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# Development of an immunoassay for the detection of carbaryl in cereals based on a camelid variable heavy-chain antibody domain

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## Abstract

**BACKGROUND:** The variable domain of camelid heavy-chain antibodies (VHH) is increasingly being adapted to detect small molecules in various matrices. The insecticide carbaryl is widely used in agriculture while its residues have posed a threat to food safety and human health.

**RESULTS:** VHHs specific for carbaryl were generated from an alpaca immunized with the hapten CBR1 coupled to keyhole limpet hemocyanin. An enzyme-linked immunosorbent assay (ELISA) based on the VHH C1 and the coating antigen CBR2-BSA was developed for the detection of carbaryl in cereals. This assay, using an optimized assay buffer (pH 6.5) containing 10% methanol and 0.8% NaCl, has a half-maximum signal inhibition concentration of 5.4 $ngmL^{-1}$ and a limit of detection (LOD) of 0.3 $ngmL^{-1}$ for carbaryl, and shows low cross reactivity (≤0.8%) with other tested carbamates. The LOD of carbaryl using the VHH-based ELISA was 36 ng $g^{-1}$ in

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SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

Conflict of interest

The authors declare that they have no conflict of interest.

rice and maize and 72 ng $g^{-1}$ in wheat. Recoveries of carbaryl in spiked rice, maize and wheat samples were in the range of 81–106%, 96–106% and 83–113%, respectively. Relative standard deviations of repeatability and intra-laboratory reproducibility were in the range of 0.8–9.2% and 2.9–9.7%, respectively.

**CONCLUSION:** The VHH-based ELISA was highly effective in detecting carbaryl in cereal samples after simple sample extraction and dilution.

### Keywords

VHH; carbaryl; ELISA; cereals; food safety

## INTRODUCTION

Pesticides play a critical role in global food production. Because of the widespread use of pesticides, concern about the toxic threat of these compounds to humans and ecosystems has increased.[1,2] Rapid and effective methods to monitor pesticide residues in the environment are generally acceptable worldwide, especially in underdeveloped regions. Immunoassays such as the enzyme-linked immunosorbent assay (ELISA) based on polyclonal antibody (pAb) or monoclonal antibody (mAb) have proven to be a high-throughput method for screening pesticides in a wide variety of matrices.[3–7] Nonetheless, these traditional pAbs and mAbs have certain shortcomings in the development of a robust immunoassay of pesticides, such as large size, low thermal stability and restriction in genetic manipulation.

Over the past decades, advancement in the production of recombinant antibodies, such as single-chain variable fragment (scFv) and variable domain of heavy-chain antibodies (VHH), has widened the application of immunoassays in different fields, including clinical diagnostics and environmental and food safety monitoring.[8–10] VHH (*ca*15 kD), known as the smallest functional antibody (nanobody), is genetically engineered from the heavy-chain-only antibody (HCAb) IgG2 or IgG3[11] that occurs naturally in both Old World camelids (e.g. dromedary and Bactrian camel) and New World camelids (e.g. alpaca and llama).[12] VHH has some advantages over traditional antibodies, such as small size, high solubility, high thermal stability, refolding capacity, and low-cost production.[13,14] It has shown promise as an emerging tool in disease diagnostics and treatment.[15,16] VHHs have been increasingly attractive for monitoring human exposure to environmental chemicals, e.g. polybrominated diphenyl ethers and tetrabromobisphenol A,[17] but they have been much less frequently used for the detection of pesticide contamination.[18]

Carbaryl (1-naphthalenyl methyl carbamate) is a broadspectrum pesticide that has been applied to over 120 different crops for insect control because of its inhibitory effect on acetylcholinesterase.[19] In China, carbaryl is used mainly in the production of cereal, oil and vegetable corps and the maximum residue limit (MRL) of carbaryl in these foods is 1.0 mg $kg^{-1}$.[20] Carbaryl remains the third-most-used insecticide in the USA for commercial agriculture, home gardening and rangeland protection.[21] Carbaryl is toxic to the immune, nervous, and endocrine systems.[22] Recent research showed that carbaryl is a structural mimic of the neurohormone melatonin and that it binds directly to the melatonin receptor,

which could seriously disrupt the homeostatic balance between the neurotransmitter and modulators.[23]

Instrumental analytical techniques, including liquid chromatography (LC) or gas chromatography (GC) coupled with fluorescence and mass spectrometry (MS) detectors,[24–27] have been developed for the determination of carbaryl in a variety of matrices. These methods are accurate, with good repeatability, but they are currently limited with respect to applications for on-site detection because of the requirement of expensive instruments, skilled operators, and complicated sample pretreatment. In addition, significant decomposition of carbaryl to its main metabolite, 1-naphthol, might occur when large numbers of samples are prepared for analysis. For these reasons, simple, sensitive and cost-effective screening tests for the detection of carbaryl residues in the environment are highly desirable.[28–31] In this study, we extended the application of VHH technology to food safety research and developed a rapid VHH-based ELISA for the detection of carbaryl in rice, maize and wheat samples.

## MATERIALS AND METHODS

### Materials

Incomplete Freund's adjuvant, keyhole limpet hemocyanin (KLH), bovine serum albumin (BSA), 3,3, 5,5-tetramethylbenzidine (TMB), polyethylene glycol 8000 (PEG 8000), isopropyl-$\beta$-D-thiogalactopyranoside (IPTG), and imidazole were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Mouse anti-M13 phage mAb-horseradish peroxidase (HRP) was purchased from GE Healthcare (Piscataway, NJ, USA). The phagemid vector pComb3X was a generous gift from Dr. Barbas (Scripps Research Institute, La Jolla, CA, USA). Electrocompetent cells of *Escherichia coli* ER2738 were acquired from Lucigen Corporation (Middleton, WI, USA). All restriction enzymes, T4 DNA ligase, and M13KO7 helper phage were purchased from New England Biolabs, Inc. (Ipswich, MA, USA). DNA polymerase was purchased from Tsingke Biological Technology Lt. (Beijing, China). Goat anti His-tag IgG and HRP conjugate was purchased from Abcam (Cambridge, MA, USA). LeukoLOCK™ Total RNA Isolation System, HisPur Ni-NTA resin, agar, yeast extract, tryptone, and Nunc MaxiSorp flat-bottom 96-well microtiter plates were purchased from Thermo Fisher Scientific Inc (Rockford, IL, USA). All pesticide standards were purchased from the Institute for the Control of Agrochemicals, Ministry of Agriculture and Rural Affairs, China.

### Animal immunization and construction of a phage-displayed VHH library

Haptens CBR1–CBR8 (Fig. 1) and hapten-protein conjugates are available from previous studies.[32,33] Hapten CBR1 conjugated to KLH was used as an immunogen, while all CBR1–CBR8 haptens coupled to BSA were used as coating antigens.

A two-year-old healthy male alpaca was immunized subcutaneously with CBR1-KLH five times biweekly. Blood lymphocytes collected after the fifth injection were used as the starting material to construct the VHH library.[34] Briefly, total mRNA was extracted according to the manufacturer's protocol for the LeukoLOCK™ Total RNA Isolation

System and was transcribed into complementary DNA. VHH fragments were amplified by polymerase chain reaction (PCR) and ligated into the plasmid pComb3X using restriction sites *Sfi* I. Ligated plasmids were transferred by electroporation into competent cells *E. coli* ER2738. After bacterial culture and addition of helper phage M13KO7, the phage was precipitated with PEG-NaCl (0.04 g $mL^{-1}$ PEG and 0.5 M NaCl) and then resuspended in phosphate buffered salt (PBS: 0.01 mol $L^{-1}$ phosphate, 0.137 mol $L^{-1}$, NaCl, 3 mmol $L^{-1}$ KCl, pH 7.4). The transformed bacterial clones were titrated on agar plates to determine the library size.

## Selection of anti-carbaryl VHHs

One well of a microtiter plate was coated overnight with 100 μL of CBR1-BSA (8 μg $mL^{-1}$) in carbonate buffer (0.05 mol $L^{-1}$ $Na_2CO_3$, 0.05 mol $L^{-1}$ $NaHCO_3$, pH 9.6) at 4 °C, and four additional wells were coated with 100 μL BSA (0.03 g $mL^{-1}$). The next day, the plate was blocked with gelatin (0.01 g $mL^{-1}$) in PBS for 1 h at ambient temperature. A 100-μL aliquot of the phage-display VHH library suspension was added into the well coated with CBR1-BSA and was incubated for 2 h with gentle shaking at ambient temperature. After washing 10 times with PBST (0.05% Tween-20 in PBS), this well was eluted with 100 μL of carbaryl (1000 ng $mL^{-1}$) in PBS for 1 h at ambient temperature under shaking. The eluent was then evenly distributed into the next four BSA-coated wells and incubated for 1 h to remove non-specific binding phages. After combination of the eluents, 10 μL was used to determine the titer and the remainder was used for amplification with helper phage M13KO7 and was put into the next round of panning. The entire panning process was repeated three times, with a gradual reduction in concentrations of the coating antigen and the elution carbaryl in each round. The concentrations of CBR1-BSA were 4, 2 and 1 μg $mL^{-1}$, respectively, while the concentrations of carbaryl were 500, 200 and 100 ng $mL^{-1}$, respectively, for the 2nd, 3rd and 4th round of panning. After four rounds of panning, phage clones were tested for their binding affinity to carbaryl by a competitive phage ELISA. VHHs were expressed and purified using a Ni-NTA resin as reported previously.[35]

## Competitive VHH-based ELISA for carbaryl

Optimal concentrations of coating antigens and VHHs were determined by a checkerboard titration method. A 100-μL aliquot of coating antigen solution (100 ng $mL^{-1}$) was used to coat a microtiter plate overnight at 4 °C. The next day, the plate was washed five times with PBST and was blocked with gelatin (0.01 g $mL^{-1}$) at ambient temperature. Serially diluted carbaryl (50 μL/well) was added, followed by 50 μL of VHH solution. The plate was incubated at ambient temperature under shaking for 1 h. After washing, 100 μL of HRP-conjugated anti-His-tag mAb (diluted at 1:25000 with PBST) was added and incubated for 1 h. After another washing, 100 μL of TMB solution (400 μL of 0.006 g $mL^{-1}$ TMB and 100 μL of 1% $H_2O_2$ diluted in 25 mL of citrate buffer, pH 5.5) was added into the plate and the reaction was stopped 10 min later by the addition of 50 μL of 2 M $H_2SO_4$. The absorbance was read at 450 nm on a microtiter plate reader (Multiskan MK3, Thermo, MA, USA). The half-maximal signal inhibitory concentration ($IC_{50}$), an indicator of assay sensitivity, and the limit of detection (LOD, $IC_{10}$) were calculated using a four-parameter logistic equation from SigmaPlot 10.0.

### Optimization of VHH-based ELISA

The VHH-based ELISA was optimized by evaluating the effects of variation of buffer pH (4.5, 5.5, 6.5, 7.5, 8.5, 9.5 and 10.5), content of NaCl (0.4%, 0.8%, 1.6% and 3.2%) and percentage of methanol (0%, 2.5%, 5%, 10% and 20%) on assay performance. With the exception of the variable, ELISAs were performed in PBS (pH 6.5) containing 0.8% NaCl and 10% methanol.

### Cross-reactivity

The specificity of the VHH was evaluated by determining its cross-reactivity (CR) with the carbaryl metabolite 1-naphthol and other important carbamate insecticides. The CR was calculated as follows:

$$\mathrm{CR}\ (\%) = [\mathrm{IC}_{50}(\text{carbaryl})/\mathrm{IC}_{50}(\text{tested compound})] \times 100 \quad (1)$$

### Sample preparation and analysis

Rice, maize, and wheat grain samples were purchased from a local supermarket in Beijing and confirmed to be free of carbaryl by a GC method.[36] After grinding and sieving through a 20-mesh screen, cereal samples were fortified with carbaryl at 200, 1000, and 2000 ng $g^{-1}$. A 10-g aliquot of sample was weighed and 1.0 g of anhydrous sodium sulfate was added, followed by 20 mL of methanol. The mixture was ultrasonicated for 20 min and was then centrifuged for 15 min at 3000 ×*g*. The supernatants were collected and diluted in PBS containing 10% methanol prior to ELISA. The ELISA results for carbaryl were validated with a GC method.

## RESULTS AND DISCUSSION

### Selection of anti-carbaryl phage-VHH

The size of the constructed library is *ca* $1 \times 10^8$ CFU $mL^{-1}$ as calculated according to the number of clones grown on the plate. The VHH gene insert ratio was estimated to be 100% on the basis of colony PCR performed on 30 randomly selected single clones. Different VHH gene sequences were identified in these clones (data not shown), indicating the high diversity of the constructed library.

Among the designed haptens (Fig. 1), CBR1, which preserves the carbamate group, has a structure similar to that of carbaryl and, therefore, was used as both immunizing and panning hapten to generate VHHs for this pesticide. In an attempt to select clones with high affinity for carbaryl, the concentration of both panning antigen CBR1-BSA and competitor carbaryl was decreased in each round of panning. The titers of output phage gradually increased with the panning process (Supporting Information, Table S1), indicative of the enrichment of phage particles binding to carbaryl. After the fourth round of panning, 96 clones were randomly selected from the plate for identification of positive clones. In total, 21 clones showing high binding affinity for CBR1-BSA (OD > 1.5) in the absence of carbaryl and strong inhibition (>50%) in the presence of 500 ng $mL^{-1}$ carbaryl were taken to be positive.

### Sequence alignment of VHHs

All 21 positive clones were sequenced and only six clones, named C1–C6, were found to possess a unique sequence (Fig. 2). The main feature of VHHs that distinguishes them from VHs is the occurrence of amino acids F, E, R, and F in the conserved region FR2 at positions 37, 44, 45, and 47, respectively. The conserved regions FR1, FR2, FR3,and FR4 were highly similar. In the hypervariable region,CDR3, C1, C2, C4, C5, and C6 possess 17 amino acids, while C3 has four less amino acids. The sequencing results indicated that C2 was a derivative of the subclass IgG3 while the others were derivatives of subclass IgG2.

### Selection of VHH and coating antigen pairs

In competitive immunoassays for small molecules, it is possible to significantly improve the sensitivity by using the competing hapten with a structure different from that of the immunizing hapten.[35] In the present study, seven heterologous haptens (CBR2–CBR8) with variations in linker structure and length were employed to improve the VHH-based ELISA. The VHHs C1–C4 could recognize almost all of the eight haptens, whereas C5 and C6 recognized less than half of them (Table 1). The pairs showing good recognition were then employed for full competitive ELISAs and the binding capacity of VHHs to carbaryl varied significantly, while pairing with different coating haptens (Table 1). In general, all VHHs demonstrated higher sensitivity to carbaryl in heterologous, as opposed to homologous, assays. Each VHH showed the highest sensitivity with heterologous pairs, i.e. C1/CBR2-BSA, C2/CBR6-BSA, C3/CBR7-BSA, C4/CBR3-BSA, C5/CBR4-BSA and C6/CBR2-BSA (Table 1, in bold). Among all heterologous assays, the C1/CBR2-BSA ELISA displayed the lowest $IC_{50}$ value (8.1 ng $mL^{-1}$), approximately 23-fold lower than the homologous C1/CBR1-BSA ELISA ($IC_{50}$ = 189 ng $mL^{-1}$). In comparison with CBR1, CBR2 has an $OCOCH_2$ substituent for OCONH and is two carbons shorter in the linker, which drastically minimizes the binding affinity of C1 for CBR2 and thereby improves the sensitivity of the assay to carbaryl. Thus, the heterologous pair of C1/CBR2-BSA was used in the rest of this study. After expression and purification, the VHH C1 was analyzed, using sodium dodecyl sulfate–polyacrylamide gel electrophoresis, which revealed one major band with an expected size of *ca*15 kD (Supporting Information, Fig. S1).

### Optimization of VHH-based ELISA for carbaryl

Buffer pH might affect the binding affinity of VHH by disturbing hydrogen bonding, surface charge, and paratope. Within the pH range of 4.5–8.5, the $IC_{50}$ of carbaryl determined with the C1/CBR2-BSA ELISA varied in the range of 6.3–15.4 ng $mL^{-1}$ [Fig. 3(a)]. The isoelectric point (pI) of the VHH C1 was estimated to be 8.0, as deduced from the protein sequence using DNAMAN software. When the assay was performed at pH 7.5–8.5, i.e., close to the pI value, some antibodies may have precipitated, and low sensitivity was observed ($IC_{50}$ = 8.4–13.9 ng $mL^{-1}$). In the buffer with a pH > 9.5, the maximum signal ($A_0$) declined sharply and an abnormal standard curve was formed. The highest sensitivity was obtained at pH 6.5 ($IC_{50}$ = 6.3 ng $mL^{-1}$), i.e., in a weakly acidic medium in which both carbaryl and antibody are stable. The binding affinity of the VHH C1 for carbaryl was dramatically reduced when the content of NaCl was higher than 0.8% [Fig. 3(b)]. Presumably, at high ionic strength, binding to the charged groups of epitopes or paratopes is

impeded to a great extent, thereby obstructing their combination and leading to a significant decrease in sensitivity.[37] A low concentration of methanol is commonly used to solubilize lipophilic compounds (e.g., carbaryl) in immunoassays. On the other hand, methanol at a high concentration may destroy the van der Waals and hydrophobic forces in play between antigen and antibody, thereby leading to the disintegration of the immunocomplexes. This assay demonstrated the highest sensitivity to carbaryl ($IC_{50}$ = 5.9 ng $mL^{-1}$) in the buffer containing 10% methanol ([Fig. 3(c)].

A typical calibration curve of the competitive C1/CBR2-BSA ELISA for carbaryl was generated using PBS (pH 6.5) containing 10% methanol and 0.8% NaCl (Fig. 4). The assay has a linear range of 0.8–38 ng $mL^{-1}$ ($IC_{20}$ −$IC_{80}$), an $IC_{50}$ of 5.4 ng $mL^{-1}$, and an LOD of 0.3 ng $mL^{-1}$. The sensitivity of the VHH-based ELISA for carbaryl is lower than those of conventional antibody-based ELISAs[38,39] because VHHs, unlike conventional antibodies, do not have the ideal surface, i.e., a pocket in which a small molecule can be bound with high affinity.[40] Additionally, VHHs are more suitable for binding to larger molecules (e.g., proteins) than for binding to haptens because the long CDR3 allows VHH interaction with concave epitopes and cryptic sites in protein antigens.[41] Despite the lower sensitivity, this assay is promising for detection of carbaryl in cereals at levels below its MRL of 1.0 mg $kg^{-1}$, as set in China (GB 2736–2016).

## Selectivity of VHH

Selectivity of the VHH C1 was evaluated by comparing the $IC_{50}$ of carbaryl with that of carbaryl structural analogs using the C1/CBR2-BSA ELISA. With the exception of the carbamate group of the tested insecticides, the remaining parts of their molecular structure are largely different from that of carbaryl (Table 2). Thus, CR between C1 and these compounds is low (≤0.8%). The substitution of the carbamate group with a hydroxyl group may result in a significant change in chemical features, resulting in negligible CR with the main metabolite 1-naphthol (0.1%). In the present study, selectivity of the VHH C1 was similar to that of an mAb, probably because both conventional antibodies and HCAbs predominately responded to the same epitope of the hapten CBR1. The high specificity of VHH C1 allows for the selective detection of carbaryl in complicated matrices, avoiding the interference of analogs, especially the main metabolite 1-naphthol.

## Sample analysis

The ELISA developed here was applied to the detection of carbaryl in spiked cereals and the accuracy and precision of the assay were evaluated. The interaction of antibody and antigen is susceptible to matrix interference, which may reduce the sensitivity and reliability of immunoassays. Dilution proved to be a simple and effective method to eliminate the matrix effect of samples on immunoassay results.[42] Generally, the overlap of assay curves constructed using PBS-diluted blank sample extracts with that constructed using PBS is indicative of the minimum matrix effect. For the proposed ELISA, at least a 60-fold dilution in PBS containing 10% (v/v) methanol was needed to eliminate the matrix effects of rice and maize extracts to yield a linear range of 96–4560 ng $g^{-1}$ and an LOD of 36 ng $g^{-1}$ for carbaryl, while at least a 120-fold dilution was required for wheat extracts to yield a linear

range of 192–9120 ng $g^{-1}$ and an LOD of 72 ng $g^{-1}$ for carbaryl (Supporting Information, Fig. S2).

The levels of carbaryl in various spiked cereals (200, 1000, and 2000 ng $g^{-1}$) were all fitted in the linear ranges. Repeatability and intra-laboratory reproducibility were determined as intra-day and inter-day precisions, respectively, by analyzing carbaryl in the spiked samples (Table 3). From the results obtained, the relative standard deviations under repeatability conditions ($RSD_r$) and intra-laboratory reproducibility conditions ($RSD_R$), as well as recovery, were calculated for each matrix according to the International Organization for Standardization (ISO) guidelines.[43] The average recoveries of carbaryl from rice, wheat, and maize were 81–106%, 96–106%, and 83–113%, respectively (Table 3). The $RSD_r$ for repeatability and the $RSD_R$ for within-laboratory reproducibility were in the range of 0.8–9.2% and 2.9–9.7%, respectively (Table 3).

In a separate series of experiments, a GC method was employed for the detection of carbaryl in the same spiked samples as above and the average recoveries of carbaryl from rice, wheat, and maize were found to be 87–97%, 96–105%, and 88–103%, respectively (Supporting information, Table S2). Both methods showed good recoveries and correspondence concerning detection of carbaryl in cereal samples. This assay was applied to real-world cereals including five rice, five maize, and six wheat samples collected from local markets in Beijing, and the levels of carbaryl were all found to be below the LOD, probably due to the decreasing usage of carbaryl in staple food production in most regions of China.

## CONCLUSIONS

This study describes the generation of VHHs specific for carbaryl and the development of a VHH-based ELISA for carbaryl detection in cereals. Six alpaca-derived VHHs with varying carbaryl-binding capacities were obtained from a diverse library and an optimal ELISA for this pesticide was based on the heterologous pair of the VHH C1/CBR2-BSA ($IC_{50}$ = 5.4 ng $mL^{-1}$). The sensitivity of the VHH-based ELISA was lower than that of a previous mAb-based ELISA, but it is considered to be sufficient for the detection of carbaryl below the MRL (1.0 mg $kg^{-1}$) in cereals. The satisfactory recovery, repeatability and reproducibility of this ELISA for carbaryl in cereal samples, after simple extraction and dilution, make it a promising method for the quantification of pesticide in surveys of food safety and, perhaps, in environmental pollution.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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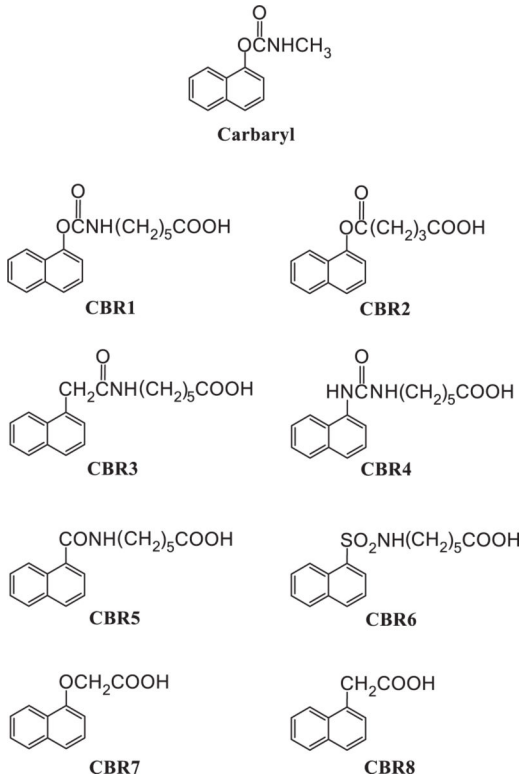



**Figure 1.**
Structures of carbaryl and the haptens. The conjugate of CBR1 with KLH was used as the immunization antigen.

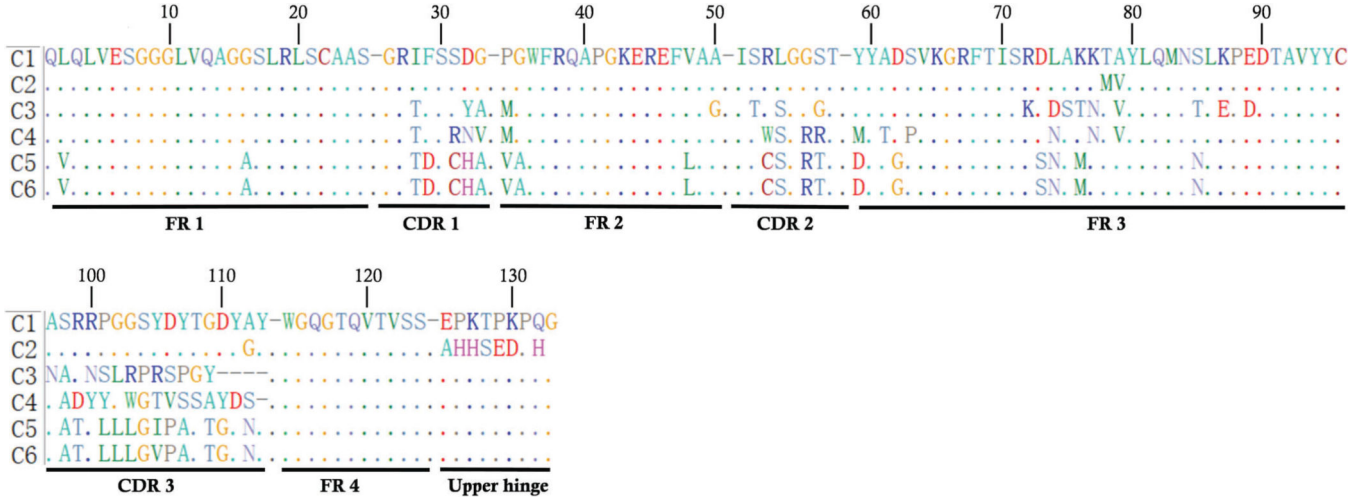



**Figure 2.**

The amino acid sequences of VHHs C1–C6. The dots indicate amino acid residues identical to those in VHH C1.

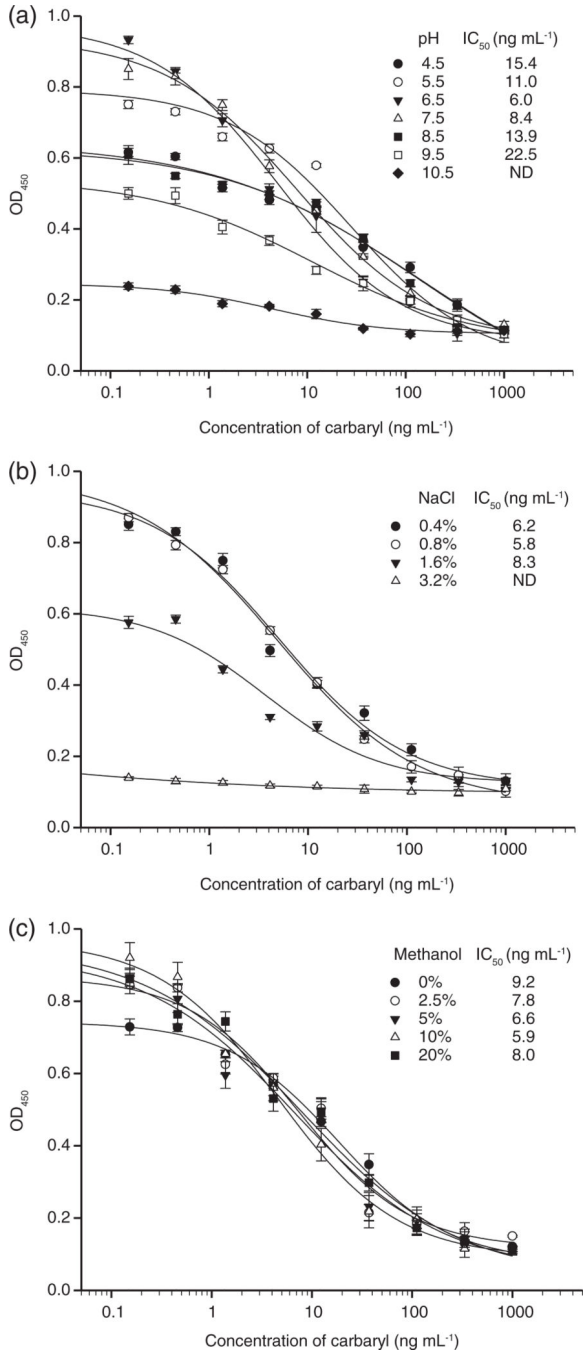



**Figure 3.**

Effect of pH (a), concentration of NaCl (b) and methanol (c) on the VHH-based ELISA for carbaryl. ND: not detectable.

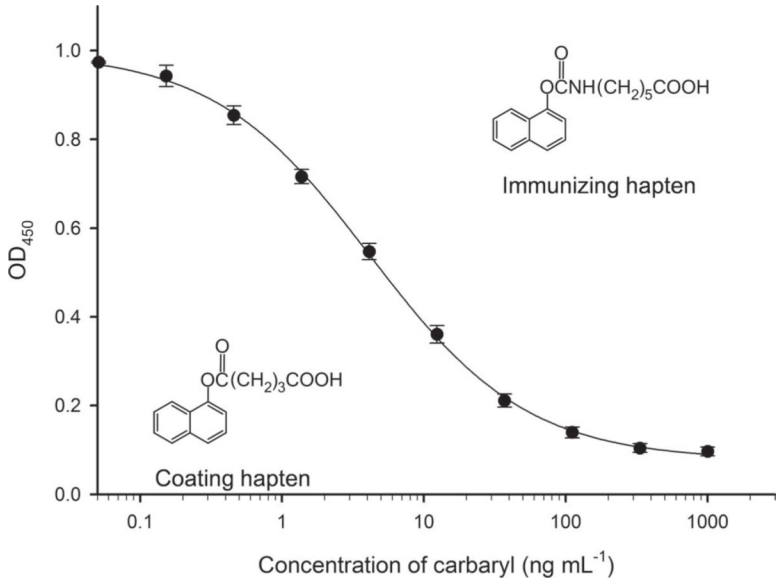



**Figure 4.**

Calibration curve of the C1/CBR2-BSA ELISA for carbaryl. Each value is the average of three replicates, with the standard deviations given.

**Table 1.**

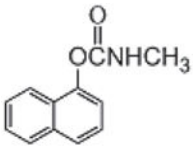
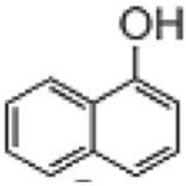
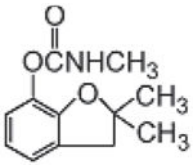
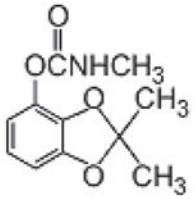
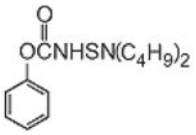
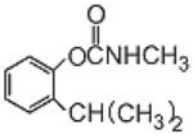
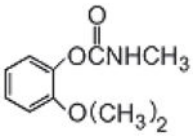
Sensitivity of ELISAs based on different combinations of VHH and coating antigen

| | $IC_{50}$ (mean ± SD) (ng $mL^{-1}$)[a], n = 3 | | | | | |
|---|---|---|---|---|---|---|
| **Coating antigen** | **C1** | **C2** | **C3** | **C4** | **C5** | **C6** |
| CBR1-BSA | 189 ± 4.0 | 238 ± 3 | 56 ± 0.6 | 37 ± 0. 6 | 302 ± 0.6 | 256 ± 0.9 |
| CBR2-BSA | **8.1 ± 0.1** | 68 ± 1.4 | 39 ± 0.7 | 103 ± 0.3 | 156 ± 0.4 | **205 ± 0.3** |
| CBR3-BSA | 68 ± 1.2 | 79 ± 0.4 | 30 ± 0.1 | **22 ± 0.8** | 227 ± 0.4 | 357 ± 0.9 |
| CBR4-BSA | 83 ± 3.2 | 167 ± 0.8 | 68 ± 0.2 | 90 ± 0.2 | **115 ± 0.8** | ND |
| CBR5-BSA | 34 ± 0.4 | 47 ± 0.2 | 72 ± 0.3 | 67 ± 0.7 | ND | ND |
| CBR6-BSA | 15 ± 0.8 | **26 ± 0.7** | 25 ± 0.3 | ND | ND | ND |
| CBR7-BSA | 70 ± 0.4 | 54 ± 0.9 | **23 ± 0.4** | 56 ± 0.3 | ND | ND |
| CBR8-BSA | 67 ± 0.6 | 59 ± 0.3 | 110 ± 0.2 | 108 ± 0.7 | ND | ND |

[a] $IC_{50}$, the values were obtained at ODs > 0.6 but could not be determined (ND) at ODs < 0.3.

**Table 2.**

Cross-reactivity between VHH C1 and carbaryl structural analogs

| Compound | Structure | Cross-reactivity (%) |
|---|---|---|
| Carbaryl |  | 100 |
| 1-Naphthol |  | 0.1 |
| Carbofuran |  | 0.4 |
| Bendiocarb |  | 0.8 |
| Carbosulfan |  | <0.1 |
| Isoprocarb |  | 0.1 |
| Propoxur |  | 0.4 |

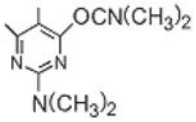
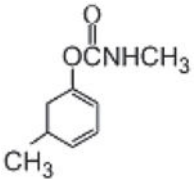
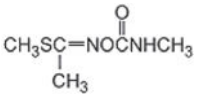
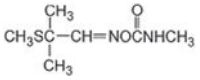
| Compound | Structure | Cross-reactivity (%) |
|---|---|---|
| Pirimicarb |  | <0.1 |
| Methylcarb |  | 0.4 |
| Methomyl |  | <0.1 |
| Aldicarb |  | <0.1 |
| Propamocarb |  | <0.1 |

**Table 3.**

Recovery, repeatability, and intra-laboratory reproducibility of ELISA for carbaryl in cereal samples

| Sample | Spike level (ng $g^{-1}$) | Average recovery (%) | $RSD_r$(%)[a] | $RSD_R$(%)[b] |
|---|---|---|---|---|
| Rice | 0 | ND[c] | | |
| | 200 | 98 | 9.2 | 9.7 |
| | 1000 | 106 | 1.3 | 3.9 |
| | 2000 | 81 | 1.6 | 5.4 |
| Maize | 0 | ND | | |
| | 200 | 106 | 4.1 | 5.7 |
| | 1000 | 96 | 2.2 | 4.6 |
| | 2000 | 106 | 0.8 | 5.9 |
| Wheat | 0 | ND | | |
| | 200 | 91 | 8.6 | 9.0 |
| | 1000 | 113 | 4.1 | 5.6 |
| | 2000 | 83 | 3.8 | 2.9 |

[a] $RSD_r$ for repeatability was determined by three extractions and analyses within a day.

[b] $RSD_R$ for intra-laboratory reproducibility was determined on three consecutive days (three extractions and analyses each day).

[c] Not detectable.