Application of Fluorescence Polarization Immunoassay for the Screening of Ochratoxin A in Unpolished Rice

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To High Throughput Screening (HTS), a homogeneous fluorescence polarization immunoassay (FPIA) was developed for the quantitative determination of ochratoxin A(OTA) using a Victor[3] (PerkinElmer). The homologous tracer, fluorescein-labelled OTA-EDF were synthesized and a specific OTA antibody has been used in the development of the method. It allowed the determination of OTA in the concentration range 0.5-200 ng/ml, with the detection limit of 0.3 ng/ml. The method developed was highly specific and reproducible. OTA spikes in unpolished rice extracts were determinable by FPIA with good recovery. For naturally contaminated unpolished rice samples some disagreement was observed between the results obtained by FPIA and HPLC, which could be related to the a little matrix effect observed for FPIA. Further research is needed to validate the procedure. On the basis of these initial results, this FPIA appears to meet the performance criteria for OTA screening of food samples without a complicated clean-up.

Key words – Fluorescence polarization immunoassay, ochratoxin A, monoclonal antibody, unpolished rice.

In today's changing world, safety and security have generally remained basic human needs. Ensuring the safety of food has been a major focus of international and national action over the last years. Both microbiological and chemical hazards are of concern. Among chemical hazards, the contamination of food and feed by mycotoxins (toxic metabolites of fungi), fishery products by phycotoxins (toxins produced by algae) and edible plant species by their significant sources of food-borne illnesses[38]. Of these three categories of natural toxins, most attention has been directed to mycotoxins until now. In several parts of the world, mycotoxins currently represent a major food safety issue.

The knowledge that mycotoxins can have serious effects on humans and animals has led many countries to establish regulations on mycotoxins in food and feed in the last decades to safeguard the health of humans, as well as the economical interest of producers and traders. The first limits for mycotoxins were set in the 1960s for the aflatoxins. By the end of 2003, approximately 100 countries had developed specific limits for mycotoxins in foodstuffs and feedstuffs, and the number continues to grow. Despite the difficulties, mycotoxin regulations has been established in many countries during the past decades, and newer regulations are still being issued[5]. National regulations have been established for a number of mycotoxins such as the naturally occurring aflatoxins, trichothecenes deoxynivalenol, diacetoxylscirpenol, T-2 toxin and HT-2 toxin, the fumonisins B_1, B_2 and B_3; agaric acid; the ergot alkaloids; ochratoxin A; patulin phomopsins; sterigmatocystin and zearalenone [2,7,12,24,25,26, 31,34,36,37]. Among them, the ochratoxin A (OTA) is a mycotoxin produced by several *Aspergillus* and *Penicillium* species on different agricultural commodities. It has carcinogenic, mutagenic, immunosuppressive, nephrotoxic and teratogenic properties. The occurrence of OTA in food and feed ha been reported worldwide[11,13,22]. The regulation of mycotoxins in Korea, the limits were established guideline levels for aflatoxin B_1, M_1 in all foods and patulin mostly in fruit products such as apple juice, but not OTA, yet[9]. But a significant increase in the number of countries that have specific regulations for OTA at levels ranging from 1 to 50 μg/kg in foods and from 5 to 300 μg/kg for animal feeds[35].

Cereals are considered the major source of human exposure to OTA. Many countries have set a limit for ochratoxin A in cereals, many others for cereal products, and

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various have set separate(different) limits for both. Setting mycotoxin regulations is a complex activity, which involves many factors and interested parties. Various factors play a role in decision-making processes focused on setting limits for mycotoxins. These include scientific factors to assess risk (such as the availability of toxicological data), food consumption data, knowledge about the level and distribution on mycotoxins in commodities, and analytical methodology. The first two factors provide the necessary information for hazard assessment and exposure assessment respectively, the main ingredients for risk assessment. The exposure assessment were undertaken within the distribution of the concentration of mycotoxins in products. So the data for the level and distribution on mycotoxins in commodities, that is an important factor to be considered in establishing regulatory. Rice (*Oryza sativa* L.) is an important food crop worldwide along with wheat and corn, and has been major food in several countries of Asia including Korea since ancient times. Rice is a staple food as well as the main source of carbohydrate in the diet of the people. The daily intake of rice by Korean was surveyed in 1998 remains as high as ever end accounts for about 19% of the Korean total diet, or more than 70% of Korean diet of cereals and grain products[10]. Despite the importance of rice as a staple food and the reported occurrence data of mycotoxins, there is only a little information on the incidence of fungal flora in Korean rice[15,21].

To determine OTA levels in foodstuffs and biological fluids, a variety of methods have been proposed[16,27,35], the most widely employed being high-performance liquid chromatography (HPLC, reversed-phase or direct), using different clean-up procedures such as liquid-liquid partition, solid-phase extraction (SPE) and immunoaffinity chromatography, detected by fluorescence (excitation 330-340 nm, emission 460-470 nm) or, more recently, by tandem mass spectrometry[1,4,14,18,29,30,33]. However, these methods for the most part are expensive, laborious, time consuming, and require sophisticated equipment and complicated clean-up procedure. Recently, analysis using immunochemical methods especially enzyme-linked immunosorbent assay (ELISA), are gaining wide acceptance for the routine use on analysis of mycotoxins for many samples because of the sensitivity, specificity, rapidity, simplicity and cost effectiveness[3,17]. However, ELISA is a heterogeneous method and separation of free and antibody-bound analyte, as well as long reaction time (1-2 h), is needed; in addition, this method involves multiple washing steps. A very promising way for the simplification of immunoassays for routine applications is a shift from heterogeneous methods(with separation) to homogeneous assays(without separation). Fluorescence polarization immunoassay (FPIA) is the most extensively used homogeneous technique, which meet the requirements of a simple, reliable, fast and cost-effective analysis. The theory and applications of these method to the determination of different compounds, including pesticides, infectious disease and mycotoxins, have been described in several books and review[6,8]. Recently, the use of FPIA for the determination of fumonisins, deoxynivalenol and aflatoxins in grains has been reported [19,20,28].

This paper focuses on the determine the occurrence of ochratoxin A in unpolished rice by developed rapid detecting method, FPIA.

Materials and Methods

Chemicals

OTA and other toxins used for cross-reactivity studies, 1-ethyl-3-(3-dimethyl-aminopropyl)carbodiimide hydrochloride (EDC), *N*-hydroxysuccinimide (NHS), fluorescein isothiocyanate (FITC, Isomer I), Tween 20, common solvents and salts were purchased from Sigma Co. (St. Louis, MO, USA). Thin-layer chromatography (TLC) plates (Silica gel 60, fluorescent, 1 mm, 20×20 cm) were obtained from Merck Co. (Darmstadt, Germany). All chemicals and organic solvents were of reagent grade or higher. Water used in all experiments was purified with Aquamax- Ultra system (Yonglin, Korea). Fluoresceinthiocarbamyl ethylenediamine (EDF) was synthesized from FITC and ethylenediamine as described previously by Pourfarzaneh *et al.* [23] with modifications[20]. Sodium borate buffer (BB, 0.05 M, pH 9.0) was used for all FPIA experiments. Standard solutions of OTA and cross-reactants were prepared by dilution of stock solutions of these compounds (1 mg/ml, in methanol).

Sample preparation

Unpolished rice samples were taken from fields in Gyeongnam and Gyeongbuk provinces, South Korea, which cultured 2005. Unpolished rice sample were first analysed by HPLC and found to be OTA negative. One gram of dried ground blank sample was spiked with up to dif-

ferent concentrations of OTA (10, 50 and 100 ng/g) and then extracted with 10 volumes (10 ml) of methanol/ultra-pure water (3:2, v/v) by shaking for 30 min at room temperature. Extracts were centrifuged at 3000 rpm for 10 min, the supernatants were subsequently diluted two-fold with the assay buffer(BB) and tested in the FPIA without further purification. Analyzed in triplicate by FPIA. Blank and naturally contaminated unpolished rice samples were prepared as described above but not spiked with OTA.

Preparation of immuno-reagents

The synthesis of fluorescein-labeled tracer, OTA-EDF and purification of OTA-EDF were performed as described by Shim *et al.*[28]. The monoclonal antibody, CVL-MAB0029-1(3C5) against OTA-bovine serum albumin(BSA) conjugate, was purchased from Axxora Life Science Inc. (San Diego, CA, USA).

Fluorescence polarization immunoassay

Apparatus Measurements of fluorescence polarization and intensity were performed using Victor^3 multilabel plate reader(PerkinElmer^{TM} Life Sciences, Boston, MA, USA). The fluorescence polarization was detected by millipolarity (mP) units and intensity were detected by conventional units using OptiPlate^{TM} 384 F (black, pinch bar design, PerkinElmer^{TM} Life Sciences, Boston, MA, USA) and provided data processing software.

Dilution curves To 30 μl tracer solution (10 nM) in the 384-well, 30 μl of monoclonal antibody in various dilutions was added, mixed and analyzed using fluorescence polarization.

Competitive FPIA procedure 10 μl OTA standard solution (or sample), 30 μl tracer solution and 30 μl monoclonal antibody solution in optimal dilution were added sequentially to the well with 12 channel micropippet and mixed followed by measurement. Standard curves were plotted as mP vs. logarithm of analyte concentration. Cross-reactivity (CR) for different mycotoxins(analyte) was determined by performing competitive assays and comparing the analyte concentration giving half-maximal inhibition (IC_{50}, ng /ml), and calculated as:

% CR = (IC_{50} for OTA ÷ IC_{50} for analyte) × 100.

OTA concentration in spiked samples was calculated after fitting the standard curve using the four-parameter logistic model using SigmaPlot(Version 9.0) and SOFTmax® PRO(version 4.0).

HPLC

For the conform of the OTA concentration, reversed-phase high-performance liquid chromatography (HPLC) method was used. The HPLC method used was an adaptation of that described by Sibanda *et al.* [30]. For HPLC, the samples were extracted with acetonitrile-water (84:16) and the sample extracts were cleaning up using NH_2 solid-phase clean-up method[30]. The HPLC system consisted of a Waters^{TM} 600 Controller and a Waters 610 Fluid Unit(Waters, Milford, MA, USA). The flow-rate was 1 ml per minute over a Spherisorb ODS II column (5 μm particule size, 250 mm × 4.6 mm i.d.) at ambient temperature. The mobile phase used was acetonitril/water/acetic acid (99:99:2). OTA detection was achieved by means of a Water 474 scanning fluorescence detector (Waters, Milford, MA, USA) set at 333 nm excitation and 460 nm emission wavelengths.

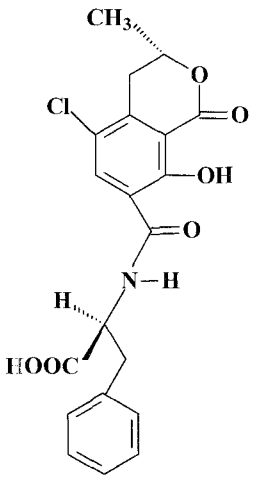
Results and Discussion

Optimization of assay conditions

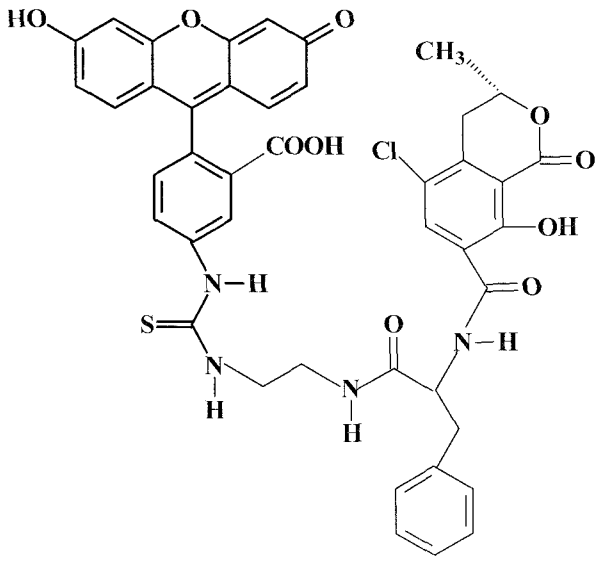
The FPIAs were very simple to perform: the sample and antibody were mixed. This solution was used as a blank, and then the OTA-fluorescein tracer (OTA-EDF) was added. After a short holding time at room temperature, the fluorescence polarization signal (mP) was measured. The interaction of the antibody with the tracer increased the polarization signal. In the presence of unlabeled OTA, the tracer and toxin competed for attachment to the antibody, and the signal decreased in relation to the toxin content. In common with the other immunochemical techniques FPIA requires the production and characterization of immunoreagents as well as the optimization and validation of an analytical system. The antibody and tracer are key components in the development of FPIA. Being a low molecular weight compound (hapten), OTA, like most of the mycotoxins, is not immunogenic and should be therefore conjugated to a carrier protein to elicit the immune response. Thus OTA molecule was conjugated via the carboxyl group to the carrier protein (BSA) and also to the amino derivative of fluorescein (EDF) to be used as the immunogen and competitor (homologous fluorescent tracer), respectively (Fig. 1).

FPIA is a homogeneous assay technique based on differ-

ences in polarization of the fluorescence labeled species in the free and bound fractions; it involves the competition between free analyte and tracer for binding to a specific antibody. Hence, the tracer concentration is one of the key parameters for the development and optimization of the FPIA procedure, which influences the assay sensitivity markedly. The tracer determines the intensity of emitted polarized light and also contributes to the competition for antibody binding. Thus, the lowest possible tracer concentration, which permits the reliable detection of a label and produces the minimum effect on the competition, should be used to develop a sensitive assay. For OTA-EDF tracer



Ochratoxin A (OTA)



OTA-EDF

Fig. 1. Chemical structure of OTA and fluorescein-labeled tracer OTA-EDF.

the lowest concentration was approximately 10 nM in the final reaction, corresponding to the total fluorescence intensity of ~ 2000 fluorescent units, what was more than 30 times higher than the background signal for the borate buffer.

The preliminary assessments of antibody binding to the tracer were performed by FPIA using the dilution curves obtained for monoclonal antibody, MAb-3C5. (Fig. 2). MAb-3C5 gave the titer value (antibody dilution corresponding to 50% binding of a tracer) of 1/5,000 and was used for further assay development.

Analytical performance of the assay

Under the assay conditions optimized, the standard curve for OTA detection by FPIA was obtained (Fig. 3). Assay sensitivity, defined as 50% inhibition (IC_{50}), was 4.24 ng/ml, while the detection limit, determined using three-fold confidence interval of zero analyte dose signal, corresponding to the average of 20 measurements, converted into the concentration value, was 0.3 ng/ml. The assay dynamic concentrations ranged from 0.5 ng/ml (corresponding to 20% inhibition) to 200 ng/ml (corresponding to 80% inhibition). The FPIA was characterized by good reproducibility; coefficients of variance (CVs) ranged from 5.9 to 12.8 % for intra-assay and from 5 to 13.7 % for inter-assay. The assay with the use of MAb was highly specific. As shown in Fig. 4, cross-reactivity to other toxins, such as patulin, zearalenone, T-2 toxin, and aflatoxin B_1 was negligible (<0.1%). This results were more sensitive, rapid, high thruput than reported by Shim *et al.*[28]. It could be the minimize of the immuno-reagent and samples, the sensitivity is more increase.

Homogeneous immunoassay techniques like FPIA are generally known to be less sensitive than heterogeneous ones. The detection limit of the method developed was higher than that for ELISA techniques or instrumental methods. For instance, ELISA for OTA detection with the use of polyclonal antibodies was reported to have the detection limit of 0.1 ng/ml and IC_{50} value of 5 ng/ml[32]. Being a rapid and simple method for screening of a large number of samples, FPIA, however, has certain advantages such as short assay time (4-6 min compared to hours); no separation or washing since everything is performed in a single step in comparison to excessive handling required in ELISA; low cost.

Organic solvent interferences

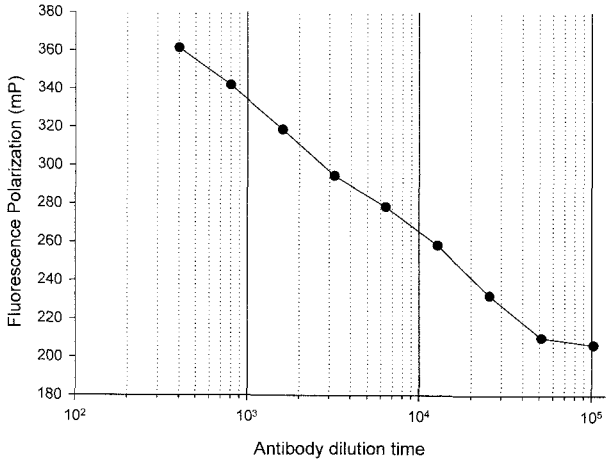


Fig. 2. Dilution curve monoclonal antibod (MAb-3C5) using tracer OTA-EDF.

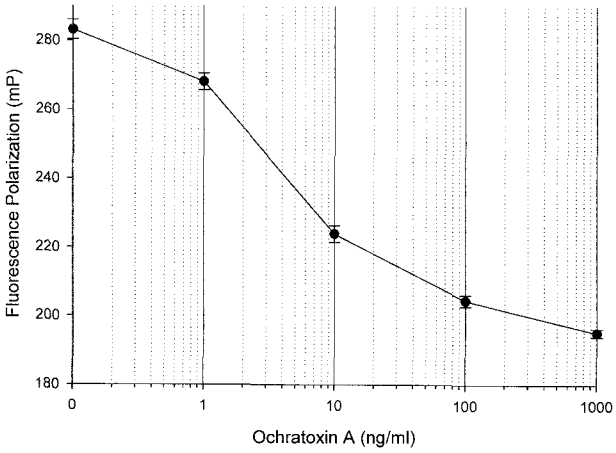


Fig. 3. Optimized FPIA standard curve for OTA detection in borate buffer with 50% methanol.
*Each point of the curve represents the mean ± S.D. (standard deviation) of three assays.

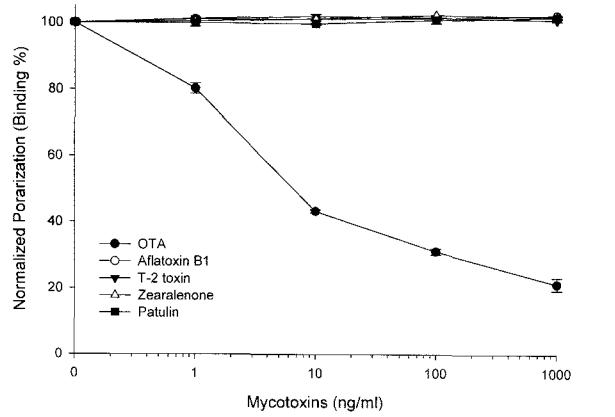


Fig. 4. Standard curves for OTA and other mycotoxins for check the coss reactivity.

To estimate the utility of the method for the analysis of extracts from different real samples such as foodstuffs and feeds, the influence of several organic water miscible solvents, commonly used for the extraction, on the assay performance was studied. As illustrated in Fig. 5(a), using OTA standard solutions in 10% organic solvents such as DMF, ethyl acetate and acetonitrile resulted in a decrease of maximum binding signal, as well as assay sensitivity, compared to the FPIA standard curves with the use of methanol and ethanol. OTA is usually extracted from grains with methanol. It was shown that the near-optimal standard curve could be obtained with the buffer containing up to 50% (v/v) methanol (Fig. 5(b)). Only using 100% methanol standard solutions caused a slight effect on the assay performance.

Application of the assay to the analysis of unpolished rice samples and correlation with HPLC

The method developed was applied to OTA detection in

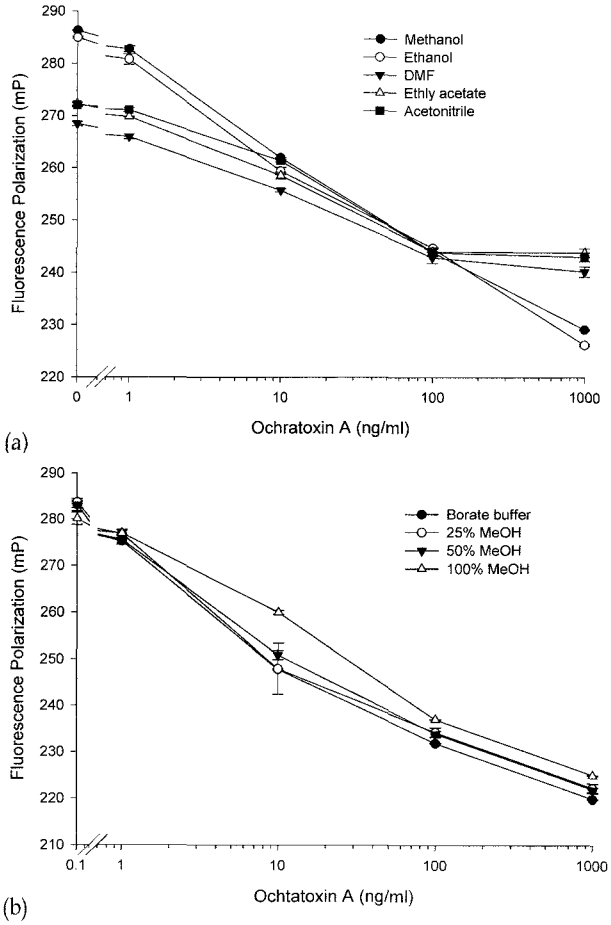


Fig. 5. Effect of organic solvents on OTA FPIA performance: (a) influence of the solvent type; (b) influence of the methanol content.

unpolished rice samples. The analytical performance of an immunochemical technique can be affected by various substances existing in complex matrices. To correct the matrix effect of the samples different approaches are preferable to (1) sample clean-up, which is laborious, time-consuming, and may affect the assay reproducibility and recovery, and to (2) dilution of the extract. One of the major advantages of FPIA technique is its simplicity, so the second approach was used. Two-fold dilution of the methanol extracts of unpolished rice samples enabled to correct for matrix interferences with the assay performance. The optimum sample volume of 10 μl was used.

Unpolished rice samples were spiked with OTA at 10, 50 and 100 ng/g level. OTA was extracted from samples with methanol/ultra-pure water (3:2, v/v), extracts were subsequently diluted two-fold with the assay buffer and submitted in triplicate to FPIA. The results (Table 1) were also compared with those obtained for the same spiked samples by HPLC. Method recoveries ranged from 110 to 84% in spiked samples (n=3 replicated triple). The detection limit for HPLC was 5 ng/ml. Regression (r^2) of peak area on concentration for both standards and spiked samples were identical and these were 0.981 and 0.977, respectively. The results of both methods correlated well and were in a good agreement with the amounts spiked, demonstrating the applicability of the developed assay to practical problems.

Sixteen contaminated unpolished rice samples were treated as described above and submitted in triplicate to FPIA and HPLC. Some results for the positive samples are presented in Table 2. In two samples OTA was detected by FPIA at the level around 12-20 ng/g, which correlated reasonably well with HPLC. In four extracts OTA concentration was found to be below the detection limit, and ten samples were considered to be negative, according to HPLC. Generally, the results obtained by FPIA for naturally contaminated samples were in rather poor agreement with those observed for HPLC. FPIA tended to overestimate results, even though good recoveries were observed for spiked samples. It could be attributed to the matrix interferences which were stronger in the case of HPLC. It should be mentioned that the standard error was usually higher for FPIA than for HPLC and the HPLC detection limit is higher than FPIA, then that could not recognize. So, in the first, the samples was screened by rapid FPIA method, if some positive samples, that could be conformed with HPLC.

Table 1. Recoveries of OTA from spiked unpolished rice samples by FPIA and HPLC methods

OTA concentration spiked (ng/g)	Recovery (%)[a]	
	FPIA	HPLC
10	108	88
50	90	84
100	110	98

[a]Data are the means of triplicates

Table 2. Detection of OTA in real unpolished rice samples by FPIA and HPLC methods

Sample No.	Ochratoxin A determined (ng/g)	
	FPIA	HPLC
Gyeongbuk 1	1.86 ± 0.82	N[a] (≤ 5)
Gyeongbuk 2	0.53 ± 0.07	N
Gyeongbuk 3	0.85 ± 0.06	N
Gyeongbuk 4	N (≤ 0.3)	N
Gyeongbuk 5	20.65 ± 1.19	17.23 ± 0.21
Gyeongbuk 6	1.21 ± 0.06	7.21 ± 0.17
Gyeongbuk 7	1.57 ± 0.16	N
Gyeongbuk 8	6.92 ± 0.15	N
Gyeongnam 1	19.66 ± 0.12	5.6 ± 0.21
Gyeongnam 2	N	N
Gyeongnam 3	N	N
Gyeongnam 4	N	N
Gyeongnam 5	2.82 ± 0.06	N
Gyeongnam 6	1.08 ± 0.17	N
Gyeongnam 7	12.82 ± 0.35	11.12 ± 0.12
Gyeongnam 8	1.44 ± 0.28	N

[a]It means that negative, which below than detection limits.

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초록 : 현미에서의 오크라톡신 A의 검색을 위한 형광편광면역분석법의 응용

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식품안전에 대한 관심이 증가되고 있는 현재, 생물학적 · 화학적 위해요소로 분류되고 있고, 현재 많은 나라에서 규제치를 설정하고 있는 곰팡이 독소인 ochratoxin A(OTA)에 대한 정량적 측정이 가능한 고속검색법을 개발하고자 단클론성 항체를 이용하여, 측정시 분리과정이 필요 없는 형광편광면역분석법(FPIA)을 개발하고 최적화시켰다. 동일구조를 가지는 형광물질 표식자인 OTA-EDF를 합성하여 OTA에 대한 특이항체와 경쟁반응을 시켜 나타나는 형광-편광도(mP)의 변화를 측정하였다. 이는 면역분석법의 특이성과 민감성을 충분히 만족하였다. OTA의 검출범위는 0.5-200 ng/ml였고, 검출한계는 0.3 ng/ml였다. 개발된 분석법은 다른 곰팡이 독소들과의 교차반응은 없었고 높은 특이성과 재현성 및 회수율을 나타내었다. HPLC 방법에 의한 회수율은 88-84%로 다소낮게, FPIA법의 회수율은 90-110%로 다소 높게 나타났다. 16점의 현미시료를 분석하였을 때, 2점이 상관관계가 높게 12-20 ppb 정도 오염된 것으로 나타났다. 4점은 FPIA 및 HPLC 모두에서 음성으로 판정되었다. 개발된 FPIA는 복잡한 전처리 방법이 필요 없는 신속한 검색이 가능하므로, 식품 및 환경에서의 OTA 잔류 검사에 유용하게 사용될 수 있을 것이다.