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Preparation of an immunoaffinity column and its application in sample cleanup for methandrostenolone residues detection

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ABSTRACT

Methandrostenolone (MA) is a steroid used as veterinary medicine on stockbreeding to promote animal growth. The use of MA has been strictly regulated because of its harmful effect on consumers. This paper describes the production of polyclonal antibody (pAb) against MA, the preparation of immunoaffinity column (IAC) and its potential application to the selective extraction of MA residues from animal tissue and feed samples. The produced pAb exhibited good sensitivity to MA with an IC_{50} value of 5.6 ng/mL. The cross-reactivity values of the antibody with MA structurally related compounds of testosterone propionate (TP) and trenbolone (TR) were lower than 0.6%. By coupling the produced antibody with CNBr-activated Sepharose 4B, an IAC was prepared. 2% methanol and 80% methanol were selected as loading and eluting solution by optimization. The maximum capacity of the column for MA was approximately 334 ng/mL gel. The average recovery of 20, 40 and 60 ng/mL MA standard solutions from IACs was 97.9% with the relative standard deviation (RSD) among columns of 6.7%. After 3 times of repeated usage, the column capacity and recovery rate still remained 82.0% and 92.6% respectively. The IACs were then challenged with MA-fortified animal tissue and feed samples, recoveries of MA were found to be in the range of 83.5–99.7%.

1. Introduction

Methandrostenolone (MA), or 1-dehydro-17a-methyltestosterone is an exogenous anabolic androgenic steroid. Due to its positive effect on muscle growth, MA has been widely used by veterinarians on livestock to increase their appetite and body weight [1]. However, the administration of MA is often associated with several adverse effects on animals and the persons who have taken them as food. It can result in liver damage, some degree of reduced fertility, hypertension, atherosclerosis, psychiatric and behavioral disorders [2,3]. Because of these deleterious side effects, MA has been strictly regulated in some regions and countries. In China, a ban on the use of anabolic steroid-related veterinary medicine on stockbreeding has been issued in 2002. To enforce the prohibition on MA abuse and monitor the MA residues, an effective, convenient and rapid method is urgently demanded.

For screening and confirmation of MA, instrumental analysis methods including micellar electrokinetic chromatography, gas chromatography–mass spectrometry (GC–MS) and liquid chromatography-tandem mass spectrometry (LC–MS/MS) have been reported in the literature [4–7]. Due to the low level of the target analyte and the complexity of the real sample matrices, a pretreatment step is always necessary before instrumental analysis, such as Soxhlet extraction, accelerated solvent extraction, liquid–liquid partitioning, or solid phase extraction. However, these methods used in sample pretreatment are far from satisfactory for analytical purpose in terms of high sensitivity, reproducibility, small amounts of extraction solvents and time-saving factors.

Immunoaffinity chromatography is a separation method that takes the advantage of the specific and reversible interaction between antibody and antigen and has been considered one of the most powerful techniques for single-step purification and concentration of target analyte from complex matrices [8,9]. The application of immunoaffinity chromatography for a variety of samples pretreatment in toxins, veterinary drugs and pesticides residues analyses have been reported [10–16]. In this work, we describe the generation and application of polyclonal antibody (pAb)-based immunoaffinity chromatography as a cleanup procedure, followed by enzyme-linked immunosorbent assay (ELISA) analysis of MA in animal tissue and feed samples. The aims of this study were to: (1) preparation of immunoaffinity column (IAC) employing anti-MA polyclonal antibody and CNBr-activated

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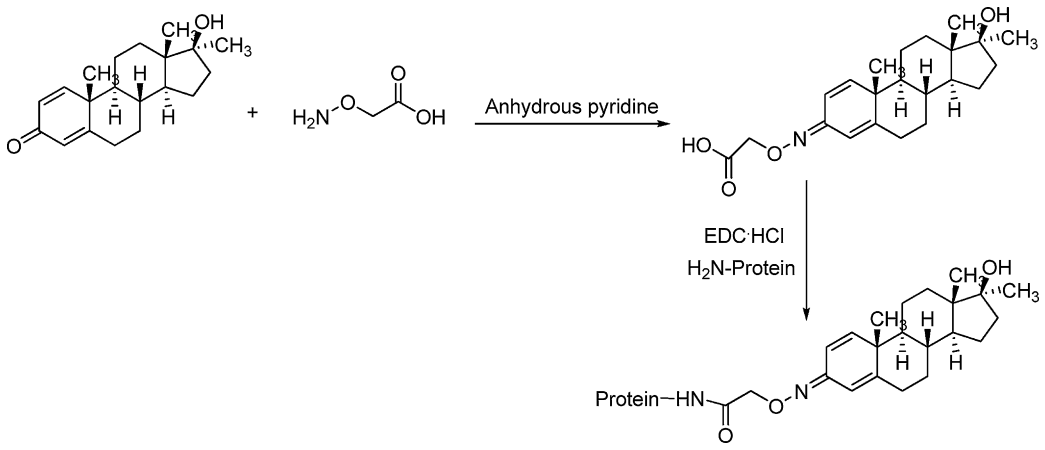

Fig. 1. Synthesis route of MA–protein conjugate.

Sepharose resin, (2) develop optimal extraction conditions for the binding and release of antibody-bound MA from the IAC, and (3) evaluation of the prepared IAC for the effective extraction of MA from actual samples.

2. Experimental

2.1. Chemicals and materials

Methandrostenolone, trenbolone (TR), testosterone propionate (TP), bovine serum albumin (BSA), goat anti-rabbit IgG-horseradish peroxidase (HRP) conjugate, N-hydroxysuccinimide (NHS), 1-ethyl-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC·HCl), and O-(Carboxymethyl) hydroxylamine hemihydrochloride were purchased from Sigma–Aldrich (St. Louis, MO). Complete Freund's adjuvant (CFA) and incomplete Freund's adjuvant (IFA) were purchased from Bio Basic, Int. (Ontario, Canada). Keyhole limpet haemocyanin (KLH) was purchased from Pierce (Rockford, IL). Protein-A Sepharose 4B and CNBr-activated Sepharose 4B were obtained from General Electric Healthcare (Uppsala, Sweden). High-performance liquid chromatography (HPLC)-grade methanol was purchased from Fisher Scientific, Inc. (Pittsburgh, PA). Thin layer chromatography silica gel glass plates GF254 (5 cm × 10 cm) were obtained from Shanghai Sanpont Co., Ltd. (Shanghai, China). All other chemicals were of analytical grade and supplied by Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

Enzyme-linked immunosorbent assay plate reader (synergy HT) was obtained from Bio Tek Instrument, Inc. (Winooski, VT). Ultraviolet-visible spectrometer (Cary 100) was from Varian Medical Systems, Inc. (Palo Alto, CA). CO_2 cell incubator (Heracell 150i) was obtained from Thermo Fisher Scientific, Inc. (Waltham, MA) and centrifuge (5415R) was purchased from Eppendorf (Hamburg, Germany). Ultrapure water system was supplied by Chengdu Ultrapure Technology Co., Ltd. (Chengdu, China).

2.2. Preparation of hapten

MA hapten was synthesized according to Eilanger [17] as shown in Fig. 1. 0.3 mmol MA was dissolved in 4 mL of anhydrous pyridine and then 0.5 mmol O-(Carboxymethyl) hydroxylamine hemihydrochloride was added to it. After reaction for 4 h at 50 °C, anhydrous pyridine was removed by rotary evaporation. The product was re-dissolved in 50 mL of ethyl acetate and washed with water for 3 times. The collected upper-layer was dried by rotary evaporation and then dissolved in methanol. The solution was purified with thin-layer chromatography using hexane/ethyl acetate (1:2, v/v) as a developing solvent. The band at the position of $R_f = 0.1$ was collected by scraping to get the MA hapten.

2.3. Preparation of hapten–protein conjugate

The hapten was covalently coupled to BSA or KLH using the modified active ester method [18,19]. As indicated in Fig. 1, 7 mg of MA hapten, 4.4 mg of NHS and 3.5 mg of EDC·HCl were dissolved in 500 μL of N,N-dimethylformamide (DMF) and then agitated for 1 h at room temperature. The solution was then added into 4.5 mL of BSA or KLH solution (4.5 mg/mL) dropwise. After reaction for 4 h at 4 °C, the products were dialyzed against phosphate buffer saline (PBS) for 2 days.

2.4. Generation and purification of polyclonal antibody

The polyclonal antibody was generated as previously described [14,20]. Briefly, two female New Zealand white rabbits (about 2 kg each) were used to generate antisera. For each rabbit, 0.4 mg of MA–KLH conjugate was dissolved in 1 mL of 0.9% NaCl solution and emulsified with CFA (1:1, v/v). The emulsion was injected intradermally at multiple sites on the back of the rabbit. For booster immunizations, 0.2 mg of immunogen was dissolved in 0.5 mL of 0.9% NaCl solution and emulsified with 0.5 mL of IFA. The booster immunizations were given every three weeks for a total of 4 booster immunizations. A week after the final booster injection, blood was drawn from ear vein and the serum was isolated by centrifugation. The antibody was purified by affinity chromatography on protein-A Sepharose 4B. To obtain the control serum, blood was drawn one week before immunization.

2.5. Specificity of the pAb

The cross-reactivity of the obtained antibody to other two MA structurally related compounds of TP and TR was estimated using the procedure outlined by Cooper et al. [21] and determined by measuring their IC_{50} values in the competitive ELISA method described below. Cross-reactivity values were calculated according to the following formula: CR = [IC_{50} of MA/IC_{50} of structurally related compound] × 100%.

2.6. Preparation of IAC

The immunoaffinity column was prepared by coupling the purified pAb with CNBr-activated Sepharose 4B according to the following procedures. About 0.1 g of CNBr-activated Sepharose 4B was swollen thoroughly in 10 mL of 1 mM HCl, and then washed

with 100 mL of 1 mM HCl and 100 mL of coupling buffer (0.1 mol/L $NaHCO_3$ containing 0.5 mol/L NaCl, pH 8.3) to remove the protecting groups. 1 mL of the obtained wet gel was mixed with 2 mL of 0.5 mg/mL purified pAb solution and gently agitated for 3 h at room temperature. The gel was then washed with 20 mL of coupling buffer to remove the excess antibody. The coupled gel was added to 10 mL of blocking buffer (0.1 M Tris–HCl, pH 8.0) and the mixture was gently agitated for 2 h at room temperature. The resultant immunosorbent was transferred to a column (30 mm × 5 mm) and stored in PBS containing 0.02% NaN_3 at 4 °C.

2.7. Application and elution of samples on IAC

To obtain high extraction efficiency, the sample loading and eluting solutions were examined according to the previous study [22]. Briefly, 100 ng of MA dissolved in either 2%, 5% or 10% methanol/water (v/v) was loaded on the IAC respectively, and followed by washing with PBS to remove the unspecific binding MA. Finally, 5 mL of eluting solution (60%, 70%, 80% or 90% methanol/water, v/v) was used to release the bound MA from the column. All fractions in the loading, washing and eluting steps were collected respectively and the MA content was determined by HPLC. A HPLC system from Shimadzu Co. (Shimadzu, Japan) with a Shimpack VP-ODS C18 column (250 mm × 4.6 mm) and a UV detector was used. The mobile phase was acetonitrile/water (80:20, v/v) at a flow rate 0.8 mL/min. The UV detection wavelength was set at 250 nm. The standard curve for MA was constructed in the concentrations of 0, 5, 10, 50, 100, 200 and 300 ng/mL. For each analysis, 20 μL of the sample was injected.

2.8. Column capacity determination

Maximum binding capacity of the column was determined according to Qiao et al. [22]. Under optimum extraction conditions, a total of 300 ng of MA dissolved in 6 mL of 2% methanol was continuously loaded in 1 mL portions on the IAC. MA content in effluents was determined by ELISA for calculating the column capacity.

2.9. Evaluation of matrix effect on IAC cleanup

In order to evaluate the matrix effect on IAC cleanup, animal tissue and feed samples were fortified by MA at the final concentration of 1.2 and 4.8 ng/g. 5 g of spiked animal tissue (pork) samples were homogenized and shaken for 5 min in 20 mL of tert-butyl methyl ether [19]. After 10 min of ultrasonic extraction, the mixture was centrifuged at 8000 rpm for 10 min. The supernatant was collected and the pellet was repeatedly extracted using tert-butyl methyl ether for 2 times followed by ultrasonic extraction and centrifugation as before. All the supernatant fractions were pooled and evaporated to dryness. The residues were dissolved in 2% methanol and loaded on the IAC. For animal feed (maize powder) samples treatment, the same procedure was applied with tert-butyl methyl ether replaced by 50% ethanol (v/v). Under optimum conditions, the eluate was collected, and MA content was analyzed by ELISA method to estimate the recovery rate in IAC cleanup step.

2.10. ELISA procedure

For ELISA, a polystyrene microtiter plate was coated with coating antigen (MA-BSA) in 0.05 M carbonate buffer (pH 9.6) followed by incubation for 1 h at 37 °C. Unoccupied sites were blocked with 2% of glycine solution for 2 h at 37 °C. After 3 times of washing, 50 μL/well of MA followed by 50 μL/well of pAb were added and incubated for 2 h at 37 °C. After the washing procedure, 100 μL/well goat anti-rabbit IgG conjugated to horseradish peroxidase was added and incubated for 2 h at 37 °C. The color development was initiated by adding 50 μL of the substrate (TMB/H_2O_2 in acetate buffer pH 5.5) for each well and incubated for 10 min at 37 °C. The absorbance values were measured at 450 nm by an ELISA reader. The inhibition curve was plotted as A/A_0 versus the MA concentration (0.001, 0.01, 1, 2.5, 5, 10, 25, 50, 100 and 500 ng/mL), where A was the absorbance of the well containing analyte and A_0 was the absorbance of the well without MA. The IC_{50} value was estimated as the concentration of MA that provides a 50% reduction of A/A_0.

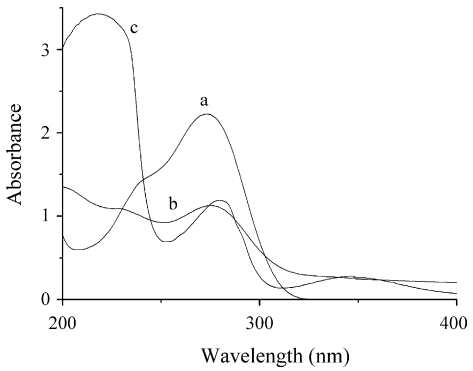

Fig. 2. UV scanning spectrums of MA, KLH and MA-KLH conjugate. (a), MA; (b), MA-KLH; and (c), KLH.

3. Results and discussion

3.1. Polyclonal antibody production and characterization

MA is a small organic molecule and it must be coupled with a carrier protein before animal immunization [23]. To synthesize the immunogen, a carboxyl group was introduced into the MA molecule by oximate reaction at first. After being purified by thin layer chromatography, the carboxyl group of the product was covalently coupled with the amino group of the carrier protein. The prepared MA–KLH conjugate was examined by UV spectroscopy to confirm the coupling reaction (Fig. 2). The curve diagram of the conjugate was the combination of hapten and KLH, which suggested successful conjugation.

The immunogen was inoculated to New Zealand rabbits and the crude antiserum was purified by affinity chromatography on protein-A Sepharose 4B. Electrophoresis analysis indicated that a highly purified pAb was obtained (Fig. 3). To characterize the produced pAb, the titer, the IC_{50} value and the cross-reactivity of the antibody were determined by ELISA method. The titer of the antibody was over 6.4×10^4. As indicated in the inhibition curve for MA (Fig. 4), the IC_{50} value was found to be 5.6 ng/mL and the limit of detection (LOD) value was lower than 0.03 ng/mL. As shown in Table 1, the antibody was very specific to MA and had little cross-reactivity to both TP and TB (<0.6%). All these results demonstrated the excellent sensitivity and high specificity of the obtained antibody.

Table 1
IC_{50} and cross-reactivity of anti-MA polyclonal antibody against structurally related compounds.

Compounds	IC_{50} (ng/mL)	Cross-reactivity (%)
Methandrostenolone	5.6	100
Testosterone propionate	>1000	<0.6
Trenbolone	>1000	<0.6

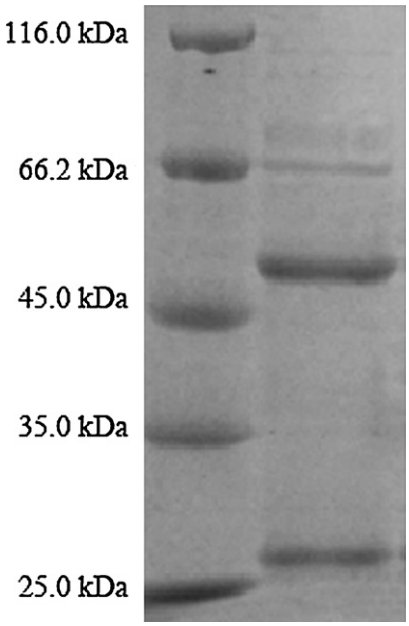

Fig. 3. SDS-PAGE of purified polyclonal antibody.

3.2. Evaluation of the IAC chromatography conditions

IAC was prepared by coupling the produced pAb specific for MA with CNBr-activated Sepharose 4B. Optimization of loading and eluting conditions is necessary to IAC cleanup because these conditions have a strong influence on the association/dissociation of analyte–antibody complex and the activity of antibody [13]. Due to the low water solubility of MA, three different loading solutions with low concentration of methanol (2%, 5% and 10% of methanol/water, v/v) were evaluated. It was observed that the concentration of methanol in loading solution has no significant effect on the IAC binding rates of MA (97.2%, 96.3% and 96.7%), thus the 2% methanol was selected as loading solution.

Selection of the most appropriate eluting conditions was made attending to (1) the recovery of the analyte, (2) the volume needed for an acceptable recovery, (3) the potential damage of the IAC after several cycles of usage, and (4) the compatibility with the immunochemical analytical method [24]. Although the use of acidic, basic buffers or solutions with high ionic strength has been reported, organic solvent/water mixtures such as methanol/water or ethanol/water have often provided the best recoveries for low molecular weight analytes from immunoaffinity columns [25,26]. Therefore, four different eluting solutions with higher concentration of methanol were examined and the data are presented in Fig. 5. As shown, with the increase of methanol concentration from 60% to 90%, the MA content in eluting fractions increased from 81.3% to 102.7%. High concentration of methanol in the eluting solution might enhance the remove efficiency, but it would be more harmful to the antibody. In this case, 80% of methanol was chosen as eluting solution and more than 95% of the MA bound on the column could be released by no more than 5 mL of this solution.

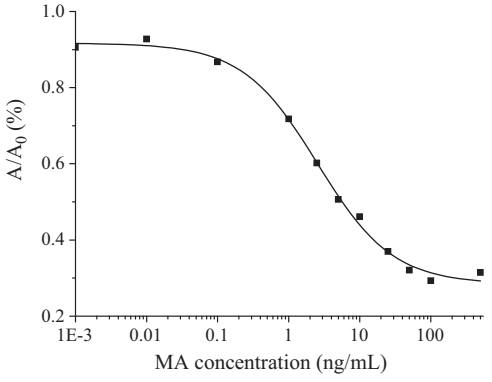

Fig. 4. Inhibition curve of anti-MA polyclonal antibody with MA as a competitor.

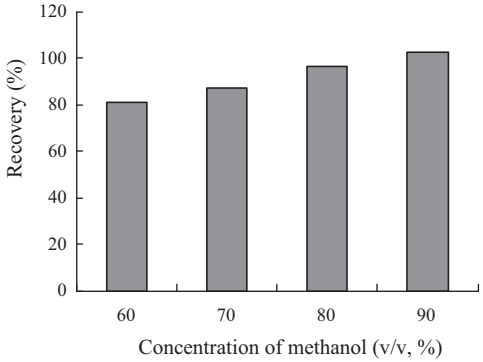

Fig. 5. Percent of MA in the elution fraction depending on the concentration of methanol in the eluting solution.

3.3. Characterization of the IAC for MA

Under optimum immunoaffinity chromatography conditions, the performance of the prepared IAC was evaluated in terms of maximum binding capacity, column-to-column variability and reusability.

The maximum binding capability of the IAC was examined by sequential application of 1 mL of MA standard solution at the concentration of 50 ng/mL to the single IAC (gel volume 0.5 mL). MA content in flow-through was determined by ELISA. Nearly all the MA in the first three 1-mL solutions was retained on the column. After the loading of fourth 1-mL of MA standard solution, about 33 ng of MA was detected in the flow-through. Therefore, the maximum binding capacity of this IAC for MA was calculated as [3 × 50 (completely retained) + 17 (partially retained)]/0.5 = 334 ng/mL gel.

To determine column-to-column variability, standard solutions were respectively applied to the six IACs. Under optimal immunoaffinity chromatography conditions, 2.5 mL of three different MA standard solutions (20, 40 and 60 ng/mL in 2% methanol) were loaded on the IACs respectively. The MA content in the fractions of eluting was detected by HPLC and compared with that of total loading. Table 2 summarizes the MA recoveries from the IACs. Recoveries of MA ranged from 90.7 to 106.7% for all columns with the relative standard deviation (RSD) among columns of 6.7%. Thus, the prepared IACs bound similar amounts of MA antibody and functioned similarly when challenged with 20, 40, and 60 ng/mL of MA solutions in 2% methanol.

Table 2
MA immunoaffinity column-to-column variability.

Column ID	Measured amount (ng)	Recovery (%)
50 ng of MA standard applied		
1	53.3	106.7
2	52.5	105.0
100 ng of MA standard applied		
3	97.2	97.2
4	93.8	93.8
150 ng of MA standard applied		
5	136.0	90.7
6	140.9	93.9
Average recovery (%)	97.9 ± 6.7	

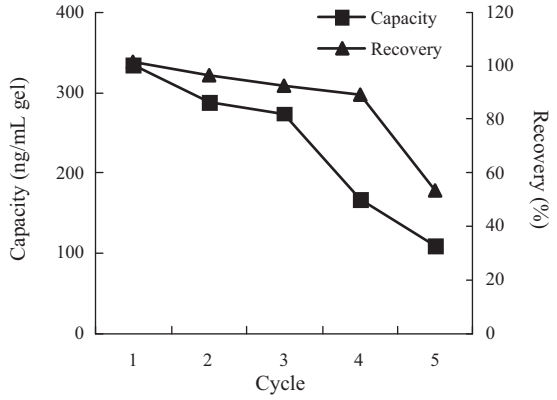

Fig. 6. Column capacity and recovery variation curves of the IAC through 5 cycles of usage.

The IAC with the maximum loading amount of 334 ng MA was subsequently tested for its reusability. The column capacity and recovery through 5 cycles of usage were examined (Fig. 6). It was observed that about 92.6% of recovery rate and 82.0% of the maximum capacity still remained after 3 repeated usages. Similar results concerning the reusability of various immunoaffinity columns have been observed in previous reports [27–29]. The possible reason for gradual decrease of recovery and binding capacity was considered to be derived from the damage of binding site of the antibodies and the detachment of the antibodies from the immunoaffinity support [28]. However, all these data obtained above suggested that the prepared IAC can provide a high extraction efficiency and suitable reusability.

3.4. Application of IAC for actual samples

In order to validate the feasibility of the prepared IACs, the columns were challenged with fortified animal tissue and feed samples. The samples were spiked with MA at the concentration of 1.2 ng/g and 4.8 ng/g according to the maximum residue level (2.0 ng/g) of its structurally related compound (trenbolone) accepted by FAO/WHO. As can be seen in Table 3, the recoveries of MA from the fortified samples were found to be in the range of 83.5–99.7% with RSD lower than 6.5%. The estimated time required for performing the IAC cleanup was about 30 min. Solid phase extraction (SPE) is commonly utilized in samples cleanup for various residues analysis [30]. Compared with Ho's work [31], in which a 65% recovery with RSD 10.5% and 1 ng/mL detection limits was obtained by SPE cleanup coupled LC/MS analysis of MA spiked urine samples, the newly established IAC cleanup coupled ELISA method provided a higher extraction efficiency and reproducibility and a similar detection limits. All these results demonstrated that the method established in this experiment could allow a reliable and high sensitive analysis for MA from various real samples.

Table 3
Extraction recoveries of MA from spiked animal tissue and feed samples by IAC cleanup ($n = 3$).

Sample	Spiked level (ng/g)	Recovery rate (%)	RSD (%)
Animal tissue	1.2	96.8	2.3
	4.8	83.5	5.6
Animal feed	1.2	99.7	6.5
	4.8	99.4	3.5

4. Conclusion

To the best of our knowledge, this is the first generation of antibody against MA and its application to IAC cleanup so far reported in the literature. In this study, polyclonal antibody against MA was generated and then an IAC was prepared by coupling the antibody with CNBr-activated Sepharose 4B resin. The produced antibody exhibited excellent sensitivity and specificity towards MA with an IC_{50} of 5.6 ng/mL and <0.6% cross-reactivity with MA structurally related compounds. We demonstrated that the MA can be applied in 2% methanol in water and the bound MA can be released quantitatively from the IAC by 80% methanol in water. The maximum binding capacity of IAC for MA was about 334 ng/mL gel. The column-to-column variability was within 6.7%. After 3 times of usage, the column capacity and recovery rate still remained 82.0% and 92.6% respectively. The prepared IAC was challenged with MA spiked animal tissue and feed and quantitative recoveries were achieved. Thus, the developed IAC cleanup procedure coupled with ELISA or HPLC analysis could be hopefully used as an alternative method for the determination of MA residues in complex actual samples.

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