

OPTIMIZATION AND EFFICIENCY IMPROVEMENT OF IN-HOUSE IMMUNOAFFINITY COLUMN KU-AF02 FOR AFLATOXIN DETECTION

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ABSTRACT

In-house immunoaffinity column for aflatoxins (AFs) detection is firstly developed by Kasetsart University since 2011 i.e. the first IAC prototype, KU-AF01. Detection of aflatoxin B₁ (AFB₁) up to 200 ng is considered significantly efficient, but the production cost is considered high. The high cost was due to the cost of monoclonal antibody specific to aflatoxin and its supporting materials i.e. CNBr-activated sepharose 4B. Therefore, this study was aimed to improve the efficiency of in-house immunoaffinity columns by replacing CNBr-activated sepharose 4B with other supporting materials and to lower the ratio of antibody to supporting materials. Response surface methodology (RSM) was applied to determine the optimum alternative supporting materials and the ratio. Results revealed that when compared with CNBr-activated sepharose 4B, all materials tested could recover higher than 80% AFB₁, except the activated CH sepharose 4B. Results also indicated that the optimum ratio was 0.4 mg anti-aflatoxin monoclonal antibody to 204 µL CNBr-activated sepharose 4B. CNBr-activated sepharose 4B was considered to provide the best precision in recovering AFB₁. KU-AF02 increased the ability to detect AFB₁ to 500 ppb. The recovery of AFs in the reference materials using KU-AF02 was more than 96% successful, with HorRat value range of 0.34 – 0.76. The reference materials used were 2 levels of AFs in peanuts, i.e. 47.68 and 72.14 ng/g. In terms of quality control in IAC production, there were no significant differences among the 5 batches produced ($p > 0.05$). KU-AF02 demonstrated stable and constant percent recovery at 4 °C for up to 12 months (tested with standard 200 ppb AFB₁). This study indicated that KU-AF02 could lower the ratio of supporting materials to the antibody and that KU-AF02 has high stability and has good ability for cleaning up AFs. Therefore, KU-AF02 is recommended to be used as an in-house immunoaffinity column for aflatoxin detection in Thailand.

Keywords: Aflatoxins, immunoaffinity column, in-house method, mycotoxin detection

INTRODUCTION

Aflatoxins (AFs) are secondary metabolites called difuranocoumarins, produced by *Aspergillus flavus* and *A. parasiticus*, commonly found in food and feed (Calvo *et al.* 2002). AFs are toxic and may have been the most investigated mycotoxin. AFs

have been associated with aflatoxicosis in livestock, domestic animals and humans worldwide. At present, the maximum limit of AFs in food is set at 20 ppb by the United States Food and Drug Administration (US FDA) (Anukul *et al.* 2013), whereas only 4 ppb is allowed in food by the European Union (European Union 2006). Countries worldwide have established their respective limit of AFs.

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Usually, the contamination of AFs has high prevalence, but low concentration at the level of part per billion (ppb). In the last couple of years, several Thai exported products were rejected due to AFs contamination; for example 40 ppb of AFs in rice (Chinaphuti & Aukkasarakul 2009) and 31 ppb of AFs in chili pouch packed together with instant flavoured noodles (Rapid Alert System for Food and Feed 2012).

AFs analysis in food is normally carried out by extracting food samples with organic solvents such as methanol, followed by AFs detection. The food extract solvent, however, may contain Afs together with other impurities which consequently will interfere with the results of analyses. To increase the reliability of analysis results, the impurities have to be removed from the food extract solvent prior to analysis, using immunoaffinity column (IAC). At present, IAC is the common requirement in mycotoxin detection and quantification. Unfortunately, in Thailand most of IAC are imported which resulted to high analysis cost.

In 2007, the first prototype of IAC developed from polyclonal antibody was conducted at Kasetsart University and was succeeded in recovering 20 ppb AFB₁ (Wongsuttichot 2007). This prototype was initially meant to be used as an in-house IAC to reduce analysis cost. Later, the polyclonal antibody of this prototype IAC was replaced with monoclonal antibody to improve the efficiency, but the production cost was still considered costly (Mahakarnchanakul *et al.* 2011). Since antibodies and supporting material contribute as a major part of the production cost, these components have to be optimized to reduce the production cost of IAC for cleaning up AFs. Lower cost production may be encouraged using KU-AF02 as in-house IAC. It is expected that the increasing success in detecting aflatoxins will result to safer food in Thailand.

MATERIALS AND METHODS

Materials and Chemicals

Cyanogen bromide-activated sepharose 4B (CNBr-activated sepharose 4B) as supporting material used for antibody immobilization was purchased from GE Healthcare (Sweden). The apparatus for IAC production such as solid phase

extraction (SPE) empty tube 1 mL, 20 µm loose frits, SPE outlet cap and SPE inlet cap (Vertical Chromatography, Thailand) were used for packing the coupled medium. Chrome grade acetonitrile and methanol were purchased from Mallinckrodt (USA). The monoclonal antibody (MAb) specific to AFs was kindly provided by Department of Plant Pathology, Faculty of Agriculture at Kamphaeng Saen, Kasetsart University, Thailand. Acetic acid, sodium chloride and sodium bicarbonate were purchased from Merck (Germany). Standard AFB₁ and sodium acetate were purchased from Sigma (Spain). Tris hydrochloride was purchased from Vivatis (USA). Ground peanuts containing aflatoxins (as the reference material) were prepared by Mycotoxin Laboratory, at Scientific Equipment and Research Division, Kasetsart University Research and Development Institute (KURDI), Kasetsart University, Thailand.

Preparation of Solution

According to CNBr-activated sepharose 4B manual (GE Healthcare 2009), coupling buffer solution (0.1M NaHCO₃ containing 0.5M NaCl, pH 8.3) was prepared by dissolving 8.40 g NaHCO₃ and 29.20 g NaCl in 1 L filtered deionized (DI) water. Then, the blocking buffer solution (0.1M Tris-HCl, pH 8.0) was freshly prepared by dissolving 15.76 g Tris hydrochloride in DI water. Before adjusting volume, pH was adjusted to 8.0. This step was followed by preparing the washing buffer solution (0.1 M Acetic acid/Sodium acetate, pH 4.0 containing 0.5 M NaCl and 0.1 M Tris-HCl, pH 8.0 containing 0.5 M NaCl) which was prepared by mixing 90 mL 0.2M NaAc with 410 mL of 0.2M Acetic acid, added with 29.20 g NaCl followed by adjusting the total volume to 1,000 mL. Phosphate buffer saline 10x (PAA, Austria) was diluted with DI water to achieve 1x PBS (pH 7.4) to re-suspend the medium. PBS with 0.02% NaN₃ was prepared by dissolving 0.02g NaN₃ in 100 mL PBS. All solutions were filtered through 0.45 µm nylon filter before usage.

Karnovsky solution, as fixative for electron microscopy, was prepared by mixing 2 g of paraformaldehyde with 25 mL of distilled water. The solution was heated in 60 °C with stirrer plate. Then the solution was added with 1M

NaOH until the solution was clear. Finally, 5 mL 50% Glutaraldehyde and 20 mL 0.2M Cacodylate buffer (pH 7.2) were added into the solution. To prepare 0.2M Cacodylate buffer (pH 7.2), 8.56 g Sodium cacodylate, 25 g Calcium chloride and 2.5 mL 0.2N HCl were dissolved with 200 mL of DI water.

Amplification of Monoclonal Antibody Specific to Aflatoxins

Monoclonal antibody (MAb) specific to aflatoxins from hybridoma clone C10 was produced using Integra Celline flasks (CL1000) based on the manual from the manufacturer (INTEGRA Biosciences AG CH-7205 Zizers, Switzerland). Briefly, the hybridoma was maintained between the space of membrane (initial cell amount was 2×10^6 cells/mL which had > 80% of viable cell) in the Hybridoma Serum Free Medium (HSFM). The flask was filled with HSFM up to 250 mL. At certain times, cells were collected, leaving 3×10^7 cells/mL to be maintained in the flask. The flask was then refilled with fresh HSFM up to 250 mL. The collected medium was then centrifuged (2,000 rpm for 5 minutes). The supernatant was purified by AKTApriime™ system (GE Healthcare, Sweden) using Hitrap protein G column (GE Healthcare, USA). The purified antibody concentration was determined by measuring OD 280 nm and calculated as the following equation. Then, MAb was lyophilized and stored at 4 °C until usage.

IAC Preparation

To prepare 50 columns of IAC, purified 50 mg MAb was dissolved in coupling buffer solution (0.1M NaHCO₃ containing 0.5 M NaCl, pH 8.3) (1 mg/mL) then coupled with 10.2 mL CNBr-activated sepharose 4B. The mixture was then shaken using orbital shaker (Boeckel scientific, USA) at 150 rpm, 4 °C, for 18 hours. At the end of the incubation period, the mixture was left at 26 °C to separate the medium (CNBr-activated sepharose 4B coupling with Mab). The supernatant was then measured at OD 280 nm, in order to determine the excess of MAb using the following formula:

$$\text{Concentration of antibody (mg/mL)} = \frac{\text{OD 280 nm value} \times \text{dilution factor}}{1.4}$$

The medium was then collected using sintered filter flask, washed from the excess antibody using ± 50 mL coupling buffer and transferred into ± 50 mL blocking buffer (0.1M Tris-HCl, pH 8.0). The medium in blocking buffer was shaken using orbital shaker for another 2 hours, washed with washing buffer (0.1 M Acetic acid/Sodium acetate, pH 4.0 containing 0.5 M NaCl and 0.1 M Tris-HCl, pH 8.0 containing 0.5 M NaCl). This was conducted for 3 cycles. Each cycle used ± 50 mL washing buffer. The medium was then resuspended in 50 mL PBS. Finally, 0.2 mL medium was packed in each SPE empty tube with polyethylene frits and the column was filled up with PBS containing ± 0.6 mL 0.02% NaN₃ in each column. (modified from GE Healthcare 2009) and stored at 4 °C until usage.

Optimization of Antibody and CNBr-Activated Sepharose 4B

To develop IAC, the major cost of IAC production depended on the amount of antibody and CNBr-activated sepharose 4B (Mahakarnchanakul *et al.* 2011). Thus, the amount of these 2 components has to be optimized. Response surface methodology was applied to determine the proper ratio. The experiment was designed as central composite design and the data obtained were analyzed using MINITAB (version 14). The alpha value was face centered. The experimental treatments are summarized and shown in Table 1.

The recovery percentage of AFB₁, standard deviation (SD) and relative standard deviation (RSD) were the parameters for response surface determination to optimize volume of antibody and CNBr-activated sepharose 4B.

The seven columns from each treatment (Table 1) were tested with 15 mL 30% methanol (containing 200 ng of AFB₁, which was prepared from stock of 10 ppm AFB₁). The solution was passed through IAC at a flow rate of 1 - 2 drops per second (dps). After the whole solution had been passed through the IAC, the column was washed twice with 10 mL deionized water (DI water) at the same flow rate. The captured AFs were eluted with 1 mL methanol at 1 dps, and then filtered (nylon syringe filter, Whatman) and subjected to high performance liquid chromatography (HPLC) evaluation.

Table 1 Coupling percentage, actual amount of antibody and recovery percentage of 200 ng AFB₁ from IAC containing different volumes of antibody and supporting material (CNBr-activated sepharose 4B)

Column	Experimental design			Results		
	Expected antibody (mg)	Volume of CNBr-activated sepharose 4B (μL)	Antibody to supporting material ratio (mg/mL)	Coupling percentage (%)	Actual antibody (mg)	AFB ₁ recovery ^{*,a} (%)
1	0.3	153	1.96	97.26	0.31	79.66 ± 2.03 ^f
2	0.4	153	2.61	90.02	0.37	82.70 ± 2.63 ^e
3	0.5	153	3.26	90.95	0.47	85.82 ± 0.83 ^d
4	0.3	204	1.47	93.29	0.29	86.64 ± 2.51 ^d
5	0.4	204	1.96	97.26	0.42	103.36 ± 2.31 ^b
6	0.5	204	2.45	90.95	0.47	88.62 ± 3.70 ^d
7	0.3	255	1.17	92.51	0.29	87.83 ± 2.13 ^d
8	0.4	255	1.46	93.14	0.38	94.80 ± 4.23 ^c
9	0.5	255	1.96	97.26	0.52	107.13 ± 1.25 ^a

Note: * = Values in the same column followed by different letters are significantly different at $p < 0.05$

^a = Mean ± SD from 7 replicates

The results were transformed to percent recovery and the optimum ratio of antibody and CNBr-activated sepharose 4B volumes was determined. Percent recovery was calculated using the following formula:

$$\text{Percent recovery} = \frac{\text{concentration of AFB}_1 \text{ after passing through the IAC column}}{\text{concentration of AFB}_1 \text{ before passing through the IAC column}} \times 100$$

After calculating the recovery percentage, the Standard Deviation (SD) and Relative Standard Deviation (RSD) were determined. RSD was calculated to describe the precision and the repeatability of each treatment. RSD was calculated using formula:

$$\text{Relative Standard Deviation (\%)} = \frac{\text{standard deviation}}{\text{mean}} \times 100$$

The recovery percentage, SD and RSD were then used as suitable variables in the response surface analysis, performed by MINITAB version 14.

High Performance Liquid Chromatography Condition

To determine the amount of AFB₁, AFB₂, AFG₁ and AFG₂, the HPLC with fluorescent detector (Separation module e2695 with Fluorescence detector 2475, Waters, USA) was used. A symmetry C18 column (5 μm, 4.6 x 150 mm) with C18 guard column (Water Corporation, USA) was used to separate the aflatoxins. Photochemical derivatization (PHRED, AURA

Industries, USA) was used to enhance the AFB₁ and AFG₁ detection by fluorescence detector. The column was equilibrated with a mixture of water:acetonitrile:methanol [60:20:20 (v/v)] which was also the mobile phase. Flow rate at 1 mL/minute was used and the column oven temperature was 35 °C. The excitation and emission wavelength of the fluorescence detector were set at 365 and 445 nm, respectively.

Improvement of the Efficiency of In-house IAC

The CNBr-activated sepharose 4B was replaced by CH-activated sepharose 4B, HiTrap NHS-activated sepharose 4B or NHS-activated sepharose 4B having spacer arm length of 8, 10 and 14 atoms, respectively. The IAC efficiency were compared by passing 200 ng AFB₁ in 15 mL 30% methanol through the IAC and then evaluated by HPLC.

Beside the recovery percentage, the distributions of antibody on the supporting material were observed by transmission electron microscopy. The coupled supporting material was then attached with gold labeled anti-mouse IgG (H+L) system (KPL, USA). The wet gel was dehydrated with rising concentrations of ethanol (30, 50, 70, 90, and 100%, v/v), absolute acetone and embedded in activated Micropal. After sedimentation of the gel particles occurred, the

mixture was cooled down to 4 °C. The lower part of the stiffened gelatin containing the sepharose beads was fixed at 4 °C in Karnovsky solution for 30 minutes, washed in cacodylate buffer (pH 7.2, 0.2 M), dehydrated and embedded in activated Micropal. Ultrathin sections were then made with the LKB Ultratome I11 (LKB-Produkter, Stockholm). The sections were viewed with electron microscope (HT 7700, Hitachi, Japan) at an accelerating voltage of 80 kV with 10,000x magnification.

IAC Capacity Testing

The capacity of the optimized IAC was tested by passing various amounts of standard AFB₁ (1, 10, 100, 200, 300, 500 and 1,000 ng) through the IAC column. Total AFs recovery was also conducted at 50 ng for each of AFB₁, AFB₂, AFG₁ and AFG₂ (total AFs amount was 200 ng). The results were then transformed into recovery percentage. RSD and Horwitz ratio (HorRat) value were then determined. The HorRat value was calculated using formula:

$$\text{HorRat} = \frac{\text{RSD from the results}}{\text{RSD predicted from Horwitz equation}}$$

The predicted RSD was calculated from Horwitz ratio (HorRat) using formula:

$$\text{Predicted RSD} = 2C^{(-0.15)},$$

where:

C = concentration found or added, expressed as dimension less mass fraction (Thompson 2007).

Ground peanuts as reference material containing AFs at 47.68 ng/g (AFB₁ 38.25 and AFB₂ 9.75 ng/g) and 72.14 ng/g (AFB₁ 56.28 and AFB₂ 15.86 ng/g) were extracted and passed through the optimized and Aflatest IAC columns based on the Aflatest HPLC instruction manual (Vicom 2009).

Briefly, 25 g reference material with 5 g methanol were blended at high speed (for 2 minutes at 26 °C) with 125 mL methanol:water (70:30), then filtered through filter paper No.1 (Whatman). Fifteen milliliters of the filtered extract was diluted with 30 mL DI water, filtered through glass microfiber filter (Whatman) and 15 mL was passed through the IAC column.

Efficiency Comparison between In-house IAC and Commercial IAC

The AFB₁ and AFs concentration resulted from reference material clean-up by the in-house IAC and commercial IAC were compared with statistical analysis including correlation (SPSS version 17), *t*-test (SPSS version 17) and Bland-Altman plot (Sigma plot version 12).

The Stability of IAC Production and Shelf-life Testing

Five batches of optimized IAC were produced in order to check the quality of producing different batches. Seven columns from each batch were tested with 200 ng AFB₁. The results were analyzed to obtain data on the quality difference among batches productions using SPSS software version 17. For shelf-life testing, IAC was produced and stored at 4 °C. The IAC was tested within batches with 200 ng AFB₁ after 1, 3, 6, 9 and 12 months of storage duration. The results were analyzed to obtain data on the difference among storage duration and acceptable range of recovery percentage using SPSS software version 17.

RESULTS AND DISCUSSION

Optimization of Materials using IAC Production

After MAb was coupled with CNBr-activated sepharose 4B, the excess amount of the antibody was measured to determine the coupling percentage. All treatments gave excellent coupling percentage (> 90%), ranging from 90.02 to 97.26% (Table 1). The amount of bound antibodies did not different from the expected amount of antibodies in each column. The highest coupling percentage (97%) was obtained when the volume ratio of the antibody to the supporting material was 300 µL:153 µL, 400 µL:204 µL, 500 µL:255 µL. Under these conditions, ratio of antibody and supporting material were much lower than the ratio recommended by the operating procedure (GE Healthcare 2009) i.e. at least 2.5 - 5 times. The operating procedure recommended 5 - 10 mg of

protein per mL of CNBr-activated sepharose 4B, which is quite large amount of antibody to be used. When the amount of antibody was higher or lower than 2 mg of antibody per 1 mL of CNBr-activated sepharose 4B, the coupling percentage decreased as shown in Table 1.

The supplier's recommended ratio of MAb and supporting material might not be suitable for specific purpose or antibody. For instance, in developing IAC for zearalenone and ochratoxin clean-up, some research found that 1 mg antibody coupled with 1 mL of CNBr-activated sepharose 4B, as supporting material, provide 40 – 50% stability toward high concentration of acetonitrile and methanol (Uchigashima *et al.* 2009; Uchigashima *et al.* 2012). The ratio used was obviously lower than the recommended ratio, but still the recovery percentage were satisfactorily ranging from 80 to 100%.

In some cases, however, the supplier's recommended ratio is useful. For example, in developing IAC for bisphenol A in serum, some research found that the ratio of the polyclonal antibody and supporting material (14 mg to 2 mL) provided coupling percentage of $86 \pm 3\%$ (Zhao *et al.* 2003). In the case of IAC for quinolone and sulfonamide clean-up, it was found that the ratio of 8 mg antibody to 1 mL supporting material provided coupling percentage of 91.1% (Li *et al.* 2008), which was lower compared to coupling percentage provided by our study (97%).

High coupling percentage, however, is not the guarantee for the IAC efficiency. Therefore, the efficiency should be evaluated as recovery percentage. According to The Codex Alimentarius Commission (1995), the acceptable recovery percentage of mycotoxin analysis is 80 – 110%.

All conditions could recover AFB₁ in the acceptable range, except the ratio of 300 μ L antibody to 153 μ L CNBr-activated sepharose 4B (Table 1). Although our study provided high coupling percentage (97%), the efficiency to recover AFB₁ was not acceptable (79%).

In terms of extraction step, basically 70 – 80% methanol or acetonitrile is sufficient to be used as the solvent for AFs extraction. To allow antibody completely capture the toxin, the organic solvent in extraction process should be diluted to 23 – 26%. The highest organic solvent concentration to be passed through IAC column was 30% (Scott & Trucksess 1997).

In our study, the specificity test was conducted by passing through 200 ng AFB₁ diluted in 15 mL 30% methanol in the IAC column. The concentration of organic solvent before being passed through the column was slightly higher than the recommendation provided by Vicam manual (23 – 26%).

When the amount of supporting materials was varied toward a constant 0.3 mg of antibody, the results showed significantly different recovery percentage ($p < 0.05$). Recovery percentage increased from 79 to 87% as a result of increasing the amount of CNBr-activated sepharose 4B from 153 to 255 μ L (Table 1), which might have been caused by the steric hindrance between the tightly packed of antibody on the surface of each bead in the column. Other possible cause was that higher amount of supporting material (at a constant amount of antibody) would provide higher surface and extended reaction time between the antibody and the toxin. Similar results were obtained when the amount of antibody was increased to 0.4 and 0.5 mg. On the contrary, equal amount of supporting materials toward varied amount of antibody would increase recovery percentage.

Increasing the amount of antibody did not increase the coupling percentage, which indicated that the excess of antibody would lose during the coupling process. Also, excess amount of antibody might cause the occurrence of steric hindrance which resulted to the less specificity to toxin.

Therefore, ratio of antibody to the supporting material is the critical factor to develop the IAC. The ratio should be balanced to achieve the best efficiency in toxin detection.

ANOVA results in our study showed that the amount of antibody and CNBr-activated sepharose 4B as supporting material had significant effect on the recovery percentage ($p < 0.05$), in which our study used 200 ng AFB₁ for testing the efficiency of IAC column. Therefore, our study chose the 400 μ L:204 μ L ratio of the antibody to the supporting material or 2 mg antibody per 1 mL supporting material as the expected optimum condition to provide high coupling percentage.

To confirm the ratio of these two coupling substances, the response surface methodology was used to determine the optimum coupling condition. Before the optimization process was

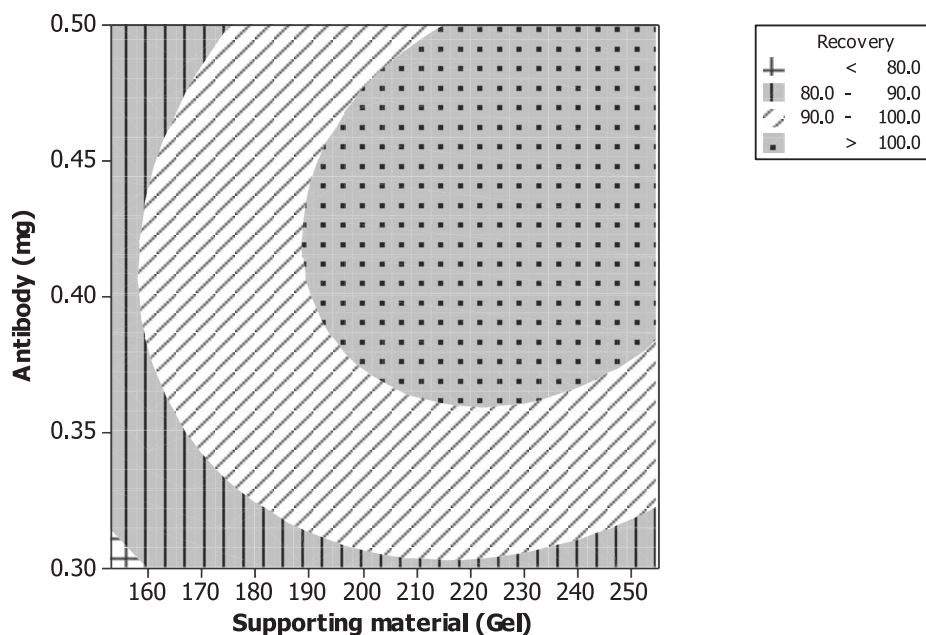


Figure 1 Contour plot of recovery percentage determined by antibody and supporting material (Gel)

conducted, the recovery percentage, standard deviation and relative standard deviation variables were analyzed to select suitable variables for plotting the response surface. Suitable variables were chosen based on the R^2 value $> 85\%$ and p value < 0.05 . The selected suitable variables had R^2 of 88.2% and p values of 0.033 for antibody and 0.049 for supporting materials. The value of recovery percentage was determined to be the optimum condition of coupling step based on contour plot of the response surface methodology (Fig. 1). Based on setting criterion of 100% , recovery percentage was selected between the areas of $400 \mu\text{L}$ of antibody and $204 \mu\text{L}$ of supporting material.

Improving the Efficiency of the In-house IAC

This experiment was aimed to improve the efficiency of IAC by varying the spacer arm length between the CNBr and sepharose 4B. Within the affinity of chromatography system, normally the binding site of the target toxin may be located at the surface. Some binding site, however, may be located deep within the molecule hindering the antibody to access the target toxin. Increasing spacer arm length may overcome the hindrance by interposing antibody and supporting material. Length of the spacer arm may provide better affinity, but the length increase must be cautiously done.

Previously, the experiment was designed to vary the spacer arm length between the sepharose 4B and CNBr, due to the limited availability of cyanogen bromide in local market, under the permission from the Thailand Ministry of Defence (Royal Thai Government Gazette 2008). This situation made it impossible to vary the length of spacer arm within the same supporting material. Therefore, we used four supporting materials available in local market i.e. CNBr-activated sepharose 4B, CH-activated sepharose 4B, Hitrap NHS-activated sepharose 4B and NHS-activated sepharose 4B. Recovery percentage of the coupling step with different supporting materials are shown in Table 2.

Coupling monoclonal antibody with three supporting materials (CNBr-activated sepharose 4B, Hitrap NHS-activated sepharose 4B and NHS-activated sepharose 4B), resulted in successful recovery of AFB_1 ($96.6 - 101.1\%$, $88.9 - 98.0\%$ and $95.9 - 103.9\%$, respectively), except the CH-activated sepharose 4B which provided much lower recovery ($59.70 - 84.47\%$). Low amount of AFB_1 was possible to be recovered using the CH-activated sepharose 4B as supporting material, which might be due to the limited length of spacer arm. Based on the specification of each supporting material, the recovery percentage may decrease depending on the number of spacer arms. The CH-activated sepharose 4B used in our

Table 2 Recovery percentage of AFB₁ recovered by IAC containing different supporting material

Supporting material	Spacer arm (n of atom)	Coupling percentage (%)	Recovery percentage ^a (%)
CNBr-actiavated 4B	- (0)	98.21	98.67 ± 1.80
CH-activated Sepharose 4B	6-aminohexanoic acid (8)	97.56	71.83 ± 9.78
Hitrap NHS-activated 4B	6-aminocaproic acid (10)	98.40	93.85 ± 3.63
NHS-activated Sepharose 4B	6-aminocaproic acid (14)	96.08	100.59 ± 3.06

Note: ^a = Mean and SD from 5 replicates

study contained 10 atoms spacer arm and provided the lowest recovery percentage. Among the other three supporting materials, CNBr-activated sepharose 4B exhibited the lowest SD value (Table 2). Therefore, CNBr-activated sepharose 4B was selected as material for IAC production.

Table 2 shows similar values of coupling percentage and quite different values of recovery percentage. CNBr-activated Sepharose 4B, Hitrap NHS-activated Sepharose 4B and NHS-activated as supporting materials had moderate active role on the surface to capture the antibody and the

target toxin, resulting to high recovery percentage (93 – 101%). The developed IAC was named as KU-AF02 and was further tested for capacity, reproducibility and stability.

The distribution of antibody on each supporting materials by Transmission Electron Microscope (TEM) was conducted to investigate the relationship between the affinity of antibody on supporting material to the coupling percentage. Unfortunately, the illustrations presented in this manuscript did not exhibit the relevance of the affinity of antibody and supporting material to the coupling percentage

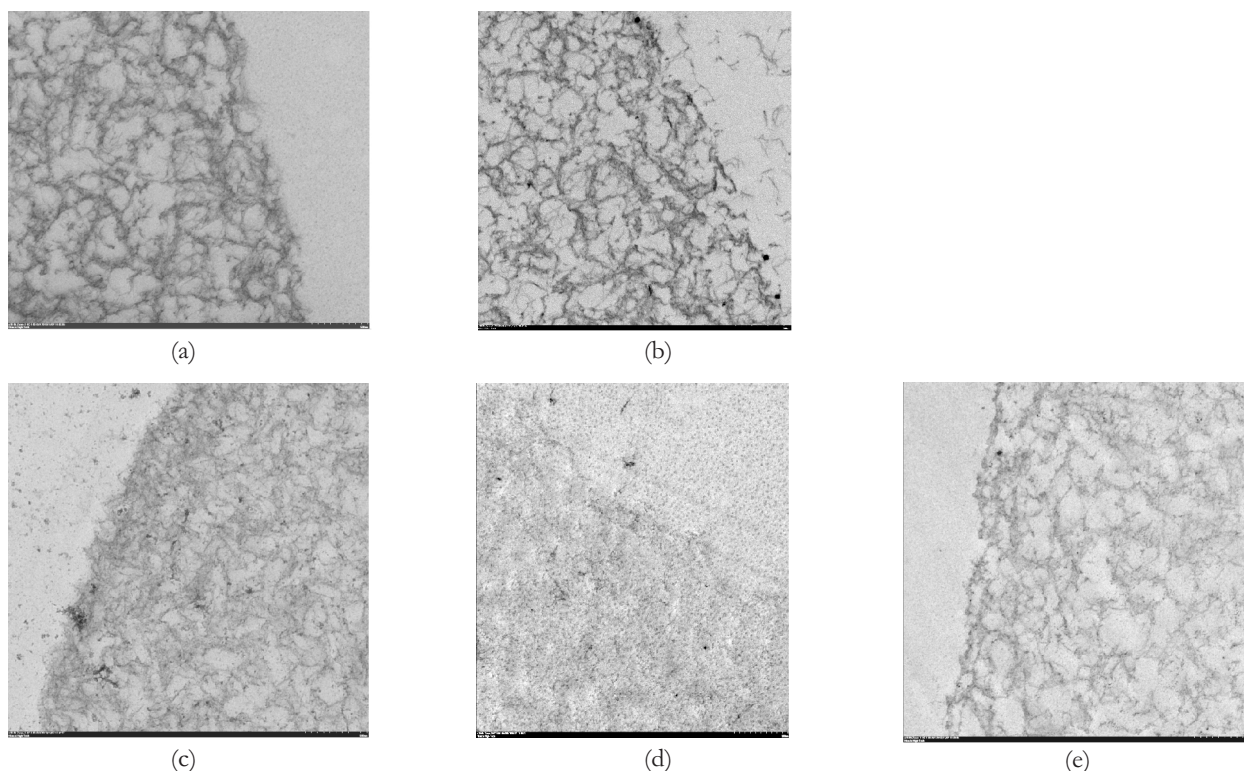


Figure 2 The transmission microscopy illustration of (a) CNBr-activated sepharose 4B and monoclonal antibody distribution on (b) CNBr-activated sepharose 4B © CH-activated sepharose 4B (d) Hitrap NHS-activated 4B and (e) NHS activated sepharose 4B at 10,000x magnification

(Fig. 2). The dark color indicates the uniform distribution of antibody (Fig. 2b), which was the coupling of antibody with CNBr-activated sepharose 4B. The condensed color was distributed from the outer surface of supporting material to the inner surface. The antibody which should have been shown as dark color was not found at the inner or the outer layer of sepharose (Fig. 2a), although the sepharose was stained with gold label. The dark color shown in Figure 2c was the coupling of antibody with CH-activated sepharose 4B, which was located at the inner or outer surface; the color was slightly toned down from the outer to the inner surface of the supporting material. The dark color shown on Figure 2c, which was the coupling of antibody with activated CH sepharose 4B, was located at the surface or outer and the color slightly dropped from the outer to the inner of supporting material. In case of Hitrap NHS-activated sepharose 4B and NHS-activated sepharose 4B (Fig. 2d and 2e), the antibody seemed to be equally distributed to the inner surface, but the color intensity was less than that in Figure 2a.

The coupling percentage of antibody on four supporting materials was shown to be similar ($> 90\%$) (Table 2), which might be presented in Figure 2c. The density of antibody on the surface of CH-activated sepharose 4B seemed to be higher than that on other supporting materials. It was expected that CH-activated sepharose 4B had potential to enhance the affinity of antibody to the supporting material. However, the steric hindrance of the condensed antibody on CH-activated sepharose 4B, caused the lower capture of the tested toxin. The steric hindrance might interfere the affinity of antibody resulting to the

lowest recovery percentage for CH-activated sepharose 4B.

The Capacity of IAC

Recovery percentages of various amount of AFB₁ (1 to 1,000 ng) in 15 mL 30% methanol obtained using the developed IAC KU-AF02 (coupling condition: 0.4 mg of antibody with 204 μ L of CNBr-activated sepharose 4B) are shown in Table 3. The capacity of KU-AF02 achieved the standard of analysis when 1 to 500 ng was tested. The recovery percentages were all above 80%. Particularly at 500 ng AFB₁, the recovery percentage was 87% with HorRat value less than 2 (Table 3). As expected, the recovery value slightly decreased when the amount of AFB₁ increased. Recovery percentage was lower than 80% when 1,000 ng of AFB₁ was loaded. At this amount of AFB₁, although the HorRat value was less than 2, the recovery percentage was considered not satisfying for the capacity of IAC. Thus, the developed IAC KU-AF02 was successful in increasing the recovery of AFB₁ up to 500 ng compared to the previous IAC, KUAF-01 (Mahakarnchanakul *et al.* 2011).

KU-AF02, was also successful in recovering other derivatives of AFs i.e. AFB₂, AFG₁ and AFG₂. The experiment was carried out by mixing 50 ng of each AFB₁, AFB₂, AFG₁ and AFG₂ in 15 mL 30% methanol and passing them through the KU-AF02. The IAC column of KU-AF02 was capable to recover 45.37 ± 1.87 ng of AFB₁, 40.64 ± 2.63 ng of AFB₂, 43.41 ± 1.93 ng of AFG₁ and 33.75 ± 2.20 ng of AFG₂, with total aflatoxin recovery percentage of 81.6%.

Table 3 Recovery percentage at different amount of AFB₁ recovered by optimized IAC

Amount of AFB ₁ (ng)	Recovery percentage ^a (%)	Calculated RSD	Predicted RSD	HorRat value
1	102.86 \pm 6.42	6.25	22.39	0.28
10	103.81 \pm 1.93	1.86	15.85	0.12
100	95.41 \pm 2.27	2.38	11.22	0.21
200	95.51 \pm 1.71	1.79	10.11	0.18
300	90.07 \pm 2.19	2.43	9.52	0.25
500	87.06 \pm 3.57	4.10	8.81	0.47
1000	75.35 \pm 6.04	8.02	7.94	1.01

Note: ^a = Mean and SD from 7 replicates

The results showed that IAC KU-AF02 recovered AFB₁, AFB₂ and AFG₁ higher than 80%, except for AFG₂ the recovery percentage was only 67%. Although preliminary test showed that our monoclonal antibody had specificity with single toxin test when loading AFB₁, AFB₂ and AFG₁ and AFG₂ separately in IAC, the recovery percentage of each test obtained was > 98% and was not significantly different ($p > 0.05$) among those recovery percentages. After mixed AFs were loaded, IAC KU-AF02 could recover the parent form of B and G aflatoxins (B₁, 90.74% and G₁, 86.81%) better than the others (B₂, 81.28% and G₂ 67.50%). Similar to the results when tested with commercial IAC, higher recovery percentage of AFB₁, AFB₂ and AFG₁ (79.18%, 72.68%, 78.39%) over AFG₂ (27.25%) were observed.

The remaining AFG₂ in test samples apparently reduced performance of total AFs detection by IAC, either in the developed IAC or the commercial IAC. Therefore, samples contaminated with high amount of AFG₂ should be carefully handled because the AFG₂ may decrease the recovery of other forms aflatoxins. AFG₂ is the highest contaminant in unpolished rice (5.5 ppb) (Jankhaikhot 2005).

Further tests were done using reference materials i.e. two-level contaminant contained in defatted ground peanuts. The reference materials were extracted and passed through optimized IAC. The results showed that the recovery

percentage of AFB₁, AFB₂ and AFs obtained from reference material no. 1, representing medium contamination (47.68 ng/g) and reference material no. 2, representing high contamination (72.14 ng/g), were between 80 - 110% with HorRat value of 0.8 and ± 0.4 , respectively. These results also showed the capacity of KU-AF02 in detecting contaminated food using peanuts as the representative. Similar to the test in pure toxin solution, developed IAC KU-AF02 recovered parent forms (B and G) of aflatoxins from the reference material better than those from the derivative forms, particularly in mixed forms.

Efficiency Comparison of In-house and Commercial IAC

Seven replicates of medium and high contaminants aflatoxins in peanut (reference materials no.1 and no.2) were quantified using HPLC to determine the amount of AFB₁ and AFs. The correlation between recovery percentage of AFB₁ and AFs using KU-AF02 IAC column and recovery percentage using commercial IAC column were determined (Fig 3).

Close correlation (R) between the obtained amount of AFB₁ and the AFs using the developed and commercial IAC columns were 0.961 and 0.962, respectively. The R² values representing the confidence values of X and Y variables were

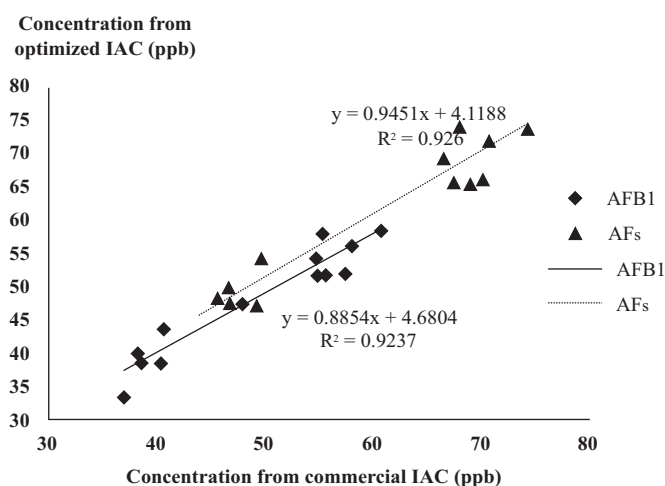


Figure 3 Correlation (linear-regression) between clean-up samples with commercial IAC column (x-axis) and optimized IAC column (y-axis) for AFB₁ (◆) and AFs (▲) detection in peanut reference materials

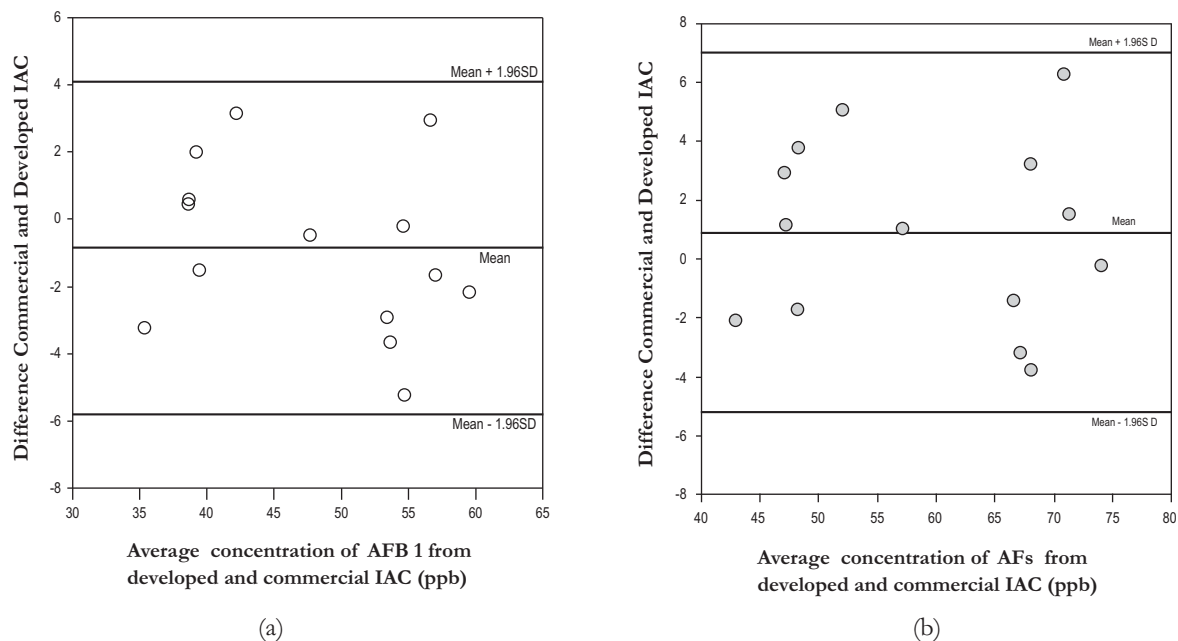


Figure 4 Bland–Altman plots of AFB₁ (a) and AFs (b) concentration after being cleaned-up using the developed IAC KU-AF02 and commercial IAC columns

0.923 and 0.926, respectively. Close correlations of the linear regression equations indicated similar trend of analysis, but might not be directly compared the efficiency of these tested two IAC columns.

The paired samples *t*-test and Bland-Altman plot were used to compare the results. The *t*-value of AFB₁ data (1.283) was lower than *t* critical two-tailed value (2.160), which meant no difference between two data sets resulting from the two tested column ($p > 0.05$). A similar result was found in case of Afs.

Apart from *t*-test, the Bland–Altman plot was used to determine the agreement between two tested methods, for example between modified and standard methods (Bunce 2009; Eken 2009; Hanneman 2008). The use of this method has been reported in the application of medical instrument (Zaki *et al.* 2013), computed tomography and ultrasound prostate volume measurements (Gloi *et al.* 2008). In 2013, Tansakul *et al.* (2013) compared between ELISA and LC-MS/MS for fumonisin detection in corn samples using Bland-Altman analysis and found no difference between these two methods at a range of 0 - 20 mg/kg.

In our study, the amount of AFB₁ and AFs obtained from the developed IAC KU-AF02 and commercial IAC columns were compared using Bland–Altman plot (Fig. 4a and 4b). None of

these data were located outside the limit line (± 1.96 SD). Thus, the clean-up of contaminated peanut samples using the developed IAC KU-AF02 or commercial IAC column provided similar results, ranging from 35 to 60 ppb for AFB₁ or ranging from 40 to 80 ppb for AFs (Fig. 4).

The Stability of Batch Production and Shelf-life of Developed IAC

The stability of IAC production was determined in 5 batches. The results showed that all batches could recover AFB₁ higher than 95% (98.32, 99.68, 101.04, 101.27 and 102.38) and there were no significant difference among the batches produced ($p > 0.05$). Thus, the quality of each batch had no effect on the IAC efficiency.

Stability of recovery percentage of KU-AF02 stored for 1 - 12 months, are shown in Table 4. Along the storage duration, recovery percentage decreased from 95 to 85%, but still greater than 80%. There were significant differences of recovery percentage among storage durations (Table 4).

The HorRat values for all storage duration were less than 2 (Table 4). Based on the result, the developed IAC KU-AF02 had shelf-life up to 12 months when stored at 4 °C. The long stability will encourage the use of the developed IAC KU-

Table 4 Recovery percentage, RSD and HorRat value of 200 ng AFB₁ obtained from recovery process using the developed IAC KU-AF02 stored at different storage duration

Storage duration (months)	Recovery percentage ^{*, a} (%)	RSD	HorRat value
1	95.01 ± 2.62 ^a	2.75	0.27
3	94.57 ± 3.73 ^a	3.95	0.39
6	93.18 ± 1.19 ^a	1.27	0.13
9	90.52 ± 0.87 ^{ab}	0.96	0.10
12	85.61 ± 5.18 ^c	6.06	0.60

Note: * = Values in the same column followed by different letters are significantly different at $p < 0.05$

^a = Mean and SD from 7 replicates

AF02, as in-house method. This developed IAC KU-AF02 may also be distributed to other laboratories.

CONCLUSIONS

The production cost of the developed IAC KU-AF02 was successfully reduced compared to the previous IAC (KU-AF01) by reducing the ratio of antibody to CNBr-activated sepharose 4B. The optimized coupling condition was determined by Response Surface Methodology (RSM) and the range of recovery percentage was acceptable. CNBr-activated sepharose 4B was determined to be the suitable supporting material in this study. The capacity of KU-AF02 was higher than that of the commercial and previous IAC (KU-AF01). KU-AF02 was successful to recover AFs in two reference materials. No significant difference was observed when five batches of KU-AF02 were produced. Optimized IAC had a shelf-life of 12 months when produced and stored at 4 °C. There were no significant difference in using the developed IAC KU-AF02 and commercial IAC to clean-up AFB₁ and AFs from ground peanuts reference materials.

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