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Isolation of alpaca anti-idiotypic heavy chain single domain antibody for the aflatoxin immunoassay

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Abstract

Anti-idiotypic antibodies recognize the antigenic determinants of an antibody, thus can be used as surrogate antigens. Single domain antibodies from camlid heavy chain antibodies with the benefit features of small size, thermostability and ease in expression, are leading candidates to produce anti-idiotypic antibodies. In this work, we constructed an antibody phage library from the mRNA of an alpaca immunized with an anti-aflatoxin monoclonal antibody (MAb) 1C11. Three anti-idiotypic VHH antibodies were isolated and applied to immunoassay towards aflatoxin as a coating antigen. The best immunoassay developed with one of these VHH antibodies shows an IC_{50} of 0.16 ng/mL towards aflatoxin B_1 and cross-reactivity towards aflatoxin B_2, G_1 and G_2 of 90.4%, 54.4% and 37.7%, respectively. The VHH-based immunoassay was successfully applied to the analysis of peanuts, corn and rice, which are the predominant commodities regularly contaminated by aflatoxins. A good correlation (r^2=0.89) was found between the data obtained from the conventional ELISA and the ELISA based on a VHH coating antigen for the analysis of aflatoxins in peanuts and feedstuff. The use of biotechnology in developing the surrogate, the absence of standard aflatoxin and organic solvents in the synthesis procedures, and the

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Author Contributions

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Supporting Information. This material is available free of charge via the Internet at http://pubs.acs.org.

reproducibility of the VHH antibody makes it an ideal strategy for replacing conventional synthesized antigens.

Immunoassays are important analytical methods in the field of clinical chemistry, home testing, safety assessment of agricultural products and environmental monitoring, due to their many attractive features including sensitivity, specificity, ease of manipulation, fast detection, cost effectiveness, high throughput and point of care analysis.[1–3] Large molecules can be easily determined by a sandwich format immunoassay. The target is captured by an immobilized antibody and then detected by the addition of a second antibody conjugated with signal-producing molecules. Small analytes, such as mycotoxins and pesticides, are not large enough to be simultaneously recognized by two antibodies, so instead of using the sandwich format, a hapten is needed to compete with the target compound for binding to the antibody, which is called a competitive immunoassay. In a heterologous immunoassay the competing antigen differs from the immunizing antigen, which influences the antibody binding such that the analyte may be detected with better sensitivity. It is common to synthesize a library of chemicals and to select one that yields the best assay. The conventional synthesis of competing antigens has several drawbacks such as the over use of organic solvent (e.g. $SOCl_2$, dimethylformamide, dioxane, $CHCl_3$), time consuming, requiring expertise in synthetic chemistry and complex separation/removal of impurities.

An easy and environmental-friendly way to obtain the competing antigen is needed to improve the development of immunoassays. In recent years, people found peptides and proteins can replace the synthetic competing antigen, serving the same function, i.e. to compete with free analytes binding to the antibody. Those peptides and proteins, termed mimotopes, can be obtained by two methods, phage-displayed technology or anti-idiotypic antibodies. A phage displayed peptide library is a vast repertoire of candidate peptides expressed on the surface of phage particles. This technique has been used to select peptide mimics of non-proteinaceous compounds, such as deoxynivalenol,[4] aflatoxin,[5–7] zearalenone,[8] ochratoxin,[9] molinate, atrazine[10] and 3-PBA.[11] However, in most of these assays, the peptide must be used linked to the phage particles, except in one report[4] where the synthetic peptide alone was sufficient for binding to the antibody. This approach leads to complex difficulties in measuring the phage-displayed peptide and quality control. In addition, the phage peptide library must be very large to increase the chance of obtaining a desirable phage peptide. Another approach to prepare immunochemical reagents is through generating the corresponding anti-idiotypic antibodies.[12–14] The variable regions of immunoglobulins possess specific antigenic determinants known as “idiotypes”.[15] Anti-idiotypic antibody is a secondary antibody that targets the idiotype of the primary antibody, thus bearing an internal image of the target compound. Numerous anti-idiotypes against both large and small molecules have been developed by monoclonal[16–21] or polyclonal[22–24] techniques and applied in clinical diagnostics, immunotherapy, and immunoassays. Recent success in generating camelid nanobodies prompted our interest in generating anti-idiotypic nanobodies.[25–28] In 1993, a group of Belgian scientists found a type of antibody in the blood of camelids (camels, llamas and alpacas) completely void of light chains (heavy chain antibody).[29] The variable domain (VH) of such heavy chain antibodies is formed by only one variable domain (VHH).[30] Recombinant expression of these heavy chain variable

domains yields a single domain heavy chain antibody, termed “nanobody”.[31] Nanobodies have the following advantages: 1) Small size, the molecular weight is about 17kDa. 2) High solubility, high thermal and chemical stability. 3) Nanobodies can be readily produced on a large scale. 4) Sequences of nanobodies can be easily manipulated. 5) The elongated nanobodies are thought to be more readily insert into narrow pits or grooves. Due to these benefits, nanobodies have been applied in many areas. Anti-idiotypic nanobodies, with these beneficial features, have also been developed and applied for diagnostic and therapeutic purposes.[32–36] However, there is no report using anti-idiotypic nanobodies in environmental immunoassays.

Aflatoxins, a group of naturally occurring mycotoxins, were chosen in order to determine the possibility of employing a heavy chain single domain anti-idiotypic antibody as the coating antigen. Aflatoxins are highly toxic and among the most potent carcinogens.[37] Efficient control of aflatoxins in food and agro-products requires precise and easily performed analytical methods. Many chromatographic methods for aflatoxin determination were developed, such as HPLC[38,39], LC-MS[40] and LC-MS/MS[41]. These methods are sensitive and accurate, however, most are time-consuming and require sophisticated equipments.[42] In addition, the requirement for rapid screening of a large number of samples has encouraged the development of immunoassays toward aflatoxins. A number of immunoassays toward aflatoxins have been reported.[43–47] There are mainly two approaches to synthesizing aflatoxin conjugated with bovine serum albumin. One (Scheme S-1 in the Supporting Information) is by first preparing aflatoxin oxime and then coupling it to BSA.[48,49] The other (Scheme S-2 in the Supporting Information) involves synthesizing AFB_1Cl_2 first and then conjugating it to BSA.[50] In these procedures, large amounts of aflatoxin standard and organic solvents (pyridine, dichloromethane) are involved which are hazardous and do not comply with the demand for eco-friendly/green approaches. With regards to safety, biologically-derived coating antigens would be a preferred reagent.

We have previously reported the generation and characterization of a monoclonal antibody, designated 1C11, which has broad selectivity for four major aflatoxins (aflatoxin B_1, B_2, G_1 and G_2)[46]. Herein, we report the generation of anti-idiotypic VHH antibodies V2-5, V2-12 and V2-29, specific for 1C11. Nanobody V2-5 has been successfully applied in a 1C11 based ELISA for aflatoxin determination in peanuts, corn and rice.

EXPERIMENTAL SECTION

Safety

Due to the high toxicity and carcinogenicity of aflatoxin, all items coming in contact with aflatoxins (glassware, vials, tubes, ELISA plates) were immersed in a 10% bleach solution for 1–2 h before they were discarded. Pure aflatoxin standard was handled in a hood with extreme caution.

A 4-year old neutered male alpaca was immunized subcutaneously with 200 μg of anti-aflatoxin MAb 1C11 mixed with Freund’s incomplete adjuvant. Total RNA was extracted from alpaca’s blood and used to synthesize first strand cDNA. The phage displayed VHH library was constructed by ligating amplified VHH genes with plasmid pComb3X. Anti-

idiotypic phage displayed VHH was selected by panning-elution procedures. After sequencing, unique phage clones were selected and transformed into TOP 10F′ cells to express soluble VHH fragments. ELISAs with soluble VHHs as coating antigen were developed to identify the assay's sensitivity. The one with the best sensitivity was chosen for sample analysis. For a more detailed experiment description, see the Supporting Information.

RESULTS AND DISCUSSION

Panning-elution selection of positive phage-displayed VHH

In order to select phage-displayed VHHs that specifically recognize variable regions of MAb 1C11, wells were coated with the monoclonal antibody and bound phage eluted with free aflatoxin B_1. Titer of the output phage was increased after each round of panning which means specific phage was enriched (Figure S-1 in Supporting Information). After the fourth round of panning, thirty clones were selected and phage affinity towards MAb 1C11 was tested by a phage ELISA. While all of them bound to the antibody, only 11 out of 30 (Figure S-2 in Supporting Information) turned out to be anti-idiotypes which showed inhibition binding to the antibody by free aflatoxin B_1.

Plasmids from the positive clones were extracted and sequenced. The VHH sequences are shown in Figure S-3 in Supporting Information. As expected, the framework regions are highly conserved in all of the sequences. We can distinguish three groups of isolated VHHs based on the composition of CDR regions. Group 1 (1-26, 1-17, 1-13, 1-18 and 2-29) and group 2 (2-8, 2-5, 2-20 and 2-27) differ in only a few amino acid residues, while 2-12 (group 3) is a unique antibody. Interestingly, group 2 are long-hinge VHHs (IgG2) and the rest are short-hinge VHHs (IgG3).[51] We decided to use a representative from each group (VHHs 2-5 2-12 and 2-29) for the large-scale protein expression and further characterization.

VHH ELISA for aflatoxin

Plasmids from the three unique clones were transformed to TOP 10F′ cells. TOP 10F′ is a nonsuppressor strain which recognized the amber stop codon between the VHH and pIII and allows VHH expression without pIII protein. VHHs have a 6×His tag so they can be purified using a Ni-NTA metal affinity column. The purity was checked on a 15% SDS-PAGE gel and was greater than 95% (data not shown). The size of the VHH is about 15 kDa, calculated using the protein information resource (PIR). Using a BCA protein kit (BioRad, Emeryville, CA), the concentrations of three VHH antibodies were determined. VHH 2-5 had the highest yield of 3.24 mg/L bacterial culture, 2-12 was 182.7 μg, and 2-29 was 868.6 μg/L. For the measurement of free aflatoxin in samples or standards, plates were first coated with VHH, after competitive binding of MAb1C11 to coated VHH or free aflatoxin, goat anti-mouse antibody-HRP was added to detect the captured antibody.

The optimal concentrations of VHH and MAb 1C11 were determined by a checkerboard titration. The sensitivity of the assay was tested using each of the VHH antibodies as the coating antigen. VHH 2-5 was the best coating surrogate with the lowest IC_{50} (Figure 1). The following assays were all conducted with VHH 2-5.

Cross reactivity

Aflatoxin B_1, B_2, G_1 and G_2 are four major aflatoxins that are usually produced simultaneously in grains. Aflatoxin M_1 is the hydroxylated metabolite of aflatoxin B_1 and usually found in milk and milk products. The cross reactivity of the VHH-based ELISA against aflatoxin B_1, B_2, G_1, G_2 and M_1 was tested (Figure 2). The ELISA results indicated that anti-aflatoxin MAb 1C11 in combination with VHH 2-5 recognizes all five aflatoxins. It shows the highest sensitivity towards aflaoxin B_1 with the IC_{50} value of 0.16 ng/mL, followed by 90.4% and 54.4% cross-reactivity with aflatoxin G_1 and B_2, respectively (% cross-reactivity=100×[IC_{50} (AFB1)/IC_{50} (cross-reacting compound)]). The assay showed weaker cross-reactivity with aflatoxin G_2 (37.7%) and aflatoxin M_1 (37.4%). In most cases, when all four aflatoxins occur, aflatoxin B_1, B_2, G_1 and G_2 exist with proportions of 1.0:0.1:0.3:0.03.[52] So relatively lower cross-reactivity towards aflatoxin G_2 does not interfere with the detection of total aflatoxins in grains or foodstuffs.

Solvent effect

Aflatoxins are relatively non-polar chemicals that have low solubility in water. Thus methanol is commonly used to extract aflatoxins from samples and to keep it in solution. Higher concentrations of methanol may interfere with the activity of antibody; however, too high dilution of the extract will result in less sensitivity of the assay. To reach a balance of sensitivity and activity of the assay, a study to optimize the concentration of methanol in the final assay buffer was conducted. A series of aflatoxin B_1 concentrations was diluted in 5%, 10%, 20% and 40% methanol-PBS (phosphate buffered saline). The IC_{50} values generated from each dilution buffer were compared (Figure 3). There were slight differences among 5%, 10% and 20% methanol-PBS, however, 40% methanol-PBS interfered with antibody binding strongly. So in the following analysis, we used 20% methanol-PBS to dilute the aflatoxin standard. Also, the sample extract was diluted to a final concentration of 20% methanol.

Matrix effect

Matrix effects can substantially interfere with the immunoanalytical measurement of a target analyte. In ELISAs, matrix effects are mainly caused by inhibition of enzyme activity, binding between antibody and analyte (aflatoxin B_1) or both.[53] Dilution of the extract is an effective way to eliminate matrix effects, however, as we mentioned above, too much dilution results in lower sensitivity for sample analysis.

Sample extracts (peanut, rice and corn) were diluted with PBS by 4, 8 and 20 times. Serial concentrations of aflatoxin B_1 in each diluted sample extract were determined and compared with those diluted with pure methanol/PBS buffer. Some extent of matrix effect was found in the three test sample matrices (Table S-1 in Supplement Information). However, the observed effects were eliminated by diluting the extract in 4% BSA (bovine serum albumin)/PBS. After this adjustment, there were no significant differences among standard curves with 25% peanut, corn or rice matrix (1 in 4 dilution of the extract with 4% BSA/PBS) (Figure 4). A 4-fold dilution of the extract is used for the sample analysis in the following study.

Method validation

To validate the assay, we carried out a spike-and-recovery analysis with the newly developed VHH-based ELISA. Peanut, corn and rice samples spiked with known concentrations of aflatoxin B_1 or mixed aflatoxins (ratio of 2:1:1:1 of aflatoxin B_1, B_2, G_1 and G_2) were tested. Good recovery results (70–120%) were obtained with samples spiked with only aflatoxin B_1. The recoveries of mixed aflatoxins spiked samples were all under 80% (Table 1), which was expected given the relatively low cross-reactivity of the assay towards aflatoxin B_2 and G_2.

Naturally contaminated samples were collected from farm in China. Both conventional ELISA and VHH-based ELISA were used to test aflatoxin concentrations in those samples. The standard curves for both assays were established using aflatoxin B_1 as the standard. The VHH-based ELISA exhibits a lower sensitivity (IC_{50}=13.8 μg/kg) compared with conventional ELISA (IC_{50}=1.2 μg/kg). The linear range calculated by 20%–80% inhibition is 10–20 μg/kg, which is suitable for monitoring total aflatoxin concentration under current regulatory limits of aflatoxin in most countries (20 μg/kg). Good correlation (r^2=0.89, removing the 3 samples for which the VHH assay could not provide a determination) was obtained between the two immunoassay methods (Table 2).

CONCLUSION

In this work, we describe a simple and straightforward strategy for developing mimotopes in immunoassay. The conventional antigen, aflatoxin B_1-BSA, was replaced with an anti-idiotypic heavy chain single domain antibody thought to be approximate the internal image of aflatoxins in mAb 1C11. This VHH antibody mimics the reaction of aflatoxin in ELISA, and thus can serve as a surrogate for aflatoxin haptens as coating antigens. The newly developed assay avoids the use of an aflatoxin standard in the synthesis of a competing antigen and as the competing analyte in the assay. Besides, taking advantage of camelid single domain antibodies, the anti-idiotypic antibody is small, stable and easy to obtain. The final assay has a good sensitivity and can be used to detect aflatoxins B_1 in agri-products. These results indicate that anti-idiotypic heavy chain single domain antibody would be an excellent tool for developing environmental-friendly immunoassays.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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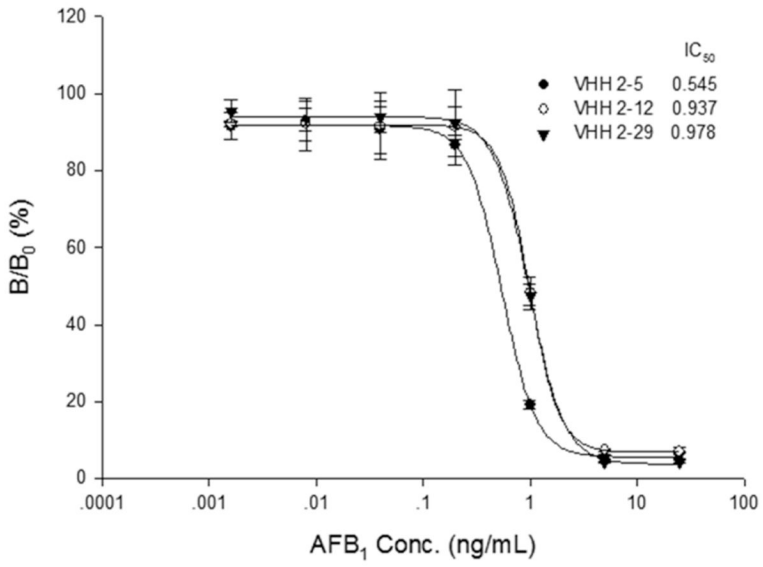

Figure 1.

Inhibition curves of VHH-based ELISA with VHH 2-5 (black circle), VHH 2-12 (write circle) and VHH 2-29 (triangle) antibodies as coating antigen. Each value is the average of three replicates ± standard deviation. %B/B0 = [Absorbance (calibrator, control, or unknown)/Absorbance (blank)] × 100.

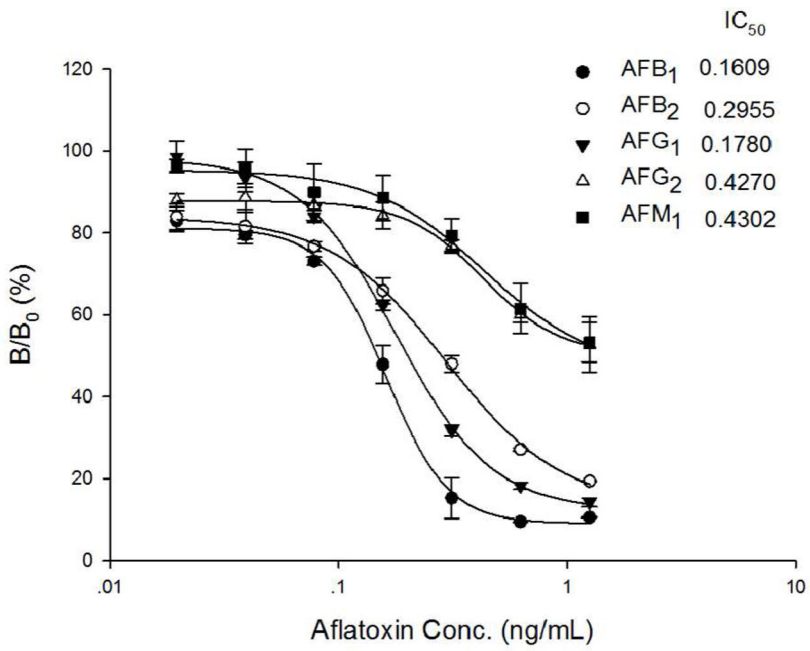

Figure 2.

Cross-reactivity of VHH-based ELISA towards aflatoxin B_1 (black circle), B_2 (white circle), G_1 (black triangle), G_2 (white triangle) and M_1 (square). Each result is represented as an average ± standard deviation of three replicates.

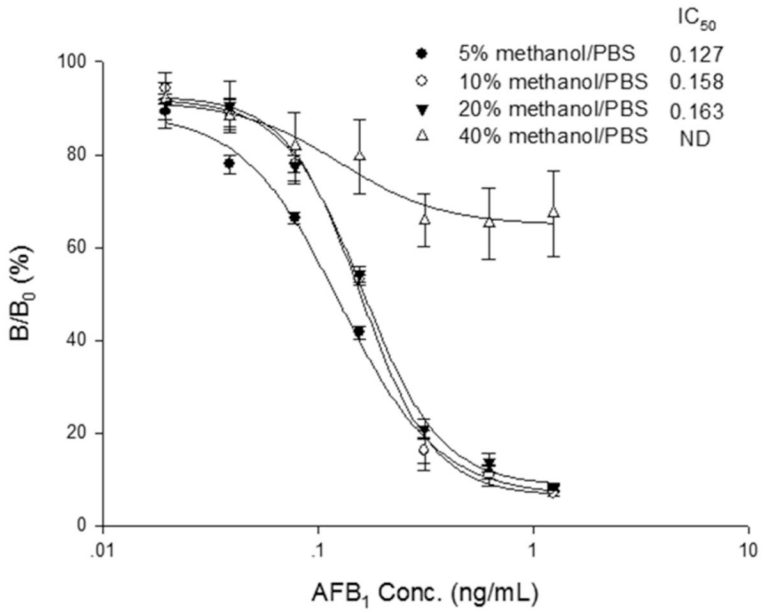

Figure 3.

Performance of the VHH-based ELISA under 5% (black circle), 10% (white circle), 20% (black triangle) and 40% (white triangle) methonal/PBS. Data are represented as an average ± standard of three replicates.

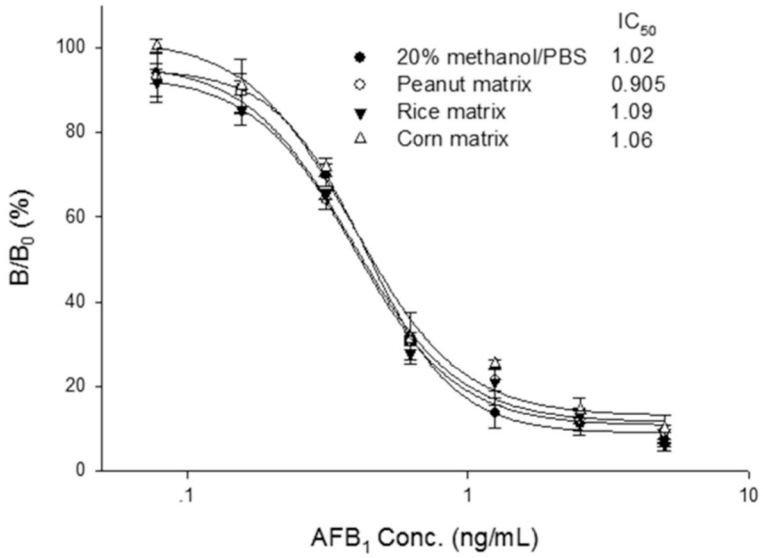

Figure 4.

Assay standard curve in 20% methanol/PBS (black circle), peanut (white circle), rice (black triangle) and corn (white triangle) matrix. Each value represents the mean value of three replicates.

Table 1

Recovery analysis of aflatoxin by VHH-based ELISA

Sample Type	Spiked Aflatoxin(s)	Spiked Level (μg/kg)	Mean±SD	Average Recovery (%)
Within assay (n=3)[a]				
Peanut	Aflatoxin B1	60[d]	71.8±6.5	120
		20	18.4±0.4	91.9
		10	11.8±0.4	118
	Mixed aflatoxins[c]	60	44.8±0.6	74.6
		20	11.3±0.6	56.6
		10	7.1±0.4	71.2
Rice	Aflatoxin B1	60	65.2±10.4	109
		20	18.8±0.8	94.1
		10	11.2±0.8	112
	Mixed aflatoxins	60	25.3±4.4	42.1
		20	11.4±0.5	57.2
		10	7.0±0.8	70.0
Corn	Aflatoxin B1	60	64.0±3.9	107
		20	17.2±0.3	85.9
		10	11.6±0.3	116
	Mixed aflatoxins	60	41.2±0.7	68.7
		20	11.9±0.4	59.5
		10	6.9±0.3	69.4
Between assay (n=5)[b]				
Rice	Aflatoxin B1	60	58.2±10.3	97.0
		20	16.5±2.5	82.2
		10	11.0±1.7	109.7

[a] Each assay was carried out in 3 replicates on the same day

[b] The assays were carried out on 6 different days

[c] Mixed aflatoxin standard was prepared with aflatoxin B_1, B_2, G_1 and G_2 at a ratio of 2:1:1:1. The results are based on using aflatoxin B_1 as the standard.

[d] Samples spiked with 60 μg/kg aflatoxin were extracted and diluted by 20 times in order to make the final concentration included in the linear range of the VHH-based ELISA.

Table 2

Detection results of conventional ELISA and VHH-based ELISA in peanuts and feedstuff

Samples	Conventional ELISA (μg/kg ± SD, n=3)	VHH-based ELISA (μg/kg ± SD, n=3)
Peanut		
1	29.8±3.2	>20
2	10.4±1.4	10.6±0.2
3	30±4.8	>20
4	15.6±1.7	15.1±0.6
5	0.8±0.3	5.8±0.3
Feedstuff		
1	8±0.5	8.4±0.3
2	30±5.4	>20
3	15±2.8	11.8±0.2
Rice		
1	21±1.5	17±2.2
2	8.4±1.8	6.0±0.5
3	15.8±0.5	14.8±1.8
4	12.2±2.9	12.8±1.0
5	8.6±0.9	8.5±0.2
6	18.3±1.2	14.1±2.1
7	20.1±1.6	12.5±2.7
8	3.5±0.7	ND[a]
Corn		
1	29.2±2.0	15.6±1.0
2	33.4±5.8	>20
3	1.8±0.2	ND
4	12.8±0.3	13.4±1.2
5	14.3±1.1	18.8±1.7

[a]ND, not detectable