Mycotoxin Analysis Using Immunoaffinity Columns

Masahiro Nakajima

Abstract

To minimize the interference of co-extracted compounds from foods and feeds in which mycotoxins occur, the conventional methods for chemical analysis of mycotoxins consume large amount of time and solvent, need several cleanup steps, and sometimes require practical experiences. From the environmental point of view, toxic solvents such as chloroform and benzene used for mycotoxin analysis should be saved from now on. The immunoaffinity column (IAC) using anti-mycotoxins antibodies are highly specific, simple and rapid, and saving toxic solvents. Moreover, recently, with the availability of commercial IACs for aflatoxins (AFs), ochratoxin A (OTA), zearalenone (ZEN), fumonisins and deoxynivalenol (DON), these IACs have become the powerful tools in cleanup stage of mycotoxin analysis. This paper describes the performances of IACs in mycotoxin analysis, reuse of IAC, and multimycotoxin analysis using linked different IACs.

Key words: immunoaffinity column, analysis, mycotoxin

Introduction

At the ending of the seventies, specific antibodies have been made against several mycotoxins such as AFs, OTA, ZEN, T-2 toxin, and DON\(^5\)-\(^6\). Using these antibodies, simple and rapid immunological methods have been developed. The major advantages of these immunological methods are highly specific, rapid and saving toxic solvents. In these immunological methods, one of the most applicable methods for mycotoxin analysis is the IAC chromatography. The first IAC for AFs was developed by Groopman et al. in 1984\(^7\), and we have first reported the IAC method for OTA in 1990\(^8\). Main procedures of IAC were shown in Fig. 1. The anti-mycotoxin antibodies are coupled covalently to an appropriate support such as agarose gel, this support suspended in phosphate buffered saline (PBS) is placed in a mini-column, and sample extract is run. The mycotoxin in sample is bound specifically to the antibody ①, while impurities other than mycotoxin are removed with water ②. The purified mycotoxin is eluted with appropriate solution such as methanol or acetonitrile ③.

Now in Japan, 4 types of commercial IACs are available (Table 1). First is -Test column from VICAM (Watertown, MA) for AFs, AFM\(_1\), OTA, ZEN, fumonisins and DON. Second is -Prep column from Rhône-Poulenc Diagnostics (Glasgow, Scotland) for AFs, AFM\(_1\) and OTA. Third is Easi-Extract column from Biocode Ltd. (York, UK, Biocode is now owned by Rhône-Poulenc Diagnostics) for AFs, AFM\(_1\) and OTA. Last is RIDA-SCREEN column form R-Biopharm (Darmstadt, Germany) for AFs and OTA. In these commercial IACs, a method for determining AFs in corn, peanuts, and peanut butter using
the AflaTest P column (VICAM) coupled with solution fluorometry with bromine or HPLC with postcolumn derivatization with iodine has been adopted by AOAC INTERNATIONAL as an Official Method. Nowadays, these IACs have been used in many countries, however, the IACs are not so popular in Japan until now. This paper describes the performances of commercial IACs such as AflaTest P, OchraTest and ZearalaTest that have been used in our department. The detail information of IAC have been well documented by Scott and Trucksess.\(^9\)

**Binding capacity of IACs**

The AflaTest P column capacity was found to be 0.8 μg of AFB₁. Above this level no increase of AFB₁ binding was observed (Fig. 2). The binding capacity of OchraTest was found to be 0.7 μg of OTA. In the case of ZearalaTest, 4.0 μg of ZEN bound to the column, and 10 μg of fumonisin B₁ bound to the Fumoni Test (Table 2). Thus, these commercial IACs are available for a wide range of mycotoxin contamination.
Fig. 2 Binding capacity of AflaTest P column.  
10 ml of each concentration of AFB₁ in methanol-phosphate buffered saline (PBS), pH 7.4 (2 + 8) was applied onto the column. After washing with water, AFB₁ was eluted with 3 ml of methanol. The eluate was diluted to 10 ml with water, then AFB₁ was determined with HPLC-fluorescence detector. Averages of duplicate measurements are represented.

Table 2 Binding capacity of commercial immuno-affinity columns.

<table>
<thead>
<tr>
<th>Column</th>
<th>AflaTest</th>
<th>OchraTest</th>
<th>ZeaaraTest</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.8 μg AFB₁</td>
<td>0.7 μg OTA</td>
<td>4.0 μg ZEN</td>
<td></td>
</tr>
<tr>
<td>Fumonistest</td>
<td>DONTest</td>
<td>N.D.*</td>
<td></td>
</tr>
<tr>
<td>10 μg FB₁</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Not determined

**Effect of solvents concentration in loading solution onto IACs**

In general, the solvents for extraction of mycotoxins from samples are methanol or acetonitrile. Because antibodies are proteins, so they are sensitive against these solvents, it is necessary to dilute the extract with water or PBS before application onto the column. There are 2 types of AflaTest, one is AflaTest P column and the other is AflaTest 10 column. AflaTest P column can bind AFB₁, AFB₂, AFG₁, AFG₂ and AFM₁. AflaTest 10 column can bind AFB₁ and AFB₂. The recoveries of AFB₁, AFB₂, AFG₁ from AflaTest P were not affected by up to 30% methanol concentration, however, when methanol concentration exceeds 25%, the recovery of AFG₁ was low (Fig. 3). OchraTest column can bind not only ochratoxin A, but also ochratoxin C (ochratoxin ethyl ester) and ochratoxin methyl ester. The binding of OTA onto OchraTest was able to withstand 30% methanol. ZeaaraTest column can bind ZEN and its analogues such as α-zearalanol, β-zeaaranol, α-zearalenol, β-zeaaranol, and zeearalanone. The binding of these toxins onto ZeaaraTest was able to withstand 40% methanol. On the other hand, IACs were able to withstand lower concentration of acetonitrile than methanol. As shown in Fig. 4, acetonitrile-water extract must be diluted to equal or less than 10% acetonitrile. Also OchraTest was able to withstand 10% acetonitrile.
Fig. 3 Effect of methanol concentration in loading solution onto AflaTest P columns. 10 ml of each concentration of methanol in PBS including 50 ng of each aflatoxin was applied onto the column. After washing with water, aflatoxins were eluted with 3 ml with acetonitrile. The eluate was evaporated to dryness under nitrogen gas. The residue was treated with trifluoroacetic acid (TFA). After evaporation, residue was dissolved with 2 ml of 20% acetonitrile. Aflatoxins were detected with HPLC-fluorescence detector. Averages of duplicate measurements are represented.

Fig. 4 Effect of acetonitrile concentration in loading solution onto AflaTest P column. 10 ml of each concentration of acetonitrile in PBS including 50 ng of each aflatoxin was applied onto the column. Averages of duplicate measurements are represented.

**Effect of pH of loading solution onto IACs**

Antibodies are sensitive not only solvents concentration, but also pH of solution. When pH of loading solution onto AflaTest P column exceeds 4 or 9, the recoveries of AFs from column were decreased extremely. On the other hand, ZearalaTest was able to withstand pH < 4, and enable to withstand pH > 9. However, OchraTest was able to withstand only neutral pH (pH 6-7). From these results, dilution of sample extract with PBS is recommended. In most samples, by dilution with PBS, the recoveries of toxins from IACs were in-
creased. However, the recoveries of toxins from some samples like black pepper were not able to increase by PBS dilution (less than 50% recoveries of each AFs). To increase recoveries of toxins from like these samples, the 10% Tween 20 dilution has been used generally. By introducing the 10% Tween 20 dilution, the recoveries of AFs was able to increased, however, the recovery of AFG2 was low (less than 50% recovery).

**Effect of loading volumes of diluted blank raw peanut extract onto AflaTest column**

If large volume of sample extract is loaded onto the IAC, lower detection limit could be achieved. In this respect, the effect of loading volumes of sample extract onto AflaTest was evaluated. Different volumes of diluted blank raw peanut extract spiked with 10 ng of each AF/loading volume were loaded onto the AflaTest P column. Up to 100 ml of peanut extract (corresponding to 10 g of sample) were used. Increasing the loading volume decreased the recoveries of AFs. When 40 ml (4 g of sample) or more extract was loaded onto the column, the elution rate was too slow. From these results, less than 40 ml loading of sample extract might be practical.

**Elution patterns of mycotoxins from IACs with methanol and acetonitrile.**

In general, AFs are eluted with methanol from IAC. However, AFs are not stable in methanol, too pure AFs like standards were decreased by evaporation of methanol. If methanol eluate must be evaporated to derivatize with trifluoroacetic acid, acetonitrile elution from IAC is recommended. More than 90% of AFs were eluted from AflaTest P column with first 0.5 ml of acetonitrile and 100% of AFs were eluted with 2 ml of acetonitrile. In the case of OchraTest column, although 100% of OTA was eluted with 2 ml of methanol, only 60% of OTA was eluted with acetonitrile. This phenomenon was also observed in FumoniTest, that is, fumonisins were not eluted from the column with acetonitrile[^13].

![Regeneration of AflaTest P column by washing with PBS.](image-url)

Fig. 5 Regeneration of AflaTest P column by washing with PBS. 25 g of blank raw peanut spiked with 250 ng of aflatoxins were extracted with 125 ml of 70% methanol in the presence of 5 g of NaCl. Filtrate was diluted 3 times with PBS. After filtration through glass microfiber filter, 15 ml (equivalent to 1 g sample) of filtrate was applied onto the column. After washing with water, aflatoxins were eluted with 3 ml of acetonitrile. 20 ml of PBS was passed through the column immediately, then stored in refrigerator for 24 hours or 48 hours. Averages of duplicate measurements are represented.
Effect of loading-rate to AflaTest column

Flow rate of loading solution also affected the recoveries of AFs from column. More than 10% of each AFs were lost by employing a flow rate of 10 ml/min. Optimum flow rate was equal to less than 1 ml/min.

Regeneration of IAC by washing with PBS

One of the disadvantages of commercial IACs is high cost per assay. To reduce the costs regeneration of IACs for reuse has been investigated\(^{29-30}\). Regeneration method is as follows. After elution with methanol or acetonitrile, wash with 20 ml of PBS immediately, then stored in refrigerator. More than 90% of binding ability of antibodies in AflaTest P column was able to restored after 30 min regeneration. Good recoveries were obtained when the columns were stored more than 24 hours regeneration. The increase of reused times decreased the recoveries of all AFs (Fig. 5). The recoveries of AFB\(_1\), G\(_1\) and G\(_2\) from the columns reused 5 times were decreased to 85%, 80% and 54% of first column’s recoveries, respectively. The recovery of AFB\(_2\) was not changed up to 6 times of reuse. However, these decreased recoveries were able to restore fully by equal to or more than 48 hours regeneration time except for AFG\(_2\).

Table 3 Recoveries of mycotoxins from blank corn sample.

<table>
<thead>
<tr>
<th>Recovery (%)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zearalenone</td>
<td>80.6</td>
</tr>
<tr>
<td>Zealalânone</td>
<td>79.4</td>
</tr>
<tr>
<td>a-earalenol</td>
<td>78.9</td>
</tr>
<tr>
<td>b-earalenol</td>
<td>82.4</td>
</tr>
<tr>
<td>a-earalanol</td>
<td>84.4</td>
</tr>
<tr>
<td>b-earalanol</td>
<td>86.1</td>
</tr>
</tbody>
</table>

\(^1\) Average of duplicate measurements

\(^2\) Spiked with each 10 ng/g toxin by multimycotoxin analysis using linked 3 immunoaffinity columns
Multimycotoxin analysis using linked different IACs

The linking of different IACs is applicable to simultaneous separation of multiple mycotoxins from the same extract (8). Three IACs of Zearala Test, OchraTest and AflaTest P were linked as shown in Fig. 6. Corn sample spiked with 10 ppb of AFs, OTA and ZENs were extracted with 70% methanol. After 5 times dilution with PBS, 20 ml of filtrate (equivalent to 1 g of corn sample) was applied onto the linked column. Toxins were eluted with 3 ml of acetonitrile from AflaTest, and 3 ml of methanol from OchraTest and ZearalaTest. AFs and Ochratoxin were detected with HPLC-fluorescence detector, ZENs were detected with HPLC-electro chemical detector. As shown in Table 3, good recoveries of these mycotoxins were obtained. Thus, this approach showed the possibility of multimycotoxin analysis from the same extract.

References
