Development of antibody-based microarray assay for quantitative detection of aflatoxin B_1

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Abstract:

BACKGROUND: Aflatoxin B1 (AFB1) is a toxic metabolite produced by *Aspergillus* species that contaminates a wide range of agricultural products. **OBJECTIVES:** This study was designed to develop a rapid and highly sensitive immunoassay method in microarray format for quantitative detection of AFB1 to evaluate the potential of microarray platform for high-throughput screening, which can be beneficial in food and feed industry. **METHODS:** Following successful optimization, using an indirect competitive immunoassay in dot blot format, AFB1-bovine serum albumin (AFB1-BSA) conjugate was contact-printed onto 16 isolated sub-arrays on multi-pad nitrocellulose coated slides; subsequently used in competitive binding assays. **RESULTS:** Using the aforementioned assay, AFB1 was determined from 15 pg/g to 3.04 ng/g working range with detection limit (LOD) of 1 pg/g. To evaluate assay performance in real food matrices, the authors spiked wheat samples with different concentration of AFB1. After extraction, working ranges of 0.11-4.15 ng/g with detection limit of 30pg/g was determined. Good recoveries (94±9%) were obtained, demonstrating accurate detection of AFB1 concentrations in wheat samples. Assay procedure completed in 3 hours. **CONCLUSIONS:** The results indicated that the proposed developed assay in microarray format could be used for rapid and sensitive detection of AFB1in wheat samples.

Introduction

Aflatoxin B_1 (AFB_1) is a toxic metabolite produced mainly by *Aspergillus flavus* and *A. parasiticus*. AFB_1 was listed as a Group I carcinogen by the International Agency for Research on Cancer (IARC) (IARC, 2002). It is a potent carcinogen, teratogen, and mutagen (Speijers and Speijers, 2004). Aflatoxins can affect a wide range of vegetable commodities such as cereals, nuts, peanuts, fruits, oilseeds, and dried fruits both in the field and during storage (Doradimos et al., 2000). The most common aflatoxin exposure is consumption of grains contaminated by aflatoxin-producing fungal strains during growth, harvest, or storage (Bakirci, 2001; Lopez et al., 2001).

European Community legislation has established a maximum level of 2 µg/kg (2ppb) of AFB_1 in foodstuffs (Anklam et al., 2002); Levels above that result in toxic manifestations, which in turn leads to liver cancer (hepatocellular carcinoma), which is the fifth most commonly occurring cancer throughout the world and the third greatest cause of cancer mortality (Parkin et al., 2001).

Aflatoxin production occurs in a wide range of foods and because of its harmful effects on humans and animals, several methods and techniques have been developed for Aflatoxin determination over the last few years. There are well-established methodologies for analyzing aflatoxins in many different foodstuffs; e.g., thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC), overpressure-layer chromatography (OPLC), immune affinity chromatography (IAC), and near infrared

spectroscopy (NIR) (Li et al., 2009).

These methods typically require skilled operators, extensive sample pretreatment, and expensive equipments (Stroka and Anklam, 2002; Papp et al., 2002).

The goal of more recent studies has been to simplify and expedite the method of detection while attempting to maintain or improve sensitivity.

Immunological techniques have been used for a long time for the detection and identification of Aflatoxin in different assays. Antibody based detection methods for AFB_1 include standard immunoassays coupled to colorimetric (Garden and Strachan, 2001; Delmulle et al., 2005; Xiulan et al., 2005), electrochemical (Ammida et al., 2004) or surface plasmon resonance (Daly et al., 2000; Dunne et al., 2005) detection, as well as enhanced immunoassays such as the enzyme-linked immunosorbent assay (ELISA) (Bhattacharya et al., 1999; Pal and Dhar, 2004; Lee et al., 2004). Dot blot is a simple technique to detect proteins. It is a quick assay in which sample proteins are spotted on a membrane and hybridized with an antibody that acts as a probe. Dot blot results give semi-quantitative measurements of the spotted proteins. Therefore, in this study, a dot blot technique has been used for initial optimization towards development of sensitive microarray format for detection of AFB_1. To achieve microarray optimization, an immunoassay was applied on the spotted polyvinylidene difluoride (PVDF) membrane. A simple dot blot technique offers significant savings in time and can be used for designing the layout of microarray. Thus, using these findings, a toxin microarray has been developed for rapid and sensitive detection of AFB_1. The efficacy of this microarray assay was evaluated in food samples using spiked wheat flour as a model of real matrices.

Materials and Methods

16-pad nitrocellulose coated slides and incubation chambers were purchased from Whatman Int. Ltd. AFB_1 standard solution (2µg/mL) in acetonitrile was purchased from Sigma. AFB_1-bovine serum albumin (AFB_1-BSA), monoclonal anti- AFB1 antibody (Mab), sheep anti mouse IgG -Cy3 (Ab2-Cy3), and goat anti mouse IgG- Alkaline phosphatase (Ab2-AP) were obtained from Sigma-Aldrich. Alexa Fluor® 647 Goat Anti-Mouse IgG was purchased from Invitrogen. All other chemicals were of analytical grade (A.R.) and purchased from Sigma-Aldrich.

Assay optimization using dot blot: One volume of AFB1-BSA (400-10 µg/mL), phosphate buffered saline (PBS) (as the negative control), monoclonal, and secondary antibodies (as internal control) were spotted on pre- activated PVDF membrane to optimize microarray layout according to the schemes in the Figure 1, 2 and 3. The spotted membranes were stored at 4ºC overnight for further application.

A simple indirect immunoassay procedure was performed on the spotted membranes. After blocking for 1h in 5% (w/v) low-fat milk prepared in tris-buffered saline -T (150mM NaCl, 10 mM Tris-HCL pH 7.5, 0.05% v/v Tween 20), membranes were washed three times with TBS-T for 5 min. Then, Mab was applied in different dilutions; 1:10 000, 1:20 000, and 1:30 000 (from 33 mg/mL concentration) and incubated for 1 h. Following 3 washes, two different detection methods were applied by using two secondary antibodies. Ab2-AP was tested in dilution series of 1:1000, 1:5000, and 1:10 000 (1mg/mL). Ab2-Cy3 was tested in dilution series of 1:500, 1:5000, and 1:10 000 (1mg/mL). The secondary antibodies were incubated for 45 min. Additional steps were applied for those Ab2-APs that were used in the detection method. The membrane was equilibrated in AttoPhos® buffer (100mM Tris-HCL pH 9.5, 1mM MgCl2) for 10 mins, then transferred to a 1:40 dilution of AttoPhos® substrate in its buffer and incubated in the dark for 5 mins. In the detection step, all the membranes were scanned using LAS3000 Fuji imager (Figure 1 and 2) or G: BOX (SYNGENE) (Figure 3).

Contact printing and immobilization of toxin microarray: Q-Array System (Genetix) was used to generate microarrays. 16-pad nitrocellulose coated FAST slides were used as reacting chips (Figure 4). An image of each identical subarray is shown in Figure 1A. The identical layout of sub-arrays is shown in Figure 1B. The printing design of each sub array consisted of 32 replicates of AFB1-BSA, 8 replicates of mouse Ab2-Cy3 (printing control), 4 replicates of monoclonal anti- AFB_1 antibody (internal control), and 4 replicates of BSA 2% in PBS (negative control). After printing, the microarray slides were stored in a slide box at 4°C for at least 24

h before use.

Microarray assay: The spotting chambers were fixed on each slide and an indirect competitive immunoassay was performed on each sub array. First, the chips were blocked with BSA 2% in PBS (100µL per sub array) to minimize the nonspecific binding of the AFB_1 to the chips. They were then incubated at room temperature for 1 h. Subsequently, the chips were washed thoroughly (100µL per sub array) two times with PBST. Standard solutions of AFB_1 at different concentrations were prepared in BSA 1% PBS 0.01% (v/v) Tween 20, mixed with monoclonal anti-AFB1 (0.19µg/mL, diluted in BSA 1% PBS 0.01% (v/v) Tween 20) and then pre-incubated at 37°C for 20 min, before application to each sub array (50 µL per sub array). The chips were incubated at 37°C for 30 min. After incubation, the chips were washed (100µL per sub array) three times with PBST (each wash for 3 min).

The secondary antibody, Alexa Fluor® 647 anti-mouse IgG (I mg/mL) was diluted 1:5000 (v/v) in BSA 1%- PBS- 0.01 % (v/v) Tween and added to the subarrays (50 µL per sub array). The chips were incubated at 37°C for 45. After three washes, the chips were centrifuged at 3000rpm for 3 min at 4°C and scanned using confocal microarray reader (Genepix 4000B) at a wavelength of 635 nm. The total assay procedure was completed in 3 hours.

Food Sample preparation: Wheat flour samples were artificially contaminated by adding 100 µL of AFB_1 standard solutions (0, 0.1, 1 and 10 µg/mL) to 5 g of sample. The extraction method used was a modification of the method used by Strachan and Garden (Garden and Strachan, 2001). To this end, 15 ml methanol-water (80: 20) was added to 5 g of sample. The suspension was vortexed for 1 min and then centrifuged at 4000g for 15 min. The aqueous layer was diluted 1 in 10 for the assays. The concentration of AFB1 in diluted sample extracts was measured by reference to a calibration curve and was used to estimate the concentration in the original sample.

Data extraction and analysis: Quantitative data was extracted using Genepix Pro 5.1 software (Axon Instruments), generating the value "mean foreground minus mean background" intensity for each spot, applied for analysis. Finally, calibration graphs were handled with Origin 6.0. Standard curves generated from one chip in parallel and were repeated two times.

Results

Dot blot optimization: Each microarray has a layout that should be set up and designed properly. Therefore, AFB_1-BSA, PBS (as negative control), and a primary and two secondary antibodies (as internal controls) were spotted to establish the optimum design for future use on a microarray platform. The concentration of 100µg/mL was selected as the optimal concentration for AFB_1-BSA, as it was the lowest concentration that was detectable using the dilution of both primary and secondary abs. (Figure 1, 2 and 3). The dilution of 1: 5000 of Mab was the only dilution that could be detected by Ab2-AP and nothing was detected by Ab2-Cy3 (Figure 1). Although all dilutions of spotted secondary antibodies have been detected with imager, using Ab2-AP as the detection method the signals were stronger. With regard to the final result of spotting dilutions, a 1:100 dilution of both Mab (330µg/mL) and secondary antibodies (10µg/mL) was chosen for the positive controls (Figure 1).

Antibodies titrations: Antibody detection always plays an important role in an indirect immunoassay system; therefore, the conditions for using two different secondary Abs, Ab2-AP, and Ab2-Cy3 were optimized (Figure 2). As It was expected, Ab2-AP was more sensitive than Ab2-Cy3, as the dilution of 1:10 000 (v/v) for Cy3 has made a weak signal but was strong enough for Ab2-AP. Thus, the dilution of 1:5000 (0.2 µg /mL) was established as the optimal detection dilution for use in final format of microarray design.

Different dilutions of Mab were applied on the final figure of designed chip. Although the Ab2-AP was more sensitive, further optimization was carried out using Ab2-Cy3 as detection method because the final microarrays format adapts to the florescent detection. The images showed that the dilution of 1:30 000 (1.1 µg/mL) can still detect AFB_1 and can be used as a starting point for further optimization on expensive microarray surfaces (Figure 3).

Utilizing the dot blot technique, the concentrations of 100 µg/mL of AFB1-BSA, 330µg/mL of Mab and 10µg/mL of secondary antibodies (as positive controls) were chosen for designing the final

Table 1. Estimation of LOD for AFB1 detection using antibody-based microarray assay.

Matrix	Standard curve	LOD (ng/g)	Working range (ng/g)
Buffer	y=2.1386+ 1.2023/(1+(x/0.3708^2.1386))-1.2023,R2=0.98	0.001	0.015 - 3.04
Wheat	y=4.0836+ 0.3898/(1+(x/1.0089^0.7033))-0.3898,R2=1	0.03	0.11- 4.15

Table 2. Measurements of AFB1 in wheat samples by antibody-based microarray assay . Each extraction value was represented the average value of 16 measurements on each sub-array. Each sub-array measurement was repeated two times.

Sample(ng/g)	AFB1 spiked	AFB1 measured after extraction (ng/g)		(Mean ± SD)	R.S.D. (%)	Recovery (%)
		First extraction	Second extraction			
Wheat	2	2.01	1.79	1.9±0.15	7.87	95.3
	20	16.02	17.99	17.01±1.39	8.19	85.06
	200	206.28	199.8	203.04±4.58	2.25	101.52

feature of microarrays on suitable surface. For developing the immunoassay, the dilution of 1:30 000(v/v) of Mab can be used as a starting point for further optimization. The dilution of 1:5000(v/v) of secondary antibodies was established as the optimum.

Optimization on microarray: To establish the working range in the microarrays format, further titration starting from 1.1 µg/mL concentration of Mab antibody was performed. The four final optimized concentrations of monoclonal antibody on the sub arrays performance were evaluated by generating standard competition curves for AFB_1 (Figure 3). All the experimental data were fitted using non-linear four-parameter logistic calibration plot. The four-parameter logistic (Fare et al., 1996) is given by the equation:

$f(x)= a-d/1+(x/c)^b+d$

in which and are the asymptotic maximum and minimum values, the value of at the inflection point (IC50), and the slope. Comparison of the calibration curves generated using the toxin microarray indicated that the dilution of 1: 170 000 (v/v) (0.19 µg/mL) is optimal for quantitative detection of AFB1. Several microarrays were screened without competitors in order to evaluate the experimental variation in spot intensities among each array. Relative standard deviation no higher than 10 % was observed. The variation across the slide was affected by the distance of the slide from microtitre plate and the exact location on the slide. Therefore, a non-contact form of printing the variation should be overcome.

Calibration curves: In Table 1, the result of calibration curves, the equations for estimation of limit of detection (LOD) values (equivalent to IC10) and working ranges are shown. Each concentration of AFB_1 had 32 replicates in a sub array and each sub array value representing two time measurements. The logistic correlation coefficient (R2), which was above 0.98, indicated the excellent analytical performance of this optimized toxin microarray assay method. The results reveal that this microarray assay can detect the pure toxin at a level of 1 pg/mL. It should be pointed out that the sensitivity of assay in wheat samples was 30 pg/mL, i.e. near 30 fold higher. The reduction of sensitivity for AFB1 detection in real samples is explainable due to food matrix effect; nonetheless, this assay achieves adequate sensitivity for applications in food samples.

Recovery in food samples: The recovery analysis of artificially contaminated wheat flour samples has been shown in Table 2. Good recoveries (94±9%), demonstrating the suitability of the proposed assay for accurate determination of AFB1 concentration in wheat samples was obtained. Each extraction value indicated the average of 16 measurements. The recovery values were represented the mean value of two extraction procedure repeated on two different days. The precision was estimated by calculating the relative standard deviation (% R.S.D) for replicate measurements.

Discussion

In recent years, the development of array-based biosensors and microarray technology has offered using them in various applications, including the study of disease, drug discovery, genetic screening, clinical diagnostic, and food screening. Antibody-based microarrays provide a powerful tool that can be used to generate rapid and detailed expression profiles of a defined set of analytes in complex samples, and they are potentially useful for generating rapid immunological assays of food contamin-

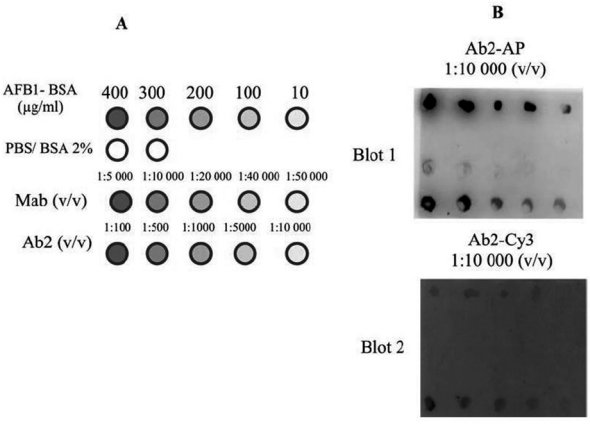

Figure 1. Optimization of microarray feature using dot blot. (A)The illustration of the spotted elements and related concentrations; AFB1-BSA, BSA 2% in PBS, Mab and Ab2 on each blot has been shown in cartoons. (B) Corresponding image of spotted blots. A 1:10 000 (v/v) dilution of Mab and Ab2-AP or Ab2-Cy3 has been applied for immunoassay detection.

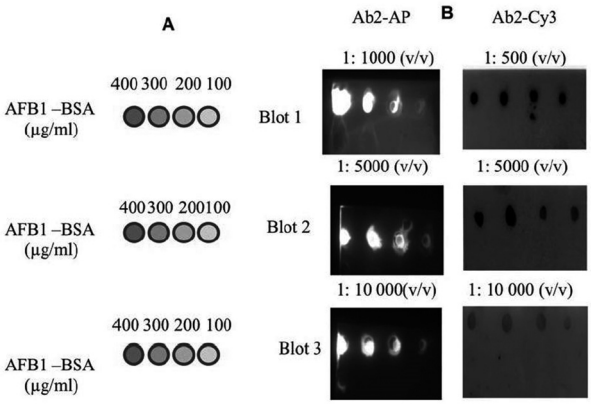

Figure 3. Primary antibody titration using dot blot. (A) The illustration of the spotted feature on each blot has been shown in cartoons. (B) Corresponding image of spotted blots. Three different dilutions of Mab were applied on each blot for immunoassay detection.

ants.

There are different issues that should be addressed for optimal microarray performance, e.g., the type of antibody molecules, solid supports and binding chemistries, detection system, blocking reagents, stability of printed antibodies, sensitivity and labeling strategies, specificity and cross-reactivity, immunoassay format, analysis of microarray data, and normalization (Parro, 2010). Considering all these factors looks challengeable and has made this technology complicated. Most of the microarray reagents and equipments (e.g., buffers, solid Support-

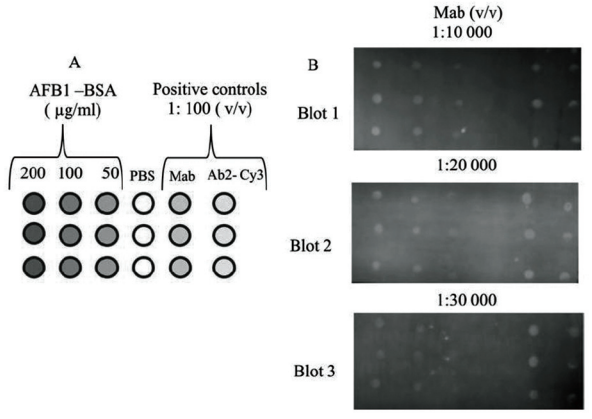

Figure 2. Secondary antibodies titration using dot blot. (A) The illustration of the spotted elements on each blot in cartoons. (B) Corresponding image of spotted blots. From left to the right, Ab2-AP and Ab2-Cy3 were applied in three different dilutions on each blot for immunoassay detection.

s, etc.) are expensive; therefore, the need to use an inexpensive method for initial optimizations prior to the actual microarray platform seems to be crucial. Dot blot is a simple technique to detect proteins. Thus, in an attempt to develop an antibody-based assay for detection of AFB_1 in microarray format, dot blot technique was applied for primary optimization. Different concentrations of AFB_1-BSA, monoclonal and secondary antibodies, sheep anti-mouse IgG-Cy3 conjugated (Ab2-Cy3), or goat anti mouse IgG-Alkaline phosphatase conjugate (Ab2-AP) (as internal control) were spotted in series of dilution to establish the optimum conditions for microarray layout. The concentration of 100µg/mL was selected as the optimal concentration for AFB_1-BSA, as it was the lowest concentration that was detectable using the optimized immunoassay. For positive internal controls, a 1:100 dilution of Mab (330µg/mL) and secondary antibodies (10µg/mL), was determined as optimal (Figure 1 and 3).

The type of antibody molecules (polyclonal-monoclonal, phage-display, Fab, affybodies, etc.) and detection systems, label-dependent (e.g. fluorescence, chemoluminiscence, enzymatic, etc.) or label free, always plays an important role for optimal antibody microarrays function. (Parro, 2010). Minimum antibody titers of 1:30 000 (1.1 µg/mL) of Mab and 1:5000 (0.2 µg/mL) for secondary antibodies were established as suitable dilutions for immunoassay. Ab2-AP was more sensitive than Ab2-Cy3 as 1:10 000 (0.1 µg /mL) of Ab2-Cy3 has made a

A

B

Anti-AFB1 Mab	Anti-AFB1 Mab	AFB1-BSA	AFB1-BSA	AFB1-BSA	AFB1-BSA
IgG-Cy3	IgG-Cy3	AFB1-BSA	AFB1-BSA	AFB1-BSA	AFB1-BSA
BSA 2%	BSA 2%	AFB1-BSA	AFB1-BSA	AFB1-BSA	AFB1-BSA
IgG-Cy3	IgG-Cy3	AFB1-BSA	AFB1-BSA	AFB1-BSA	AFB1-BSA

Figure 4. Image of a microarray chip. (A) An image of a Nitrocellulose coated slide containing 16 physically isolated subarrays assembled with reaction chamber has been shown. (B) A representing order of the layout of each subarray has been shown in table. Every subarray was consisted of two replicates of this printing layout.

weak signal but was strong enough for Ab2-AP. (Figure 2). For the final titration of monoclonal antibody, only Ab2-Cy3 was used because of the advantage of one step detection.

In all of the studies designed to detect mycotoxins, the LOD, which reflects sensitivity, is an important parameter e.g., the LOD of AFB_1 was 3.00 ng/ml using surface plasmon resonance (Daly et al., 2000), 12.5 ng/g by ELISA in food stuffs (Saha et al., 2007), 0.16 ng/mL by HPLC (Ghali et al., 2009) and 1.00 ng/mL using LC/APPI-MS/MS (Capriotti et al., 2010); 1 ng/mL by novel selective immunochromatographic assay (Zhang et al, 2011); and 1 mg/kg by lateral flow immunoassay (Anfossi et al., 2011).

In comparison with the current published methods for aflatoxin detection, the LOD of our developed method was 1 pg/mL, indicating the high sensitivity of developed assay, which is more sensitive than the currently available commercial methods. Total assay time was 3h, which indicates the rapid detection ability of the proposed method. The performance of the microarray assay in commodities was evaluated using spiked wheat flour samples. The sensitivity of this method was determined as 30 pg/g. The reduction of sensitivity in actual food samples is explainable by the effect of food matrices. Therefore, further investigation needs to be carried out to address this issue. A good recovery (98±11%) indicates the accuracy of the proposed assay for AFB1 detection in real food samples. In conclusion, microarray technology has the potential to be used as a screening tool for monitoring food samples on a large scale. Using this method, small quantities of reagents and samples are required. In addition, parallel assays can be performed for multiple analyses. Dot blot is a simple and inexpensive technique which confirms the presence or absence of a biomolecule. A successful dot blot optimization can be considered as a significant cost benefit step toward designing an effective microarray. Future work can focus on larger scale application of this method in commercial foodstuffs. In addition, this method could be extended to detect other foodborne hazards (such as food borne pathogens, bacterial toxins, chemicals, antibiotics residues, etc.) on a single chip format.

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توسعه سنجش ریز آرایه‌ای آنتی بادی برای تشخیص کمی آفلاتوکسین B_1

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چکیده

زمینه مطالعه: آفلاتوکسین B_1 یک متابولیت بسیار سمی است که توسط گونه های آسپرژیلوس تولید گردیده و طیف وسیعی از محصولات کشاورزی راآلوده می کند. **هدف:** هدف از این مطالعه توسعه یک روش ایمونو اسی سریع و حساس در فرمت ریز آرایه ای به منظور اندازه گیری کمی آفلاتوکسین B_1، در جهت ارزیابی کارایی این روش به منظور توسعه روش های غربالگری باکارایی بالا برای استفاده در صنایع مواد غذایی انسانی و دامی بوده است. **روش کار:** به دنبال بهینه سازی سنجش با استفاده از تکنیک دات بلات و روش ایمونواسی رقابتی غیرمستقیم، غلظت بهینه از آفلاتوکسین متصل شده به آلبومین سرم گاوی بر روی ۱۶ساب اری بر روی اسلایدهای پوشیده شده با نیتروسلولوز پرینت گردیده و سنجش ایمونواسی رقابتی اعمال گردید. **نتایج:** با استفاده از این روش، سنجش آفلاتوکسین در طیف کاری برابر ۱۵pg/g تا ۳/۰۴ng/g با حد تشخیص ۱pg/g تعیین گردید. به منظور ارزیابی کارایی این میکرواری تولیدشده در مواد غذایی، آرد گندم به صورت مصنوعی با مقادیر مختلف توکسین آلوده گردید و پس از استخراج، طیف کاری برابر ۰/۱۱ng/g-۴/۱۵ با حد تشخیصی برابر ۳۰pg/g درآرد گندم حاصل گردید. میانگین تخمین بازیافت ۹٪±۹۴تعیین گردید که نشان دهنده صحت سنجش با استفاده از این روش می باشد. این سنجش در مدت زمان ۳ ساعت تکمیل گردید. **نتیجه گیری نهایی:** این روش سنجش مطرح شده در فرمت ریز آرایه‌ای می تواند به عنوان روش اندازه‌گیری سریع با حساسیت بالا برای سنجش دقیق آفلاتوکسین B1 در نمونه های گندم به کار رود.

واژه های کلیدی: آفلاتوکسین B_1، دات بلات، ایمونواسی، ریز آر، بهینه‌سازی اولیه

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