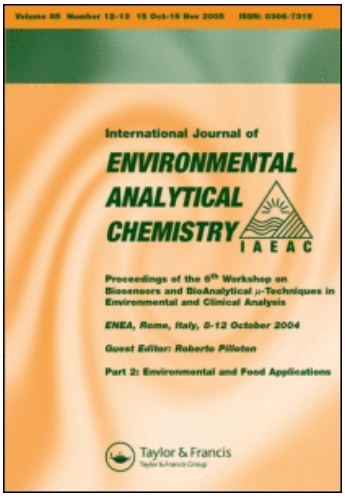

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RAPID METHOD FOR ON-SITE DETERMINATION OF ATRAZINE RESIDUES IN WATER SAMPLES. ASSAY OPTIMISATION

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A rapid and sensitive dipstick immunoassay for atrazine has been developed. The optimisation of different parameters, such as substrate dilution, assay time and simultaneous versus sequential protocol, by means of an immunofiltration device is described. The assay is based on the competitive principle and uses a polyclonal antibody immobilized on an Immobilon-P membrane and alkaline phosphatase as enzyme tracer. The test takes about 10 min and has a visual detection limit for atrazine of $10\,\mu g\,L^{-1}$. Cross-reactivity of polyclonal sera to atrazine and propazine are similar, decreasing 10-fold for simazine and terbuthylazine and 100-fold for prometryn and ametryn. Based on the good reproducibility obtained (90% of positive response) the developed dipsticks can be very useful as a qualitative or semiquantitative on-site test for atrazine determination in water samples.

Keywords: Immunofiltration; Membranes; Dipstick; Atrazine; Water

INTRODUCTION

Presently, pesticides are widely used to ensure high crop yields in order to satisfy food demands. However, exposure to pesticide or consuming contaminated water or foodstuffs may result in hazards to human health and living resources. In this sense, consumers claim the assessment, management and control of pesticides.

For this reason, the European Union Council[1] and other public institutions (EPA, FDA, etc.) have issued a set of directives dealing with the monitoring of pesticide residues in food and environmental matrices, establishing maximum residue limits (MRL) for each pesticide on the basis of its toxicity, persistence and impact parameters[2]. In general, European MRLs range from 10^{-4} to $10\,mg\,L^{-1}$ (or $mg\,kg^{-1}$). Also, for high toxicity pesticides, tolerance zero (absence of pesticide residues) is applied. Thus, each state member should establish a surveillance system and inspection programs to guarantee the circulation in its territory of commodities that do not exceed the fixed MRLs.

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In fact the increased world-wide demand for pesticide residue monitoring has led to a need for rapid and reliable analytical methods that allow the screening of a large number of samples.

The time and expenses involved in classical analytical methods (i.e., sampling, sample preparation, and laboratory analysis) limit the number of samples that can be studied in survey programs (SP). In this sense, there is a real need for developing fast, easy to use, robust, sensitive, cost-effective and field-analytical techniques. Immunoassays (IAs) meet these requirements and many pesticides can be analysed and monitored at regulatory levels without any or with minimal sample preparation[3].

The IA systems used in residue analysis are mostly based on microtitre plates, test tube or magnetic particles. They have allowed the quantification of analytes such as pesticides but generally require between 1 and 3 h to obtain results and tedious sample and reagent manipulations.

Currently, conventional laboratory methods are increasingly (but not fully) replaced by rapid-response analytical tools that can be used at the sampling place, providing a binary yes/no response which would indicate whether the target analytes are present above or below a pre-set threshold concentration[4]. The confirmatory analyses at the laboratory can then be restricted to those samples giving a positive on-site test.

Strip tests have characteristics that make them well suited for non-laboratory applications: the test strip is a convenient vehicle for assay reagents and enables simple preparation and visualisation steps (visual colour interpretation)[5]. Moreover, the fact of carrying out *in situ* analysis and at real time is an important advantage for taking further decisions that will allow suitable performances.

In the mid-1970s initial immunoassays on membranes were developed in order to solve established EIAs drawbacks (high skill, sophisticated equipment and timing). In recent years there has been an enormous increase in the development and application of membrane based rapid tests that are used in many fields all over the world. There are more than 250 commercial tests using the membrane format which are based on: antigen detection sandwich assays, antibody detection assays and competitive hapten assays protocols. These tests are available for human and veterinary diagnostics, agriculture testing and environmental screening. These low-cost alternatives to expensive instrumental methods become more attractive as the number of applications daily increases[6–11].

Presently, the most common formats used to develop rapid diagnostic test (RDT) are based on: specific immunoadsorpion (dipstick), immunoconcentration (flow-through) and immunochromatography (later flow) devices[6–11].

The aim of this research is the development of a dipstick analytical screening assay to detect atrazine residues in aqueous samples. In that configuration, a membrane coated with anti-atrazine antibody is placed into a solution containing sample and tracer. Due to the flow-through characteristics of the membrane, this brings the immunoreagents rapidly into close contact with the reactive sites on the membrane. The kinetics of reaction between immobilised and soluble reactants closely approaches the kinetics of binding in solution. Thus, assay time is significantly shorter[12].

Development and setting-up strip tests include a set of steps such as support selection, reactive immobilisation, study and optimisation of assay formats, tracer selection, detection mode and immunoreagents running. As a first steps, our goal was directed to optimising strip tests in order to determine atrazine residues in water samples.

EXPERIMENTAL SECTION

Chemicals

Analytical standards of atrazine, propazine, simazine, terbuthylazine, prometryn and ametryn were purchased from Ciba-Geigy (Barcelona, Spain), Dr. Ehrenstorfer (Augsburg, Germany) and Riedel de Häen (Seelze-Hannover, Germany). Stock solutions of pesticides were prepared in methanol (MeOH) and kept at −20°C. Alkaline phosphates (AP), poly (oxyethylensorbitan) monolaurate (Tween 20), bovine serum albumin, fraction V (BSA) and 5-bromo-4-chloro-3-indoyl phosphate (BCIP)/nitroblue tetrazolium (NBT) liquid substrate system were purchased from Sigma Chemical Co., (St. Louis, MO). *N*-hydroxysuccinimide (NHS) and *N,N′*-dicyclohexylcarbodiimide (DCC) used for hapten–protein conjugation were from Boehringer (Mannheim, Germany). A poly(vinylidenedifluoride) 0.45 μm pore-sized membrane (Immobilon-P) was from Millipore Corp. (Bedford, MA). Before the immobilisation step, the membrane was preconditioned as suggested by the manufacturer. All other reagents used were analytical or biochemical grade.

Buffers

The coating buffer (CB) is 50 mM carbonate–bicarbonate buffer pH 9.6. A Tris buffer (TBS) solution was made of 21 mM trishydroxymethylaminomethane, 137 mM NaCl, 37 mM KCl, pH 8.0. TBS-T is TBS with 0.05% Tween-20.

Immunoreagents

Rabbit anti-atrazine polyclonal serum R10 and 2d hapten *N*-(4-chloro-6-isopropylamine-[1,3,5]triazin-2-il)-6-aminohexanoic acid) used for enzymatic hapten conjugate preparation, were kindly provided and characterised by Gascón *et al.*[13].

Goat Anti-Rabbit linked to AP enzyme (GAR-AP) was from Sigma Chemical Co., (St. Louis, MO).

Preparation of Enzyme–Hapten Conjugate

Hapten 2d was covalently attached to AP using this NHS ester method as described by Schneider and Hammock [14] with slight modifications. Briefly, 12 mg of hapten (45–55 μmol) were dissolved in 65 μL of DMF. Also, 170 μL of DCC and 95 μL of NHS solutions (50 mg mL^{-1}) in DMF were added. The reaction mixture was stirred for 4 h at room temperature until appearance of the urea precipitate. The reaction volume (hapten activation) was 330 μL with a reactive concentration of 150 mM. The precipitate was separated by centrifugation (15 min at 12,000 rpm). The supernatant (145 μL) was taken to 200 μL with DMF and then slowly added to a solution of AP (20 mg/0.8 mL) in CB and stirred in the dark for 4 h at room temperature. Finally, protein–hapten conjugate was purified by gel filtration on Sephadex G-25 (Pharmacia, Uppsala, Sweden) using TBS as eluant. Tracer was aliquoted (50 μL, 255 mg L^{-1} in TBS) and stored at −20°C.

Instrumentation

An immunofiltration system –ELIFA– from Pierce Chemical Co. (Rockford, IL) was used for parameter selection and optimisation of working conditions. Flow-rate was controlled by a single-channel peristaltic pump, Minipuls-3 (Gilson, Villiers LeBel, France). Basically, the ELIFA device is composed of a sample application plate, a 96-transfer cannula set, and a collection chamber. These three pieces are sealed with silicone gaskets to provide constant flow rates in all wells. The membrane (12×7 cm) is placed between the gaskets and the sample application plate in the typical 8×12 microtiter plate format. Ninety-six individual cannula emptied the filtrated solutions into a waste chamber. A silicone tube connected the vacuum valve of the device to a peristaltic pump, which controlled the flow rate and provided the vaccum for pulling the reagents through the membrane.

Autoclavable polypropylene reservoirs (plastic boxes) of eight and one channel from Labsystems Oy (Helsinki, Finland) were used as reagent vessels in the competition, washing, colour development and stop steps.

ASSAY OPTIMISATION

Support selection

Attending to a previous selection and characterisation study of several membranes regarding sensitivity, high binding capacity and lower unspecific adsorptions after active center blockage, Immobilon-P membrane was selected and used throughout this work[15].

Previous Tests: General Assay Performances

Polyclonal sera (R10) was randomly immobilised on the membrane by simple passive adsorption since it is an attractive, simple and fast procedure[16]. Briefly, 50 µL of 1/100 R10 solution in CB was added with a micropipette onto a plate well to coat a circular surface of Immoblion-P membrane. In order to prevent immunoreagent diffusion away from the reactive zone (5 mm diameter), it was labelled by a hydrophobic permanent marker. After 1 h at room temperature, the strip was washed for 1 min with TBS-T and blocked for 15 min by immersion in a solution of BSA 1%(w/v) in TBS buffer. Next, the strip was dried at room temperature and 50 µL of 10 mg L^{-1} solution of 2d-PA was added onto the reactive zone. One hour later, the membrane was washed again and immersed in BCIP/NBT substrate for 15 min. Finally, the dot colour was visually appreciated.

Based on the good results obtained in previous experiments, significant changes in the dispersing protocol are required to achieve consistent deposition in order to gain reproducibility.

Thus, by using the immunofiltration system, up to 96 results could be checked simultaneously in a controlled way. Basically, the membrane is placed within the ELIFA device. An R10 serum solution in CB was added (200 µL/well) and filtered through the membrane, and the remaining binding sites were blocked with BSA 1% (w/v) in TBS buffer (200 µL/well). The membrane was then taken away and dried. The reactive zone would be determined by the ELIFA well size (4 mm diameter). The dried

membrane (12 × 7 cm) was cut into 12 pieces (1 × 7 cm) that were then adhered onto a Nunc plastic self-adhesive sealing tape for easy handling. The coated strips could be stored in plastic vacuum-sealed bags at room temperature in the dark, for several weeks.

As can be seen in Fig. 1, each comb-shaped strip test (strip arrays) used for assay optimisation includes eight suitably isolated checking spots in order to achieve complete immersion into every plastic box compartment for the competition step. In addition, for washing and colour development steps, strip arrays were immersed sequentially into every plastic box compartment.

The optimal immunoreagent (R10 : 2d-PA) concentrations to be used in strip tests were determined by checkerboard titration. Once immunoreagent assay conditions were established, competitive assays would be carried out.

In order to ascertain the assay sensitivity for visual determinations, the atrazine concentrations that mark a colour decreasing or disappearing when BCIP/NBT substrate reacts with PA should be established.

Ideally, this goal could be achieved by a sigmoidal curve with high slope. In this sense, working parameters such as contact, incubation and washing times and substrate dilution had to be optimised.

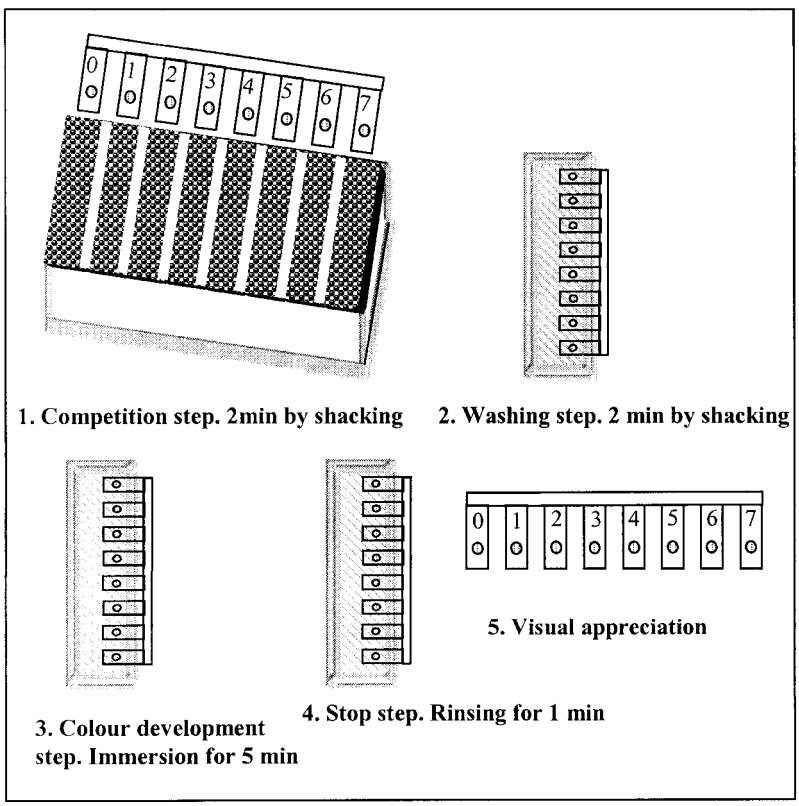

FIGURE 1 General Assay Protocol. Total assay time 10 min.

Thus, dots were identified as follows: 0 without sera acting as blockage control and numbers from 1 to 7 corresponding to different points of a serial atrazine solution (0, 0.1, 1, 10, 100, 1000 and 10,000 $\mu g\,L^{-1}$).

In essence, the assay consists of a competition step, washing with TBS-T, colour development by immersion in substrate solution and colour stopping with distilled water before visualisation.

For competition steps, each compartment of plastic box contains a mixture of 1 mL of previously optimised tracer concentration and 1 mL of each point of serial atrazine solution (simultaneous protocol).

Also, in order to assure effective contact in the washing, colour development and stop steps, 10 mL of TBS-T, BCIP/NBT substrate and distilled water, respectively, were introduced into individual plastic boxes.

Semi-Quantitative Assays

The colour must be considered under many different aspects: like simple physico-optical phenomenon necessary to carry out and precisely interpret a subjective measurement, or like a sensation in the observer of physico-psychological nature produced by coloured objects or the light of certain characteristics. Sensory colour measure is based on a visual perception made by one or different people who compare a perceived stimulus with a reference. In this way, sensory analysis will be carried out by a panel of trained referees in order to achieve similar and homogeneous information for the establishment of a visual standard value.

Therefore, the dot colour intensity was visually compared to that of the negative control which showed the most intense red colour, since atrazine concentration is inversely related to colour intensity.

Sequential Protocol

Once assay conditions were optimised, the competition step was to be carried out in a sequential way. Strip arrays were immersed in a solution of tracer for 2 min and dried for 1 h at room temperature before washing. Afterwards, strip arrays were put in contact with correspondent atrazine standard solutions for 2, 5 and 10 min. Finally, washing, colour development and stopping steps were performed.

In order to establish assay selectivity, standard curves for atrazine, propazine, simazine, terbuthylazine, prometryn and ametryn were prepared. Thus, from a pre-coated membrane, two strip arrays for each analyte were obtained following general assay procedure described before for atrazine. Finally, assay selectivity was visually established.

Preparation of Dipsticks

For routine use, strips should include only two test-points: one as negative control and the other for analyte determination. Thus, immobilisation step was slightly modified. Basically, 200 μL/well of 1/10,000 GAR-PA solution was added to even rows and 200 μL/well of optimised R10 solutions to odd rows. The remaining protocol steps (blockage and drying) were performed as previously described. In the last step,

0.5×1.5 cm pieces were cut and coupled to an inert plastic support for handling ease. At this stage, 48 strip tests were ready for use.

In order to test dipstick feasibility, tap water samples were fortified with atrazine at 0, 1, 10 and 1000 $\mu g\,L^{-1}$. Tap water was also checked by the ELISA method for atrazine contamination according to Gascón *et al.* [13].

Tubes were labeled from 1–4 and provided as blind samples to four trained referees for visual determination. Twelve dipsticks, TBS-T, BCIP/NBT substrate and distilled water were also supplied as part of the kit.

RESULTS AND DISCUSSION

In order to develop a dipstick field test for the determination of atrazine, optimal immunoreagent concentrations (R10 : 2d-PA) were selected as a compromise between a clear colour appreciation in dot-test, with no colour development in blockage control –without R10– and minimum immunoreagent consumption. For this purpose, twelve serial solutions of R10 polyclonal sera (from 0–1 : 1000) and four of enzymatic tracers (from 0.01 to 10 $mg\,L^{-1}$) were tested. Thus, concentrations of 1/200 and 0.1 $mg\,L^{-1}$ for R10 and 2d-PA, respectively, were selected. Higher tracer concentrations ($> 0.1\,mg\,L^{-1}$) originated colour development in negative control.

Competitive Assays

In this assay, atrazine and tracer compete for binding to specific sites of immobilised Ab. The optimisation of this assay (contact, incubation and washing times, substrate dilution, etc.) was accomplished with a sequential process based on univariate method.

(a) Substrate Dilution

For this experiment, competition time and washing step were fixed at 10 and 2 min, respectively. Strip tests were immersed for 15 min in eight aqueous substrate dilutions (from 1/1 to 1/128) (v/v) and dried at room temperature for 1 h. As shown in Table I, 1 : 16 (v/v) solution maintained good sensitivity (around 10 μgL^{-1}). However, for substrate dilutions above 1 : 8 (v/v), visual appreciation was difficult since colour intensity decreases proportionally as substrate dilution increases.

TABLE I Optimisation of substrate dilution (v/v)

[Atrazine] ($\mu g\,L^{-1}$)	*1 : 1*	*1 : 2*	*1 : 4*	*1 : 8*	*1 : 16*	*1 : 32*	*1 : 64*	*1 : 128*
Control	ncd	ncd	ncd	ncd	ncd	ncd	ncd	ncd
0	−	−	−	−	−	+	+	+
0.1	−	−	−	−	−	+	+	+
1	−	−	−	−	−	+	+	+
10	−	−	−	−/+	+	+	+	+
100	−	−/+	+	+	+	+	+	+
1000	+	+	+	+	+	+	+	+
10,000	+	+	+	+	+	+	+	+

ncd, no colour development; −, absence of atrazine; +, presence of atrazine.

(b) Assay Time

Competition step was optimised being 2 min for washing step and 15 min for contact time with 1 : 8 BCIP/NBT (v/v) substrate solution. Seven strip arrays were immersed during 2, 4, 6, 8, 10, 20 and 30 min into different slots of the plastic box. Each slot cointained 1 mL of serial atrazine solution and 1 mL of 0.1 mg L^{-1} 2d-PA. As shown in Table II assay sensitivity was affected by competition time. Short contact times of 2, 4 and 6 min gave colour changes between 0.1 and 1 μg L^{-1}. On the contrary, larger incubation time decreased assay sensitivity due to an increase in colour intensity.

An optimum competition time of 2 min was established to achieve sensitive, reproducible, stable and faster tests.

The next step was the optimisation of contact time with BCIP/NBT substrate. From previous studies, larger substrate dilutions made necessary larger contact times in order to obtain precipitates with visible colour. In addition, a new step together with dipstick immersion in BCIP/NBT was included to avoid colour over-development produced when the reaction was not stopped. In this sense, rinsing dipsticks with distilled water would be included in order to stop colour development.

After competition and washing steps, strip arrays were immersed into the following substrate dilutions: 0 (undiluted), 1 : 1, 1 : 2, 1 : 4 and 1 : 8 (v/v). Previous to stop, dipsticks were fully covered with BCIP/NBT for 5, 10 and 15 min. Also, 1 and 2.5 min were included for undiluted and substrate (1 : 1, v/v).

Based on premises described above –sensitivity, colour intensity and assay time– good results were obtained with both, undiluted and 1 : 1 (v/v) substrate, since it was only necessary 5 min. for colour development (Table III).

Hence, the experiments that followed were carried out using optimised assay conditions: R10 1/200, 2d-PA 0.1 mg L^{-1}, 2 min for competition time, 2 min for washing step with TBS-T, 5 min for colour development by immersion into undiluted BCIP/NBT substrate and subsequent stop of colour development by immersion in distilled water. A representative dipstick obtained with optimised assay conditions is shown in Fig. 2.

(c) Semi-Quantitative Assays

In order to establish the visual sensitivity of the proposed methodology, 30 dipsticks were prepared using optimised assay conditions. Each strip was identified with a character and numbers from 0, without R10 as control blockage, to 7, corresponding to

TABLE II Optimisation of competition assay time (min)

[Atrazine] (μg L^{-1})	*2*	*4*	*6*	*8*	*10*	*20*	*30*
Control	ncd	ncd	ncd	ncd	ncd	ncd	ncd
0	−	−	−	−	−	−	−
0.1	−	−	−	−	−	−	−
1	+	+	+	−/+	−/+	−	−
10	+	+	+	+	+	+	−/+
100	+	+	+	+	+	+	+
1000	+	+	+	+	+	+	+
10,000	+	+	+	+	+	+	+

ncd, no colour development; −, absence of atrazine; +, presence of atrazine.

TABLE III Optimisation of contact time with substrate at several dilutions

[Atrazine] ($\mu g\,L^{-1}$)	Undiluted[a]					1 : 1					1 : 2			1 : 4			1 : 8		
	1[b]	2.5	5	10	15	1	2.5	5	10	15	5	10	15	5	10	15	5	10	15
Control	ncd	ncd	ncd	ncd	ncd	ncd	ncd	ncd	ncd	ncd	ncd	ncd	ncd	ncd	ncd	ncd	ncd	ncd	ncd
0	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−/+	−	−
0.1	−	−	−	−	−	−/+	−	−	−	−	−	−	−	−/+	−/+	−/+	+	+	−/+
1	−/+	−/+	−	−	−/+	−/+	−/+	−/+	−/+	−	−/+	−/+	−/+	+	+	+	+	+	+
10	+	+	+	−/+	−/+	+	+	+	−/+	−/+	−/+	+	−/+	+	+	+	+	+	+
100	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
1000	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
10,000	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

[a]Dilution (v/v). [b]Time (min). ncd, no colour development; −, absence of atrazine; +, presence of atrazine.

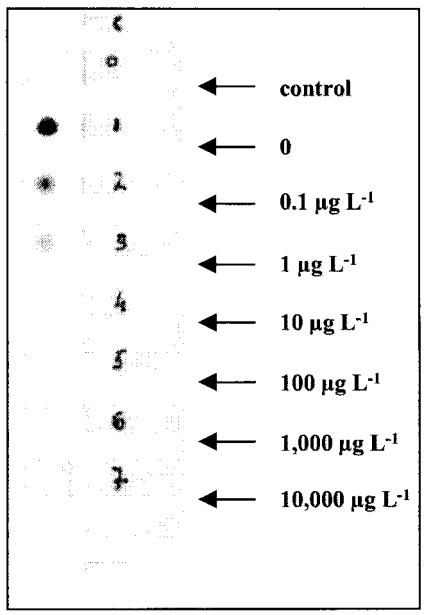

FIGURE 2 Representative dipstick obtained in optimised assay conditions.

different atrazine concentrations. When the assay protocol was concluded, dipsticks were ready for sensory analysis. A panel of 22 trained referees carried out naked eye visual appreciations in order to achieve similar and homogeneous information to establish a sensitive value.

Referees should indicate in every strip, the number that gave colour absence. Results obtained are shown in Fig. 3. Thus, the visual detection limit was selected as the point corresponding to a concentration threshold that ensured 95% of positive responses[17].

Based on the results obtained (11%, 74% and 13% corresponding to atrazine concentrations of 0.1, 1 and 10 $\mu g\,L^{-1}$, respectively), a concentration value of 10 $\mu g\,L^{-1}$ was fixed as the visual detection limit (98% of positive responses).

Regarding assay selectivity (Table IV), the developed methodology is able to establish a cut-off for atrazine and propazine between 1 and 10 $\mu g\,L^{-1}$. The assay sensitivity

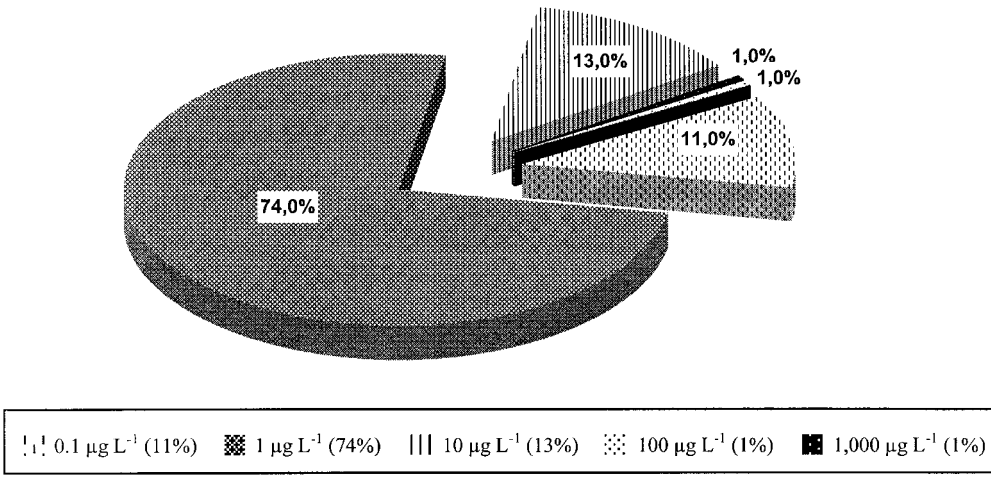

FIGURE 3 Percentages obtained in visual appreciations.

TABLE IV Dipstick selectivity for s-triazines

[Atrazine] ($\mu g\,L^{-1}$)	*Ametryn*	*Atrazine*	*Propazine*	*Prometryn*	*Simazine*	*Terbuthylazine*
Control	ncd	ncd	ncd	ncd	ncd	ncd
0	−	−	−	−	−	−
0.1	−	−	−	−	−	−
1	−	−	−	−	−	−
10	−	+	+	−	−	−
100	−	+	+	−	+	+
1000	+	+	+	+	+	+
10,000	+	+	+	+	+	+

ncd, no colour development; −, absence of atrazine; +, presence of atrazine.

decreased 10-fold for simazine and terbuthylazine and 100-fold for prometryn and ametryn.

In order to avoid potential errors – immunoreagent handling or pippeting-, a set of strips with all immunoreagents (R10 and 2d-AP) were prepared. Experiments carried out demonstrated that atrazine was unable to displace antibody-tracer union, even at high concentrations. The reason for this behaviour could be that the displacement is only possible in a few cases, since Ab-tracer binding is almost irreversible, and it is only reversed by harsh conditions (low pH and high ionic strength).

Finally, assay performance was studied in tap water samples. Four trained referees had to indicate as negative (−) or positive (+) the unappreciable or appreciable difference of colour with respect to the control dot. As mentioned in the experimental section, each dipstick had two points, a negative control and the reactive one. Also, from 12 dipsticks delivered, referees had to introduce three in each blind sample. In this sense, each determination was done in triplicate.

If we considered as erroneous hesitant results (−/+), only five results were in disagreement. Therefore, 43 out of 48 answers were in agreement (Table V), indicating that the developed methodology is suitable in reproducibility (90%) and sensitivity

TABLE V Results of analysis of tap water samples

Sample	*[Atrazine]* ($\mu g\,L^{-1}$)	*1*			*2*			*3*			*4*		
1	0	−	−	−	−	−	−	−	−	−	−	−	−
2	1	−	−	−/+	−	+	−	−	−	−	−/+	−	+
3	10	+	+	+	+	−/+	+	+	+	+	+	+	+
4	1000	+	+	+	+	+	+	+	+	+	+	+	+

−, absence of atrazine; +, presence of atrazine.

cut-off ($10\,\mu g\,L^{-1}$), and is a fast tool (results in 10 min) for atrazine determination in water samples.

CONCLUSION

The proposed dipstick assay seems to be a fast and reliable field test for a preliminary screening of water samples. On the basis of the referee positive responses obtained from visual appreciations of thirty strips, assay reproducibility was 95%.

The use of the developed application allows us to reach an acceptably low detection limit for atrazine and propazine determinations, suitable for analysing water samples or aqueous extracts as a practical screening tool. It can be assumed that the dipstick could serve as a field test to determine on-site potential triazine contamination.

In order to improve assay sensitivity and its application to food samples, further developments including instrumental colour measurements are necessary. In addition, the employment of automatic devices for antibody immobilisation will be considered for reproducible strip preparation.

Although the test described can be improved and will still have to be evaluated further by analysing vegetable samples, the preliminary results are very promising and give lead to further research into the applicability of this field test for pesticide residue analysis.

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