the average ± the standard deviation (SD) and the relative standard deviations (RSD) of two independent experiments, respectively.

3.2.4.3 Precision

The method's precision means the closeness of agreement between independent test results obtained under stipulated conditions. It can be evaluated in 3 levels: repeatability, intermediate precision

and reproducibility [93]. The precision of the BSA-agarose method was evaluated through the repeatability using the data obtained from the quantification of the red wine samples supplemented with five different concentrations of this mycotoxin in the range of 0.05-3.0 μg L^{-1}. The tests with the replicates were performed with about 2 days apart in homogenous conditions (same laboratory, same reagents, same equipment and same analyst).

According to ISO 5725:1, at least 10 replicates per sample should be performed and the procedure should be repeated for several concentrations within the defined range for the method's application. In the current work, only 2 replicates of each sample in a range of 5 concentrations of OTA (0.05-3.0 μg L^{-1}) were performed. Using the chromatograms obtained, OTA was quantified in each sample and the relative standard deviations were calculated for each concentration (Table 3.7). The values are presented in the form of percentages.

Table 3.7. Relative standard deviations (RSD) obtained with the BAC method.

OTA (μg L^{-1})	RSD (%)
0.05	9.3
0.5	3.6
1.0	3.8
2.0	1.3
3.0	0.2

The European Commission has also established guidelines for the RSD values in terms of the repeatability of a method's performance. According to their indications, the percentages of RSD must be ≤ 40% and ≤ 20% for the concentrations below and above 1 μg L^{-1}, respectively [100]. Since the values of RSD obtained for the developed method fit in a range of 0.2% to 9.3%, the obtained results are in accordance with the recommendations of this organization.

3.2.4.4 Selectivity

Selectivity is defined as the capacity that a method has to distinguish the analyte under study and other substances that may be present in the sample. That means that an analytical method is considered selective when there are no overlap of peaks or co-elution of other compounds with the analyte of interest [101]. Thus, the selectivity of the developed method was evaluated by visual inspection of the analytical chromatograms of extracts obtained from wine spiked with OTA. An example of these chromatograms is presented in Figure 3.9A. The analytical chromatogram of the same sample obtained with the IAC method is also presented for comparison purposes (Figure 3.9B).

As expected, because the antibodies present in IACs are highly specific towards OTA, the chromatograms obtained had a very clear background with a base line with few interferents particularly close to the OTA peak. The chromatograms obtained with BSA-agarose method are not so clear and show many peaks in the first 12 minutes of the run. Also, close to the OTA peak they can be observed two peaks very close to OTA. Nevertheless, there are no overlaps and the chromatographic separation of the three peaks is achieved. Thus, although the BAC method is not as specific and selective as the IAC method, the use of BACs to extract OTA from wine in combination with the C18-AQ chromatographic separation gives enough selectivity to the full method and allows a reliable quantification of OTA in this matrix.

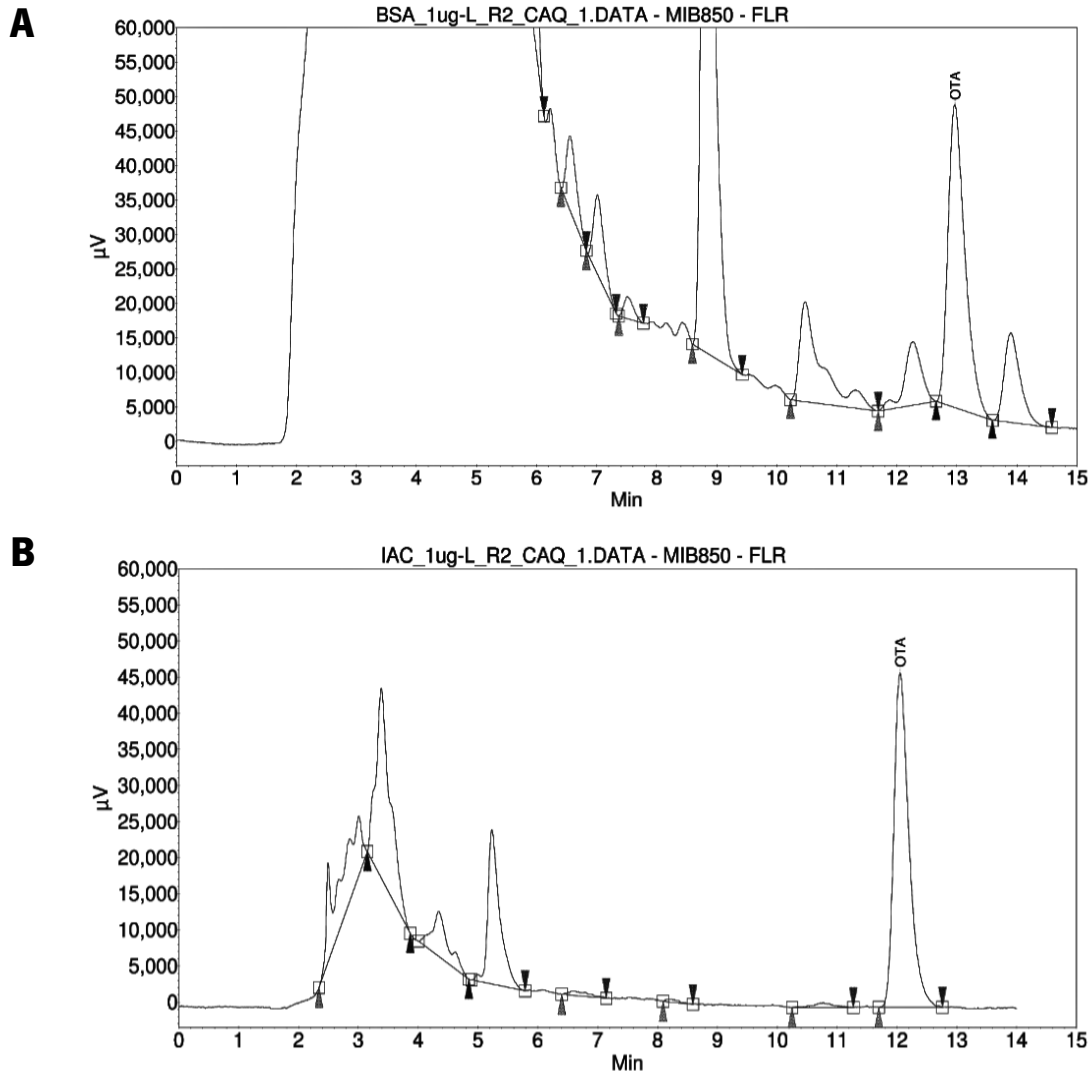

Figure 3.9. HPLC-FL chromatogram obtained for the wine sample spiked with 1 µg L^{-1} of OTA and analyzed by the (A) BAC method and (B) IAC method.

3.2.4.5 Limits of detection and quantification

The analytical limits of detection (LOD) and quantification (LOQ) of the BAC and IAC method were determined using the values of the standard deviation (Sy/x) and the slope (m) of the linear regression consisting of the OTA peak areas plotted against the concentrations of OTA determined by the HPLC instrument (Annex IV) using the formulas described in Section 3.1.4.5. The values obtained are presented in Table 3.8.

Table 3.8. LOD and LOQ (μg L^{-1}) values for the BAC and IAC methods.

Condition	BAC	IAC
LOD (μg L^{-1})	0.02	0.01
LOQ (μg L^{-1})	0.05	0.03

As expected, due to the higher cleanness of chromatograms base line, which allow a higher sensitivity in the measurements, the IAC method presented lower limits of detection and quantification. Still, using the BAC method, wine samples with 0.05 μg L^{-1} of OTA can be quantified, which is exactly the lowest concentration tested, while samples containing 0.02 μg L^{-1} of OTA can be detected but not quantified. It is important to note that these values are lower than the ones reported in the literature for traditional SPE based methods. In 2006, a solid-phase microextraction (SPME) using polydimethylsiloxane/divinylbenzene fiber was performed on wine samples with LOD and LOQ values of 0.07 and 0.22 μg L^{-1}, respectively [102]. In 2013, a method based on C18 cartridges (AccuBond II otadecyl) was employed to determine OTA in wine with LOD and LOQ values of 0.03 and 0.1 μg L^{-1} [103]. More recently, in 2015, a method for OTA determination in wine based on the stationary phases Amberlite IRC-50 and Lewatit CNP105 functionalized with a hexapeptide (Ser-Asn-Leu-His-Pro-Lys) reported LOD and LOQ values of 0.45 and 1.5 μg L^{-1}, respectively [68].

3.2.4.6 Comparison of BAC and IAC methods

A direct comparison of the results obtained by the BAC and IAC method was performed. Since the IAC method is already validated for the determination of OTA in wine [62], this comparison is a good form of confirming the good performance of the BSA developed method.

As seen in Table 3.9, the concentrations of OTA determined by both BAC and IAC methods were similar at all OTA levels studied. Furthermore, the variance between the mean values obtained for each OTA concentration with the two methods was statistically analysed performing a two-way ANOVA (α=0.05). The analysis revealed that no significant differences were found in the results for the

concentrations of 0.05, 0.5, 1.0 and 2.0 μg L^{-1} of OTA ($p > 0.05$). Only the results for the assays performed with 3 μg L^{-1} OTA were significantly different ($p \leq 0.01$).

Table 3.9. Values of OTA concentration determined using the BAC and IAC methods.

OTA added (μg L^{-1})	OTA (μg L^{-1})*	
	BAC	IAC
0.05	0.054 ± 0.005	0.046 ± 0.008
0.5	0.490 ± 0.018	0.496 ± 0.018
1.0	0.986 ± 0.037	0.959 ± 0.076
2.0	1.981 ± 0.025	1.894 ± 0.040
3.0	2.966 ± 0.006	2.799 ± 0.059

*The values of OTA represent the average ± the standard deviation (SD) of two independent experiments.

Further, the agreement of results obtained by both methods was also demonstrated by the linear relationship ($R^2 = 0.9998$) that was obtained when the concentration of OTA determined with these two methods were plotted one against the other (Figure 3.10A). A better agreement of the results would occur if the slope obtained for this linear relationship (m = 0.9412) was closer to the unit. Also, the respective residuals plot was depicted (Figure 3.10B) and the random distribution of the values proves, once again, the linear relationship between the two methods. Thus, the good performance of the developed method is once again proved.

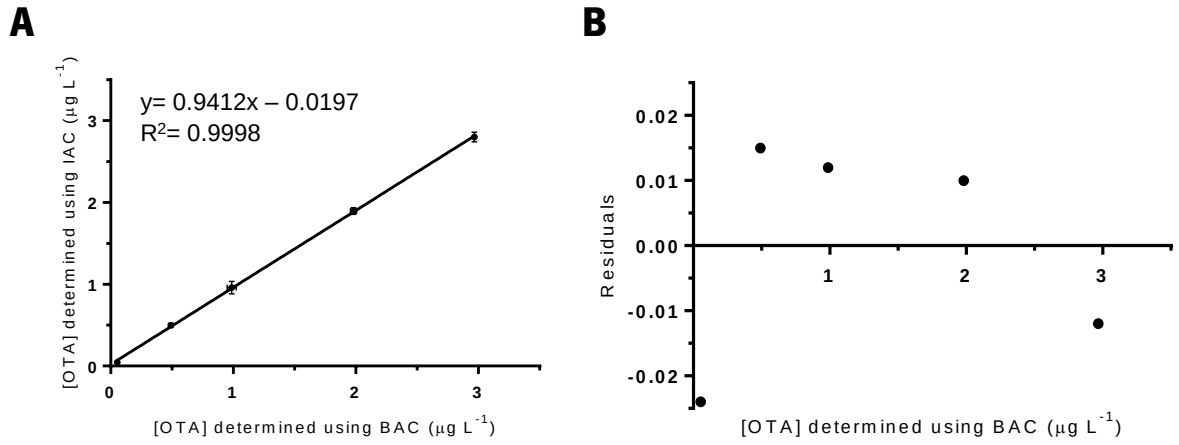

Figure 3.10. (A) Relationship between OTA determined in wine by HPLC-FL using the IAC and BAC methods and (B) the respective residuals plot. The error bars in the linear regression represent the standard deviations (SD) of two independent experiments.

3.2.5 OTA determination in naturally contaminated wines

Finally, after optimization and validation, OTA presence in six commercial red wines was analyzed using both the BAC and IAC extraction methods. The selected wines had different ethanol contents and were produced between 2015 and 2017 in different wine regions (Douro, Lisbon, Alentejo and Dão). OTA concentrations of each wine determined by both methods are shown in Table 3.10.

Table 3.10. Determination of OTA concentration in Portuguese wines using the BAC and IAC methods.

Wine sample	Ethanol (%)	Year	Region	OTA determined by BAC ($\mu g\ L^{-1}$)	OTA determined by IAC ($\mu g\ L^{-1}$)
1 (Farmer)	ni	2015	Douro	0.17	0.17
2	13.5	2016	Lisbon	0.06	0.08
3	13.5	2017	Alentejo	-	< LOQ
4	13.5	2015	Alentejo	0.06	0.08
5	13.5	2017	Alentejo	-	-
6	13.0	2016	Dão	-	-

"ni" – no information; "<LOQ" – below limit of quantification; "-" – not detected; The values presented are result of single assays.

Regarding the analysis using BAC, OTA was found in wines number 1, 2 and 4 at a concentration of 0.17, 0.06 and 0.06 $\mu g\ L^{-1}$, respectively. The assays using IAC also allowed the quantification of OTA in those three same wines with the results of wine number 1 being the same with both methods and the results of wines 2 and 4 being similar. The wine number three revealed the presence of OTA using IAC, although below the limit of quantification, but not using BAC. The differences in the LOD values, which are lower in the IAC method, may explain the non-detection of the mycotoxin in this wine using the developed BAC method. Nonetheless, in all cases, detected OTA was below the European legal limit of 2 $\mu g\ L^{-1}$ in all wines.

CHAPTER IV.

RECOMBINANT PRODUCTION OF THE DOMAIN II OF BSA IN *ESCHERICHIA COLI* AND EVALUATION OF ITS INTERACTION WITH OTA

4.1 Materials and methods

4.1.1 Sterilization procedure

The sterilization of all the materials and culture media used was done by autoclaving at 121 °C during 20 minutes at a pressure of 1 bar. On the other hand, the thermolabile solutions were sterilized by filtration through a 0.2 μm sterile polyethersulfone (PES) syringe filter.

4.1.2 Strains and plasmids

E. coli NZY5α competent cells (NZYTech) were used as the bacterial cloning host for the DNA manipulations while the recombinant protein production was conducted using *E. coli* BL21 (DE3) (NZYTech) and Origami™ 2 (DE3) (Novagen). This last strain has the particularity of having mutations in both the thioredoxin reductase (trxB) and glutathione reductase (gor) genes, which greatly enhance the formation of disulphide bonds in the cytoplasm [104]. Its selection is done with tetracycline 12.5 μg mL^{-1} and streptomycin 50 μg mL^{-1}.

The two plasmids used in this work, pETM10 (5528 bp) and pETM20 (6123 bp) (EMBL), contain the T7 lac promoter, a tag of six histidines (6xHis tag) and are selected, respectively, with 50 μg mL^{-1} kanamycin and 100 μg mL^{-1} ampicillin. The vector pETM20 has the particularity of encoding a thioredoxin fusion protein (TrxA) that may improve the solubility of the target protein [105]. This fusion partner can be cleaved trough the Tobacco Etch Virus (TEV) cleavage site, also present in the vector. Each one of these two plasmids was ligated with the gene of interest, the coding sequence of the domain II of BSA (BDII), and were transformed in the two *E. coli* strains used for the recombinant protein production, *E. coli* BL21 (DE3) and Origami 2 (DE3), giving rise to 4 different strains (Table 4.1).

Table 4.1. *E. coli* strains constructed in this work for the recombinant production of BDII and their respective drug resistances.

E. coli strain	Drug resistance
BL21 (DE3) + pETM10 + BDII	Kan (50 μg mL^{-1})
BL21 (DE3) + pETM20 + BDII	Amp (100 μg mL^{-1})
Origami™ 2(DE3) + pETM10 + BDII	Tetr (12.5 μg mL^{-1}) + Strept (50 μg mL^{-1}) + Kan (50 μg mL^{-1})
Origami™ 2(DE3) + pETM20 + BDII	Tetr (12.5 μg mL^{-1}) + Strept (50 μg mL^{-1}) + Amp (100 μg mL^{-1})

4.1.3 Storage conditions and culture media

During this work, all microorganisms were regularly spread in adequate selective medium and the inverted agar plates were sealed with parafilm and maintained at 4 °C. All strains were cultured in Luria-Bertani (LB) medium (1% w/v tryptone, 1% w/v NaCl, 0.5% w/v yeast extract at pH 7.5) with the adequate antibiotics for each strain (Table 4.1).

4.1.4 Recombinant proteins

In the current work, three versions of the recombinant domain II from BSA (BDII) were used. *E. coli* BL21 (DE3) and Origami 2 (DE3), both transformed with pETM10 or pETM20 containing the BDII gene, were used to produce recombinant BDII and TrxBDII, respectively. Also, the BDII protein free of the TrxA and 6xHis tag (cBDII) was obtained by the cleavage of TrxBDII from *E. coli* Origami 2 (DE3) by the TEV protease. In Table 4.2 are represented some characteristics of all the BDII versions produced, such as molecular weight (Mw) and isoelectric point (pI). These characteristics were calculated based on the amino acid sequences of each (Annex V) using the online *ExPASy ProtParam* tool.

Table 4.2. Characteristics of the produced recombinant BDII versions.

Protein designation	Expression vector	Purification method	Nº of Amino acids	Molecular Weight (kDA)	Theoretical pI
BDII	pETM10	IMAC-Ni	208	23.8	6.0
TrxBDII	pETM20	IMAC-Ni	332	37.0	5.54
cBDII	pETM20	Reverse IMAC-Ni	201	22.7	5.45

4.1.5 Construction of *E. coli* strains expressing recombinant domain II of BSA

The DNA coding sequence for BDII with codons optimized for *E. coli* expression, flanked by the restriction sites for *NcoI* and *XhoI*, was purchased from a specialised company. The gene (608 bp) was delivered in the *E. coli* cloning vector pUC57 (2710 bp, 0.5-1 µg in 20 µL of distilled deionized water (ddH_2O)).

4.1.5.1 Transformation of *E. coli* NZY5α by the heat-shock method

The vector pUC57, containing BDII coding sequence, was transformed and propagated in *E. coli* NZY5α competent cells by the heat-shock method. Thus, the plasmid DNA was gently mixed with 100 µL of competent cells and incubated in ice for 30 minutes. Afterwards, the cells were submitted to a heat-

shock during 40 seconds in a 42 °C water bath before being, once again, incubated in ice for 2 minutes. Afterwards, a volume of 900 µL of super optimal broth with catabolite repression (SOC) (2% w/v tryptone, 0.5% w/v yeast extract, 0.0025 M KCl, 0.01 M NaCl, 0.01 M $MgCl_2.6H_2O$, 0.01 M $MgSO_4.7H_2O$ and 0.02 M glucose) was added to the cells which were further incubated at 225 rpm and 37 °C for 1 hour. Finally, the cell suspension was spread on LB agar plates with 100 µg mL^{-1} ampicillin, in different dilutions, and incubated overnight at 37°C. Some transformants were further picked up in new LB agar plates with 100 µg mL^{-1} ampicillin.

4.1.5.2 Plasmid DNA purification

Plasmid DNA was purified using the commercial GenElute™ Plasmid Miniprep Kit (Sigma) and the procedure was performed according to the manufacturer's protocol.

4.1.5.3 DNA quantification

The concentration of the purified plasmid DNA was determined in a NanoDrop 1000 Spectrophotometer (ThermoFisher Scientific). A volume of 2 µL of sample was loaded and the absorbance at 260 nm was measured and converted to DNA concentration (ng μL^{-1}).

4.1.5.4 DNA digestion with restriction enzymes

In order to obtain BDII gene isolated from the pUC57 vector, the purified plasmid DNA was submitted to double digestions with the restriction enzymes *NcoI* and *XhoI* (New England Biolabs). Digestion was performed overnight at 37 °C with a final volume of reaction of 40 µL containing 1 µL of each restriction enzyme and CutSmart™ Buffer 10x. The same restriction enzymes were also used to digest the vectors pETM10 and pETM20.

4.1.5.5 DNA electrophoresis

The DNA fragments obtained from the digestions were separated and analysed by electrophoresis in agarose gel. The agarose gel (1.5% w/v) was prepared in 1x TAE buffer (Table 4.3) with 0.006% (v/v) of Green Safe premium (NZYTech) for staining of nucleic acids. The DNA samples were mixed with 1x loading dye purple (New England Biolabs) (Table 4.3) and loaded to the gel wells. The DNA molecular weight marker used, NZYDNA Ladder III (NZYTech), gives rise to a pattern composed of 14 bands that range from 200 to 10 000 bp (Table 4.4). Running of the gel was made with 1x TAE buffer at 80 V for 1 hour. Finally, the gel was visualized and photographed in the Molecular Imager ChemiDoc™ XRS + Imaging System (Bio-Rad) and analysed using the Image Lab 4.0 software.

Table 4.3 Composition of the running and sample buffers used in DNA electrophoresis.

Reagent	Composition
50x TAE buffer	2 M Tris-base 0.05 M EDTA pH 8.0
6x Loading dye	25% w/v glycerol 0.02 M EDTA 0.25% w/v Bromophenol blue

Table 4.4. Bands from the NZYDNA Ladder III (NZYTech) and their respective molecular sizes.

Band	Molecular size (bp)
1	10 000
2	7 500
3	6 000
4	5 000
5	4 000
6	3 000
7	2 500
8	2 000
9	1 400
10	1 000
11	800
12	600
13	400
14	200

4.1.5.6 DNA purification from agarose gel

The digested BDII gene and vectors pETM10 and pETM20 separated by gel electrophoresis were recovered from the agarose gel using QIAquick® Extraction Gel Kit (QIAGEN) using the manufacturer's protocol.

4.1.5.7 Ligation of BDII to pETM10 and pETM20

The ligation of the digested BDII gene to the digested vectors pETM10 and pETM20 was performed overnight at 4 °C with T4 DNA Ligase (Promega) to obtain pETM10+BDII and pETM20+BDII. The quantity of insert used in the ligation reaction was calculated using the next formula:

$$\textbf{(1)}\ ng\ of\ insert = \frac{ng\ of\ vector \times size\ of\ insert\ (Kb)}{size\ of\ vector\ (Kb)} \times molar\ ratio\ of\ \left(\frac{insert}{vector}\right)$$

The molar ratio used was 2:1 for about 69 ng of pETM10 and 63 ng of pETM20. Ligation reaction contained 1 µL of Ligase 10x buffer (Promega) and 1 µL of T4 DNA Ligase (Promega) and the final volume of reaction was adjusted to 10 µL with ultrapure water (uH_2O).

4.1.5.8 Positive clones identification

The total volume of each resulting ligation reactions was used to transform *E. coli* NZY5α competent cells as described before in Section 4.1.5.1. The cell suspensions of the transformations of *E. coli* NZY5α with pETM10 and pETM20, containing BDII, were spread on LB agar plates with 50 µg mL^{-1} kanamycin and 100 µg mL^{-1} ampicillin, respectively, in different dilutions, and incubated overnight at 37 °C.

Some randomly chosen transformants were further picked up new LB agar plates containing 50 µg mL^{-1} kanamycin or 100 µg mL^{-1} ampicillin and their plasmid DNA was purified and quantified as described in Sections 4.1.5.2 and 4.1.5.3, respectively. Purified DNA was further digested with *NcoI* and *XhoI* (Section 4.1.5.4), separated and analysed by DNA electrophoresis (Section 4.1.5.5) in order to identify clones with the BDII gene present.

4.1.5.9 Transformation of purified pETM10+BDII and pETM20+BDII in *E. coli* BL21(DE3) and Origami 2 (DE3)

About 50 nanograms of purified plasmid DNA from the positive colonies were used to transform commercial competent *E. coli* BL21 (DE3). This strain was transformed with plasmids pETM10+BDII and pETM20+BDII using the protocol described at Section 4.1.5.1. The *E. coli* Origami 2 (DE3) cells were made competent and transformed as described in the next section.

A) Preparation and transformation of *E. coli* Origami 2 (DE3) competent cells

Origami 2 (DE3) cells were incubated overnight at 37 °C and 200 rpm with 10 mL of LB medium containing 12.5 µg mL^{-1} tetracycline and 50 µg mL^{-1} streptomycin. A volume of 0.5 mL of the previous inoculum was used to inoculate 50 mL of LB medium containing the same antibiotics at 37 °C and 200 rpm until the optical density at 600 nm (OD_{600}) was between 0.4 and 0.6. The cells were then cooled in ice for 10 minutes and 40 mL of the culture were centrifuged (4 000 rpm, 10 minutes at 4 °C). The supernatant was discarded, and cells were resuspended in 20 mL of cold sterile 0.1 M $MgCl_2$ before being centrifuged again. The supernatant was discarded, and the cell pellet was resuspended in 2 mL of cold sterile 0.1 M $CaCl_2$. After incubation in ice for 2 hours, the transformation was done by mixing 100 µL of cells with 200 nanograms of pETM10+BDII and pETM20+BDII. This mixture was incubated for 10

minutes in ice and the cell suspension was spread on a single LB agar plate with the adequate antibiotics. After incubation overnight at 37 °C, some transformants were further picked up new LB agar plates containing the same antibiotics.

4.1.6 Evaluation of recombinant BDII expression in small-screening assays

Preliminary tests were performed with each strain to evaluate their capacity to perform a soluble expression of the recombinant BDII. Thus, *E. coli* BL21 (DE3) and Origami 2 (DE3) containing pETM10+BDII and pETM20+BDII were cultured in 15 mL of LB medium with adequate antibiotics at 37 °C and 200 rpm until the OD_{600} reached the values of 0.5-0.6. Then, 5 mL of each flask were transferred to new flasks and Isopropyl β-D-1-thiogalactopyranoside (IPTG, NZYTech) was added to a final concentration of 0.2 mM. The four flasks were then incubated overnight at 18°C and 150 rpm. The cell pellets of the 5 mL of culture were harvested (Section 4.1.7.1), lysed (Section 4.1.7.2) and both the total lysate (total fraction) and the cleared lysate (soluble fraction) were analysed by SDS-PAGE (Section 4.1.8).

4.1.7 Production and purification of recombinant BDII and TrxBDII

Pre-cultures ranging from 10 mL to 20 mL of *E. coli* BL21 (DE3) and Origami 2 (DE3) containing pETM10+BDII and pETM20+BDII were grown overnight at 37 °C and 150 rpm in LB medium supplemented with adequate antibiotics. Next, flasks with volumes of LB medium ranging from 250 mL to 500 mL containing the adequate antibiotics were inoculated with their respective pre-cultures. The volume of pre-culture inoculated was 1:100 of the total culture volume. The cultures were further incubated at 37 °C with continuous shaking at 200 rpm until OD_{600} reached values between 0.5-0.6. Protein production was induced with the addition of IPTG (NZYTech) to a final concentration of 0.2 mM and cell cultures were incubated overnight at 18 °C and 150 rpm.

4.1.7.1 Cell pellets harvesting

A volume of 100 µL of the cultures was centrifuged (13 200 rpm, 10 minutes at 4 °C) and the supernatant was discarded. The cell pellet was frozen at -20 °C until SDS-PAGE analysis. In the SDS-PAGE gel, this sample will account for the total cell fraction, consisting of the total lysate upon sample boiling, which contains the proteins in both soluble and insoluble forms. The culture volume was then centrifuged (10 000 rpm, 15 minutes at 4 °C) and the supernatants were discarded. Cell pellets were frozen at -20 °C until being lysed (Section 4.1.7.2).

4.1.7.2 Cell lysis of *E. coli*

Cell pellets were resuspended in NZY Bacterial Cell Lysis Buffer (Nzytech) (1 mL of buffer per each 5 mL of culture), Lysozyme, DNaseI (Nzytech, 2 µL per each mL of buffer user) and phenylmethylsulfonyl fluoride (PMSF) (Sigma, final concentration of 1 mM) by pipetting or vortexing. Afterwards, cell suspensions were incubated at 150 rpm during 20 minutes at room temperature. Finally, insoluble cell debris was removed by centrifugation (10 000 rpm, 15 minutes at 4°C) in order to obtain the cleared lysate (soluble fraction) which was analysed by SDS-PAGE (Section 4.1.8). The soluble fraction consists of the cleared lysate and contains only the soluble proteins.

4.1.7.3 Recombinant BDII and TrxBDII purification

Protein purification was done by immobilized metal affinity chromatography (IMAC). This technique is widely used in the purification of his-tagged recombinant proteins and is based on the interaction of the histidine residues on the surface of the proteins with divalent metal ions, such as Ni^{2+}, immobilized via a chelating ligand [106]. The purification of the produced recombinant proteins by IMAC-Ni was possible due to the 6xHis tag in their sequence. Thus, recombinant BDII and TrxBDII from *E. coli* BL21 (DE3) were purified from pellets originated from 240 mL and 330 mL of culture, respectively, while both *E. coli* Origami 2 (DE3) recombinant proteins were purified from cell pellets originated from 40 mL of culture.

The purification was performed in a 5 mL HisTrap™ HP column (GE Healthcare) operated with a peristaltic pump. Initially, the column was washed with 50 mL of uH_2O with a flow rate of 1.5 mL min^{-1} and was charged with nickel by loading a solution of 10 mL of 0.1 M $NiSO_4.6H_2O$ into the column with a flow rate of 1 mL min^{-1}. Then, the column was washed again with 50 mL of uH_2O and equilibrated with 30 mL of binding buffer (0.05 M Tris pH 8.0, 0.15 M NaCl, 0.02 M Imidazole). Before being loaded into the column, the imidazole concentration of the cleared lysate was adjusted to 0.02 M to enhance the efficiency of the purification. Cell extracts were further applied to the column with a flow rate of 0.5 mL min^{-1}. Afterwards, the column was washed with 50 mL of washing buffer (0.05 M Tris pH 8.0, 0.15 M NaCl, 0.04 M Imidazole) to remove unbound material with a flow rate of 1 mL min^{-1}. The elution of the proteins was performed with elution buffer (0.05 M Tris pH 8.0, 0.15 M NaCl, 0.3 M Imidazole) and the proteins were collected in fractions of 2.5 mL. Finally, the column was loaded with 10 mL of 0.01 M EDTA to remove nickel and washed with 10 mL of uH_2O before being stored at 4 °C in EtOH 20% (v/v).

Samples of the flow-through of the lysate, washing steps and elution steps fractions were collected and further analysed by SDS-PAGE (Section 4.1.8). Furthermore, the concentration of protein in each

eluted fraction was quantified by a Bradford assay method (Section 3.1.1.1) and the yields of production (mg of produced protein per litre of culture) were calculated considering the culture volume from which the protein was purified.

A) Cleavage of TrxBDII with TEV protease

Recombinant TrxBDII purified from 220 mL of *E. coli* Origami 2 (DE3) was cleaved with the TEV protease in order to obtain the BDII protein free of the fusion partners TrxA and 6xHis tag (cBDII). Prior to the cleavage, recombinant TrxBDII was submitted to a buffer exchange from the elution buffer to the digestion buffer (0.01 M Tris pH 7.0) (Section 4.1.7.4). Finally, the protein was incubated overnight at 4 °C with 100 µL of TEV protease (1 mg mL^{-1}). The purification of cBDII was further done using HisPur Ni-NTA resin (ThermoFisher Scientific). Briefly, the storage solution of 0.5 mL of resin suspension was removed by centrifugation (13 200 rpm, 2 min at 4°C) to obtain 0.25 mL of dry resin. Then, the resin was washed with 1 mL of 0.01 M Tris at pH 7.0 before being, once again, centrifuged to remove the buffer. This cycle of washing steps and centrifugations was repeated three times. Finally, the solution of the cleaved protein was incubated with the resin during 30 minutes at room temperature and the supernatant, containing only cBDII, was collected and stored at 4°C.

Samples of the purified TrxBDII after buffer exchange to the digestion buffer, digestion mixture after overnight incubation and purified cBDII were analysed by SDS-PAGE (Section 4.1.8). The concentration of cBDII was quantified by a Bradford assay method (Section 3.1.1.1) and the yield of production (mg of pure cBDII per mg of fusion protein) was calculated.

4.1.7.4 Protein buffer exchange

The elution buffer in which the purified recombinant TrxBDII was diluted after the purification protocol was exchanged to the digestion buffer (0.01 M Tris pH 7.0) using PD-10 Desalting Columns (GE Healthcare). Thus, the columns were equilibrated with 25 mL of the new buffer before applying 2.5 mL of the protein sample in the elution buffer. Finally, proteins were eluted with 3.5 mL of the new buffer and stored at 4 °C until further use. The columns were washed with 25 mL of uH_2O and stored in 20 % (v/v) EtOH at 4 °C.

4.1.8 Protein analysis by SDS-PAGE

Protein analysis was performed under denaturing conditions by dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). This technique is used for the separation of proteins based on their ability to move in the gel within an electrical current. This ability is a function of the length of their polypeptide

chains or molecular weight [107]. The gel system used was the Laemmli's system [108] which consists of two gels, the stacking and the resolving gel, with different acrylamide percentages (Table 4.5).

Table 4.5 Composition of the stacking gel and resolving gel for SDS-PAGE analysis of proteins.

Reagent	Stacking gel (4%)	Resolving gel (15%)
40% Acrylamide (v/v)	480 µL	3.6 mL
2% Bis-Acrylamide (v/v)	260 µL	2.0 mL
1.5 M Tris-HCL pH 8.8	-	2.5 mL
0.5 M Tris-HCL pH 6.8	1.25 mL	-
10% SDS (w/v)	50 µL	100 µL
10% APS (w/v)	25 µL	50 µL
TEMED (v/v)	2.5 µL	5 µL
dH_2O	2.933 mL	1.745 mL
Final volume	5 mL	10 mL

Sample preparation was done by mixing 16 µL of the sample with 4 µL of 5x SDS-PAGE sample loading buffer (NZYTech). Total cell fraction samples were, however, prepared in a different form: the pellets from 100 µL of culture (Section 4.1.7.1) were mixed with 40 µL of uH_2O and 10 µL of 5x SDS-PAGE sample loading buffer. All samples were incubated during 10 and 5 minutes at 100 °C, respectively, and finally, the gel well was loaded with 15 µL of each prepared sample. Gel running was made in 500 mL of 1x Running Buffer (0.021 M Tris base, 0.1% w/v SDS and 0.192 M glycine) at 15 mA until the tracking dye reached the base of the gel. The protein molecular weight marker used was PageRuler™ Unstained Broad Range Protein Ladder (ThermoFisher Scientific) (Table 4.6).

Table 4.6. Bands from PageRuler™ Unstained Broad Range Protein Ladder and their respective molecular weight sizes.

Band	Molecular size (kDa)
1	250
2	150
3	100
4	70
5	50
6	40
7	30
8	20
9	15
10	10
11	5

Finally, staining was performed by incubation of the gels under gentle shaking covered in the protein stain BlueSafe (NZYTech) during about 30 minutes. After washing in dH_2O, gels were visualized and photographed in Molecular Imager ChemiDoc™ XRS + Imaging System (Bio-Rad) and analysed using the Image Lab 4.0 software.

4.1.9 Interaction studies of recombinant BDII and TrxBDII with OTA by fluorescence spectroscopy

The interaction of the produced recombinant proteins with OTA was studied by fluorescence spectroscopy. Thus, the three-dimensional spectra of a solution of 1 µM OTA in 0.01 M Tris pH 7.0 was recorded with the Spectro fluorimeter Horiba Aqualog 800 to determine the wavelengths of the maximum excitation and emission. Then, purified proteins produced by *E. coli* BL21 (DE3) (bl-BDII and bl-TrxBDII) and Origami 2 (DE3) (ori-cBDII and ori-TrxBDII) were submitted to buffer exchange to Tris 0.01 M pH 7.0 (Section 4.1.7.4) and each protein, in different concentrations (Table 4.7), was mixed with 1 µM OTA. The protein-mycotoxin mixtures were incubated at room temperature under gentle shaking for 5 minutes. Next, the three-dimensional spectre of each protein-OTA mixtures was recorded to determine the wavelengths of the maximum excitation and emission of OTA upon addition of the recombinant proteins. Furthermore, the fluorescence intensities were analysed by tracing the fluorescence emission spectra with excitation at the wavelength of the maximum excitation previously determined. A positive control was made for each assay with BSA in concentrations proportional to the BDII part in each recombinant protein. Data regarding changes in the wavelength maxima and fluorescence intensities maximum were analysed using OriginPro 2015 software.

Table 4.7. Concentrations of the recombinant proteins, produced by *E. coli* BL21 (DE3) and Origami 2 (DE3), incubated with 1 µM OTA at pH 7.0.

Protein source	Protein	Concentration [µM]
E. coli BL21 (DE3)	bl-BDII	7.0
E. coli BL21 (DE3)	bl-TrxBDII	6.0
E. coli Origami 2 (DE3)	ori-cBDII	10.0
E. coli Origami 2 (DE3)	ori-TrxBDII	6.0

4.1.10 Performance of BDII and TrxBDII-immobilized SPE of OTA in buffer solutions

In order to study the capacity of the recombinant BDII and TrxBDII produced from the Origami strain to capture OTA, empty SPE cartridges of 3 mL were packed with IMAC-Ni resin containing the

recombinant proteins immobilized and were employed in OTA capture assays in 0.01 M Tris at pH 7.0. The resins containing the recombinant proteins were prepared by immobilizing 0.75 and 3.7 mg of ori-BDII and ori-TrxBDII, respectively, in HisPur™ Ni-NTA from purified stocks or crude cell extracts.

4.1.10.1 Construction of BDII-immobilized SPE columns

Recombinant BDII (produced from a volume 40 mL of *E. coli* Origami 2 (DE3) culture) was initially purified in 0.25 mL of HisPur™ Ni-NTA resin. Briefly, the resin storage buffer from 0.5 mL of resin suspension was removed by centrifugation (13 200 rpm, 2 minutes at 4 °C) to obtain the 0.25 mL of dry resin which were further equilibrated with 1 mL of binding buffer (0.05 M Tris pH 8.0, 0.15 M NaCl, 0.02 M Imidazole). Then, the cell extracts were incubated with the resin during 20 minutes in a rotating mixer. The supernatant was removed by centrifugation (4 000 rpm, 2 minutes at 4 °C) and the resin was then washed with 1 mL of washing buffer (0.5 M Tris pH 8.0, 0.15 M NaCl, 0.04 M Imidazole). Once again, the supernatant was removed by centrifugation and this washing step was repeated until no protein was detected using a Bradford protein assay method (Section 3.1.1.1). Finally, the recombinant proteins were eluted with cycles of addition of 0.5 mL of elution buffer (0.5 M Tris pH 8.0, 0.15 M NaCl, 0.3 M Imidazole) and centrifugation (4 000 rpm, 2 minutes at 4 °C) until no protein was detected, once again, by the Bradford protein assay method. The purified recombinant proteins were then submitted to a buffer exchange to the binding buffer following the protocol described in Section 4.1.7.4. The resin used in the purification of recombinant BDII was, once again equilibrated with 1 mL of binding buffer and the supernatant was removed by centrifugation (13 200 rpm, 2 minutes at 4 °C). This cycle was repeated 3 times before incubating the resin with the purified protein during 30 minutes in a rotating shaker at room temperature. The resin was then directly packed in empty 3 mL SPE cartridges which were further equilibrated in binding buffer.

Samples of the flow-through of the lysate, washing steps and elution steps fractions were collected and further analysed by SDS-PAGE (Section 4.1.8). The elution fractions were also analysed by the Bradford assay method (Section 3.1.1.1) to estimate the protein concentration. Also, the presence of protein immobilized in the resin was confirmed by taking a sample of 100 µL of resin. The binding buffer was removed from this sample by centrifugation (4 000 rpm, 2 minutes at 4 °C) and 50 µL of elution buffer were mixed with the resin and analysed by SDS-PAGE.

4.1.10.2 Construction of TrxBDII-immobilized SPE columns

On the other hand, recombinant TrxBDII (produced from 160 mL of *E. coli* Origami 2 culture) was immobilized in 0.25 mL of resin directly from the cell extract. Thus, 0.5 mL of resin suspension were

packed in the empty SPE cartridges and the storage buffer was drained out to obtain the 0.25 mL of dry resin. The equilibration was performed by applying 2 mL of binding buffer to the column. The cell pellet was lysed (Section 4.1.7.2) and the cleared lysate was loaded into the column. The flow-through was collected and re-applied into the column to ensure that all protein was bound. Finally, the column was washed with 25 mL of washing buffer to remove unbound protein and further equilibrated in binding buffer.

Samples of the flow-through of the lysate and washing steps fractions were collected and further analysed by SDS-PAGE (Section 4.1.8). Protein presence in the resin was confirmed as described above (Section 4.1.10.1).

4.1.10.3 OTA capture from buffer solutions by BDII and TrxBDII-immobilized columns

Considering the optimized conditions of the assays performed in BAC method with buffer solutions (Section 3.2.2), each one of the constructed columns was equilibrated with 5 mL of 0.01 M Tris pH 7.0. Then, 1 mL of a solution of 10 μg L^{-1} of OTA in 0.01 M Tris pH 7.0 was applied into the columns which were further washed with 5 mL of the same buffer. The mycotoxin elution was performed with 3 mL of the mobile phase used in OTA determination by HPLC-FL (acetonitrile/dH_2O/acetic acid (99/99/2, v/v/v)). Throughout the protocol, each millilitre was saved separately and kept at 4 °C until OTA quantification via HPLC-FL (Section 3.1.7).

4.1.11 Interaction studies of immobilized recombinant BDII and TrxBDII with OTA in eppendorfs

Recombinant BDII and TrxBDII from cultures of 125 mL of *E. coli* Origami 2 (DE3) were purified by IMAC-Ni (Section 4.1.7.3) and submitted to a buffer exchange to 0.01 M Tris pH 7.0 (Section 4.1.7.4). Purified proteins in Tris buffer were quantified by a Bradford protein assay method (Section 3.1.1.1) and about 3 mg of each recombinant protein were further immobilized in 0.3 mL of HisPur™ Ni-NTA resin according to the protocol in Section 4.1.10.2. Next, portions of 0.1 mL of resin were distributed in three eppendorfs. A negative control was performed with resin without any protein immobilized. A volume of 0.8 mL of 0.01 M Tris pH 7.0 containing OTA in different concentrations (2.0, 5.0 and 10 μg L^{-1}) was added to the tubes which were further incubated at room temperature under gentle shaking. Samples of 0.2 mL of the liquid phase were taken before incubation and after 30 minutes and 2 hours of incubation. Finally, the last sample was taken after the overnight incubation. Each sample was stored at 4 °C until OTA quantification via HPLC-FL (Section 3.1.7).

4.2 Results and discussion

4.2.1 Construction of *E. coli* strains expressing recombinant domain II of BSA

Studies regarding the interactions between various drugs and HSA revealed the existence of two important drug-binding sites named Sudlow's site I and Sudlow's site II, which are located in the hydrophobic cavities of the subdomains IIA and IIIA, respectively [109,110]. In the case of OTA, it has been shown that this mycotoxin binds primarily the Sudlow's site I and also to Sudlow's site II, although with less affinity [86]. Furthermore, two arginine (R) residues located in the positions 218 and 257 of subdomain IIA, have been reported responsible for the stabilization of the complex HSA-OTA due to their interaction with the carboxy and hydroxy groups of the mycotoxin, respectively [111]. Comparison of BSA and HSA revealed not only an high amino acid sequence identity (76%) but also a very similar three-dimensional structure [112]. In addition, the sequence alignment of the subdomain II of both proteins (Annex VI) shows that the two arginine moieties present in HSA, R218 and R257, are also present in BSA, as referred in the literature [111]. This suggests that the main OTA binding site in BSA is similar to that in HSA.

With the aim of developing OTA-extraction platforms based on the domain II of BSA (BDII), this domain was recombinantly produced in two different *E. coli* strains: BL21 (DE3) and Origami 2 (DE3). Thus, the DNA coding sequence for BDII with codons optimized for *E. coli* expression, flanked by the restriction sites for *NcoI* and *XhoI*, was purchased from a specialised company. The gene was delivered in the vector pUC57, which was transformed and propagated in *E. coli* NZY5α competent cells cultured in LB medium containing ampicillin (100 μg L^{-1}). Then, the plasmid was purified (Section 4.1.5.2) and digested with *NcoI* and *XhoI* (Section 4.1.5.4) to isolate the BDII coding sequence. Resulting DNA fragments were separated by agarose gel electrophoresis (Figure 4.1).

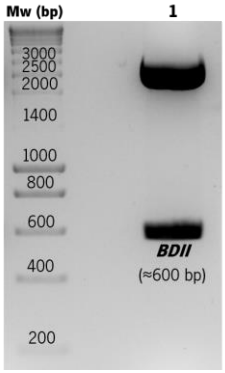

Figure 4.1. Agarose gel (1.5% w/v) of the isolation of BDII gene from the transport vector (pUC57) by enzymatic restriction with NcoI and XhoI. Legend: 1 – Digestion products, pUC57 (2704bp) and BDII gene (602 bp); Mw – molecular weight marker Ladder III (NZYTech).

The presence of a band with ≈600 bp in the agarose gel (Figure 4.1) confirms the separation of the BDII gene (which has 602 bp) from the vector pUC57.

Afterwards, BDII gene was purified from the agarose gel (Section 4.1.5.6) and, using T4 DNA ligase, was ligated to the plasmids pETM10 and pETM20 (Section 4.1.5.7), which were previously digested with the same restriction enzymes. Both plasmids contain the T7 lac promoter and a tag of six histidines (6xHis tag) for metal-affinity purification. The vector pETM20 also contains the coding sequence of thioredoxin protein (TrxA), which behaves as a solubility enhancer of the fused recombinant protein [105]. This fusion partner can be cleaved from the target protein through digestion with TEV protease, since the coding sequence for this cleavage site is located in pETM20 between the TrxA and the multiple cloning site. The ligation reactions were transformed into competent *E. coli* NZY5α. Some randomly chosen colonies were picked up in LB agar plates containing appropriated antibiotics for plasmid DNA purification (Section 4.1.5.8). DNA was further digested with *NcoI* and *XhoI* and analysed by agarose gel electrophoresis to verify if the BDII gene was indeed present in the constructs (Figure 4.2). This screening allowed the identification of two positive clones, one containing the pETM10 and other the pETM20, both carrying the BDII gene. The resulting plasmids were designated by pETM10+BDII and pETM20+BDII (Figure 4.3).

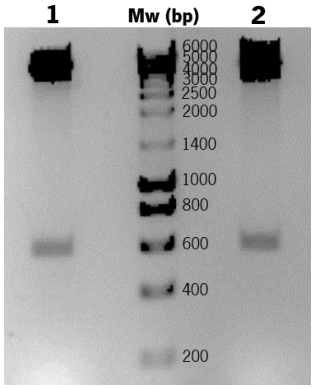

Figure 4.2 – Confirmation in agarose gel (1.5% w/v) of the insertion of the BDII gene into the expression plasmids by enzymatic restriction with *NcoI* and *XhoI*. Legend: 1 – digestion products for pETM10+BDII construct; 2 – digestion products for pETM20+BDII construct; Mw – molecular weight marker Ladder III (NZYTech).

The constructed plasmids were transformed into *E. coli* BL21 (DE3) and Origami 2 (DE3). The first strain was chosen because it is one of the most widely used strains for recombinant protein expression using T7 lac promoter plasmids. On the other hand, the second strain was chosen because it has been recently used in the expression of soluble and functional HSA [88]. The Origami 2 (DE3) strain has the particularity of providing an oxidizing intracellular environment which greatly enhances disulphide bonds formation in the cytoplasm [113]. This environment might be important for the production of

functional BDII since six disulphide bonds can be formed within this protein, as predicted using the bioinformatic tool DiANNA [114–116]. Of noting, the analysis of the entire BSA amino acid sequence in DiANNA resulted in 17 predicted disulphide bonds, which is the number reported for both HSA and BSA [88,117], two of which established between cysteines of domain II and eight of which established between cysteines of this domain and other cysteines in the protein.

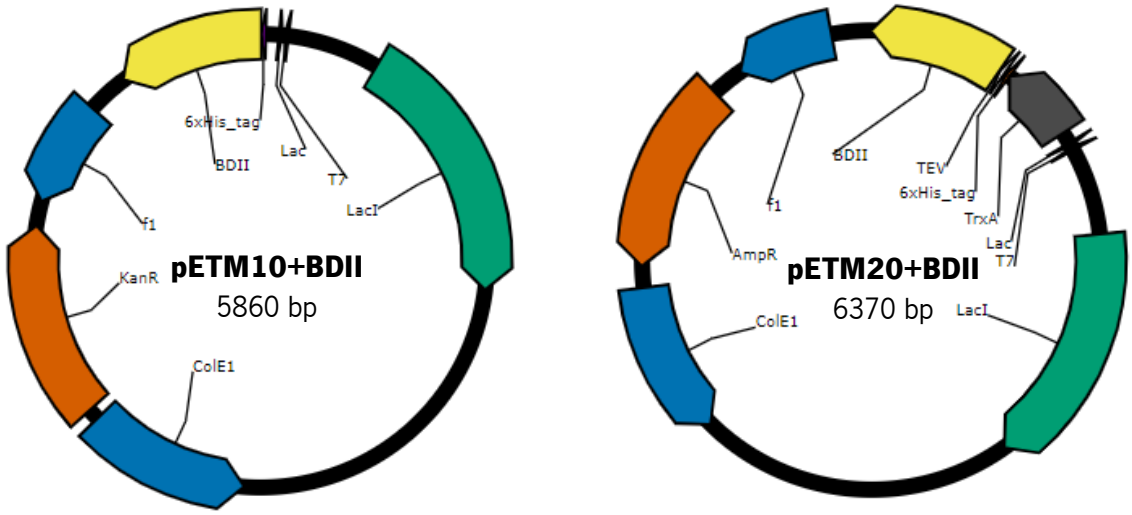

Figure 4.3 – Vector maps of the constructed plasmids pETM10+BDII (5860 bp) and pETM20+BDII (6370 bp) obtained using PlasmaDNA software. Both vectors contain the gene BDII (yellow), the coding sequence of the Lac repressor (green) and the replication origins ColE1 and f1 (blue). The resistance sequences (orange) present in pETM10+BDII and pETM20+BDII are, respectively, for Kanamycin and Ampicillin. TEV cleavage site and TrxA (gray) gene are present only in pETM20+BDII.

4.2.2 Evaluation of recombinant BDII expression in small-screening assays

The soluble expression of recombinant BDII by the two *E. coli* strains was firstly evaluated in small-screening assays (Section 4.1.6). For that, cells were cultured at 37 °C in 5 mL of LB medium containing the appropriated antibiotics and protein expression was induced with IPTG. After incubation overnight at 18°C, the pellet was lysed and both the total lysate (total fraction) and the cleared lysate (soluble fraction) were analysed by SDS-PAGE (Figure 4.4).

As seen in Figure 4.4A, recombinant *E. coli* BL21 (DE3) strains were capable of producing BDII and BDII in fusion with TrxA (TrxBDII), as indicated by the presence of intense protein bands with the molecular weight predicted for each protein (23.8 and 37.0 kDa, respectively). Nevertheless, only a small part of the produced proteins (lanes 1 and 4 in Figure 4.4A) were recovered in the soluble fraction (lanes 2 and 3 in Fig. 4.4A). This means that, most protein was produced in insoluble form, either in the presence or absence of the TrxA fusion partner. On the other hand, production of TrxBDII in *E. coli* Origami 2 (DE3) can be seen in the gel (Figure 4.4B, lanes 3 and 4), but not production of BDII (Figure 4.4B, lanes 1 and 2), as compared with the protein pattern of the negative control (untransformed cells) (Figure

4.4B, lanes 5 and 6). Moreover, TrxBDII is produced in soluble form (Figure 4.4B, lane 3). The expression of the recombinant proteins from each strain/plasmid was further evaluated in production cultures of higher volumes (Section 4.2.3).

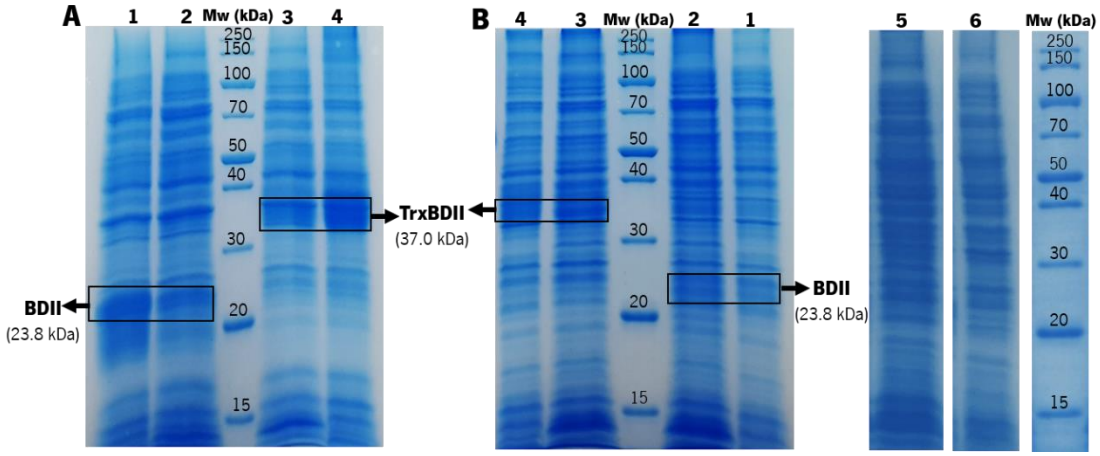

Figure 4.4. SDS-PAGE analysis (BlueSafe stained) of the expression of recombinant BDII and TrxBDII from *E. coli* BL21 (DE3) (A) and *E. coli* Origami 2 (DE3) (B) in cultures of 5 mL. Legend: 1 and 2 – total and soluble fractions, respectively, for expression of BDII from pETM10; 3 and 4 – soluble and total fractions, respectively, for expression of TrxBDII from pETM20; 5 and 6 – soluble and total fractions, respectively, of *E. coli* Origami 2 (DE3) with no expression plasmid transformed (negative control); Mw – Molecular weight marker PageRuler (ThermoFisher Scientific). The arrows/boxes indicate the location of the recombinant proteins in the gels and, between parentheses, their respective predicted molecular weights.

4.2.3 Production and purification of recombinant BDII and TrxBDII

Recombinant proteins were produced from culture volumes ranging from 250 to 500 mL using the same induction conditions as in the small screening assays. Recombinant BDII and TrxBDII were purified by IMAC-Ni from both strains *E. coli* BL21 (DE3) and Origami 2 (DE3), following the protocol described in the materials and methods section (Section 4.1.7).

4.2.3.1 Recombinant BDII purification

The purification of recombinant BDII from both *E. coli* strains was analysed by SDS-PAGE (Figure 4.5) and the results were compared.

Despite the increase of culture volume, recombinant BDII from *E. coli* Origami 2 (DE3) is still not easily detected in the soluble fractions of the cultures (Figure 4.5B, lane 2), mainly because the corresponding protein band is not a typically defined band but it is a diffuse and large band instead. Nevertheless, the purification of the recombinant BDII from both *E. coli* strains was well succeeded, confirming the production of the recombinant proteins. Namely, as seen in Figure 4.5A and B (lanes 4 to 5), protein bands with the molecular weight predicted for recombinant BDII (23.8 kDa) are present in the

elution fractions of the purification protocol. Furthermore, although not shown in the presented gels, no protein loss occurred in any of the washing steps.

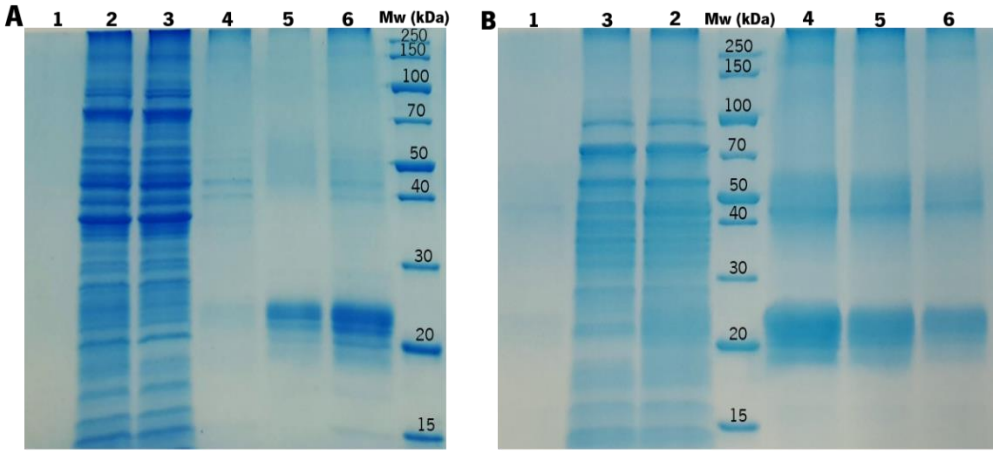

Figure 4.5. SDS-PAGE analysis (BlueSafe stained) of the IMAC-Ni purification of recombinant BDII produced from *E. coli* BL21 (DE3) culture of 240 mL (A) and *E. coli* Origami 2 (DE3) culture of 40 mL (B). Legend: 1 – last CV wash of the column; 2 – soluble fraction of the culture applied into the column; 3 – column flow-through of soluble fraction; 4 to 6 – elution fractions of recombinant BDII, corresponding to the 3rd and 4th fractions for BL21 strain and 5th to 7th fractions for Origami strain; Mw – Molecular weight marker PageRuler (ThermoFisher Scientific).

It is important to note that the elution of the recombinant proteins from the purification column was very different depending on the producing strain. The recombinant BDII from BL21 strain was released from the purification column in the third and fourth elution fractions (Figure 4.5A, lanes 4 to 6), while the BDII from *E. coli* Origami 2 (DE3) was released from the fifth to the seventh fractions (Figure 4.5B, lanes 4 to 6). Thus, BDII from the BL21 strain seems to have less affinity towards the purification column than BDII from the Origami strain, which could indicate that the two proteins have a different structure, or oligomerization degree, which may interfere with the accessibility of the 6xHis tag to the nickel ions.

Recombinant BDII from both strains seems to be degraded, as indicated by the smear present below the bands of the protein in the SDS-PAGE gel (Figure 4.5A and B, lanes 4 to 6). In addition, in the purified BDII from the Origami strain, another smeared band appears with approximately the double molecular weight expected (Fig. 4.5B, lanes 4 to 6). This led to the assumption that a dimer was formed, in spite of the denaturing conditions of the SDS-PAGE analysis.

Purification yield of recombinant BDII, estimated by a Bradford protein assay method (Section 3.1.1.1), was found to be c.a. 4.2 mg per litre of *E. coli* BL21 (DE3) culture and 18.7 mg per litre of *E. coli* Origami 2 (DE3) culture. Thus, regardless the fact that BDII was produced in equal induction conditions from the same expression vector, the yield obtained in the Origami strain was about 5 times higher than that obtained in the BL21 strain. This difference reflects the effect of the strain used in the

production of soluble recombinant BDII. As mentioned before, the oxidizing environment provided in the cytoplasm of *E. coli* Origami 2 (DE3) is reported to greatly enhance the disulphide bond formation, and thus the solubility of the protein [113]. Matter of fact, higher production yields of proteins in soluble form obtained using the Origami strain, in comparison with the BL21 strain, have been widely reported [88,118–120].

4.2.3.2 Recombinant TrxBDII purification

The purification of recombinant TrxBDII from both *E. coli* strains was also analysed by SDS-PAGE (Figure 4.6) and the results were compared.

Recombinant TrxBDII purification was well succeeded in both strains, confirming the production of the recombinant proteins. As seen in Figure 4.6A and B (lanes 4 and 5), protein bands with the molecular weight predicted for recombinant TrxBDII (37.0 kDa) are present in the elution fractions of the purification protocol. However, the obtained proteins are not completely pure, as indicated by the presence of other bands besides the recombinant protein band. Also, once again, although not shown in the presented gels, no recombinant protein was lost in any of the washing steps.

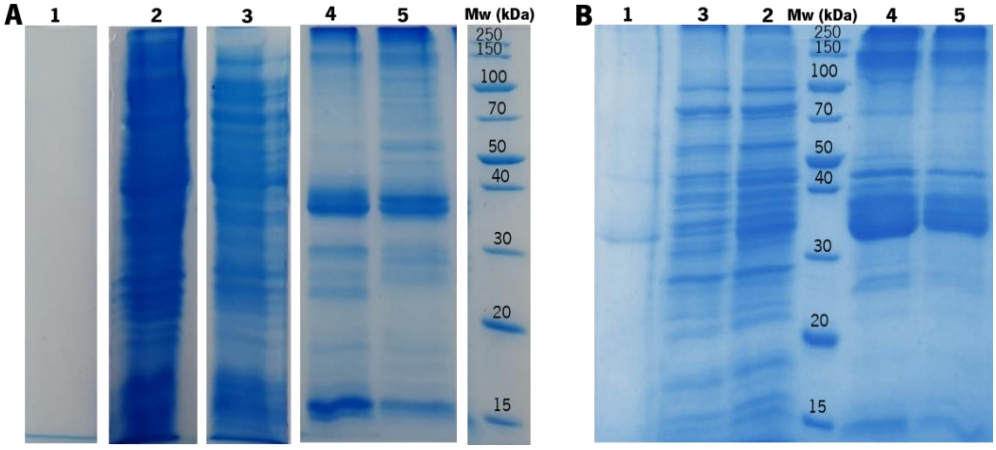

Figure 4.6. SDS-PAGE analysis (BlueSafe stained) of the IMAC-Ni purification of recombinant TrxBDII produced from *E. coli* BL21 (DE3) culture of 330 mL (A) and *E. coli* Origami 2 (DE3) culture of 40 mL (B). Legend: 1 – last CV wash of the column; 2 – soluble fraction of the culture applied into the column; 3 – column flow-through of the soluble fraction; 4 and 5 – elution fractions of recombinant TrxBDII, corresponding to the 2nd and 3rd fractions for the protein of BL21 strain and 5th and 6th fractions for the protein of Origami strain; Mw – Molecular weight marker PageRuler (ThermoFisher Scientific).

Elution profile of the recombinant protein was similar as observed before for BDII, with TrxBDII from the BL21 strain eluting more rapidly than that from the Origami strain. Also, the fused protein seems to be less degraded than recombinant BDII, as indicated by the absence of a smear below the protein band. This may indicate that the TrxA fusion partner increases the stability of the recombinant protein. Matter of fact, it has been reported that the properties of TrxA, such as its thermal stability, can be

retained by their fusion proteins [121]. Particularly, two strong bands appear close to the molecular weight predicted for TrxBDII from *E. coli* BL21 (DE3), which may indicate that, regardless the addition of PMSF during the lysis, the protein was susceptible to proteolysis.

The yield of production was calculated as in the previous sub-section and the results showed that *E. coli* BL21 (DE3) produced 7.8 mg of soluble TrxBDII per litre of culture while *E. coli* Origami 2 (DE3) produced 23.4 mg of soluble TrxBDII per litre of culture. Thus, as seen in the purification of recombinant BDII, *E. coli* Origami 2 (DE3) also has a higher yield of production of soluble TrxBDII than *E. coli* BL21 (DE3). Furthermore, considering that BDII accounts for 60% of the molecular weight of the fusion protein TrxBDII, the yield of BDII produced alone and attached to the TrxA partner are practically identical either in BL21 or Origami strain. This indicates that the TrxA did not enhance BDII solubility but, it is important to emphasize that, as mentioned before, since the TrxBDII protein seems less degraded than BDII, this fusion partner does seem to enhance the protein stability.

A) Cleavage of TrxBDII with TEV protease

Recombinant TrxBDII produced and purified from *E. coli* Origami 2 (DE3) was cleaved with the TEV protease in order to obtain the BDII free of the fusion partners TrxA and 6xHis tag (cBDII). Interaction studies of cBDII with OTA are important to be conducted to evaluate if the presence of the TrxA and 6xHis tag in the fusion protein TrxBDII can affect BDII activity, that is, its affinity towards the mycotoxin. Therefore, after the IMAC-Ni purification of TrxBDII from *E. coli* Origami 2 (DE3), and buffer exchange from the elution buffer (0.05 M Tris pH 8.0, 0.15 M NaCl, 0.3 M Imidazole) to the digestion buffer (0.01 M Tris pH 7.0), the protein was digested with TEV protease (Section 4.1.7.3A). Since the recognition site for TEV is located between the BDII sequence and the sequence of the fusion partners (Annex V), the cleavage reaction results in two products: the cleaved BDII, i.e. cBDII (22.7 kDa) and the TrxA having a C-terminal 6xHis tag (14.3 kDa). Finally, pure cBDII was obtained by performing a reverse IMAC-Ni purification. This purification is based on the fact that both the TrxA and the TEV protease (that also has a 6xHis tag) bind to the resin while cBDII remains in solution. Thus, after incubation of the digestion mixture with the resin, pure cBDII was obtained by collecting the supernatant.

The main steps of the cleavage reaction were analysed by SDS-PAGE (Figure 4.7). In the digestion mixture it is possible to identify all expected products of digestion, namely, TrxA (also seen in the fusion protein TrxBDII; Figure 4.7, lane 1), and cBDII, but also the TEV protease (≈50 kDa), and remaining fusion protein not cleaved (Figure 4.7, lane 2). Despite the presence of small amounts of digestion sub-

products, purification of cBDII was quite effective. The yield obtained was roughly 0.47 mg of pure cBDII per mg of fusion protein.

Nevertheless, the purified cBDII appears as two smeared bands (Figure 4.7, lane 4) (as previously seen in the purification of TrxBDII from *E. coli* BL21 (DE3); Figure 4.6), which could reflect that, once again, despite the presence of the PMSF during the purification protocol, the protein was susceptible to proteolysis. Thus, the use of other (more expensive) proteases inhibitors, targeting other *E. coli* proteases (e.g. cysteine proteases) could have reduced BDII proteolysis.

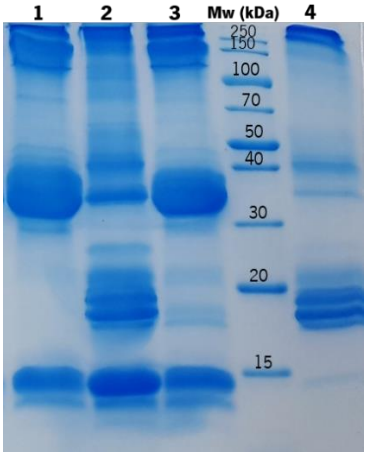

Figure 4.7. SDS-PAGE analysis (BlueSafe stained) of the cleavage of purified TrxBDII with TEV protease to obtain cBDII. Legend: 1 – Purified recombinant TrxBDII; 2 – Digestion products of TrxBDII cleavage reaction; 3 – TrxBDII after buffer exchange to the digestion buffer; 4 – Recombinant cBDII after reverse purification; Mw – Molecular weight marker PageRuler (ThermoFisher Scientific).

4.2.4 Interaction studies of recombinant BDII and TrxBDII with OTA by fluorescence spectroscopy

OTA is a molecule that possesses fluorescence properties. These properties are highly dependent on the protonation state of the molecule and it has been reported that the excitation maximum for the mono-anion (OTA^{-1}) and di-anion (OTA^{-2}) forms of OTA are, respectively, 334 and 380 nm. On the other hand, at both wavelengths, OTA has a maximum of emission around 450 nm [84]. In addition, it has been shown that upon addition of serum albumins, namely BSA and HSA, the excitation maximum of OTA shifts to around 395 nm and the fluorescence intensity suffers a major increase, thus revealing the interaction between these proteins and OTA [95,96,122]. These changes in the fluorescence properties can be used to analyse a possible interaction between OTA and the recombinant proteins produced in this work.

Firstly, the three-dimensional fluorescence spectre of OTA (1 µM) at pH of 7.0 was traced (Figure 4.8) to verify its wavelength maxima of excitation. This type of spectra provides the emission fluorescence

intensities at sequential different excitation wavelengths [123]. The values of the fluorescence intensities are represented by the colour schemes. As expected, at pH 7.0, OTA presented two maxima of excitation: one at 333 nm (close to the 334 nm reported), which is the one that results in more emission of fluorescence, and the other at 380 nm, which results in less emission of fluorescence. The emission of fluorescence maximum for these two excitation wavelengths was 450 nm. The two maxima of excitation can be explained by the presence of the two protonated forms of OTA, OTA^{-1} and OTA^{-2}, which have different excitation maxima (334 and 380 nm, respectively). The pKa values of OTA for the carboxy and hydroxy groups are, respectively, 4.2-4.4 and 7.0-7.3 [84] and, therefore, at the pH 7.0 both excitation maxima are exhibited because both forms of OTA are present [26]. Also, the higher fluorescence intensity seen for excitation at 333 nm indicates that, at pH of 7.0, OTA is mainly present as OTA^{-1}.

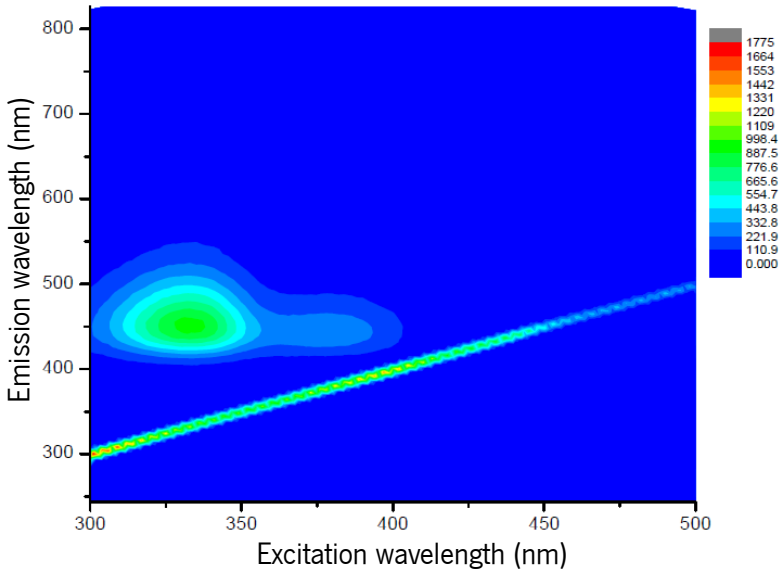

Figure 4.8. Three-dimensional fluorescence spectre of OTA (1 µM) in 0.01 M Tris pH 7.0. Colour scheme represents the intensity of fluorescence emission for each excitation wavelength.

The three-dimensional fluorescence spectra of OTA in the presence of BSA and each one of the recombinant BDII proteins produced and purified from different *E. coli* strains was also traced (Section 4.1.9) to analyse their interaction with OTA. The excitation and emission wavelengths maxima recorded for each case are presented in Table 4.8.

While the excitation maximum for free OTA was found to be 333 nm (as depicted from Fig. 3.8 data), in the presence of saturating amounts of BSA (100% bound toxin), OTA showed a maximum at 393 nm (Table 4.8), as reported in the literature [122]. At this excitation wavelength, the emission maxima wavelengths of OTA and the complex OTA+BSA were all close to 450 nm. In the presence of the recombinant BDII proteins produced in the BL21 strain, no significant changes were seen in the excitation and emission maxima (Table 4.8), which were identical to the maxima of excitation and emission of free

OTA. This absence of changes indicates that there is no interaction between OTA and these proteins, and therefore that the proteins are not functional. On the other hand, in the presence of the recombinant BDII proteins produced in the Origami strain, the OTA excitation maxima suffered a shift from the 333 nm to 383 nm. This shift, although different from that observed when BSA is present, still reflects an interaction between OTA and the recombinant proteins. In fact, Il'ichev *et al.* (2002) observed this same shift when studying the interaction of OTA with the domain II of HSA produced in *Pichia pastoris* [87]. Therefore, both proteins produced by *E. coli* Origami 2 are functional, as they interact with the mycotoxin. Furthermore, no differences are seen between the cleaved BDII (ori-cBDII) and the fusion protein ori-TrxBDII, indicating that the presence of the TrxA fusion partner does not hinder the OTA-BDII interaction.

Table 4.8. Fluorescence properties of OTA (1 μM) in the presence of BSA (10 μM) and the recombinant BDII proteins (6-10 μM) produced and purified from different *E. coli* strains.

Protein designation	Protein source	Excitation maxima (nm)	Emission maxima (nm)
BSA	Bovine	393	450
bl-BDII	BL21	334	451
bl-TrxBDII	BL21	333	451
ori-cBDII	Origami 2	383	440
ori-TrxBDII	Origami 2	383	441

The emission spectra of OTA in the presence of the recombinant proteins produced by the Origami strain was run at the fixed excitation maximum wavelength of 383 nm (Figure 4.9) to analyse the changes in the fluorescence intensity, comparing with data reported for BSA [122].

As seen in Figure 4.9, in the presence of the recombinant proteins ori-cBDII and ori-TrxBDII, the fluorescence increased significantly (≈ 56 and 61%, respectively) in comparison with that of free OTA. This increase in fluorescence intensity, although smaller than that observed when BSA is present (≈ 320%), also shows that there is indeed an interaction between BDII produced in the Origami strain and OTA. The differences in the increase of fluorescence between BSA and the recombinant proteins may be due to differences in their affinity towards OTA. The OTA-binding constants for BDII proteins are certainly lower than that for BSA, as happened for the domain II of HSA [84,87].

Given the positive results in the fluorescence assays with the recombinant proteins produced in the Origami strain, the interaction of these proteins with OTA was further evaluated in subsequent studies.

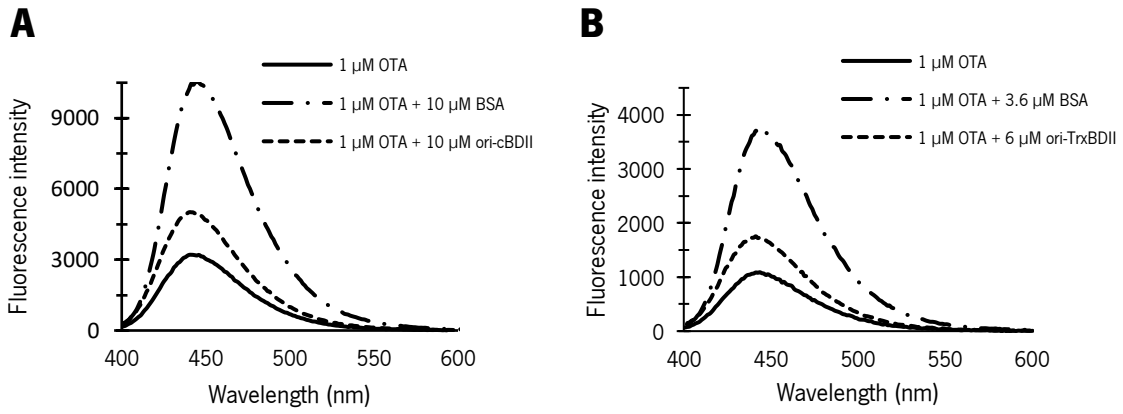

Figure 4.9. Fluorescence emission spectra of OTA (1 µM) at pH 7.0 in the absence and presence of 10 µM ori-cBDII (A) or 6 µM ori-TrxBDII (B) produced in *E. coli* Origami 2 (DE3). BSA was used as a positive control at the same molarity of BSA domain in each recombinant protein. Excitation wavelengths were 393 nm for the BSA assay and 383 nm for recombinant protein assays and free OTA. The differences observed between fluorescence in (A) and (B) are due to the use of different OTA stock solutions, which although at the same concentration, presented different intensity of fluorescence.

4.2.5 Performance of BDII and TrxBDII-immobilized SPE of OTA in buffer solutions

The potential of using both recombinant proteins BDII and TrxBDII produced by *E. coli* Origami 2 (DE3), i.e. ori-BDII and ori-TrxBDII, as extraction ligands of OTA from buffer solutions was evaluated mimetizing the SPE method previously developed for BSA (Chapter III). For that, the recombinant proteins (0.75 and 3.7 mg, corresponding to 3.0×10^{-2} µmol and 6.2×10^{-2} µmol of BDII in ori-BDII and ori-TrxBDII, respectively) were immobilized in IMAC-Ni resin (0.25 mL), which was packed into empty SPE columns (Section 4.1.10.1 and 4.1.10.2).

Thereafter, a solution of 10 µg L^{-1} OTA in 0.01 M Tris at pH 7.0 was passed through the constructed BDII and TrxBDII-immobilized columns and the mycotoxin was quantified in each fraction collected (Section 4.1.10.3). A negative control was performed with columns packed with the IMAC-Ni resin without any protein immobilized. The results obtained are presented in Figure 4.10.

As expected, in the negative control all OTA loaded in the column was washed out in the flow-through (32%) and in the two first washing steps (65 and 3%, respectively) (Figure 4.10). Nevertheless, all OTA was also washed out from the columns containing the proteins immobilized. In fact, the results from the assay in the column ori-BDII are very similar to the negative control, indicating that no stable interaction between the protein and the mycotoxin occurred. However, in the column ori-TrxBDII, only 7% of loaded OTA was lost in the column flow-through step, which is a much lower percentage than that observed in the negative control (32%).

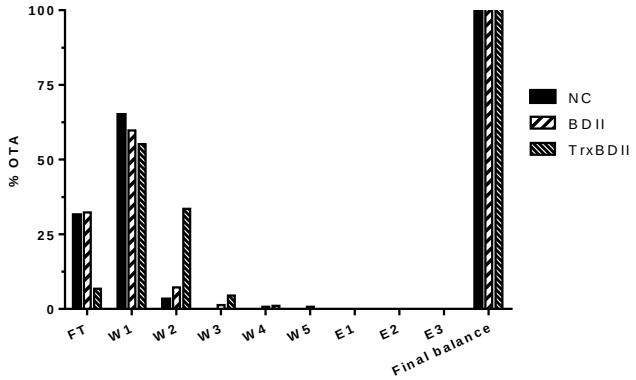

Figure 4.10. Capture assays of OTA from 0.01 M Tris pH 7.0 solution containing 10 μg L^{-1} of mycotoxin using the constructed ori-BDII and ori-TrxBDII-immobilized columns. The negative control (NC) refers to the assay performed in a column containing resin without protein immobilized. Each bar represents the % of OTA quantified in each collected fraction (FT– flow-through of the OTA solution; W1-W5 – washing steps; E1-E3 – elution steps), excepting the final balance bars, which correspond to the sum of % OTA in all fractions in each assay. Results for single assays are presented.

The delay observed in the washing out of OTA from the ori-TrxBDII column could mean that there is an interaction of OTA with this protein. Furthermore, the fact that a delay in OTA washing was observed in the column with ori-TrxBDII, and not observed in the column with ori-BDII, was not in accordance with the results obtained by fluorescence spectroscopy, where both free recombinant proteins, TrxBDII and cBDII (assuming that ori-BDII and ori-cBDII interact similarly with OTA) led to an identical change in the fluorescence properties of OTA, thus suggesting a similar interaction with this mycotoxin (Section 4.2.4). These results could be due to the fact that the quantity of BDII in the columns containing ori-TrxBDII immobilized was about 2.1 times higher than the quantity of BDII in the columns containing ori-BDII. On the other hand, in these SPE assays the proteins are immobilized, and the exposure of BDII may be compromised. The different results obtained in these assays for the two proteins could also be due to a higher exposure of the BDII in ori-TrxBDII than in ori-BDII. Matter of fact, while the ori-BDII protein has only one amino acid residue between the 6xHis tag and the BDII sequence, ori-TrxBDII has eleven amino acid residues. Thus, the BDII protein in ori-TrxBDII is not as close to the resin support, which may allow more accessibility to interact with OTA. Anyway, remaining OTA was fully recovered in the washing steps, which indicates that the interaction observed was not strong enough to retain the mycotoxin inside the column and thus, to allow an effective capture of OTA. In fact, in 2002, the interaction of OTA with the different recombinant domains of HSA was studied [87]. The binding constants of OTA towards the entire HSA ($K= 5.2 \times 10^{6}$ M^{-1}) and towards each domain were compared. The binding constant determined for the domain II of HSA was 7.9×10^{5} M^{-1}, which means that there was a decrease of the binding constant

by seven-fold in comparison with the constant determined for the entire HSA. Thus, based on this decrease observed in the binding constant in the case of HSA, the binding constant for BDII could also be inferior at least in the same order of magnitude in comparison to the entire BSA ($K = 3.2 \times 10^5\ M^{-1}$) [96]. However, these binding constants (in the order of 10^4) should be enough for the application of the produced recombinant proteins in SPE protocols. Matter of fact, in 2007 Giraudi *et al.* reported a SPE method based on the immobilization of an hexapeptide that exhibited a binding constant of $3.4 \times 10^4\ M^{-1}$ towards OTA and obtained recoveries of up to 98% of OTA in wine [30]. Thus, the incapacity of the produced recombinant proteins to retain OTA in the SPE columns may not be due to the expected decrease in their binding constants, relatively to BSA affinity constant. Probably the optimal conditions for OTA binding to BDII and TrxBDII are not the same as those determined for BSA, used in this assay (Chapter III). As such, optimization may be required for an effective capture of the mycotoxin.

4.2.6 Interaction studies of immobilized recombinant BDII and TrxBDII with OTA in eppendorfs

The interaction of OTA (2.0, 5.0 and 10 μg L^{-1}) with the recombinant proteins BDII and TrxBDII produced by *E. coli* Origami 2 (DE3), i.e. ori-BDII and ori-TrxBDII, immobilized in IMAC-Ni resin was evaluated in incubation assays performed in eppendorfs with gentle shaking at pH 7.0 (Section 4.1.11). The OTA in solution (% of free OTA) was monitored in the different assays by HPLC-FL over the incubation time (Figure 4.11).

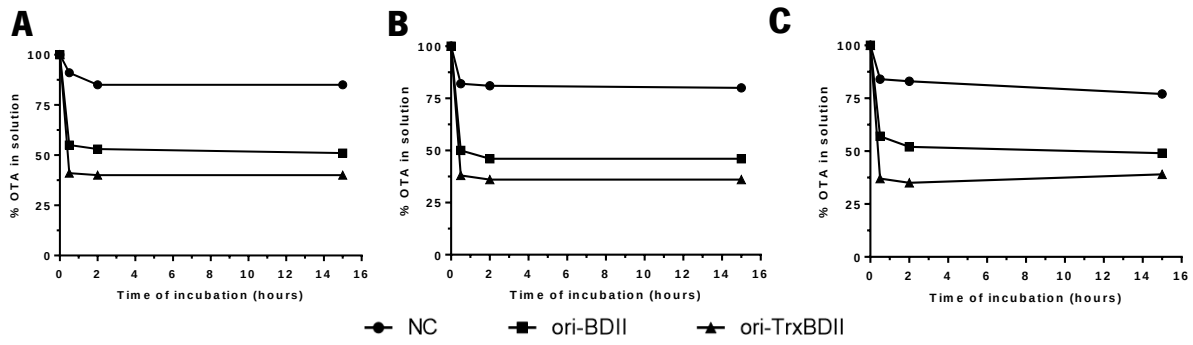

Figure 4.11- Profile of free OTA (%) over time in incubation assays of 2.0, 5.0 and 10 μg L^{-1} of OTA (A, B and C, respectively) with 1 mg of ori-BDII and ori-TrxBDII immobilized in IMAC-Ni resin. The negative control (NC) refers to the assay conducted with resin having no protein immobilized.

As seen in Figure 4.11, in the assays with recombinant proteins immobilized the percentage of OTA in solution decreases significantly, regardless the concentration of OTA tested. Moreover, this effect is higher for ori-TrxBDII (59-63% of OTA reduction in solution) than for ori-BDII (45-54% of OTA reduction

in solution). Thus, this means that ori-TrxBDII may have higher OTA capture capacity than ori-BDII, as already seen in the SPE experiments (Section 4.2.5). Furthermore, contrary to the previous assays, the quantity of immobilized ori-BDII was 2.5 times higher than the quantity of BDII in the immobilized fusion protein ori-TrxBDII (1 mg of each, corresponding to 4.0×10^{-2} µmol and 1.6×10^{-2} µmol of BDII in each recombinant protein, respectively). This suggests that the higher capture capacity observed for TrxBDII is not an effect of the quantity of protein used in these assays but may be due to a higher exposure of BDII in the fused protein than in ori-BDII. OTA-protein interaction equilibrium was rapidly established since the values for the percentage of OTA in solution were maintained almost constant from the 30 minutes of incubation until the end of the assay.

In the negative control (assay with no protein immobilized), a decrease of the percentage of OTA in solution (up to 23%) also occurred (Figure 4.11). Thus, since this resin has no protein immobilized, this means that part of OTA adsorbs on the resin. Nevertheless, the differences obtained in the capture percentages of OTA between the negative control assays and the assays with ori-BDII and ori-TrxBDII indeed indicate the existence of an interaction between OTA and both recombinant proteins produced from *E. coli* Origami 2.

CHAPTER V.

CONCLUSIONS AND FUTURE PERSPECTIVES

Given the emerging needs to control the occurrence of mycotoxins, such as OTA, in wines, the development of fast, efficient and affordable techniques for their detection is becoming increasingly important. Focusing on this, the current work aimed the development of new platforms for OTA clean-up from wine using proteins as mycotoxin ligands.

In a first approach, the strong affinity of the protein BSA towards OTA was used in the development of SPE columns for the extraction of the mycotoxin from wine, where BSA was immobilized in activated agarose. The assays of OTA capture in buffer solutions with this system allowed to conclude that the optimal binding of the mycotoxin to BSA occurs at pH values in the range of 7.0-8.0 and at low ionic strengths. The method conditions were further optimized in order to obtain the total recovery of the mycotoxin in wine and finally, the method, which was named BAC method, was validated. The validation of the method focused on the evaluation of some parameters such as the linearity, recovery rates, precision, selectivity and limits of detection and quantification, which allowed the confirmation of the suitability of the developed method for OTA detection in wine. The BAC method was shown to be linear and precise in the ranges of concentrations studied, allowing recovery rates superior to 98%. Although not as selective and specific as the reference IAC method, the BAC method gave enough selectivity to allow a reliable quantification of OTA in wine, having LOD and LOQ values lower than many SPE methods reported in the literature. Furthermore, the comparison of the developed method performance with the IAC method revealed no significant differences in comparison to the IAC method in concentrations up to 2 μg L^{-1} of OTA. The mycotoxin determination in naturally contaminated wines produced in various wine regions of Portugal using the developed BAC method originated results very similar to the IAC method and revealed that those wines were in accordance with the regulatory limit imposed for OTA presence in wine by the European entities (2 μg L^{-1}).

The validation procedure of the BAC method can always be submitted to further improvements, namely the number of replicates can be increased, since they were few in the current work and it is vital to confirm the method's reliability. Also, the increase of the number of real wines samples analysed would be advantageous for a better evaluation of the method performance. Furthermore, the evaluation of other parameters such as the cost-benefit of the method would be very important to estimate the rentability of the method and thus, the suitability for its future implementation.

In a second approach, the interaction between OTA and the domain II of BSA (which contains the primary binding site of OTA) was studied and its use as ligand in platforms for mycotoxin extraction was evaluated. For that, the protein BDII was recombinantly produced, fused with a 6xHis tag, with and without the fusion partner TrxA, in two different *E. coli* strains, BL21 (DE3) and Origami 2 (DE3). Both strains

were capable of producing soluble BDII and TrxBDII, but the Origami strain provided higher yields of production. Also, the fusion of TrxA did not allow the enhancement of the protein solubility but, the fact that TrxBDII produced by both strains was less susceptible to degradation allowed to conclude that the fusion partner caused the increase of the recombinant protein stability during purification. The fluorescence spectroscopy studies revealed that only the recombinant proteins produced from the Origami strain could interact with OTA, confirming the importance of the oxidizing cytoplasm provided by this strain in the formation of the disulphide bonds and thus, on the protein folding and activity.

The ability of the recombinant proteins produced in the Origami strain to capture OTA was firstly evaluated in SPE assays, with the proteins immobilized in nickel resin via the 6xHis tag, which led to the conclusion that, no effective capture of the mycotoxin could be achieved using the experimental conditions of the previously optimized BAC method. Thus, optimization of these assays should be conducted in order to find the conditions in which the capture of OTA is effective and results in total mycotoxin recovery.

Finally, the possibility of employing the nickel-immobilized BDII and TrxBDII produced by the Origami strain in a different system of OTA extraction, based on the process of wine clarification, was evaluated by studying the interaction of the recombinant proteins with the mycotoxin in incubation assays performed in eppendorfs. The decrease observed in the concentration of OTA in solution by more than a half allowed to conclude that there was, indeed, an interaction between both recombinant proteins and OTA. Furthermore, due to a possible higher exposure of the BDII in the TrxBDII when immobilized via the 6xHis tag in the nickel resin, this fusion protein established a stronger interaction with the mycotoxin and originated higher percentages of OTA capture. Once again, optimization of the conditions used in these assays, such as the increase of the quantity of resin and/or protein immobilized should be conducted in order to achieve higher captures of the mycotoxin.

Altogether, these results open perspectives for the development of OTA extraction platforms based on the recombinant BDII, particularly by means of purification systems designed for the process of wine clarification. Furthermore, the fusion of BDII with specific purification tags will allow its immobilization in matrixes less expensive than the agarose used in the first approach (e.g. fusion with a cellulose-binding module, such as CBM3, allows a direct printing of the recombinant protein in cellulose).

As a future work, in order to increase the affinity of extraction platforms of OTA based on recombinant proteins, the production of the entire HSA in *Pichia pastoris* can be conducted. This approach is very promising, not only due to the higher affinity of HSA towards OTA in comparison to BSA, but mainly because of the success observed in the use of this protein as a fusion partner in the production of various proteins in this host [124,125].

CHAPTER VI.

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CHAPTER VII.

ANNEXES

ANNEX I – CALIBRATION CURVE USED TO CALCULATE BSA CONCENTRATION

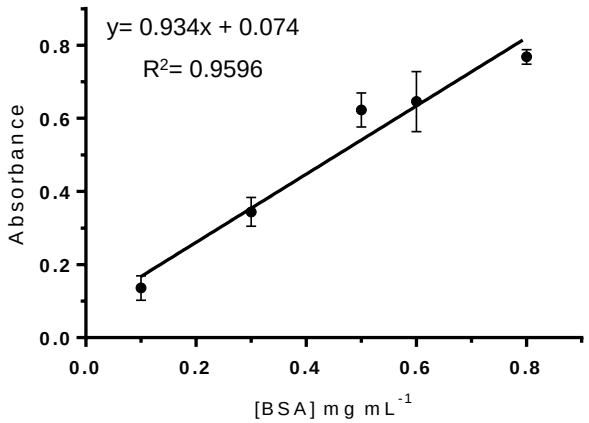

Figure I. Calibration curve used for BSA determination. The absorbance of BSA standards with concentrations between 0.1 and 0.8 mg mL^{-1} was measured in a microplate reader (Citation™ 3 from Biotek) in 96 wells plate at a wavelength of 595 nm. Absorbance of each standard was plotted against the respective protein concentration. The results were fitted to a linear regression (R^2 = 0.9596) to obtain the equation Y = (0.934 ± 0.352)x + (0.074 ± 0.183).

ANNEX II – HPLC-FL CALIBRATION CURVE USED TO CALCULATE OTA CONCENTRATION

The calibration curve for OTA determination by the HPLC-FL apparatus was constructed with twelve standard solutions of OTA (0.05, 0.1, 0.25, 0.5, 1.0, 2.5, 5, 10, 25, 50, 75 and 100 μg L^{-1}), considering the y intercept (b) null. The standard deviation (SD) of the slope (m) of the curve was used to calculate errors. Using the t table values for a confidence value of 95% (α=0.05) and n=12 (number of samples in the calibration curve), the interval of confidence for the slope and y intercept were determined.

Table I. OTA calibration curve parameters for HPLC-FL.

Calibration curve parameters	
Slope (m)	1392.076
Correlation coefficient (R^2)	1.00
Upper limit (95%)	1387.756
Lower limit (95%)	1378.570
m ± (Upper limit (95%) – Lower limit (95%))/2	1392.076 ± 4.593

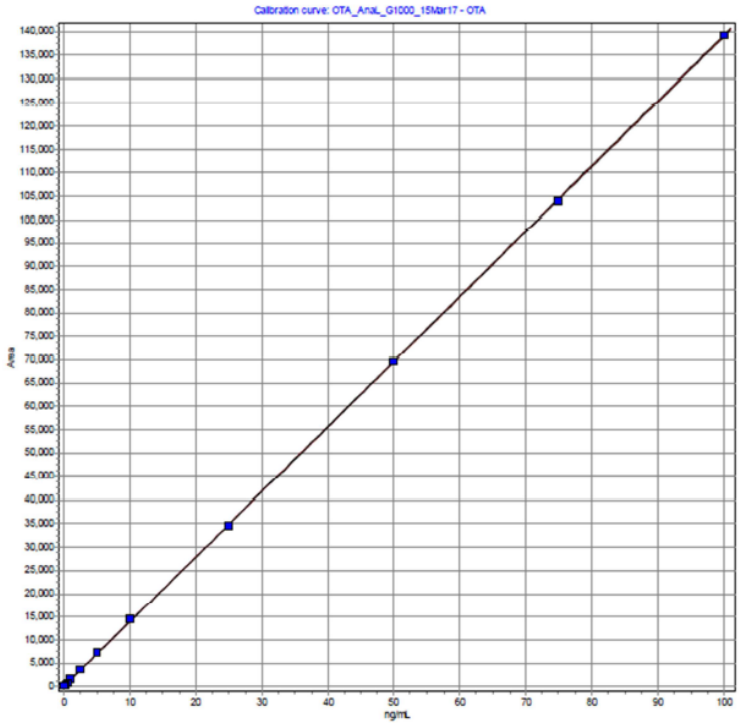

Figure II. Calibration curve obtained by the analysis of OTA standards in the range of 0.5-100 μg L^{-1} in the HPLC-FL. The peaks area for each standard was plotted against its respective concentration of OTA. The results were fitted to a linear regression (R^2 = 1.00) to obtain the equation Y = (1392.076 ± 4.593)x.

ANNEX III – PARAMETERS OBTAINED BY HPLC-FL FOR THE VALIDATION OF BSA-AGAROSE COLUMNS

Table II. Determination of OTA in red wine using the BSA-agarose columns for the linearity studies, recovery rates, precision, LOQ and LOD. The values represent the average ± the standard deviations (SD) of two independent experiments.

OTA supplemented (μg L^{-1})	OTA determined by the instrument (μg L^{-1})	Real OTA in wine (μg L^{-1})*
0.05	1.28 ± 0.05	0.05 ± 0.00
0.5	5.64 ± 0.18	0.49 ± 0.02
1	10.60 ± 0.37	0.99 ± 0.04
2	20.55 ± 0.25	1.98 ± 0.03
3	30.40 ± 0.06	2.97 ± 0.01

*The real concentration of OTA in wine was calculated dividing OTA determined by the instrument by 10 (since the final extracts of the assay are 10-fold concentrated).

Table III. Determination of OTA in red wine using the immunoaffinity columns for the studies of LOQ and LOD. The values represent the average ± the standard deviations (SD) of two independent experiments.

OTA supplemented (μg L^{-1})	OTA determined by the instrument (μg L^{-1})	Real OTA in wine (μg L^{-1})
0.05	1.20 ± 0.08	0.05 ± 0.01
0.5	5.70 ± 0.18	0.50 ± 0.02
1	10.33 ± 0.76	0.96 ± 0.08
2	19.68 ± 0.40	1.89 ± 0.04
3	28.73 ± 0.59	2.80 ± 0.06

*The real concentration of OTA in wine was calculated dividing OTA determined by the instrument by 10 (since the final extracts of the assay are 10-fold concentrated).

ANNEX IV – LINEAR REGRESSIONS USED TO CALCULATE LOQ AND LOD

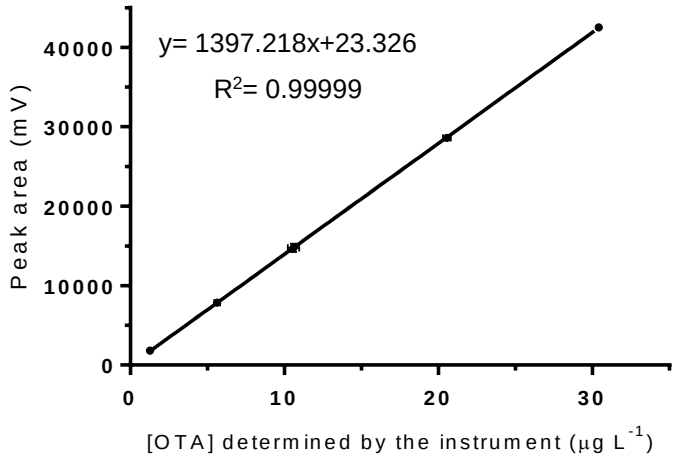

Figure III. Relationship between the peak area and OTA determined by HPLC-FL upon extraction by the BAC method. The results were fitted to a linear regression (R^2 = 0.99999) to obtain the equation Y = (1397.218 ± 9.653)x + (23.326 ± 166.751). The error bars in each point represent the standard deviations (SD) of two independent experiments.

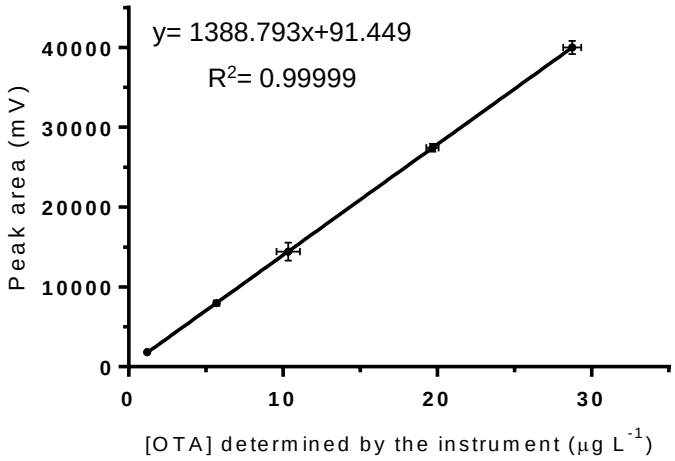

Figure IV. Relationship between the peak area and OTA determined by HPLC-FL upon extraction by the IAC method. The results were fitted to a linear regression (R^2 = 0.99999) to obtain the equation Y = (1388.793 ± 5.450)x + (91.449 ± 89.654). The error bars in each point represent the standard deviations (SD) of two independent experiments.

ANNEX V – SEQUENCES OF THE PRODUCED RECOMBINANT PROTEINS

In the current work, the recombinant domain II of BSA (BDII) was produced from two *E. coli* strains in two different versions, depending on the vector in which the sequence was inserted: BDII and TrxBDII were produced from *E. coli* BL21 (DE3) and Origami (DE3) containing the vectors pETM10 and pETM20 carrying BDII coding sequence. Furthermore, the TrxA and 6xHis tag parts of TrxBDII were cleaved through a TEV cleavage site to give rise to cleaved BDII (cBDII). The sequences for each protein version are presented below:

BDII

MKHHHHHHPMVLASSARQRLRCASIQKFGERALKAWSVARLSQKFPKAEFVEVTKLVTDLTKVHKECCHGDLL
ECADDRADLAKYICDNQDTISSKLKECCDKPLLEKSHCIAEVEKDAIPENLPPLTADFAEDKDVCKNYQEAKDAFL
GSFLYEYSRRHPEYAVSVLLRLAKEYEATLEECCAKDDPHACYSTVFDKLKHLVDEPQN*

TrxBDII

MSDKIIHLTDDSFDTDVLKADGAILVDFWAEWCGPCKMIAPILDEIADEYQGKLTVAKLNIDQNPGTAPKYGIRGIP
TLLLFKNGEVAATKVGALSKGQLKEFLDANLAGSGSGHMHHHHHHSSGENLYFQGAMVLASSARQRLRCASIQ
KFGERALKAWSVARLSQKFPKAEFVEVTKLVTDLTKVHKECCHGDLLECADDRADLAKYICDNQDTISSKLKECC
DKPLLEKSHCIAEVEKDAIPENLPPLTADFAEDKDVCKNYQEAKDAFLGSFLYEYSRRHPEYAVSVLLRLAKEYEAT
LEECCAKDDPHACYSTVFDKLKHLVDEPQN*

cBDII

GAMVLASSARQRLRCASIQKFGERALKAWSVARLSQKFPKAEFVEVTKLVTDLTKVHKECCHGDLLECADDRADL
AKYICDNQDTISSKLKECCDKPLLEKSHCIAEVEKDAIPENLPPLTADFAEDKDVCKNYQEAKDAFLGSFLYEYSR
RHPEYAVSVLLRLAKEYEATLEECCAKDDPHACYSTVFDKLKHLVDEPQN*

Description:

BDII sequence

6xHis tag

TrxA sequence

TEV recognition sequence

ANNEX VI – SEQUENCE ALIGNMENT OF THE DOMAIN II OF HSA AND BSA

```
HSA_DII   GKASSAKQRLKCASLQKFGERAFKAWAVARLSQRFPKAEFAEVSKLVTDLTKVHTECCHG 60
BSA_DII   VLASSARQRLRCASIQKFGERALKAWSVARLSQKFPKAEFVEVTKLVTDLTKVHKECCHG 60
          ****:***:***:*******:***:******:******.**:**********.*****

HSA_DII   DLLECADDRADLAKYICENQDSISSKLKECCEKPLLEKSHCIAEVENDEMPADLPSLAAD 120
BSA_DII   DLLECADDRADLAKYICDNQDTISSKLKECCDKPLLEKSHCIAEVEKDAIPENLPPLTAD 120
          *****************:***:*********:**************:* :* :** *:**

HSA_DII   FVESKDVCKNYAEAKDVFLGMFLYEYARRHPDYSVVLLLRLAKTYETTLEKCCAAADPHE 180
BSA_DII   FAEDKDVCKNYQEAKDAFLGSFLYEYSRRHPEYAVSVLLRLAKEYEATLEECCAKDDPHA 180
          *.*.******* ****.*** *****:****:*:* :****** **:***:***  ***

HSA_DII   CYAKVFDEFKPLVEEPQN 198
BSA_DII   CYSTVFDKLKHLVDEPQN 198
          **:.***::* **:****
```

Sequence alignment of the sequences of domain II from HSA and BSA was performed using the tool Multiple sequence alignment from EMBL-EBI and resulted in 79% identity and 93% similarity. The arginine residues highlighted in yellow correspond to R218 and R257 from HSA which are reported to be responsible for OTA binding. These residues are also present in BSA.