

F(ab')₂ Fragment of Monoclonal Antibody onto Magnetic Nanoparticles for Ochratoxin A Detection

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Abstract: Ochratoxin A (OTA) is a fungal metabolite found in various foods. The toxin is a public health risk. Therefore, a rapid and sensitive immunoassay for OTA determination on the base of immobilized F(ab')₂ fragments of monoclonal anti-OTA antibody onto magnetic nanoparticles (MNPs) was developed. The self-fluorescence ability of the toxin was studied. F(ab')₂ fragment was obtained by hydrolysis with the enzyme pepsin. Differences in the absorbance characteristics of whole and fragmented antibodies were determined and discussed. The conjugate OTA-OVA-ATTO620 was obtained in our laboratory and then was purified. MNPs with immobilized fragments or antibodies and the OTA-OVA-ATTO620 conjugate were used for competitive immune analysis performing. The presented assay was discussed and compared with other authors. The linear range, obtained with immobilized F(ab')₂ fragments, was from 0.002 to 100 ng/mL OTA in milk. The immunoassay with immobilized F(ab')₂ fragments had the lowest detection limit (0.001 ng/mL OTA) than that from other authors and methods available on the up-to-date web. The recovery rates and the relative standard deviations of the method were presented. The assay was precise and had good repeatability.

Keywords: ochratoxin A; F(ab')₂ fragment; monoclonal antibody; magnetic nanoparticles; immunoassay; gel filtration.

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1. Introduction

Ochratoxins (type A – OTA, type B – OTB, type C – OTC) are fungal metabolites. They are produced from several species: *Aspergillus ochraceus*, *A. carbonarius*, *A. niger*, *Penicillium verrucosum* [1,2]. OTA consists of a para-chlorophenolic moiety containing a dihydroisocoumarin group linked to L-phenylalanine [3]. The toxin poses the greatest threat to human and animal health [1,4]. OTA is at group 2B in the International Agency for Research on Cancer due to its ability to be a possible human carcinogen. There is evidence that it damages the liver (hepatotoxicity), can disturb the embryo/fetus development (teratogenicity), causes reduction of the immune system (immunosuppression), damages the function, and has a carcinogenic effect on the kidney (nephrotoxicity and nephro-carcinogenicity), based on in vitro animal studies [4-7]. OTA exposition is associated with Balkan Endemic Nephropathy (BEN). It is described as a chronic progressive disease (from 6 to 9 years period) that leads to irreversible kidney failure. It is noticed that upper urinary tract tumors in endemic regions in Bulgaria are 90-fold higher compared to that of non-endemic regions [4]. At the beginning (the 1960s), BEN was described in limited areas – Yugoslavia, Romania, and Bulgaria [1], but

nowadays, the effects of OTA have been established in many other countries around the world. OTA also acts primarily like a neurotoxin and teratogen in hamsters, rats, and mice. It inhibits protein synthesis and forms OTA-DNA adducts [1,3,8]. There is evidence of the toxin role in Tunisian Nephropathy, gastric and esophageal tumors, and testicular cancer [4]. OTA was reported in human blood samples in Croatia, Czechoslovakia, Czech Republic, Denmark, France, Germany, Hungary, Italy, Norway, Poland, Portugal, Spain, Sweden, Switzerland, UK, Yugoslavia, Canada, Japan, Lebanon, Pakistan, Turkey, Algeria, Ivory Coast, Morocco, Sierra Leone, Tunisia, Argentina, Chile, Costa Rica. Also, the toxin was detected in human milk in Germany, Hungary, Italy, Norway, Poland, Slovakia, Spain, Sweden, Switzerland, Sierra Leone, Egypt, Iran, Turkey, Brazil, and Chile [9].

OTA is detected mainly in the animal feed, and consequently, it retains in food products from animals, such as milk, eggs, meat [10]. Mycotoxin is found in cereal grains and their products [8], so it can be seen as a direct threat to humans, not just by consuming food from compromised animals. Heating, boiling, and baking are not enough for total OTA degradation [11,12], and processed foods contain significant amounts of the toxin.

There are a lot of methods involving the use of specific antibodies for OTA detection in animal feed, food, and drink detail examined and presented by Meulenbergh [13]. The immunoreaction is specific antibody-antigen binding by interacting amino acids at the paratope-epitope interface [14]. There are already established methods in that class for the determination of OTA by radioimmunoassays (with radioactive tracers ^3H , ^{14}C , ^{125}I), enzyme-linked immune sorbent assay: ELISA (predominantly with horseradish peroxidase: HRP, and alkaline phosphatase), chemiluminescent immunoassay (with luminol), fluorescent immunoassay (with a fluorophore as a label), time-resolved fluorescent immunoassay (with fluorophores with longer lifetime), fluorescence-polarization immunoassay [15] (based on observed polarization changed by fluorophore tumbling motion), fluorescence resonance energy transfer (FRET) immunoassay (with donor and acceptor for the transfer), fluorescent microarray immunoassay (with NeutrAvidin functionalized glass slide), lateral flow immunoassay (strip tests with Au-nanoparticles coupled to antibody), flow-through immunoassay (with enzyme-labeled OTA and antibody coupled to the membrane) [13]. By 2021, the newest trend is the use of nanomaterials. Jiang and colleagues [16] reported for metal and carbon nanomaterials, up-conversion and magnetic nanoparticles (MNPs), and quantum dots included in the OTA analyses. Their properties were useful tools, and they were related to their nano-size and high surface area to volume ratio [16- 19].

Consequently, there are methods for detecting toxins in foods based on MNPs and immunoreaction due to their many advantages. But up to date, there is no reported method with F(ab')_2 fragments of monoclonal antibody (MAb) for OTA. Therefore, this study is focused on F(ab')_2 fragments of Mab for OTA coupled to MNPs and fluorescent-labeled OTA.

2. Materials and Methods

2.1. Materials.

Ochratoxin A (from *Petromyces albertensis*), albumin from chicken egg white (OVA), Sephadex G 25, 2,4,6-trinitrobenzenesulfonic acid solution (TNBS), ATTO620, pepsin (from porcine gastric mucosa ≥ 250 units/mg solid), Tris, sodium dodecylsulfate (SDS), glycerol, bromophenol blue, Sephadex G75, Vivaspin 500 (5000 MWCO), (3-aminopropyl)-triethoxysilane (APTES), glutaraldehyde, anti-mouse IgG (whole molecule) – peroxidase

antibody produced in goat (secondary antibody), Tween 20, N-N-dimethylformamide (DMF), 3,3',5,5'-tetramethylbenzidine (TMB) were from Sigma-Aldrich (Germany).

2.2. *F(ab')₂ fragment obtaining.*

Monoclonal antibody to OTA was fragmented to obtain *F(ab')₂* fragment [20]. The fragmentation procedure was developed by the endopeptidase enzyme – pepsin. First, pepsin (1 mg) was activated in 1 mL of 20 mM sodium acetate buffer with pH 4 for 30 min at room temperature (RT) on a shaker. After that, 1 mL of monoclonal anti-OTA antibody (concentration 2 mg/mL in saline) was added to 200 µL of the activated enzyme. The final ratio of pepsin/antibody was 1/10. The reaction was proceeded at 37°C for 2 h in a shaker and then overnight at 4°C.

The next day, size-exclusion chromatography was performed to obtain the *F(ab')₂* fragment fraction. The column was filled with Sephadex G75 (10 × 240 mm), and the flow rate was 0.5 mL/min with eluent – saline. The resulting fractions (2.2 mL each) were analyzed spectrophotometrically (Jenway 6900 UV/Vis spectrophotometer) at wavelengths: 240 nm, 260 nm, 280 nm. The fraction with *F(ab')₂* fragment was concentrated using centrifuge tubes with filter Vivaspin 500 (5000 MWCO), and the protein content was determined by the Bradford method [21].

The fragmentation of the antibody by pepsin and also the obtained fragment fraction was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) for the determination of the actual size of the protein in the collected sample. The procedure was as described previously [22] by SE400 vertical electrophoresis chamber (Amersham Biosciences), but with some modifications. The resolving gel was 15% T, and the current was 55 mA. The samples (15 µL each) were pretreated with sample buffer (15 µL for each sample). The sample buffer contained 0.125 M Tris, 4% SDS, 20% glycerol, 0.3 mM bromophenol blue, and pH was adjusted to 6.8 by 1N HCl.

2.3. *ELISA with F(ab')₂ fragment and whole monoclonal anti-OTA antibody.*

ELISA was used to analyze the activity of the *F(ab')₂* fragment and whole monoclonal anti-OTA antibody. The antigen in the assay mode was OTA coupled to OVA (OTA-OVA coupling was described in our previous paper – [22]). The wells of a microtiter plate were loaded with 100 µL of OTA-OVA at concentrations from 0.1 to 2.0 µg/mL. The antigen was prepared in 50 mM carbonate-bicarbonate buffer pH 9.6. After 60 min incubation in a shaker at 37°C, the wells were washed three times with 250 µL of 50 mM phosphate buffer saline (PBS) pH 7.4. Then, a blocking solution containing 1% OVA in the washing buffer was loaded in the wells at 200 µL. After 60 min incubation at the same conditions, the wells were washed again. In the next step, 100 µL of the whole monoclonal anti-OTA antibody (2 µg/mL in blocking solution containing 0.05% Tween 20) or *F(ab')₂* fragment (2 µg/mL in blocking solution containing 0.05% Tween 20) were loaded for 60 min, at 37°C, in the shaker. Another washing step was performed, and a secondary antibody was added (100 µL per well). Incubation for 60 min and washing steps were performed as described above. For the color reaction, 100 µL of the substrate solution was loaded in each test well. The substrate solution contained (for 5 mL total volume): 4.5 mL of 50 mM citrate buffer pH 5.0, 0.5 mL of TMB in DMF at concentration 1 mg/mL, 15 µL of 3% H₂O₂. The microtiter plate was put in the shaker at 37°C for 40 min to react to the reagents. The color reaction was stopped with 50 µL of 2M

H₂SO₄. The absorbance signals of the test wells were read at wavelength 450 nm in a microplate reader Rayto RT-2100C.

2.4. Immobilization of F(ab')₂ fragments and monoclonal anti-OTA antibody onto MNPs.

MNPs were modified with APTES to introduce amino groups and were used as solid carriers in the presented immunofluorescence assay. They were prepared in our laboratory as described previously [23]. The immobilization procedure was mentioned in our previous papers [22,24]. Briefly, APTES-modified MNPs (2 mg) were activated with 400 µL 5% glutaraldehyde in 50 mM sodium phosphate buffer (PB), pH 8. The activation was for 2 h at RT on the shaker. The MNPs were washed two times with 50 mM PB pH 8 and five times with 10 mM PB pH 7.4. The washed MNPs were divided into two equal parts – 1 mg each. Two parallel immobilizations were performed – one with whole monoclonal anti-OTA antibody and the other with F(ab')₂ fragments from the monoclonal anti-OTA antibody. The antibody or F(ab')₂ fragment was added to the activated and washed MNP at a ratio 100 µg antibody (or fragment) to 1 mg MNPs. The reaction was performed at RT for 85 min on the shaker. The excess of the unbound antibody (or fragment) was collected, and the protein content was determined by the Bradford method [21]. The MNPs with immobilized whole monoclonal antibody (MNP-mAb) and MNPs with immobilized F(ab')₂ fragment from the whole monoclonal antibody (MNP-F(ab')₂) were processed with 5% OVA in 10 mM PB pH 7.4, 200 µL for each, for 60 min, RT, on the shaker, for blocking the vacant groups onto activated MNPs. Finally, the MNPs with immobilized antibodies or fragments were washed with 10 mM PB pH 7.4 and suspended in the same buffer to a final concentration 5 mg/mL. The suspensions were stored at 4°C.

2.5. OTA fluorescent conjugate preparation.

OTA was coupled with OVA (OTA-OVA) by carbodiimide method and purified by gel filtration with Sephadex G25 column. The degree of coupling was determined by color reaction with TNBS, and 12 amino groups were included in the coupling from total 20 amino groups in OVA molecule. The detailed procedures were described in our previous paper [22]. OTA-OVA complex was used for fluorescent dye binding (ATTO620). The OTA-OVA-ATTO620 conjugate was prepared according to the manufacturer's instructions (ATTO-TEC, Germany) and the protein to dye ratio in the reaction mixture was 10/1. The procedures were more detailed described in our previous paper [20]. The absorbance of both conjugates was detected by Jenway 6900 UV/Vis spectrophotometer. The wavelengths for OTA-OVA-ATTO620 conjugate were 280 nm, 380 nm, and 619 nm.

2.6. Optimization of the amount of F(ab')₂ fragments and monoclonal antibody for the immunoassay.

The amount of the MNPs with an immobilized monoclonal antibody or with F(ab')₂ fragments was determined using three different concentrations of OTA: 100 ng/mL, 10 ng/mL, and 1 ng/mL. The OTA solutions were prepared the day before with 10 mM PB pH 7.4 and were stored at 4°C. Each OTA solution (200 µL in a vial) was added to three different immobilized MNP amounts: 0.375 mL, 0.250 mL, and 0.125 mL. The obtained suspensions were left to react 20 min at 37°C in the shaker. After that, fluorescent-labeled OTA was added to the samples (250 µL to a vial) for the competitive reaction. The optimal concentration of the

fluorescent conjugate was determined previously: 2.5 µg/mL [20]. The mixtures were incubated once more for 20 min under the same conditions. Finally, the liquid fractions of the reaction mixtures were collected by separating the MNPs with a permanent magnet. Those fractions contained the excesses of the fluorescent-labeled OTA (OTA-OVA-ATTO620). They were analyzed by fluorescent spectrophotometer Cary Eclipse (Varian) at 619/643 nm and 800V. Each sample in the analysis was triple measured. The average value of the resulting fluorescent signal is directly proportional to the OTA concentration in the sample.

2.7. Immunoassay of OTA standard solutions in buffer and milk.

First, the MNP-based immunofluorescence analysis was performed with standard solutions of OTA in buffer – 10 mM PB pH 7.4. A variety of concentrations of the toxin was prepared on the day before the assay. The used concentrations of OTA were from 0.0001 to 200 ng/mL. The MNPs with an immobilized whole monoclonal antibody or F(ab')₂ fragments were loaded in new vials (optimal amount in each vial). The liquid was discarded by a pipette and the permanent magnet. Then was added the defined OTA solution 200 µL in each vial, and the suspensions were loaded in the shaker at 37°C for 20 min. After that, the OTA-OVA-ATTO620 (2.5 µg/mL) was added to all of the reaction mixtures (250 µL), and they have incubated once again at the same conditions for 20 min. Finally, vials were placed in a magnetic separator, and the liquid and the solid phases were separated. The liquid was the excess of the unbound OTA-OVA-ATTO620 conjugate, and its amount was determined by fluorescent spectrophotometer Cary Eclipse (Varian) at 619 nm excitation, 643 nm emission, and 800V. Each sample in the analysis was measured five times. The average value of the obtained fluorescent signal was normalized (NS) for clearer presentation by the following equation:

$$NS = \frac{B_0 - B}{B_0 - B_x} \times 100, \%$$

B₀ was the fluorescent signal of the initial OTA-OVA-ATTO620 conjugate, B was the fluorescent signal of the spiked sample with OTA, and B_x was the fluorescent signal of the sample without OTA.

The same analyses were performed with OTA standard solutions in milk. The milk used in the study was UHT with 3% fat and 2.9% protein. The initial milk was tested for OTA residues using the commercial ELISA kit (RIDASCREEN, Art No: R1311) and Rayto RT-2100C reader via the competitive inhibition enzyme immunoassay method based on the manufacturer's procedure. The UHT milk was diluted with 10 mM PB pH 7.4 (1:1) to reduce the matrix effect. The milk samples were prepared on the day before analysis. All of the other following steps were the same with standard solutions in buffer, as described above.

2.8. Analytical yield determination.

The performed method was tested for analytical yield determination. The UHT milk (1:1 dilution) with three different concentrations of OTA (0.1, 1.0, and 5 ng/mL) were used. The samples were stored overnight at 4°C. After that, the analysis was performed as described previously (in section Analysis of OTA standard solutions in buffer and milk). The concentration of the OTA in each sample was determined by the obtained standard curve of OTA in milk and its equation. Consequently, the analytical yield was determined, and the corresponding standard deviations (SDs) and coefficient of variations (CVs).

3. Results and Discussion

3.1. Preparation of $F(ab')_2$ fragment of the monoclonal anti-OTA antibody.

$F(ab')_2$ fragments were prepared by pepsin hydrolysis of monoclonal anti-OTA antibody. The $F(ab')_2$ fragment was purified from the other reaction molecules by Sephadex G75 gel chromatography. All of the fractions were analyzed with a spectrophotometer at three different wavelengths (240 nm, 260 nm, 280 nm) to be examined eventual differences due to different content (Figure 1).

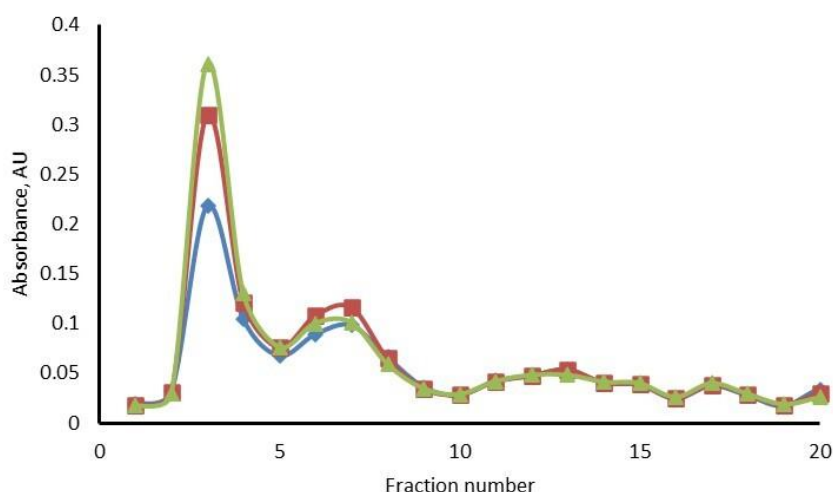


Figure 1. Sephadex G75 gel chromatography of the mixture obtained by pepsin hydrolysis of monoclonal anti-OTA antibody (♦ 240 nm, ■ 260 nm, ▲ 280 nm).

The chromatogram of the $F(ab')_2$ fragment purification has two well-defined peaks (Figure 1). The first peak (fraction 3) contains the whole antibody, the highest molecular weight (150 kDa). The second peak (fractions 6 and 7) contains molecules with lower molecular weight - $F(ab')_2$ fragments (~110 kDa). Other compounds, such as the Fc fragment (~50 kDa) and pepsin (36 kDa), are retained in the stationary phase for a longer time, so they elute after the fifteenth fraction. Similar results were obtained by other authors [25,26]. Differences in the fractions contained were determined not only by retention time but also by the absorbance of the samples. The whole monoclonal antibody had the highest absorbance at wavelength 280 nm, and the $F(ab')_2$ fragment – 260 nm (Figure 1). The reason for that was probably due to the compositions of the molecules in the sample.

It is known that aromatic amino acids are responsible for absorbing protein substances. Phenylalanine has a maximum of nearly 260 nm, tyrosine has nearly 275 nm, and tryptophan – has nearly 280 nm [27-29]. Consequently, the whole antibody molecule likely has a greater amount of the amino acid tyrosine and/or tryptophan. The $F(ab')_2$ fragment of the same antibody has a greater amount of phenylalanine. Therefore, the absorbance at wavelength 260 nm was set as an additional marker for $F(ab')_2$ fragment in the fraction. Absorbance analyzes (from 200 nm to 400 nm) were made with fraction 3 (whole monoclonal antibody) and fraction 7 ($F(ab')_2$ fragment). The results confirmed the differences in wavelength of fragment and antibody absorbance maximums (data not shown). After that, fractions 6 and 7 (with $F(ab')_2$ fragment) were mixed and concentrated using a centrifugal membrane concentrator (5 kDa).

Evidence for the presence and purity of the $F(ab')_2$ fragment was the electropherogram of SDS-PAGE under non-reducing conditions (Figure 2). At the set conditions, the $F(ab')_2$

fragment had an elongated bold line near the 90 to 110 kDa MW. The other reaction molecules were presented as very light shadows; the first was a thin line near the 140 kDa marker that corresponded to the whole antibody, the second was about 50-60 kDa (probably for Fc fragment), and the third was 36 kDa (for pepsin). The presence of the whole antibody in the analyzed sample was negligible, so the mixture of fractions 6+7 was used for F(ab')₂ fragment in subsequent analyzes.

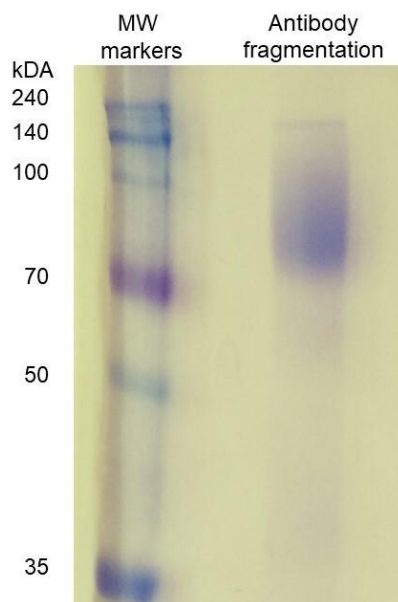


Figure 2. Electropherogram of SDS-PAGE of F(ab')₂ fragment from monoclonal anti-OTA antibody fragmented by pepsin.

ELISA was used to determine F(ab')₂ fragment activity (Figure 3). Concentrations of OTA were varied between 0.1 – 2 µg/mL. The obtained results were compared with the analogical results for the activity of the initial monoclonal anti-OTA antibody.

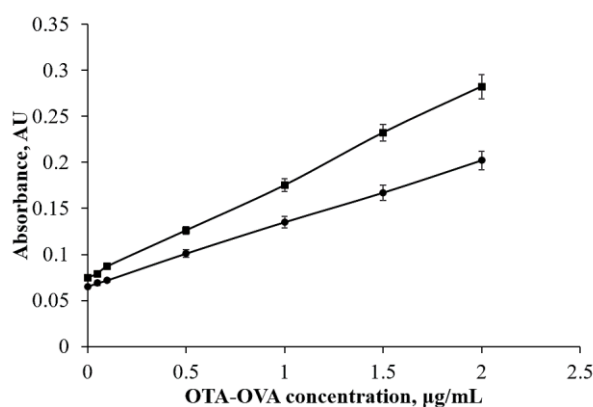


Figure 3. Activity of F(ab')₂ fragments (■) and monoclonal anti-OTA antibody (●) measured by ELISA. Vertical error bars indicate SD ± 3.0 to 5.0% (n = 3).

Figure 3 shows that both bioagents have activity against ochratoxin A. As the toxin concentrations increase, the measured absorbance by ELISA increases. A greater increase was observed using the F(ab')₂ fragment, indicating that it was slightly more sensitive to ochratoxin A than the whole monoclonal antibody. The reason for this is the smaller size and easy diffusion of the fragment over the whole antibody.

3.2. Determination of optimal amount of $F(ab')_2$ fragments and monoclonal antibody for OTA immunoassay.

For the preparation of immunoassay, fourth elements were used: modified magnetic nanoparticles, immobilized $F(ab')_2$ fragments of anti-OTA mAb on MNPs, and competitive fluorescent conjugate – antigen-fluorescent dye. The MNPs were used as solid carriers to immobilize $F(ab')_2$ fragments. A detailed description of the preparation and modification of MNPs has been described in previous papers [22-24]. The number of amino groups on APTES-modified MNPs was 0.017 mg eqv NH_2 groups per 1 g of MNPs. They were determined with Traut's reagent and Ellman's method [30,31]. The immobilization of $F(ab')_2$ fragments was carried by glutaraldehyde [22,32,33]. The amount of immobilized protein was determined by the Bradford method: 17.8 μ g $F(ab')_2$ fragments per 1 mg MNPs. For comparison, the mAb was immobilized on MNPs by glutaraldehyde. The amount of immobilized monoclonal antibody was 20 μ g per 1 mg MNPs.

The optimal amount of $F(ab')_2$ fragment and whole monoclonal antibody for immunoassay were determined by indirect ELISA (Figure 4). It was found that the optimal amount of $F(ab')_2$ fragment is 0.125 mg and of the whole antibody – 0.25 mg.

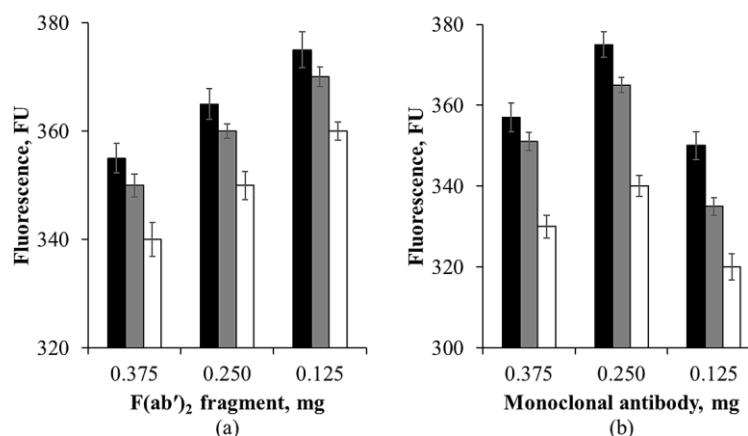


Figure 4. Optimization of the amount of $F(ab')_2$ fragments (a) and anti-OTA monoclonal antibody (b) for conducting an immunoassay, OTA concentration: black 100 ng/mL; gray 10 ng/mL; white 1 ng/mL. Vertical error bars indicate $SD \leq \pm 1.0\%$ ($n = 3$).

3.3. Basic characteristics of OTA fluorescent immunoassay with $F(ab')_2$ fragments and anti-OTA mAb.

The developed pseudo homogeneous immunoassay (Figure 5) consists of the following steps: (a) binding of the Ochratoxin A (OTA) from the sample to the immobilized antibody or immobilized $F(ab')_2$ on MNPs; (b) competitive interaction of OTA labeled with a fluorescent dye (OTA-OVA-ATTO conjugate) with the free antigen-binding sites of the immobilized monoclonal anti-OTA antibody, or $F(ab')_2$ fragment of the same antibody on the surfaces of MNPs; (c) magnetic separation; (d) determination of the excess concentration of OTA-fluorescent dye in the supernatant by measuring the fluorescence intensity.

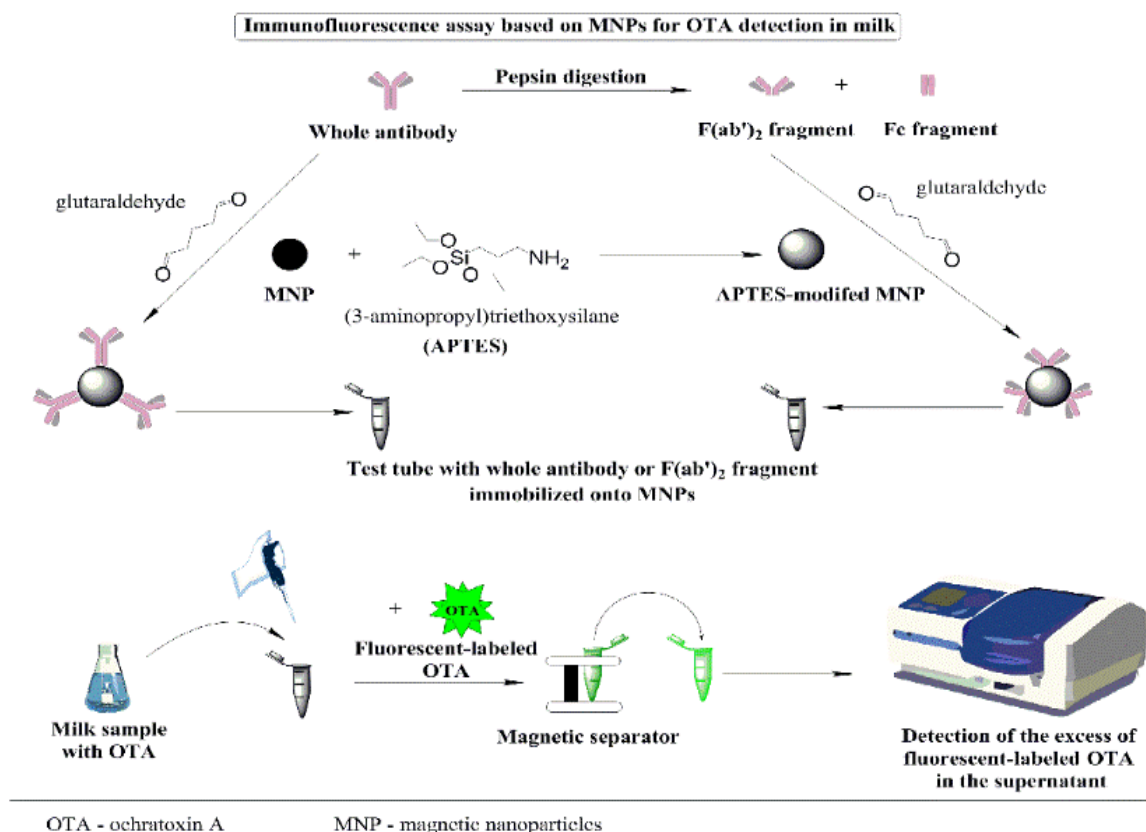


Figure 5. MNP-based immunofluorescence assay of OTA with whole or fragmented monoclonal antibody.

It has been reported that OTA generates blue fluorescence (465 nm) at acidic conditions [34]. Therefore, OTA was analyzed for self-fluorescence. Acidic conditions were made by 10 mM PBS pH 6, and fluorescence of various concentrations of OTA was determined by single read (excitation 380 nm, emission 465 nm, 800V). The results are presented in Figure 6. There was a linear range from 0.2 $\mu\text{g/mL}$ to 2 $\mu\text{g/mL}$ OTA. Those results were obtained from OTA in buffer, but OTA in food would have decreased intensity. Also, the European Commission set regulatory limits on OTA in corn (5 $\mu\text{g/kg}$) and corn products (3 $\mu\text{g/kg}$) [35]. According to European standards, the maximum allowed amount of ochratoxin in food and products for children is 2 ng/L (equal to 2 pg/mL). Consequently, those liner range concentrations were not low enough for our detection goads, so fluorescent labeling of the toxin was preferred.

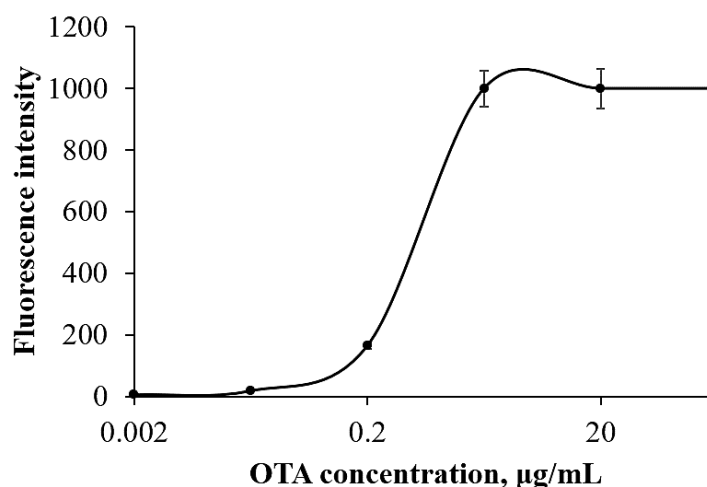


Figure 6. Ochratoxin A self-fluorescence in the acidic buffer. Vertical error bars indicate SD $\pm$ 4.2 to 7.0% (n = 3).

The fluorescent competitive conjugate was prepared between antigen OTA and fluorescent dye ATTO620. Ochratoxin A is a small molecule that does not have chemically active groups for direct binding to a fluorescent dye. That's why the OTA–OVA couple was prepared. Then, the OVA from the couple reacts directly with fluorescent dye ATTO620. The obtained fluorescent conjugate OTA-OVA-ATTO was purified by gel chromatography and proved by fluorescent analysis.

3.4. MNP-immunoassay application with buffer and milk samples.

Fluorescence immunoassay based on MNPs for OTA in buffer (PB, pH 7.4) was performed (Figure 7).

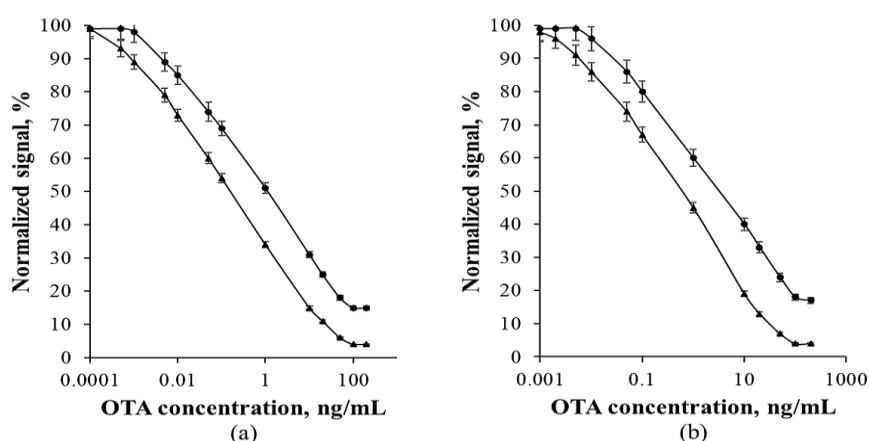


Figure 7. MNP-based fluorescence immunoassay for determination of OTA in buffer (a) and milk (b) carried by immobilized F(ab')₂ fragments – 0.125 mg (▲) and by immobilized monoclonal anti-OTA antibody - 0.25 mg (●).

The linear range of the calibration curve obtained with the immobilized F(ab')₂ fragment on MNPs, the linear range of the calibration curve is from 0.0005 to 100 ng/mL OTA (Figure 7a), SD from $\pm 2.5\%$ to $\pm 4.0\%$ and linear equation $y = -7.504 \ln(x) + 35.717$; $R^2 = 0.9938$. LOD of the immunoassay with F(ab')₂ fragment is 0.4 pg/mL OTA. The obtained results were compared with the results obtained by monoclonal antibodies. The linear range of the calibration curve obtained with the monoclonal antibody immobilized onto MNPs is from 0.001 - 100 ng/mL OTA, SD from $\pm 3.0\%$ to $\pm 4.0\%$ and linear equation $y = -7.326 \ln(x) + 48.869$; $R^2 = 0.9929$. LOD of the immunoassay with a monoclonal antibody immobilized on MNPs is 0.9 pg/mL OTA.

The same experiments were performed with standard solutions of OTA in UHT milk with similar concentrations as in the experiments in the buffer. The initial kinds of milk were diluted with buffer (10 mM PB pH 7.4) in a ratio of 1:1. It was found that the two calibration curves are similar to the curves obtained in buffer, but there is a shift of the linear intervals to higher concentrations of OTA (Figure 7b). The reason for the shifting in the curves is due to the matrix effect of the milk. With immobilized F(ab')₂ fragment on MNPs, the linear range is from 0.002 to 100 ng/mL OTA (Figure 7b), SD from $\pm 3.1\%$ to $\pm 5.0\%$ and linear equation $y = -9.109 \ln(x) + 43.277$; $R^2 = 0.9946$. The linear range of the calibration curve obtained with the immobilized monoclonal antibody on MNPs in milk is from 0.005 to 100 ng/mL OTA, SD from $\pm 3.5\%$ to $\pm 5.5\%$ and linear equation $y = -8.395 \ln(x) + 58.262$; $R^2 = 0.9955$. The LOD of immunoassay with the fragment on MNPs is 0.001 ng/mL OTA, and with the whole monoclonal antibody on MNPs F(ab')₂ is 0.004 ng/mL OTA.

To assess the accuracy and reproducibility of the analysis, the analytical yield of OTA in the initial UHT milk without OTA (checked by ELISA kit) was determined. Different concentrations of OTA (0.1, 1.0, and 5.0 ng/mL) were added to milk samples. Samples were analyzed three times using the developed method using an immobilized F(ab')₂ fragment of the same antibody on MNPs, and the results are shown in Table 1. The analytical yield was between 99.4% and 118.0%; also, the SDs were from 6.5 to 7.7%. Consequently, the MNP-based fluorescence immunoassay was accurate and had good reproducibility. The same OTA spiked UHT milk samples were determined by an alternative method – commercial ELISA – OTA RIDASCREEN kit. The obtained results (0.120, 1.100, and 4.950 ng/mL) were similar to the results of the developed immunofluorescent method. The OTA magnetic nanoparticle-based fluorescent immunoassay was faster (40 min) than ELISA (2 h).

Table 1. Analytical yield and coefficient of variation (CV) of different concentrations of OTA in spiked UHT milk samples determined by immunoassay with F(ab')₂ fragment.

Concentration of OTA, ng/mL		Analytical yield, % (Average value $\pm$ SD ^a)	CV ^b , %
Added concentration	Determined concentration		
0.1	0.118	118 $\pm$ 9	7.7
1.0	1.050	105 $\pm$ 7	6.5
5.0	4.970	99 $\pm$ 7	6.8

^a SD – Standard deviation (n = 3), ^b CV – coefficient of variation

3.5. Comparison of the results obtained with monoclonal, polyclonal, and fragmented antibodies for OTA.

OTA immunoassay's analytical characteristics with F(ab')₂ fragments and whole monoclonal antibody were compared with our previous results obtained from OTA immunoassay with polyclonal anti-OTA antibody [22]. The obtained results show that the developed fluorescence immunoassays using F(ab')₂ fragments of the monoclonal antibody and the polyclonal antibody have a higher sensitivity than the analyzes based on whole anti-OTA antibodies (Table 2). The reason for this is the smaller size of the fragment compared to its antibody and the better diffusion and penetrating ability. LODs for OTA when using the fragment are lower than assays with their whole antibodies, which convincingly shows the better sensitivity of these assays. On the other hand, the sensitivity of the monoclonal antibody immunoassays and its F(ab')₂ fragment was significantly higher than that of the polyclonal antibody assays and its F(ab')₂ fragment. Table 2 shows that the linear interval of OTA in the analysis of milk samples has shifted to higher concentrations due to the matrix effect of milk. In addition, the LODs in milk samples are slightly higher than that in the buffer. Again, in dairy samples, the LODs in the immunoassay with F(ab')₂ fragment were lower than those with the immobilized antibodies.

Table 2. Comparison of the characteristics of fluorescence immunoassays for the determination of OTA in buffer and in milk obtained with anti-OTA antibodies and their F(ab')₂ fragments.

Bioagents	OTA in buffer		OTA in milk	
	Linear interval, ng/mL	LOD*, ng/mL	Linear interval, ng/mL	LOD*, ng/mL
antibody/fragment				
monoclonal	0.001 – 100	0.0009	0.005 – 100	0.004
monoclonal F(ab') ₂	0.0005 – 100	0.0004	0.002 – 100	0.001
polyclonal	0.1 – 5	0.1	0.1 – 2.5	0.1
polyclonal F(ab') ₂	0.1 – 10	0.08	0.1 – 7.5	0.08

* LOD – limit detection

3.6. Comparison of the presented method with the currently used methods.

The analytical parameters of the developed fluorescence immunoassay based on immobilized whole monoclonal anti-OTA antibody and its F(ab')₂ fragment were compared with results obtained by other authors (Table 3). The obtained results show that the presented method has higher sensitivity than ELISA and similar sensitivity to liquid chromatographic methods with mass spectrometry (LC-MS/MS system). The developed OTA immunoassay provided higher sensitivity than the immunofluorescence assay results from other authors [36-38], probably because magnetic nanoparticles provide efficient mass transfer of the antibody with the antigen and conjugate the magnetic nanoparticles to minimize the matrix effects of milk.

Liquid chromatographic methods with mass spectrometry are generally considered the most accurate instrumental analytical methods for determining toxins. However, this method is greatly limited due to the lengthy analytical process, cumbersome pre-processing method, the difficulty of operation, and expensive instruments. ELISA methods have advantages due to their simplicity, but they are less accurate and have relatively low sensitivity and low efficiency than other chromatography techniques. The developed MNP-based immunofluorescence method for OTA is a simple, efficient, and sensitive detection method with combined advantages of instrumental detection and immunological analysis. It is a promising analytical method that combines the advantages – of simple, fast, and efficient in situ analysis, generating a low LOD, similar to the LC-MS/MS system.

Table 3. Comparison of the analytical parameters of the developed OTA fluorescence immunoassay in cow milk with results obtained by other authors.

Method	Linear interval, ng/mL	LOD, ng/mL	Reference
LC-MS/MS system	0.20 – 2.00	0.050	[39]
ELISA test kit (RIDASCREEN)	0.05 – 0.50	0.050	[40]
LC-MS/MS system	0.10 – 200.0	0.030	[41]
Immunoenzymatic method	0.02 – 0.50	0.0197	[42]
Immunochromatographic test strips	0.05 – 10	0.036	[43]
Electrochemical immunosensor	0.5 – 20	0.096	[44]
Gold@silver nanodumbbell based inter-nanogap aptasensor	0.01 – 50	0.007	[45]
Fluorescence assay with AFB1 + OTA aptamers	0.5 – 50	0.100	[36]
Immunofluorescence assay with CuS nanoparticles	0.1 – 100	0.010	[37]
CdSe@CdS quantum dots based electrochemiluminescence aptasensor	1 – 100	0.890	[46]
Fluorescence immunoassay using tetramethylrhodamine-labeled OTA	0.8 – 12	0.400	[38]
Fluorescence immunoassay with F(ab') ₂ fragment of polyclonal antibody on MNPs	0.10 – 7.50	0.080	[22]
Fluorescence immunoassay with the whole polyclonal antibody on MNPs	0.10 – 2.50	0.100	[22]
Fluorescence immunoassay with F(ab') ₂ fragment of monoclonal antibody on MNPs	0.002 – 100	0.001	In this study
Fluorescence immunoassay with the whole monoclonal antibody on MNPs	0.005 – 100	0.004	In this study

* LOD – limit detection; ELISA – enzyme-linked immunosorbent assay; HPLC – high-performance liquid chromatography; MNPs – magnetic nanoparticles; LC-MS/MS – Liquid chromatography-tandem mass spectrometry.

4. Conclusions

The F(ab')₂ fragment was prepared by cleaving the monoclonal antibody using the enzyme pepsin. The modified MNPs used in the developed immunoassay serve as a solid matrix through which the formed immune complexes can be easily and quickly separated from the unbound components by a magnetic field. The main advantages of MNPs in the developed analysis are the easily detachable matrix, the large surface for immobilization of antibodies, and the uniform distribution in the whole volume of the reaction medium. The developed OTA fluorescent immunoassays in milk using F(ab')₂ fragment of the monoclonal antibody had higher sensitivity than the analysis based on the whole monoclonal anti-OTA antibody, respectively LOD 0.001ng/mL and 0.004 ng/mL. The reason for this is the smaller size of the fragment compared to its antibody and the better diffusion and penetrating ability. The developed method, based on MNPs, provides accurate and reproducible results in a short time (40 minutes). This method is suitable not only for ochratoxin A but can also be modified for other analytes determining low concentrations of mycotoxins, antibiotics, drugs, and pesticide residues.

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Conflicts of Interest

The authors declare no conflict of interest. The funders had no role in the study's design, in the collection, analyses, or interpretation of data, in the writing of the manuscript, or in the decision to publish the results.

References

1. Klingelhöfer, D.; Braun, M.; Schöffel, N.; Oremek, G.; Brüggmann, D.; Groneberg, D. Ochratoxin—Characteristics, influences and challenges of global research. *Food Control* **2020**, *114*, <https://doi.org/10.1016/j.foodcont.2020.107230>.
2. Janik, E.; Niemcewicz, M.; Ceremuga, M.; Stela, M.; Saluk-Bijak, J.; Siadkowski, A.; Bijak, M. Molecular aspects of mycotoxins—A serious problem for human health. *International Journal of Molecular Sciences* **2020**, *21*, 8187, <https://doi.org/10.3390/ijms21218187>.
3. Gil-Serna, J.; Vázquez, C.; Patiño, B. Genetic regulation of aflatoxin, ochratoxin A, trichothecene, and fumonisin biosynthesis: A review. *International Microbiology* **2020**, *23*, 89-96, <https://doi.org/10.1007/s10123-019-00084-2>.
4. Kőszegi, T.; Poór, M. Ochratoxin A: molecular interactions, mechanisms of toxicity and prevention at the molecular level. *Toxins* **2016**, *8*, <https://doi.org/10.3390/toxins8040111>.
5. Marin S.; Ramos, A.J.; Cano-Sancho, G.; Sanchis, V. Mycotoxins: Occurrence, toxicology, and exposure assessment. *Food Chem Toxicol* **2013**, *60*, 218–237, <https://doi.org/10.1016/j.fct.2013.07.047>.
6. Bui-Klimke, T.R.; Wu, F. Ochratoxin A and human health risk: A review of the evidence. *Crit Rev Food Sci Nutr* **2015**, *55*, 1860–1869, <https://doi.org/10.1080/10408398.2012.724480>.
7. Li, X.; Ma, W.; Ma, Z.; Zhang, Q.; Li, H. Recent progress in determination of ochratoxin a in foods by chromatographic and mass spectrometry methods. *Crit Rev Food Sci Nutr* **2021**, <https://doi.org/10.1080/10408398.2021.1885340>.
8. Abramson, D. Mycotoxins, Toxicology. In: *Encyclopedia of food microbiology*. Richard, K.R. Ed.; Elsevier, **1999**; pp. 1539-1547, <https://doi.org/10.1006/rwfm.1999.1150>.

9. Ferrufino-Guardia, E.; Chavez-Rico, V.; Larondelle, Y. Ochratoxin a in human breast milk, maternal and placental blood from Cochabamba-Bolivia. *Rev. Toxicol* **2019**, *36*, 116-125.
10. Posea, C.; Sonea, A.; Birtoiu, A.; Roman, M.; Vasile, M. Ochratoxins - fooder contaminants an impact on animals and human health. *Scientific Papers, Animal Science, Series D* **2012**, *55*, 87-89.
11. Dickman, K.G.; Grollman, A.P. 7.18 - Nephrotoxicity of Natural Products: Aristolochic Acid and Fungal Toxins. In: *Comprehensive Toxicology*. 2nd ed.; Charlene A.M. Ed.; Elsevier, **2010**; pp. 433-458, <https://doi.org/10.1016/B978-0-08-046884-6.00823-X>.
12. Kunz, B.M.; Voß, A.; Dalichow, J.; Weigel, S.; Rohn, S.; Maul, R. Impact of experimental thermal processing of artificially contaminated pea products on ochratoxin A and phomopsin A. *Mycotoxin Research* **2021**, *37*, 63-78, <https://doi.org/10.1007/s12550-020-00413-9>.
13. Meulenbergh, E.P. Immunochemical methods for ochratoxin A detection: a review. *Toxins* **2012**, *4*, 244-266, <https://doi.org/10.3390/toxins4040244>.
14. Akbar, R.; Robert, P.A.; Pavlović, M.; Jeliakov, J.R.; Snapkov, I.; Slabodkin, A.; Weber, C.; Scheffer, L.; Miho, E.; Haff, I.; Haug D.; Lund-Johansen, F.; Safonova, Y.; Sandve, G.; Greiff, V. A compact vocabulary of paratope-epitope interactions enables predictability of antibody-antigen binding. *Cell Reports* **2021**, *34*, <https://doi.org/10.1016/j.celrep.2021.108856>.
15. Huang, X.; Tang, X.; Jallow, A.; Qi, X.; Zhang, W.; Jiang, J.; Li, H.; Zhang, Q.; Li, P. Development of an Ultrasensitive and Rapid Fluorescence Polarization Immunoassay for Ochratoxin A in Rice. *Toxins* **2020**, *12*, 682, <https://doi.org/10.3390/toxins12110682>.
16. Jiang, C.; Lan, L.; Yao, Y.; Zhao, F.; Ping, J. Recent progress in application of nanomaterial-enabled biosensors for ochratoxin A detection. *TrAC Trends in Analytical Chemistry* **2018**, *102*, 236-249, <https://doi.org/10.1016/j.trac.2018.02.007>.
17. You, J.; Wang, L.; Zhao, Y.; Bao, W. A review of amino-functionalized magnetic nanoparticles for water treatment: Features and prospects. *Journal of Cleaner Production* **2021**, *281*, <https://doi.org/10.1016/j.jclepro.2020.124668>.
18. Farzin, A.; Etesami, S.A.; Quint, J.; Memic, A.; Tamayol, A. Magnetic nanoparticles in cancer therapy and diagnosis. *Advanced healthcare materials* **2020**, *9*, <https://doi.org/10.1002/adhm.201901058>.
19. Liu, S.; Yu, B.; Wang, S.; Shen, Y.; Cong, H. Preparation, surface functionalization and application of Fe₃O₄ magnetic nanoparticles. *Advances in colloid and Interface Science* **2020**, *281*, <https://doi.org/10.1016/j.cis.2020.102165>.
20. Becheva, Z.R.; Ivanov, Y.L.; Godjevargova, T.I.; Tchorbanov, A.I. Simultaneous determination of ochratoxin A and enterotoxin A in milk by magnetic nanoparticles based fluorescent immunoassay. *Food Addit Contam Part A* **2021**, *38*, 1-19, <https://doi.org/10.1080/19440049.2021.1914866>.
21. Kielkopf, C.L.; Bauer, W.; Urbatsch, I.L. Bradford assay for determining protein concentration. *Cold Spring Harbor Protocols* **2020**, *2020*, <https://doi.org/10.1101/pdb.prot102269>.
22. Becheva, Z.R.; Atanasova, M.K.; Ivanov, Y.L.; Godjevargova, T.I. Magnetic nanoparticle-based fluorescence immunoassay for determination of ochratoxin A in milk. *Food Anal Methods* **2020**, *13*, 2238-2248, <https://doi.org/10.1007/s12161-020-01848-7>.
23. Godjevargova, T.; Becheva, Z.; Ivanov, Y.; Tchorbanov, A. Immunofluorescence assay using monoclonal and polyclonal antibodies for detection of staphylococcal enterotoxins A in milk. *Open Biotech J* **2019**, *13*, 137-141, <https://doi.org/10.2174/187407070190130137>.
24. Becheva, Z.R.; Ivanov, Y.; Gabrovska, K.; Godjevargova, T. Rapid immunofluorescence assay for staphylococcal enterotoxin A using magnetic nanoparticles. *Int J Food Sci Technol* **2019**, *54*, 916-922, <https://doi.org/10.1111/ijfs.14018>.
25. Li, Z.; Krippendorff, B.F.; Sharma, S.; Walz, A.C.; Lavé, T.; Shah, D.K. Influence of molecular size on tissue distribution of antibody fragments. *mAbs* **2016**, *8*, 113-119, <https://doi.org/10.1080/19420862.2015.1111497>.
26. Jones, R.G.A.; Landon, J. Enhanced pepsin digestion: a novel process for purifying antibody F (ab')₂ fragments in high yield from serum. *Journal of immunological methods* **2002**, *263*, 57-74, [https://doi.org/10.1016/S0022-1759\(02\)00031-5](https://doi.org/10.1016/S0022-1759(02)00031-5).
27. Hammond, B.R.; Johnson, B.A.; George, E.R. Oxidative photodegradation of ocular tissues: beneficial effects of filtering and exogenous antioxidants. *Experimental eye research* **2014**, *129*, 135-150, <https://doi.org/10.1016/j.exer.2014.09.005>.
28. Okoronkwo, N.E.; Mba, K.C.; Nnorom, I.C. Estimation of protein content and amino acid compositions in selected plant samples using UV-Vis spectrophotometric method. *Am. J. Food Sci. Health* **2017**, *3*, 41-46.
29. Russell, B.A. *Protein Encapsulated Gold Nanoclusters for Biological Applications*. University of Strathclyde, Glasgow, 2 Jun **2017**; <https://doi.org/10.13140/RG.2.2.36594.40645>.
30. Ngo, T.T. Colorimetric determination of reactive amino groups of a solid support using Traut's and Ellman's reagents. *Applied Biochemistry Biotechnology* **1986**, *13*, 213-219, <https://doi.org/10.1007/BF02798459>.
31. Tyllianak, P.E.; Kakabakos, S.E.; Evangelatos, G.P.; Ithakissios, D.S. Colorimetric determination of reactive primary amino groups of macro- and microsolid supports. *Applied Biochemistry and Biotechnology* **1993**, *38*, <https://doi.org/10.1007/BF02916409>.

32. Mo, H.; Qiu, J.; Yang, C.; Zang, L.; Sakai, E.; Chen, J. Porous biochar/chitosan composites for high performance cellulase immobilization by glutaraldehyde. *Enzyme and Microbial Technology* **2020**, *138*, <https://doi.org/10.1016/j.enzmictec.2020.109561>.
33. Sahin, S.; Ozmen, I. Covalent immobilization of trypsin on polyvinyl alcohol-coated magnetic nanoparticles activated with glutaraldehyde. *Journal of pharmaceutical and biomedical analysis* **2020**, *184*, <https://doi.org/10.1016/j.jpba.2020.113195>.
34. Frenette, C.; Paugh, R.J.; Tozlovanu, M.; Juzio, M.; Pfohl-Leszkowicz, A.; Manderville, R.A. Structure–activity relationships for the fluorescence of ochratoxin A: Insight for detection of ochratoxin A metabolites. *Analytica chimica acta* **2008**, *617*, 153–161, <https://doi.org/10.1016/j.aca.2007.12.030>.
35. EC (2006) Commission Regulation (EC) No 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs. *Eur Comm Off J Eur Union L* **2006**, *364*, 5–24.
36. Xiong, Z.; Wang, Q.; Xie, Y.; Li, N.; Yun, W.; Yang, L. Simultaneous detection of aflatoxin B1 and ochratoxin A in food samples by dual DNA tweezers nanomachine. *Food Chemistry* **2021**, *338*, <https://doi.org/10.1016/j.foodchem.2020.128122>.
37. Chen, R.; Sun, Y.; Huo, B.; Zhao, X.; Huang, H.; Li, S.; Bai, J.; Liang, J.; Gao, Z. A copper monosulfide-nanoparticle-based fluorescent probe for the sensitive and specific detection of ochratoxin A. *Talanta* **2021**, *222*, <https://doi.org/10.1016/j.talanta.2020.121678>.
38. Li, Y.; Zhang, N.; Wang, H.; Qiang Zhao, Q. An immunoassay for ochratoxin A using tetramethylrhodamine-labeled ochratoxin A as a probe based on a binding-induced change in fluorescence intensity. *The Analyst* **2020**, *145*, 651–655, <https://doi.org/10.1039/C9AN01879D>.
39. Flores-Flores, M.E.; González-Peñas, E. Analysis of Mycotoxins in Peruvian Evaporated Cow Milk. *Beverages* **2018**, *4*, <https://doi.org/10.3390/beverages4020034>.
40. Turkoglu, C.; Keyvan, E. Determination of Aflatoxin M1 and Ochratoxin A in Raw, Pasteurized and UHT Milk in Turkey. *Acta Sci Veter* **2019**, *47*, <https://doi.org/10.22456/1679-9216.89667>.
41. Zhang, X.; Li, M.; Cheng, Z.; Ma, L.; Zhao, L.; Li, J. A comparison of electronic nose and gas chromatography–mass spectrometry on discrimination and prediction of ochratoxin A content in *Aspergillus carbonarius* cultured grape-based medium. *Food Chem* **2019**, *297*, <https://doi.org/10.1016/j.foodchem.2019.05.124>.
42. Cuciureanu, M.; Tuchiluş, C.; Vartolomei, A.; Tamba, B.I.; Filip, L. An Immunoenzymatic Method for the Determination of Ochratoxin A in Biological Liquids (Colostrum and Cow's Milk). *Toxins* **2021**, *13*, <https://doi.org/10.3390/toxins13100673>.
43. Sun, J.; Li, M.; Xing, F.; Wang, H.; Zhang, Y.; Sun, X. Novel dual immunochromatographic test strip based on double antibodies and biotin-streptavidin system for simultaneous sensitive detection of aflatoxin M1 and ochratoxin A in milk. *Food Chemistry* **2021**, *375*, <https://doi.org/10.1016/j.foodchem.2021.131682>.
44. Kunene, K.; Weber, M.; Sabela, M.; Voiry, D.; Kanchi, S.; Bisetty, K.; Bechelany, M. Highly-efficient electrochemical label-free immunosensor for the detection of ochratoxin A in coffee samples. *Sensors and Actuators B: Chemical* **2020**, *305*, <https://doi.org/10.1016/j.snb.2019.127438>.
45. Ma, X.; Shao, B.; Wang, Z. Gold@ silver nanodumbbell based inter-nanogap aptasensor for the surface enhanced Raman spectroscopy determination of ochratoxin A. *Analytica Chimica Acta* **2021**, *1188*, <https://doi.org/10.1016/j.aca.2021.339189>.
46. Jia, M.; Jia, B.; Liao, X.; Shi, L.; Zhang, Z.; Liu, M.; Zhou, L.; Li, D.; Kong, W. A CdSe@ CdS quantum dots based electrochemiluminescence aptasensor for sensitive detection of ochratoxin A. *Chemosphere* **2022**, *287*, <https://doi.org/10.1016/j.chemosphere.2021.131994>.