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Research Article

Immunofluorescence assay of pesticides on the base of immobilized multi-polyclonal antibody

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Abstract

The sensitive competitive immunofluorescence method for simultaneous determination of paraoxon and dichlorvos with immobilized multi-polyclonal antibody on magnetic nanoparticles was developed. The multi-polyclonal antibody was obtained after the immunization of mixture of two prepared immunogens dichlorvos-cBSA and paraoxon-BSA (1:1). The immunogens dichlorvos-cBSA and paraoxon-BSA were synthesized preliminary. Multi-polyclonal antibody against dichlorvos and paraoxon was covalently coupled on magnetic nanoparticles. The competitive fluorescence conjugates dichlorvos-cBSA-FITC and paraoxon-BSA-ATTO 620 were synthesized. Two typical calibration curves of immunofluorescence assay for determination of dichlorvos and paraoxon in buffer solutions were obtained. The linear interval from 2 to 200 ng.mL^{-1} for these two pesticides was determined. Then the calibration curves for dichlorvos and paraoxon were obtained in cow milk solutions. The linear range of pesticides in cow milk was determined (from 5 to 300 ng.L^{-1}) and the detection limit for paraoxon (3.5 ng.mL^{-1}) and dichlorvos (4 ng.mL^{-1}) was found. The obtained results for cow milk samples were compare with results in UHT, pasteurized cow milk, sheep and goal milk. Quite different are the results when analyzing paraoxon and dichlorvos in standard solutions prepared in sheep's milk. The linear working range for the two pesticides is between 7 and 300 ng.mL^{-1} .It is obviously, that there is a shift of all analytical characteristics up to higher values. The reason for this is the high fat content of sheep's milk. The obtained results were showed that the developed method was 2 time more sensitive than method with the results obtained with mixture (1:1) of two separated antibodies – anti-paraoxon and anti-dichlorvos. These results confirmed the potential of the immunoassay for quantitative simultaneous screening of both dichlorvos and paraoxon. Practical applications: The use of immunofluorescence assay based on immobilized multi-polyclonal antibody on magnetic nanoparticles allows us to detect simultaneous paraoxon and dichlorovos in raw milk and other dairy products

Keywords: dichlorvos, paraoxon, multi-polyclonal antibody, immunofluorescence assay, magnetic nanoparticles

Abbreviations:

APTES - 3-Aminopropyl) triethoxysilane
BSA - bovine serum albumin
cBSA - cationized bovine serum albumin
DDVP - dichlorvos
ELISA - enzyme-linked immunosorbent assay
FITC – Fluorescein isothiocyanate
PBS - phosphate buffer saline
TEM - Transmission electron microscopy
UHT milk - Ultra-high temperature processing milk
UV/Vis spectrophotometer

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Introduction

The pesticide residues can be spread in milk, milk products and other food (Bentabo and Jordal, 1995; Willes et al. 1993; Fei et al. 2015). Nowadays the organophosphorus pesticides are among the most widely employed pesticides. The pesticides are very toxic because they are inhibitors of cholinesterase enzymes involved in muscle fibre ablation and pulse transfer in the nervous system. It is necessary to carry out continuous control of pesticide residues in food. Immunological methods using monoclonal or polyclonal antibodies are one of these alternatives (Szurdoki et al. 1995, Li et al. 2009; Limei et al. 2008). There are many ELISA for determination of low concentrations of pesticides. However, these methods are slow; requiring repeated washing, measured separated pesticides, independently, one at a time. Fluorescence detection immunoassays have the greatest utility for determining a toxic compound since they have several major advantages such as rapid analysis, signal amplification, determination of more than one pesticides in the same sample, and subsequently potentially increased sensitivity of the method (Biagini et al. 2002). The choice of solid phase is important to enhance the sensitivity in heterogeneous immunoassays. The use of magnetic nanoparticles as a solid phase is very suitable. It has been reported that the use of magnetic nanoparticles may assist in minimizing matrix interferences, reduce the immobilization time of antibodies on magnetic nanoparticles to 2.5 h and a comfortable separation of bound and free fraction of the tracer (Yamaura et al. 2004). Current trends are to use an assay to identify more than one toxin at the same sample (Spinks 2000). To achieve this, it is possible to increase the number of antibodies that recognize individual pesticides in the same sample. Typically, multi-immunoassays are developed using a combination of several antibodies and several competitive conjugates with different fluorescent dyes. Appropriate fluorescent dyes are selected without risk of overlapping wavelengths. A more economical alternative, however, is to create a single multi antibody that has a broad specificity and which is able to identify several analytes in a same sample. For this purpose in this work a multi-polyclonal antibody was prepared by injecting a mixture of immunogens DDVP-cBSA and paroxone-BSA in a ratio of 1: 1. The resulting multi-polyclonal antibody was immobilized onto magnetic nanoparticles. The immobilized antibody used to develop a simultaneous analysis of dichlorvos and paroxon in the same sample. Two fluorescence conjugates DDVP-cBSA-FITC and paroxone-BSA-ATTO, labelled with two different fluorescent dyes were prepared. The main objective of this work was to develop a simple, fast, inexpensive, selective and sensitive fluorescent immunoassay based on immobilized multi-polyclonal antibody onto magnetic nanoparticles for simultaneous determination of paraoxon and dichlorvos in the same milk sample.

Materials and Methods

Materials

Raw materials. For the preparation the fluorescent conjugates were used dimethylformamide, fluorescein 5 (6) -isothiocyanate, isomer I, 98% and ATTO 620 NHS purchased from Sigma-Aldrich (Taufkirchen, Germany). For purification of fluorescent conjugates were used Sephadex G-25 from GE Healthcare (Sweden). Bovine serum albumin (BSA), Bradford reagent, Tween 20 and APTES were purchased from Sigma-Aldrich (Taufkirchen, Germany). Glutardialdehyde 25% were supplied from Merck. Buffers were prepared according to standard laboratory procedure. All solutions were prepared using deionized water (ELGA, Buckinghamshire, UK).

Improvers. The aim of the studying is to develop multi-immunofluorescent alternative method of chromatographic methods for analysis and control of various toxic components in food and water, in order to ensure food safety. This method has great advantages over other instrumental methods that are used in this field - sensitive, accurate, selective, fast, inexpensive, suitable for screening tests and for the simultaneous determination of several components in one sample, suitable for the development of portable apparatus for measuring samples on site.

The possibility of simultaneously determining several components in the same sample is a significant advantage to conduct rapid screening. Immunoassays are quick, easy, suitable for screening and for creation of portable devices for conduction of analyses on-site. Low molecular weight compounds such as pesticides occur in food products. Residues of such compounds with low molecular weight in food are harmful to human health. Therefore, conducting quantitative analysis of such compounds with low molecular weight is essential to ensure human health, preventing their illegal use, as well facilitation of government regulation and monitoring.

Instrumentation. The ultraviolet–visible spectra of hapten-conjugates were recorded by Spectrophotometer 6900 (Jenway, England) in wavelength interval from 200 to 500 nm. Infrared spectra of hapten-conjugates were measured by Fourier transformation infrared spectrophotometer Tenzor 27-Bruker, Germany. The fluorescence intensity in immunoassay was detected by a Perkin Elmer LS 45 fluorescence spectrophotometer (USA).

Immunization of immunogens and purification of multi-polyclonal antibody. All animal experiments were performed in accordance with EU Directive 2010/63/EU for animal experiments. Three sheep were immunized with 1:1 dichlorvos–cationized bovine serum albumin and paraoxon–bovine serum albumin as immunogens. The preparation of immunogens (paraoxon-bovine serum albumin conjugate and dichlorvos-cationized bovine serum albumin conjugate) were carried out by methods described in our early paper (Yaneva et al. 2017). A total of 0.5 mg of the immunogen mixture dissolved in 0.5 mL of physiological solution was emulsified with Freund's complete adjuvant in a 1:1 volume ratio and injected intradermally at multiple sites on the back of each animal. Two weeks after the initial injection, each animal received booster injections three times at 2-week intervals with an additional 0.3 mg of the corresponding conjugate emulsified with Freund's incomplete adjuvant. The last dose was 0.2 mg of the conjugate emulsified with Freund's incomplete adjuvant. The collected whole blood were purified by affinity chromatography AKTAprime Plus (GE Healthcare, Uppsala, Sweden, column HiTrap Protein G HP) 210 and by column HiTrap Desalting (5 mL) for buffer exchange. The anti-body solution was dried under vacuum at −70°C to obtain the antibody powder.

MNPs-aminosilane obtaining and characterizing

Magnetic nanoparticles (MNPs) were obtained in our laboratory by methods described in paper (Gabrovska et al. 2013) with a chemical reactor (from Sirius Company). Fe_3O_4 nanoparticles were modified with aminosilane for formation of amino groups onto the MNPs (Ivanova and Godjevargova 2015). The average diameter of uncoated and aminosilane coated MNPs was determined by transmission electron microscopy (TEM) (JEM-1400 PLUS).

Immobilization of multi-polyclonal antibody onto the magnetic nanoparticles. The immobilization of multi-polyclonal antibody was carried out by covalent method. 5 mg of the magnetic nanoparticles (MNPs) functionalized with APTES were activated with glutardialdehyde. The nanoparticles were collected with a magnet, the solution was taken out and the nanoparticles were re-suspended in 1 ml glutardialdehyde (5 % w/v) in 50 mM PBS, pH 8 and were incubated for 2 hours at room temperature. Then the nanoparticles were washed once with 50 mM PBS pH 8 and five more times with 10 mM PBS pH 7.4. The MNPs were re-suspended in 1.0 mL 10 mM PBS pH 7.4 containing 1 $mg.mL^{-1}$ multi-polyclonal antibody and were incubated for 2 hours at 37°C. After that the nanoparticles were washed three times with 10 mM PBS pH 7.4 and the free active NH_2 – groups were blocked by addition of 10 mM PBS pH 7.4 containing 1 % BSA and 0.05 % Tween 20. The antibody - nanoparticles were stirred for one hour at room temperature, washed three times and re-suspended in the same buffer to final concentration 5 $mg.mL^{-1}$. The efficiency of the coupling strategies was evaluated by analyzing the antibody concentration in the supernatant before and after the coupling by the Bradford method (Bradford 1976).

Preparation of FITC labelled DDVP-cBSA conjugate. Fluorescein 5(6)-isothiocyanate (FITC), as a fluorescent marker, was used. DDVP-cBSA conjugate (15 mg) was dissolved in deionized water (1.5 mL). Then 500.0 μL FITC solution (2 $mg.mL^{-1}$ in dimethylformamide) were added slowly and reaction mixture was incubated overnight at 4 ºC. The labelling was carried out in brown vial to prevent photo-degradation of the fluorescent compound. The obtained conjugate (DDVP-cBSA-FITC) was purified by gel filtration through Sephadex G-25 column (1.5 x 30cm). The column was equilibrated with PBS (10 mM pH 7.4). The column flow rate was 1 mL/min. The fractions of 2.0 mL were collected. The absorbance was measured, using a Jenway 6900 UV/Vis spectrophotometer. Spectrophotometry was used to evaluate the composition of the conjugate. The resulting product was lyophilized and stored at -20 °C.

Preparation of ATTO labelled Paraoxon-BSA conjugate. Paraoxon-BSA conjugate (10mg) was dissolved in 250.0 μL 0.1 M sodium bicarbonate buffer with pH 8.3. 0.5 mg ATTO 620 NHS was dissolved in 50.0 μL dimethylformamide. ATTO 620 NHS was added drop-wise to the first solution, while constantly stirring. The solution left for 1 hour with continuous stirring at room temperature and finally left overnight at 4° C. After the incubation time elapsed, the conjugate was purified from unreacted compounds by gel chromatography. Sephadex G-25 and 10 mM PBS, pH 7.4 were used. The resulted product was lyophilized and stored at -20° C.

Determination of the optimum concentrations of conjugates and immobilized antibodies for immunofluorescence assay. Four different concentrations of the DDVP-cBSA-FITC and paroxon-BSA-FITC conjugates (1.5, 3, 7.5 and 12 $\mu g.mL^{-1}$) were used. Each concentration of the conjugate is combined with four different concentrations of Ab-MNPs - 0.125, 0.25, 0.375 and 0.5 mg. Each sample of antibody-magnetic nanoparticles described above was placed in a micro centrifuge tube. Then, 67 μL of the antigen (50 $\mu g.mL^{-1}$) was added to it and 135 μL of each concentration of the conjugate described above was added to the samples. The incubation step is followed for 15 min at 37° C. The particles were collected with a magnet, the residual fluorescence intensity in supernatant was measured by Perkin Elmer LS 45 fluorescence spectrophotometer.

Immunofluorescence assay with immobilized multi-polyclonal antibody on MNPs. 75 μL of 5 $mg.mL^{-1}$ Ab-MNPs (multi-polyclonal antibody) were transferred to tube and collected by a magnet. The supernatant was pipetted. 67 μL of antigen (DDVP and paraoxon) with concentration (0-300 $ng.mL^{-1}$ in various type of milk) were added to the Ab-MNPs and incubated for 15 min at 37˚C. Then a 135 μL of each conjugate solutions, paraoxon-BSA-ATTO (22 $\mu g.mL^{-1}$) and DDVP-cBSA-FITC (18 $\mu g.mL^{-1}$) were added to MNPs-Ab and incubated for 15 min at 37˚C. Both antigen in the sample and fluorescent conjugate competed for the immobilized antibody. After the incubation, the MNPs-Ab-pesticides were separated from the supernatant on the side wall of the micro-centrifuge tube by a magnet. The clear supernatant, containing unbound tracer, was separated. The fluorescence signal of this supernatant was measured by Perkin Elmer LS 45 fluorescence spectrophotometer at 494/518 nm (for FITC marker) and 620/642nm (for ATTO marker). Experimental signals were normalized using the following expression:

$$\text{Normalized signal} = (B_0 - B)/(B_0 - Bx).100, \%$$

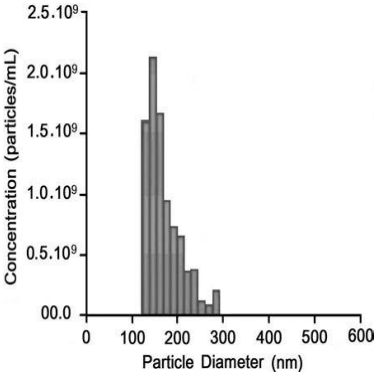
where B is the signal (intensity of fluorescence) measured in the presence of the increasing analyte concentrations; Bx is the signal in absence of pesticides; and B_0 is the signal of the initial conjugate solution.

Preparation of buffer and milk standards. Pesticides (DDVP and paraoxon) working standards containing 0, 1.0, 5.0, 50.0, 100.0, 150.0, 200.0, 250.0 and 300.0 $ng.mL^{-1}$ were prepared from a 1 $mg.mL^{-1}$ stock solution by dilution with the appropriate volume of 10 mM PBS (pH 7.4). Fresh full-cream cow's, goat's and sheep's milk (pesticide-free milk) were used for preparation of standard milk solutions. Pesticide concentrations in milk were the same as in the buffer standard solutions. Three types of cow milk UHT, raw milk and pasteurized cow milk were employed for

immunoassay validation and to obtain the pesticide calibration curves. It should be noted that it was not necessary to perform any sample pretreatment to the milk samples.

Results and Discussion

Typically, multi-immunoassays are being developed using a combination of several antibodies and several competitive conjugates with different fluorescent dyes. Appropriate fluorescent dyes are selected without risk of overlapping wavelengths. Current trends are a technique to be used to identify more than one pesticide at the same time (Spinks 2000). To achieve this, it is possible to increase the number of antibodies that recognize individual pesticides that are included in a single test. A more economical alternative, however, is to create a single multi-antibody which has a broad specificity and which is able to identify several analytes in a single test For this purpose, a multi-polyclonal antibody was obtained by injecting a mixture of immunogens DDVP-cBSA and paroxon-BSA in a ratio of 1: 1 (in section 1.3). Multi-polyclonal antibody against dichlorvos and paraoxon was covalently coupled on magnetic nanoparticles. The magnetic nanoparticles (Fe_3O_4) were synthesized by a wet chemical method through the co-precipitation of Fe_{2+} and Fe_{3+} aqueous salt solutions. They were modified by 3-(aminopropyl) triethoxysilane as the coating material to introduce amino groups (Smith et al. 2008; Wu et al. 2007). The sizes of initial and functionalized magnetic nanoparticles were determined by IZON qNano particle analyzer (England), respectively 182 and 292.5 nm (Fig. 1).



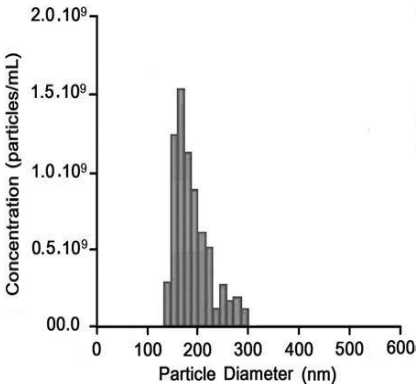


Figure 1. Size distribution of unmodified and modified magnetic nanoparticles.

The nanoparticles' size with immobilized antibody (292.5 nm) allows the possibility of handling the particles in suspension. The simple collection of magnetic particles by a permanent magnet allows separating bound and free fraction of the tracer. Multi-polyclonal antibody against dichlorvos and paraoxon was covalently coupled to the functionalized magnetic nanoparticles with amino groups by random immobilization with glutaraldehyde. The efficiency of the coupling was evaluated using the Bradford method (Bradford 1976). It was found that the amount of immobilized multi-polyclonal antibody on MNPs was 0.023 mg Ab per mg magnetic nanoparticles. The resulting immobilized multi-polyclonal antibody was applied to develop a simultaneous analysis of dichlorvos and paraoxon. Two fluorescent labelled conjugates with two different fluorescent dyes - DDVP-cBSA-FITC and paraoxon-BSA-ATO were synthesized and used. The calibration curves of immunoassay with multi-polyclonal antibody in raw cow's milk and buffer solution, Figure 2, were investigated. Optimal concentrations of the DDVP-cBSA-FITC and paroxon-BSA-ATTO conjugates and the studied multi-antibody, respectively, were determined by variation of predefined, respectively DDVP-cBSA-FITC - 18 $\mu g.mL^{-1}$, paraoxon-BSA-ATTO 620 – 22 $\mu g.mL^{-1}$ and antibody-magnetic nanoparticles - 0.375 mg. Immunofluorescence analyses were performed with a multi-polyclonal antibody for the simultaneous determination of paraoxon and dichlorvos in pasteurized milk and in UHT milk.

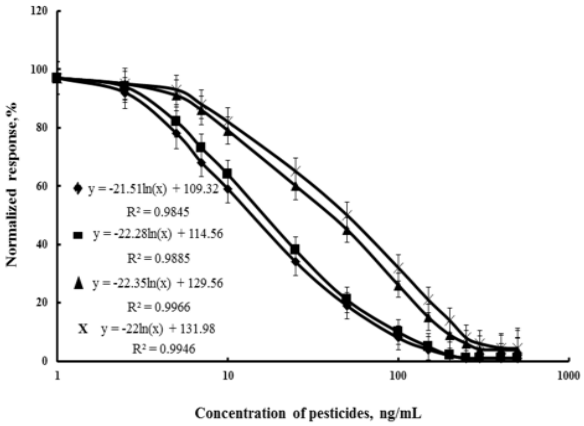


Figure 2. Calibration curves of an immunofluorescence assay with immobilized multi-polyclonal antibody on MNPs for determination of paraoxon (♦); dichlorvos (■) in 10 mM PBS pH 7.4 and paraoxon (▲), dichlorvos (x) in cow milk. Each point represents the average of five experiments, CV = 4-7%.

Table 1. Analytical Characteristics of Immune-Fluorescence Assay with Immobilized Multi-polyclonal Antibody on MNPs for Determination of Paraoxon and Dichlorvos in Cow Milk, Buffer, UHT, Pasteurized Milk

Milk	**Paraoxon**			**Dichlorvos**		
	IC50, ng/mL	Linear range, ng/mL	Detection limit, ng/mL	IC50, ng/mL	Linear range, ng/mL	Detection limit, ng/mL
Cow milk	43	5-300	3.5	45	5-300	4.00
Buffer	28	2-200	0.95	30	2-200	1.00
Pasteurized milk	35	3-300	1.50	38	3-300	1.70
UHT milk	33	2.5-250	1.30	35	2.5-250	1.50

Table 2. Analytical Characteristics of Immune-Fluorescence Assay with Immobilized Multi-polyclonal Antibody on MNPs for Determination of Paraoxon and Dichlorvos in Cow Milk, Cow, Goat and Sheep Milk

Milk	**Paraoxon**			**Dichlorvos**		
	IC50, ng/mL	Linear range, ng/mL	Detection limit, ng/mL	IC50, ng/mL	Linear range, ng/mL	Detection limit, ng/mL
Cow milk	43	5-300	3.5	45	5-300	4.0
Goat milk	39	3.5-300	2.5	41	3.5-300	2.7
Sheep milk	49	7-300	5.0	58	7-300	5.5

From Table 1 it is obvious that the analytical characteristics of the paraoxon and dichlorovos immunoassays performed in two kinds of milk have intermediate values between those obtained in buffer and those in cow's milk. The reason for this is that the matrix effect of these milks is weaker since they have been heat-treated and have lower fat content (1.5%) than fat content in cow milk (3.4%). The results obtained from the immunoassays for the two pesticides in pasteurized milk and UHT milk were very similar (Table 1). Quite different are the results when analyzing paraoxon and dichlorvos in standard solutions prepared in sheep's milk. Table 2 shows that in sheep's milk the IC50 value for paraoxon was 49 ng/mL and the detection limit was 5.0 $ng.mL^{-1}$. Respectively IC50 for DDVP was 58 $ng.mL^{-1}$ and the detection limit was 5.5 $ng.mL^{-1}$. The linear working range for the two pesticides is between 7 and 300 $ng.mL^{-1}$. It is obviously, that there is a shift of all analytical characteristics up to higher values. The reason for this is the high fat content of sheep's milk (7.8 %) as compared to the cow's milk fat content (3.8 %). The analytical characteristics of the immunoassays for paraoxon and dichlorvos in goat milk have intermediate values between those in sheep's and cow's milk. Table. 2 shows that for the paraoxon the IC50 is 39 $ng.mL^{-1}$, the detection limit is 2.5 $ng.mL^{-1}$ and for the DDVP - IC50 is 41 ng/mL and the detection limit is 2.7 $ng.mL^{-1}$. The linear operational interval for both pesticides in goat milk is between 3.5 and 300 $ng.mL^{-1}$.

Conclusions

The sensitive and rapid immunofluorescence analysis was developed with a multi-polyclonal antibody for the simultaneous determination of paraoxon and dichlorvos in cow milk. The linear range of pesticides in cow milk was determined (from 5 to 300 ng/L) and the

detection limit for paraoxon (3.5 ng/mL) and for dichlorvos (4 ng/mL) was found. The developed analysis was suitable for determination of pesticides in UHT, pasteurized cow milk, sheep and goal milk.

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