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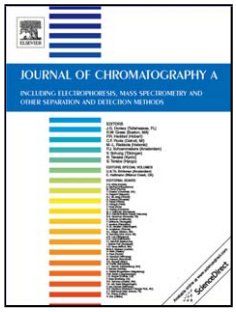

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Development of the custom polymeric materials specific for aflatoxin B1 and ochratoxin A for application with the ToxiQuant T1 sensor tool

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ABSTRACT

Two polymers were computationally designed with affinity to two of the most abundant mycotoxins aflatoxin B1 (AFB1) and ochratoxin A (OTA) for application in the ToxiQuant T1 System. The principle of quantification of AFB1 and OTA using the ToxiQuant T1 instrument comprised of a fluorimetric analysis of mycotoxins adsorbed on the polymer upon exposure to UV light. High affinity of the developed resins allowed the adsorption of both toxins as discrete bands on the top of the cartridge with detection limit as low as 1 ng quantity of mycotoxins.

Keywords: Mycotoxins, aflatoxin B1, ochratoxin A, computational modelling, solid-phase extraction.

1. Introduction

The name mycotoxin is a combination of the Greek word for fungus “mykes” and the Latin word “toxicum” meaning poison. The term “mycotoxins" is reserved for the toxic chemical products which are mainly produced by five fungal genera *Aspergillus*, *Penicillium*, *Fusarium*, *Alternaria* and *Claviceps* (1) that readily colonise crops either in the field or after harvest. These compounds pose a potential threat to human and animal health, through the ingestion of food products prepared from these commodities. Each mycotoxin is produced by one or more specific fungal species.

Among the most significant mycotoxins are aflatoxins, a group of toxins produced by the fungi *Aspergillus flavus* and *A. parasiticus* and ochratoxins, a group produced by some species of *Aspergillus* (*A. ochraceus)* and by *Penicillium verrucosum.*

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Aflatoxins, particularly AFB1, have received great attention due to their acute toxicological effects in humans. The International Agency for Research and Cancer (IARC) included AFB1 as a primary group of carcinogenic compounds (2). Many countries have strict regulatory limits on commodities intended for human and animal consumption. The legal limits for aflatoxins for human consumption are 0-50 ng g^{-1} (3) and for animal feed are 1-20 µg g^{-1} (4). Ochratoxin A (OTA) is a weak organic acid which is also classified by IARC as a compound which is carcinogenic to humans and animals (2). According to European Commission regulations the maximum limit OTA in food should be restricted to 5 or 10 µg l^{-1} (roasted and instant coffee correspondingly) and 2 µg l^{-1} (grape juice and wine) (5).

Since the mere presence of *Aspergillus* or *Penicillium* does not always mean the presence of toxins in the substrate the determination of toxins in the food samples is essential. The main methods for detection of aflatoxins and ochratoxins in food which are high performance liquid chromatography (HPLC) equipped with immunoaffinity columns and fluorescent detectors and thin layer chromatography (TLC) (6, 7). The lowest level of aflatoxin quantification using HPLC method is 0.1 ng g^{-1}. For quantitative testing of multiple samples radioimmunoassay and enzyme-linked immunosorbent assays (ELISA) are also used. According to Association of Official Analyst Chemists International (AOAC International) the detection limit of AFB1 using ELISA method is 9-20 ng g^{-1} (7). Despite sufficient sensitivity and high analytical efficiency HPLC quantification requires expensive equipment and highly trained personnel. Although ELISA could be considered as a sensitive and economical solution, it is difficult to perform it in the “point- of- care” situation where very rapid but affordable tests are mandatory. Market research shows that there is a high demand for a simple, rapid and affordable testing tool in order to provide a simple and quantitative analysis of the food for presence of toxins throughout the global food chain. The ToxiQuant T1 system has been developed to meet the demands of this niche of diagnostic market (Toximet Ltd. UK) (Fig. 1).

The ToxiQuant T1 instrument consists of a UV light source, an automatically adjustable cartridge holder, a detector, necessary optics, mechanics and software. The samples are loaded onto cartridges using standard SPE manifold equipped with a vacuum pump or manually, using a syringe. The mobile phase is filtered through the adsorbent which was designed to adsorb the analytes of interest (particularly

fluorescent mycotoxins) as a band on the top of the packed resin. The ToxiQuant T1 instrument scans the semi-transparent plastic cartridge under conditions which stimulate the fluorescence of mycotoxin adsorbed on the top of the polymeric resin and provides a quantitative measurement of the concentration of toxin present in the sample (Fig. 2). The requirements for the polymeric material are high affinity towards AFB1 and OTA and low background fluorescence.

2. Experimental

2.1 Chemicals and materials

AFB1, OTA and deoxynivalenol (DON) were obtained from Sigma (Sigma-Aldrich, UK). N,N′-methylene bisacrylamide (MBAA), diethylaminoethylmethacrylate (DEAEM), itaconic acid (IA) ethylene glycol dimethacrylate (EGDMA) and 1,1-azobis (cyclohexanecarbonitrile) were purchased from Aldrich (Sigma-Aldrich, UK). Dimethylformamide (DMF), HPLC grade methanol and HPLC grade water were obtained from Acros (Fisher Scientific, UK). 1-ml empty SPE cartridges were purchased from Supelco (Sigma-Aldrich, UK).

2.2 Computational modelling

The molecular modelling was made using a workstation from Research Machines running the CentOS 5 GNU/Linux operating system. The workstation was configured with a 3.2GHz core 2 duo processor, 4 GB memory and a 350 GB fixed drive. This system was used to execute the software packages SYBYL 7.0^{TM} (Tripos Inc., St. Louis, Missouri, USA). The LEAPFROG algorithm was used to screen the library of functional monomers for their possible interactions with the template resulting in a table ranking the monomers with the highest binding score (kcal mol^{-1}) as the best candidates for polymer preparation. The library contained 21 functional monomers commonly used in molecular imprinting which possess polymerisable residues and residues able to interact with a template through ionic and hydrogen bonds, van der Waals' and dipole–dipole interactions (8). The more detailed description of the molecular modelling protocol and functional monomers library (Fig. 1S) is included in the Supplementary Information chapter. Energy minimisation was performed on each of the monomers in the database to a value of 0.001 kcal mol^{-1}. The screening was conducted "in the vacuum" which was determined by carrying the simulation at dielectric constant equal 1. The goal of the screening was to select

the functional monomer which has sufficient binding energy towards the toxin. The result of the modelling was a virtual prediction of a molecular complex which could be formed between the toxin and selected functional monomer. Usually, several functional monomers were selected for polymer preparation and a choice of the best one was determined by laboratory testing under conditions which will be required for the practical application."

2.3 Polymers preparation

The polymers were prepared from methacrylate functional monomers and cross-linker by free-radical polymerisation approach (9). Based on the computational modelling, MBAA was selected for the preparation of the polymer specific for AFB1 (P1) and a mixture of DEAEM and IA was selected for preparation of polymer specific for OTA (P2). P1 composition: 10 g of the polymer with 0.5 g of MBAA, 9.5 g of cross-linker (EGDMA) and 100 mg of 1,1-azobis (cyclohexanecarbonitrile) as an initiator. P2 composition: 1 g of IA with 1g of DEAEM, 8 g of EGDMA and 100 mg of 1,1-azobis (cyclohexanecarbonitrile) as initiator. 10 g of dimethylformamide was used in both cases as a porogen. Polymers were polymerised on ice at +4 °C using Cermax UV lamp (PerkinElmer, UK). After synthesis both polymers were ground and sieved using Ultracentrifuge Mill and Shaker (Retsch, UK). Fractions with size particles size 25-63 μm, 63-125 μm and 125-200 μm were collected. The polymer were thoroughly washed with methanol using Soxhlet extraction, dried and used for SPE.

2.4 SPE protocol and regeneration of polymers

75 mg of the polymers (P1 or P2) were packed in the 1-ml SPE cartridges and were conditioned with 2 ml of HPLC-grade water. 1 ml of 80% methanol which was used for extraction of aflatoxin was spiked with 1-200 ng of AFB1. Before the loading the spiked extraction solution was diluted 4 times with water and loaded into the cartridge. For analysis of OTA adsorption the extraction solution, which consisted of 60% aqueous acetonitrile, was spiked with 1- 300 ng of OTA. Before the loading the spiked extraction solution was diluted 4 times with water to decrease the acetonitrile content to 15% and 4 ml were filtered through the cartridges packed with P2 polymer. 1 ml of 15% acetonitrile (OTA) or 20% methanol (AFB1) was used to wash the cartridges before the measurement using the ToxiQuant-T1 instrument. The

protocols for regeneration of the P1 and P2 polymers were optimised. In order to regenerate the P1 polymer it was washed with 4 ml of methanol followed by 4 ml of water. For regeneration of the P2 polymer the following washing steps were conducted: 2 ml of water, 4 ml of 50% methanol containing 0.1 M NaOH, 4 ml of water, 4 ml of 0.1 M HCl, 4 ml of methanol, 4 ml of water. These treatments were sufficient for complete regeneration of the polymers and preparation for the next loading experiment.

3. Results and discussion

3.1 Computational modelling

Molecular structures of AFB1 and OTA were drawn, minimised and screened against a virtual library of the functional monomers using the LEAPFROG algorithm resulting in tables ranking the monomers with the highest binding score (Suppl. Inform., Table 1S). It was found that MBAA is a functional monomer which could provide a high binding towards AFB1 (binding energy- -32.26 kcal mol^{-1}). This monomer forms two hydrogen bonds with two oxygens of the furan and coumarin rings of the molecule of AFB1 (Fig. 3). Among other polymers which were also screened for aflatoxin adsorption were polymers based on acrylamide, allylamine, ethylene glycol methacrylate phosphate (EGMP) and methacrylic acid. MBAA-based polymer demonstrated superior adsorption properties towards AFB1 and was selected for the future experiments.

The screening of OTA against the virtual library of functional monomers showed that charged DEAEM demonstrated the highest binding towards OTA (binding energy: -61.10 kcal mol^{-1}). Hydrogen bonds were formed between the charged amino group of DEAEM and carboxyl group of the phenylalanine moiety of ochratoxin A (Fig. 4). Since the pKa of the carboxylic group of OTA is 4.4 (10), it means that OTA in the neutral loading solution is negatively charged. In order to induce the positive charge of the DEAEM monomer and to increase the OTA binding, a second functional monomer, IA (binding energy: -26.74 kcal mol^{-1}) was selected for the polymer preparation. Molecular modelling showed that IA formed bonds with hydroxyl and carbonyl groups of the benzopyran moiety of OTA (Fig. 4). Based on the results of molecular modelling IA and DEAEM were selected for polymer preparation.

Among other polymers which were screened for OTA adsorption were polymers based on acrylamide, methylene bisacrylamide (MBAA), 4-vinyl pyridine (4-VP) and IA. Since polymer based on the combination of DEAEM and IA demonstrated superior adsorption properties towards OTA, it was used for the future experiments.

3.2 Polymers testing

The P1 and P2 polymers were prepared as described in the Experimental section. During the polymer's preparation special efforts were made to comply with requirements of the ToxiQuant T1 instrument and produce polymers with a low background reading. Although cross-linked methacrylate polymers do not have intrinsic fluorescent properties, some light scattering occurs when they are scanned using the ToxiQuant T1 instrument. In order to reduce the background signal, polymers were prepared using UV polymerisation at low temperature (below +4 ° C). Low-temperature polymers demonstrated significantly lower background value when used in the ToxiQuant T1 instrument than polymers prepared by thermo-polymerisation. This observation could be explained by the lower polymerisation rate, at low temperature, which resulted in more regular homogeneous gel-like morphology polymers (11). Also selection of the particle size with the lowest background reading was carried out. Several fractions with different sizes were tested. Fraction 63-125 µm demonstrated the lowest background reading and was selected for future work.

The composition of the loading solution was also optimised. It was found that although P1 and P2 polymers could adsorb the corresponding toxins directly from the extraction solution (80% methanol for AFB1 and 60% of acetonitrile for OTA) the peaks were too wide and could not be used for quantification using the ToxiQuant T1 instrument. Four-time dilution of the extraction sample resulting in 20% methanol content for the loading of AFB1 and 15% acetonitrile for OTA loading was considered as optimal. This ensured that the toxin band was situated on the top of the polymer and generated a strong signal when scanned by the ToxiQuant T1 instrument.

In order to test the polymers for adsorption of AFB1, 75 mg of P1 polymer (fraction 63-125 µm) was packed into 1-ml SPE tubes and conditioned with 2 ml of HPLC grade water using a vacuum manifold. The optical absorbance of the pre-conditioned cartridges was measured and used as a background value for the quantification of AFB1 in the samples. 4-ml aliquots of 20% methanol containing

different concentrations of AFB1 were loaded into SPE cartridges and measured using the ToxiQuant T1 instrument. A calibration curve was made by plotting the height values of the peaks measured by the ToxiQuant T1 instrument for different amounts of AFB1 obtained after subtraction of the baseline value (Fig. 5). This calibration was linear in the range between 10 and 200 ng of AFB1.

In order to assess the possibility of pre-concentrating the samples and assess the limit of detection using the ToxiQuant instrument, 10 ml of 20% methanol were spiked with 1 ng of AFB1 and loaded onto P1 polymer. A photograph of the control cartridge which did not contain toxin and a cartridge loaded with 1 ng of AFB1 was taken under UV light using a transilluminator Gene Genius Bio Imaging system (Synoptics Ltd, USA). It is possible to see a band of 1 ng of AFB1 which is adsorbed by P1 polymer (Fig. 6). Based on the observation that the aflatoxin was adsorbed on the top of the polymer it is possible to assume that the polymer has high affinity towards aflatoxin and could be used in combination with the ToxiQuant instrument platform. The minimal limit of detection was estimated as 1 ng of AFB1.

In order to test the polymers for adsorption of OTA, 75 mg of the P2 polymer (fraction 63-125 μm) was packed into 1-ml SPE tubes and pre-conditioned with 2 ml of HPLC-grade water on the vacuum manifold. 4-ml aliquots of 15% acetonitrile were spiked with different amounts of OTA and loaded onto SPE cartridges and measured using the ToxiQuant instrument. A calibration curve was plotted using the height of the peaks for different concentration of OTA after subtraction of the baseline value. This calibration was linear in the range between 10 and 300 ng of OTA (Fig. 7).

It was shown that the P2 polymer is able to adsorb 1 ng of OTA from 10 ml of diluted solution (Fig. 8). It also shows that the P2 polymer has sufficient affinity towards OTA in the wide range of concentrations. The limit of quantification of OTA (S/N=10) was under 1 ng.

Since the detection of ToxiQuant T1 sensor is based on the measurement of fluorescent compounds, it was expected that non-fluorescent toxins or other contaminants could not affect the quantification of AFB1 or OTA using ToxiQuant T1 sensor. The cross-reactivity of the developed polymers was tested with toxin deoxynivalenol (DON) under conditions similar to AFB1 and OTA adsorption. It was found that DON was not adsorbed by either P1 or P2 polymer."

The protocol for washing and regeneration of both polymers was optimised. It was found that it was possible to regenerate and to re-use the cartridges up to 10 times without losing the polymer's binding capacity to their respective targets.

4. Conclusions

Two rationally-designed polymers were developed for adsorption of aflatoxin B1 and ochratoxin A for the application with the ToxiQuant T1 instrument. It was found that both polymers have high affinity towards their corresponding toxins and could be used for detection of 1 ng of the toxins. Since the principle of operation of the ToxiQuant instrument did not require the elution of the toxin from the SPE column, it minimised the danger of contamination when using the toxin and decreases the time of the analysis. Future study will be directed towards the validation of the ToxiQuant software for quantification of mycotoxins and for application of the developed polymers for extraction of the AFB1 and OTA from complex food matrices.

It is important to emphasise that the ToxiQuant T1 System, in combination with designer resins which are custom-produced for specific analytes, could be used for a broad range of applications which require an inexpensive and rapid quantification of the compound of interest, such as environmental pollutants, drugs of abuse and counterfeit drugs.

Acknowledgement

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Figure captions:

Figure 1. The ToxiQuant T1 prototype instrument

Figure 2. The principle of toxin quantification using the ToxiQuantT1 sensor.

Figure 3. 3-D molecular complex of AFB1 with MBAA functional monomer (top picture; oxygen atoms are shown in red, carbon atoms are white and the light blue atoms are hydrogen) and 2-D structure of aflatoxin B1 (bottom picture).

Figure 4. Molecular complex between negatively charged OTA and functional monomers DEAEM and IA (top picture; oxygen atoms are shown in red, carbon atoms are white and the light blue atoms are hydrogen) and 2-D structure of OTA (bottom picture).

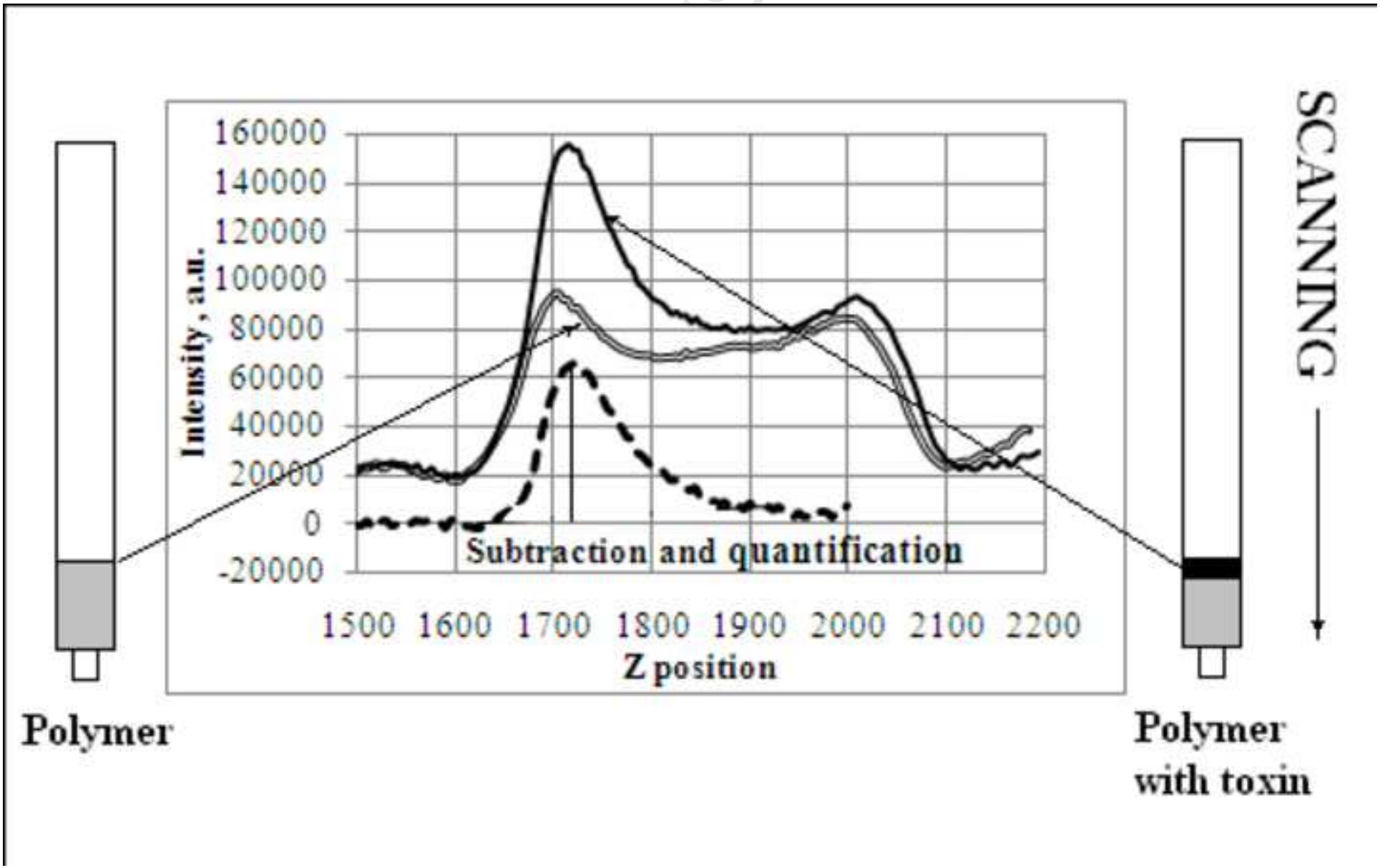
Figure 5. Calibration curve for quantification of AFB1 using the ToxiQuant T1 instrument.

Figure 6. P1 polymer with adsorbed 1 ng of AFB1 (on the right), on the left- control cartridge without toxin.

Figure 7. Calibration curve for quantification of OTA using the ToxiQuant System.

Figure 8. P2 polymer with 1 ng of adsorbed OTA (on the right), on the left- control cartridge without toxin.

Figure 2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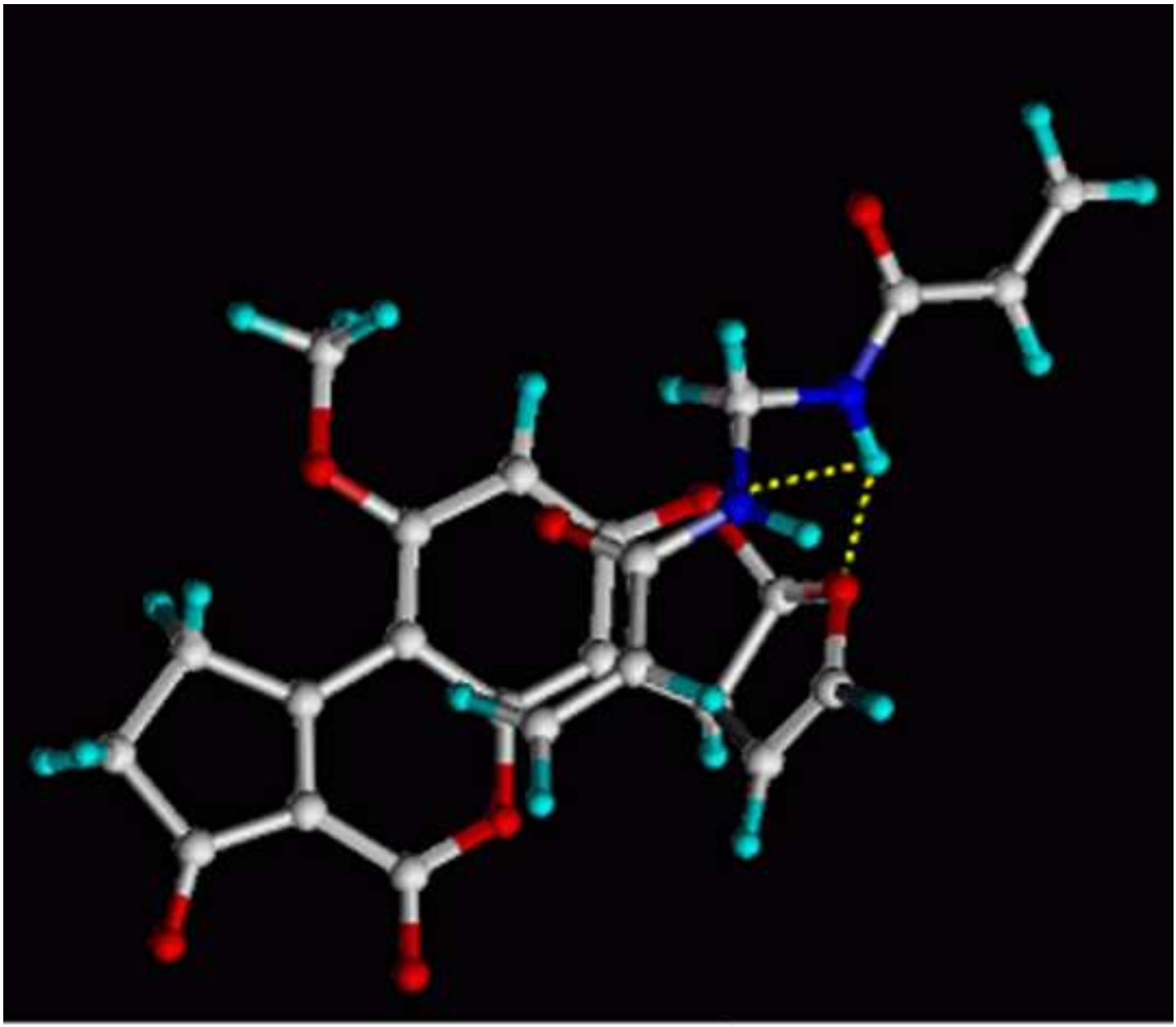
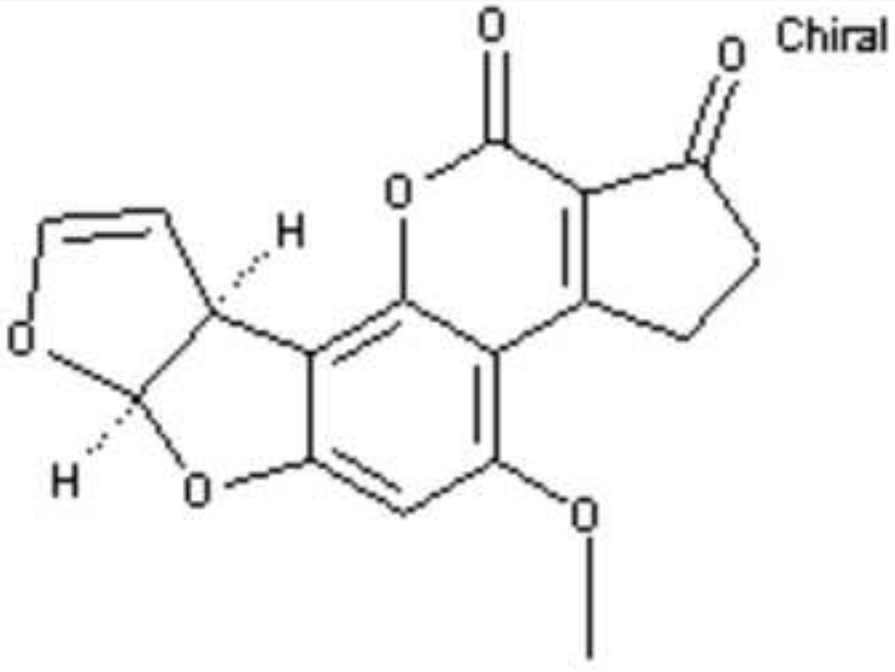
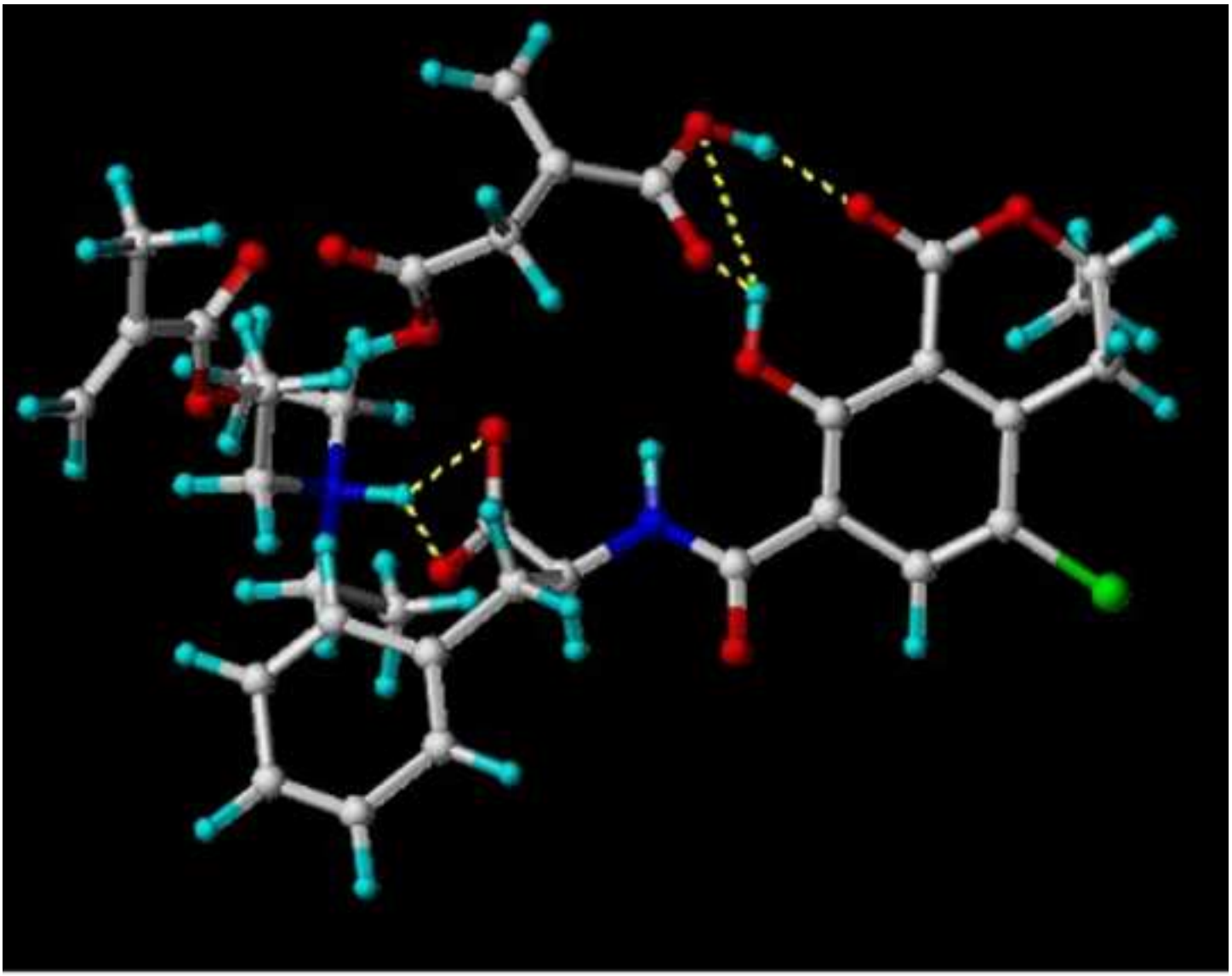






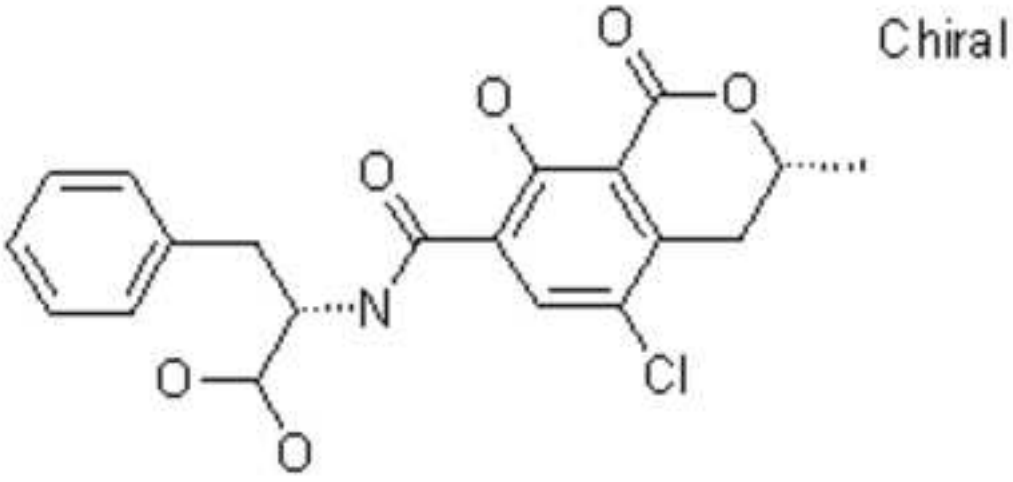
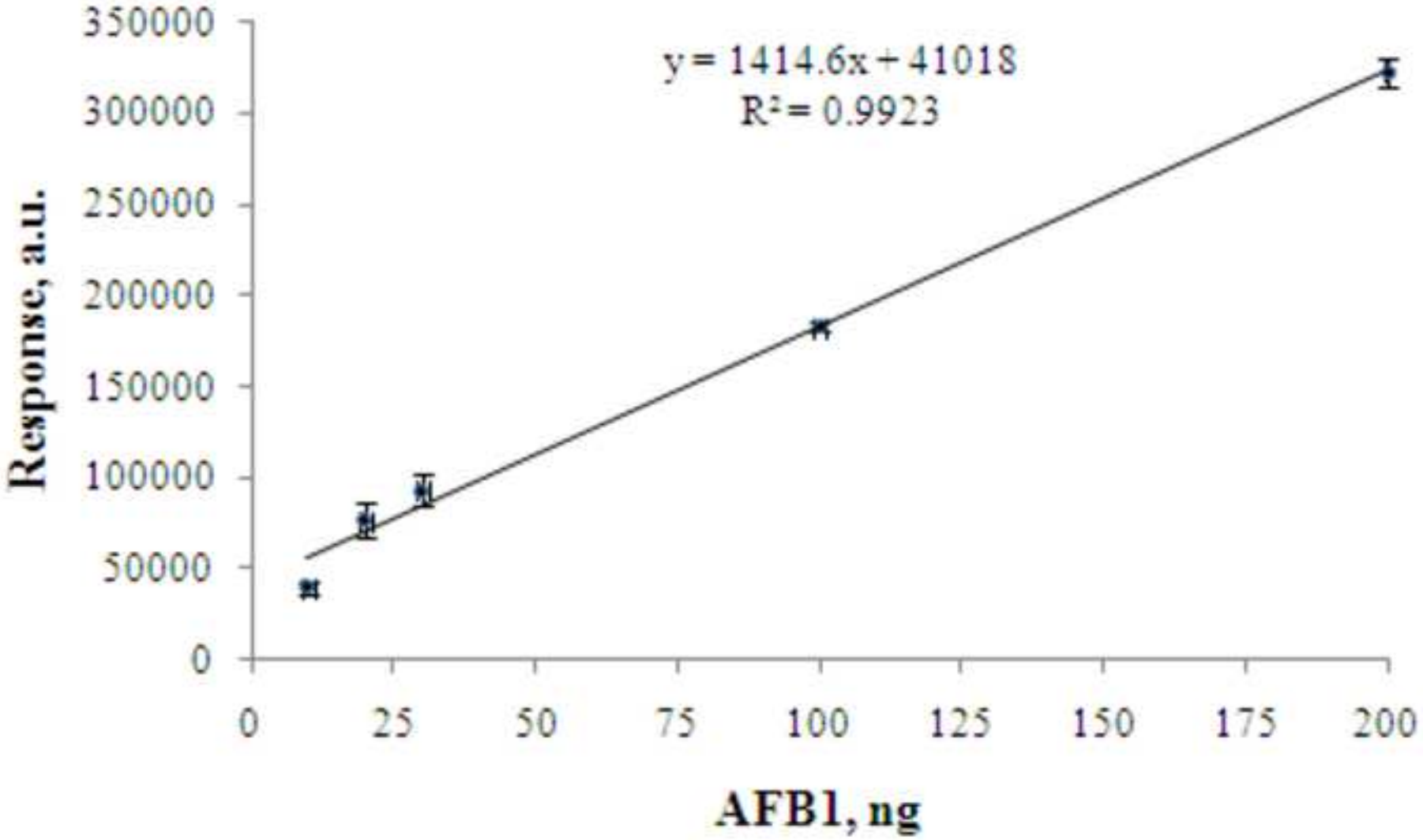


Figure 5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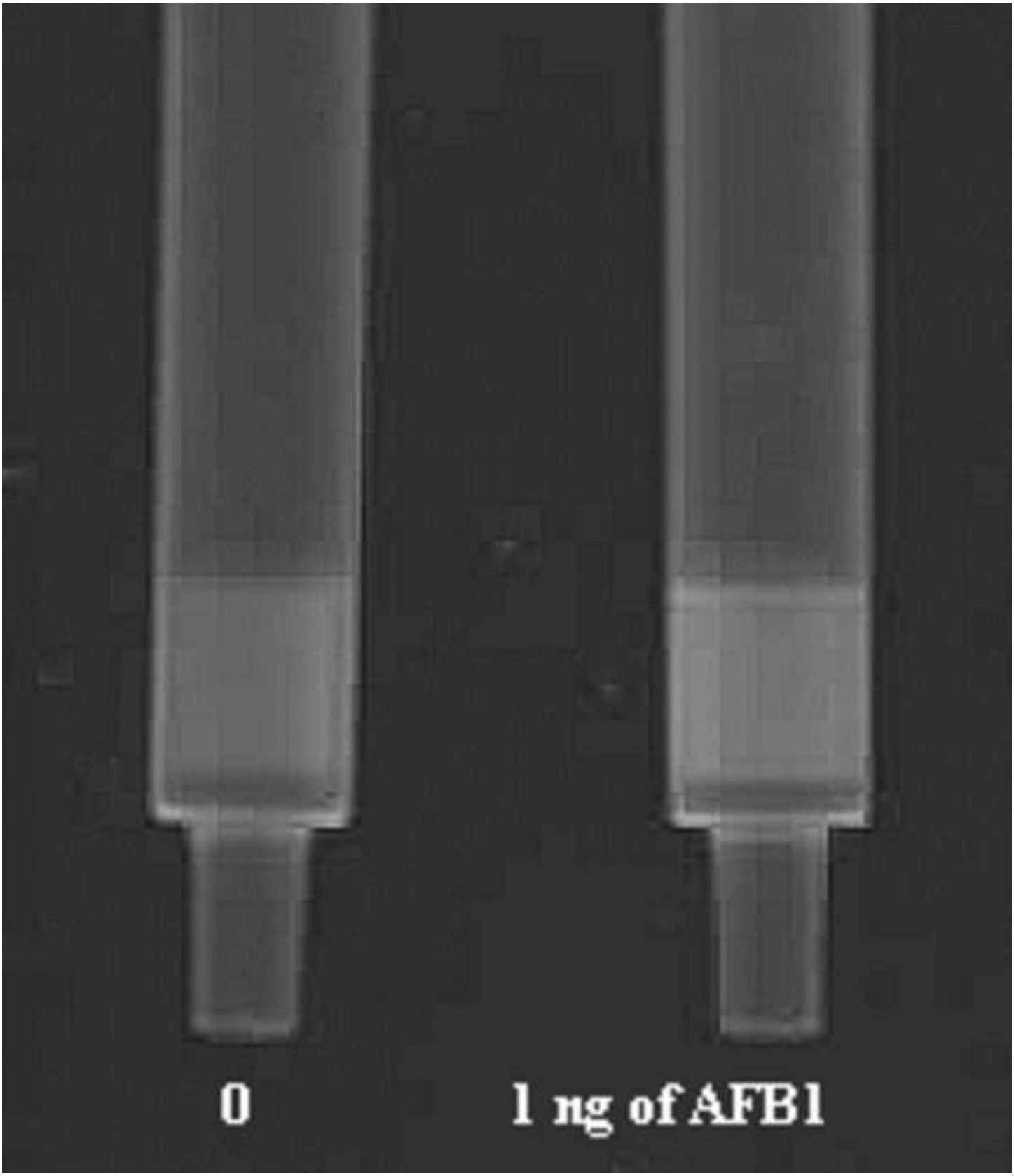
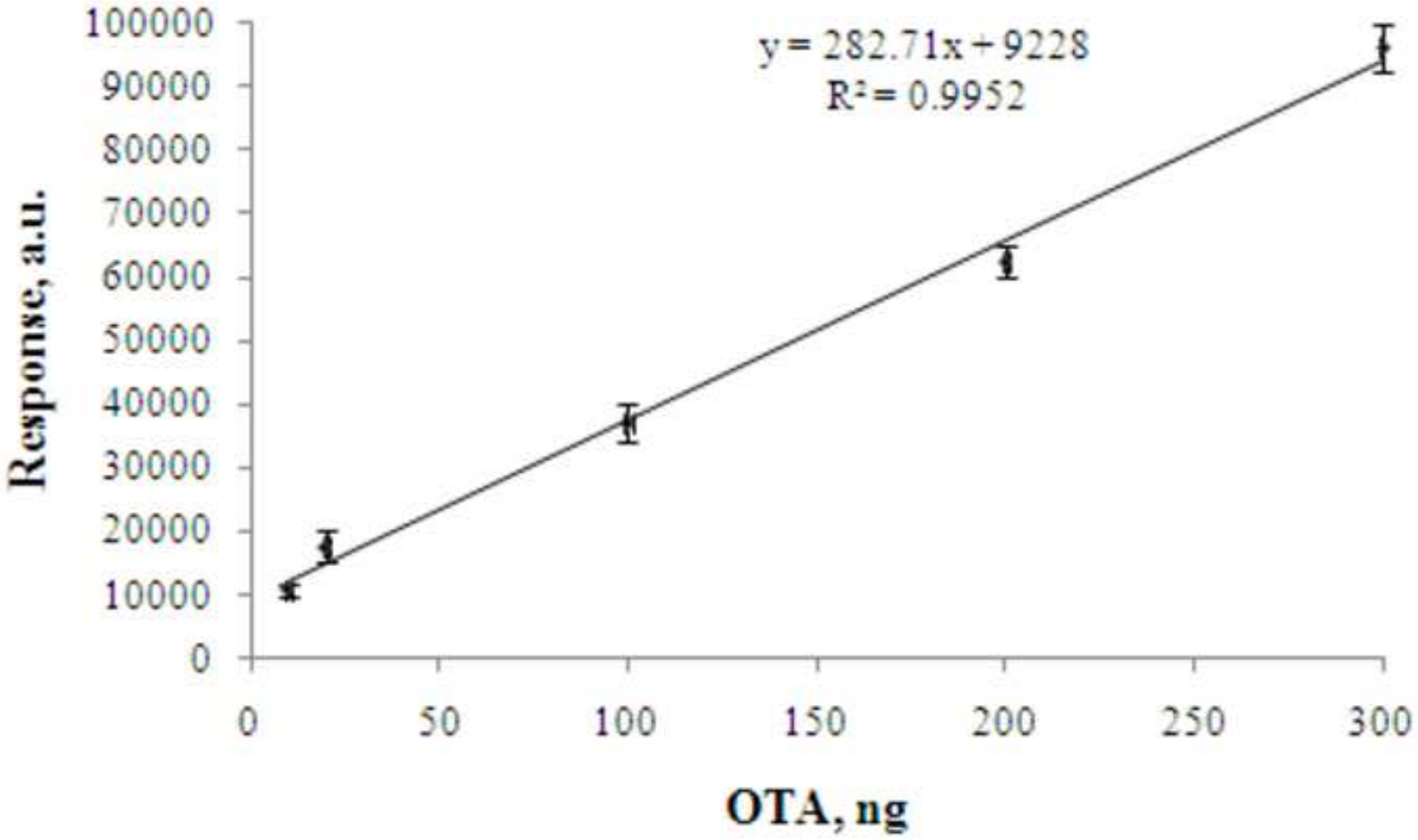


Figure 7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