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Sensitive Rapid Immunochromatographic Detection of the Herbicide Atrazine: Comparison of Labels and Assay Formats

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Abstract

Immunochromatographic methods for determination of the herbicide atrazine have been comparatively characterized. The pre-incubation of the sample and labelled antibody allowed decreasing a visual detection limit in 10 times as compared with a standard immunochromatographic test-system based on gold nanoparticles. By this way, up to 5 ng/mL of atrazine can be detected. Use of silver staining cannot improve the assay. Using alternative labels (coloured latex particles) reduced the detection limit to 2 ng/mL, the lowest value from the compared variants.

Keywords: immunochromatography; test strips; signal enhancement; nanoparticles; herbicide; atrazine

1. INTRODUCTION

Interest in food quality control has grown significantly in recent years. This has been caused by an expansion of the list of controlled compounds, changes in the maximum permissible levels for a number of contaminants, as well as by active public interest in food safety issues and the possibilities of its rapid confirmation [1].

To date, the most effective analytical approach that combines high performance and speed is immunochromatography. In carrying out immunochromatography, the contact of the test-strip with the sample initiates all subsequent interactions between the analyte and immunoreactants without requiring additional reagents or operator involvement. Due to this, the testing may be implemented directly at the sampling place for 5 to 15 minutes, and the subsequent decision may be made promptly on the basis of the assay result [2]. These advantages have led to successful commercialization and widespread use of immunochromatography in different fields of medical diagnostics [3-5].

In recent years, immunochromatography has also been used increasingly in the control of food quality as a means of monitoring all stages of the processing chain "from farm to fork". Immunochromatographic methods are applied actively for different classes of toxic contaminants in food products [1]. The current state of these methods' developments is summarized in a number of special reviews focused on the detection of mycotoxins [6], phytotoxins [6, 7], infectious agents [8], food allergens [9], etc.

Herbicides are an important class of contaminant, for which rapid control tools are required. The use of herbicides can increase crop yields and reduce labour costs for their cultivation. However, they convey toxic effects through food to humans and animals [10, 11]. In this connection, there is a need to monitor herbicides in soil, water, agricultural products and foodstuffs. Modern screening methods should provide a highly sensitive and rapid non-laboratory determination of herbicides in a variety of complex matrices.

The herbicide atrazine was selected as an investigational antigen. It is widely used to control weeds and can easily get into food. The negative effect of atrazine on humans and animals is determined mainly by its influence on endocrine system [12, 13]. The maximum residue level of atrazine in water is 10 ng/g [12]. Rapid determination of atrazine in liquid samples (drinking water, juice, milk) is important because these liquids have no need for complex sample preparation and results can be obtained in a minimum amount of time after the sampling.

The aim of this work was to develop a simple, rapid, and highly sensitive immunochromatographic analysis of atrazine. Currently a number of solutions in immunochromatography provide increased sensitivity. Unfortunately, existing publications are focused on assessment of individual solutions, whereas the comparison of different variants is practically absent in the literature [14, 15]. In this regard, this paper discusses common format of immunochromatography and three variants for reducing the detection limit of immunochromatographic assay; these are based on the use of:

1) The aggregation of silver ions on the surface of gold nanoparticle (GNPs) labels,
2) alternative label – latex particles (LPs), and
3) separating of the specific and detecting reactions by labelling of anti-species antibodies with GNPs and the sample pre-incubation with native specific antibodies.

2. MATERIALS AND METHODS

2.1. Reagents and Materials

Anti-atrazine rabbit antiserum was obtained earlier at the A. N. Bach Institute of Biochemistry (Moscow, Russia), as described in [16]. Sheep anti-rabbit immunoglobulins were from Imtek (Moscow, Russia). Goat anti-rabbit immunoglobulin G (GARI) were from Arista Biologicals (Allentown, USA).

Gold chloride, N-(4-dimethylaminopropyl)-N′-ethylcarbodiimide (EDC), N-hydroxysulfosuccinimide (NHS), N-hydroxysulfosuccinimide (sulfo-NHS) and N,N′-dimethylformamide (DMFA) were from Fluka (St. Louis, USA). Non-ionic detergent Tween 20 and bovine serum albumin (BSA) were from Sigma-Aldrich (St. Louis, USA). Blue latex particles were from Magsphere (Pasadena, USA).

CNPF-backed nitrocellulose membranes and pre-treated TYPEGBF-R7L sample pads were from Advanced Microdevices (Ambala Cantt, India). CFSP223000 adsorption pads, and fiberglass macroporous CFCP203000 conjugate pads were from Millipore (Billerica, USA).

Amicon Ultra 100 kDa centrifugal filter units were from Millipore (Billerica, USA).
All solutions for syntheses were prepared using purified water obtained using Milli-Q system (Millipore, Billerica, USA).

2.2.Methods

2.2.1. Syntheses of herbicide–protein conjugates

The conjugate of atrazine and bovine serum albumin (Atr–BSA) was synthesized by the carbodiimide method described in our earlier work [16]. The atrazine carboxylate derivative (N-(6-(N-isopropylamino)-2-chloro-1,3,5-triazin-4-yl)-6-aminocaproic acid) (0.05 mmol) was diluted in 0.5 ml of dimethylformamide, then 0.1 mmol N-hydroxysuccinimide and 0.1 mmol 1-ethyl-3(3-dimethylaminopropyl)carbodiimide were added, and the mixture was stirred for 2 h at room temperature. Then, the solution of the activated hapten was cooled to +4°C and added to the cooled protein solution (4 mg BSA) in 0.5 ml of 0.02 M Na-carbonate buffer, pH 9.5, containing the same volume of dimethylformamide. The resultant mixture was incubated with stirring for 1 h at room temperature and for 16 h at +4°C. The conjugates were separated from low molecular weight compounds by gel-filtration on Sephadex G-25 (Pharmacia, 1 × 20 cm column, in PBS) and/or by dialysis.

2.2.2. Preparation of particle–antibody conjugates

GNPs were prepared by citrate reduction of gold salt, as described in [17]. Antibodies (GARI and anti-atrazine ones) were immobilized on GNPs by physical adsorption, according to [18, 19]. Potassium carbonate solution (0.1 M) was added to the GNP solution (D_{520} = 1.0) until it reached pH 8.5, and then the antiserum was added to a concentration of 50 μg/mL (basing on a total content of IgG in antisera) and GARI solution at a concentration of 8 μg/mL. The mixture was incubated for 30 min with

stirring at room temperature. Then, the BSA solution (10%) was added to a final concentration of 0.25%.
The LP–antibody conjugate was prepared according to [20] by covalent immobilization through carboxyl groups. The molar LP:IgG ratio was 1:90. The concentration of NHS was 9.2 mM. The mixture was incubated for 2 h with stirring at room temperature. The solution was blocked with 10% BSA, and then activators were removed by centrifugation.
The diameter of GNPs, according to transmission electron microscopy, was 25±3 nm, LPs – 340±10 nm.

2.2.3. Preparation of immunochromatographic test strips

Reagents were applied onto membranes using an IsoFlow dispenser (Imagene Technology, Lebanon, USA). Conjugate GNP was dispensed with an optical density of 4.0 (wavelength 520 nm) and conjugate LP was dispensed with a concentration of 0.5%; the conjugate load was 32 μL per 1 cm of strip width. The test zone was formed by the Atrazine–BSA conjugate (2.0 mg/mL), and the control zone was formed by GARI (0.25 mg/mL in PBS). Two microliters of both reactants were applied per 1 cm of strip width. All concentrations and dispensing modes were chosen based on the earlier data [21].
After dispensing reagents and assembling the membrane components, the lists were cut into strips 3.5 mm wide using an Index Cutter-1 (A-Point Technologies, Gibbstown, USA). Each test strip was 75 mm in length. Each test strip was packaged in laminated aluminium foil using a FR-900 mini-conveyor (Wenzhou Dingli Packing Machinery, Wenzhou, Zhejiang, China), using pre-packaged silica gel (0.5 g) as desiccant. Splitting and packing were carried out at 20–22ºC in a special room with relative humidity under 30%.

2.2.4. Preparation of immunochromatographic test strips with silver–enhanced staining [14]

Two sheets of PT-R7 membrane were washed three times with distilled water and dried at 60°C for at least 20 h. One sheet was impregnated with a 0.3% aqueous solution of silver lactate, and the other was impregnated with a 3.0% hydroquinone solution in 0.5 M citrate buffer, pH 4.0; the membranes were dried in the dark at room temperature for at least 20 h and cut into strips of 20 × 3.5 mm in size.
Membranes impregnated with silver lactate and hydroquinone were sequentially applied to the working membrane of a test strip containing gold-stained zones after the immunoassay and moistened with 100 μL distilled water.

2.2.5. Immunochromatographic assay

The assay was carried out at room temperature. The test strip was vertically submerged into a sample for 10 min. Afterwards, the assay results were recorded.

2.2.6. Immunochromatographic assay with silver–enhanced staining

The assay was carried out under the same conditions (see 2.2.5). After 10 minutes of incubation, membranes containing silver lactate and hydroquinone were removed, and the assay results were recorded.

2.2.7. Immunochromatographic assay with separated specific and detection stages

The assay was carried out at room temperature. The samples were prepared in a microwell plate by mixing 25 µl of 0.04–10 ng/mL serial dilutions with 25 µl of anti-atrazine antiserum (1:500) in a 50 mM phosphate buffer, pH 7.4, containing 0.1 M NaCl and 0.05% Triton X-100 (PBST). Each test-strip was vertically immersed in the sample solution and incubated for 5 min. The test-strip was then washed by immersing it in PBST for 5 min, and incubated for 5 min in a solution of GNP–GARI (D_{580} = 2.0 in Tris-buffer, pH 7.5, containing 1% BSA, 0.25% Tween-20, 1% sucrose and 0.1 sodium azide). After 5 min of washing by immersion in PBST, the test-strip was scanned.

2.2.8. Immunochromatographic data processing

Digital images of binding zones on the working membrane of the test strip were registered with the CanoScan LiDE 90 scanner. The colour intensity of the membrane zones was calculated with TotalLab v2.01 software as described in [22] (TotalLab, Newcastle upon Tyne, UK).

The general form of the equation of the calibration curve is:

$$Y = \{(A - D)/[1 + (x/C)^B]\} + D,$$

where A is the asymptotic maximum; B is the point of inflection of the curve in the semi-logarithmic coordinates; C is the concentration of the analyte at the inflection point of the curve; and D is the asymptotic minimum (intensity of the background signal) [22].

The limit of detection (LOD) was calculated as the concentration yielding a signal reducing three times the standard deviation of the background signal for the sample without atrazine.

3. RESULTS

3.1. Development and characterization of a standard immunochromatographic system

First, a standard immunochromatographic system using antiserum as a capture reactant for determining atrazine was developed. The following parameters were varied for selection of the optimal completion of the test system:

- concentration of antiserum;
- concentration of the conjugate Atr-BSA applied in the test zone;
- quantity of the detecting conjugate between GNPs and specific antiserum;
- composition of the reaction medium.

The antiserum concentration (calculated on the base of total protein amount) in the course of the synthesis of the conjugates ranged from 10 to 50 µg/mL. Excess of antisera (50 µg/mL) was selected due to a large number of ballast proteins like albumins and nonspecific immunoglobulins.

The concentration of the Atr-BSA conjugate in the test zone was varied from 0.5 to 2.0 mg/mL. Maximum colour intensities and the minimum relative errors (<12.5%) were reached for the conjugate concentration equal to 2 mg/mL.

An optimum working nitrocellulose membrane with a pore size of 8 μm allows for testing non-viscid samples [19, 23].

With the use of the selected completion of the test system, we obtained the calibration curve that is shown in Fig. 1.

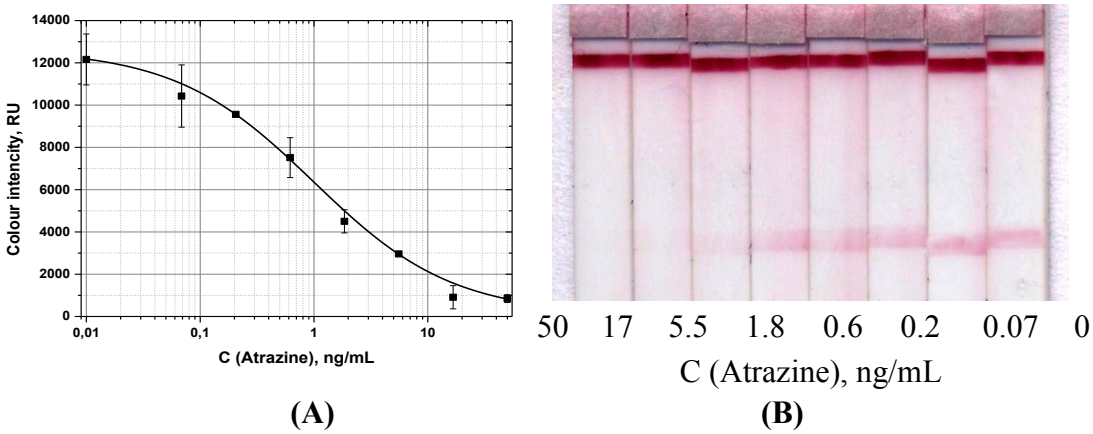


Figure 1: The calibration curve and the appearance of test strips for standard immunochromatographic system for detection of atrazine

The test system is characterized by the visual detection limit of 50 ng/mL, and colorimetric detection limit of 0.2 ng/mL. Relative error of atrazine determination in the working range of 1−100 ng/mL does not exceed 10.9%. However, this test system is characterized by a significant background colouration and low colour intensity of the test zone, and the limit of detection is high.

3.2. The development of enhanced immunochromatographic test systems

The known methods for sensitivity-increasing of immunochromatographic test systems are based on the increase in the colour intensity of the test zone. We have compared the most widely used of such methods. Three variants of test systems for atrazine have been developed:

A - test system based on the GNPs with silver-enhanced staining;
B - test system using latex particles as labels;
C - test system with pre-incubation of the sample with native specific antibodies and the labelling of anti-species antibodies.

Variant A is based on the aggregation of silver ions on the GNPs surface. It is widely used in electron microscopy [24, 25]. According to the existing publications, this

method allows for reducing the limit of detection of immunochromatographic assays from 10 to25 times [26, 27].

Variant B is based on the change of colloidal label. Thus, the replacement of GNPs to fluorescent markers reduces the detection limit by more than 20 times [28]. The use of carbon nanotubes can reduce the detection limit by 50 times due to the greater contrast of this label [29]. At the same time, coloured latex particles (cheap and mass-produced) have great practical potential and are not characterized in comparative terms.

Variant C allows increasing the time of analyte reaction with antibodies. It is noted [30] that, because of this, the detection limit decreases by approximately 10 times.

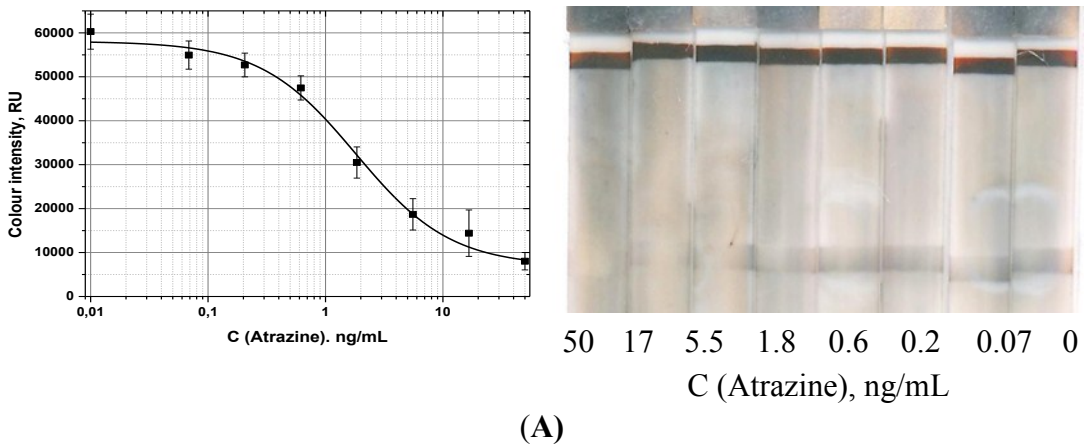
For each of these variants, optimization of immunochromatography conditions was conducted by the same way as it was done (and given above) for the standard analysis. Table 1 shows selected parameters of each of the test systems.

Table 1. Optimal configurations of the enhanced immunochromatographic systems for atrazine detection

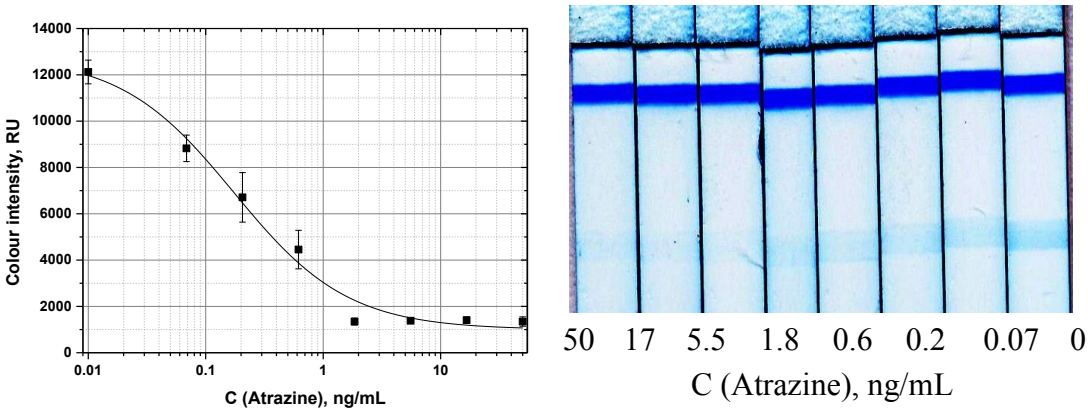
Parameter	Detection system		
	A	B	C
Working membrane	CNPF 8 μm	CNPF 8 μm	CNPF 8 μm
C (Atr-BSA), mg/mL	2	2	0.25
A_{520} (GNP-antibody)	4.0	-	2.0
C (LP-antibody), %	-	0.1	-
Antiserum dilution	1:8,000	1:3,000	1:1,000

3.3. Comparison of the analytical characteristics of the enhanced immunochromatographic test systems

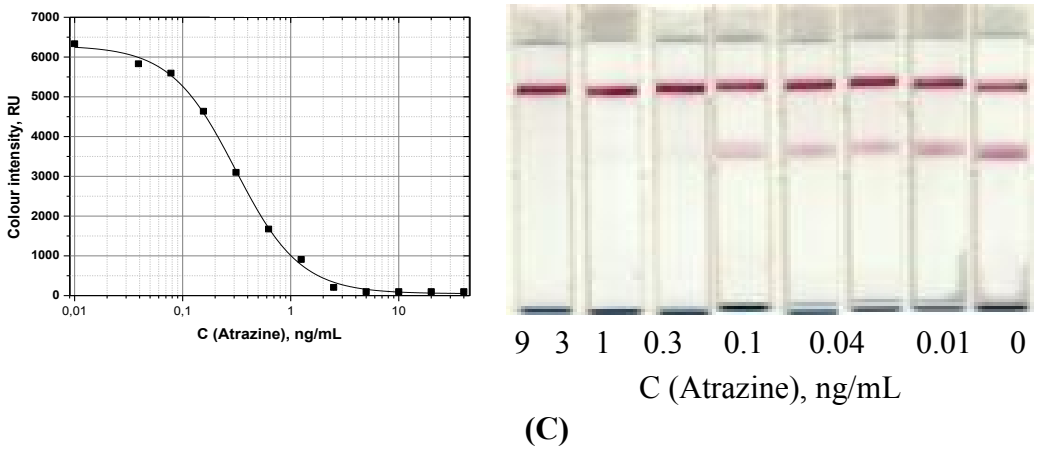
Based on the obtained data, immunochromatographic systems for atrazine detection have been made and tested. Fig. 1 shows the calibration curves and the appearance of strips for different variants of enhanced analysis.



(A)



(B)



(C)

Figure 2: Calibration curves (A, C, E) and appearance (B, D, F) of the enhanced immunochromatographic test-systems for atrazine detection: A, B – test-system with silver–enhanced staining; C, D – test-system based on LPs; E, F – test-system with pre-incubation.

A four-parameter sigmoidal function was used to approximate the calibration curves of the competitive immunoassay as recommended in [22]. The coefficient values of approximating curves are shown in Table 2. The relative error of quantitative detection is less than 12.5%, except for the system with silver–enhanced staining, for which it is increased to 20−25%.

Table 2. The coefficients of the equation approximating $Y = \{(A - D)/[1 + (x/C)^B]\}+D$ for calibration curves of the developed test systems to atrazine

	Coefficients				R^2
	A	B	C	D	
Standard	12637.8	0.71	0.99	99.0	0.999
Enhanced variant A	58078.6	1.1	1.8	6893.7	0.992
Enhanced variant B	12816.7	0.9	0.2	997.0	0.977
Enhanced variant C	6320.5	1.4	0.3	-0.9	0.998

Visual and colorimetric detection limits (DL) using the developed test systems were evaluated. The corresponding parameters are summarized in Table 3.

Table 3. Visual and colorimetric limits of atrazine detection using the developed test systems

	Visual DL, ng/mL	Colorimetric DL, ng/mL
Standard	50	0.2
Enhanced variant A	20	0.5
Enhanced variant B	2	0.02
Enhanced variant C	5	0.08

3.4. Analyte recovery in the juice samples

The evaluation of the atrazine detection efficiency for real food matrixes was implemented in additional experiments. Repeated measurements were carried out to determine inter-assay variability.

Standard immunochromatographic system and enhanced system based on blue LPs as labels were tested using commercial apple juice diluted twice with PBST with different concentrations of alled atrazine. As can be seen from Table 4, for test-system based on LPs high recovery (95–103%) of the target antigen was achieved for all samples. The recovery for standard immunochromatographic system is worse (90-110%) due to low colour intensity.