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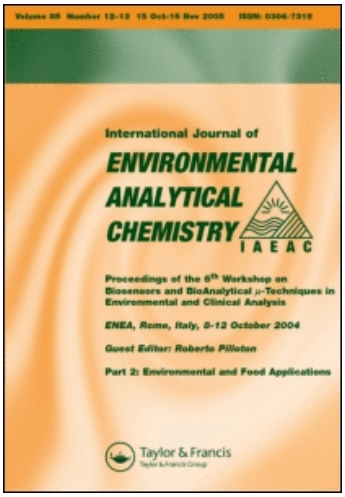

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Determination of Atrazine in Vegetable Samples Using a Dipstick Immunoassay Format

José Antonio Gabaldón[a]; Angel Maquieira[a]; Rosa Puchades[a]
[a] Departamento de Química, Universidad Politécnica de Valencia, Valencia, Spain

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DETERMINATION OF ATRAZINE IN VEGETABLE SAMPLES USING A DIPSTICK IMMUNOASSAY FORMAT

JOSÉ ANTONIO GABALDÓN, ANGEL MAQUIEIRA and ROSA PUCHADES*

Departamento de Química, Universidad Politécnica de Valencia, Camino de Vera s/n, 46071, Valencia, Spain

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A dipstick assay format for atrazine analysis in vegetable samples is described. The analytical method consists in a fast extraction procedure followed by a test based on the use of Immobilon-P strips as antibody coating support. The atrazine quantification was carried out measuring the dot colour by a spectrophotometer. Thus atrazine could be detected in a concentration range 0.16–475.0 $\mu g\,L^{-1}$ with an I_{50} of 2.04 $\mu g\,L^{-1}$. For direct quantification of vegetable samples, those were extracted by blending 5 g in 10 mL of MeOH for 10 min followed by a vacuum filtration through 0.45 μm nylon filters. To avoid erroneous atrazine results, all samples and standards were run in 50% of MeOH which decreased the assay sensitivity by ten fold ($I_{50} = 21.09\,\mu g\,L^{-1}$). Therefore, the proposed methodology was able to perform atrazine analysis under established EU MRL. The samples could be measured directly without any prior concentration or clean-up steps. Recoveries (75–105%) were in agreement with those obtained by a reference method (multiresidue extraction–GC/MS quantification). The feasibility of automated immunoreagent dispenser was also demonstrated.

Keywords: Immunoassay; Dipstick; Atrazine; Vegetables

INTRODUCTION

The use of pesticides is essential in modern agricultural practices in order to satisfy the food demand of our society. However, pesticides by definition are toxic chemicals that present health, environmental and socio-economic disruptions. After Ciba Geigy put the s-triazine atrazine on the market in 1958, it became the most commonly used herbicide worldwide[1]. S-triazines are widely used as selective herbicides for the control of annual grasses and broad-leaved weeds. As a result, herbicide residues contaminate crops, wells and streams due to spills, spraying on run-off [2].

*Corresponding author. Fax: +34-963-877349; E-mail address: rpuchade@qim.upv.es

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The increasing consumer concern about food safety implies that producers must ensure safety and quality standards of their products to achieve consumer confidence and for legal requirements. For these reasons, the food industry has established elaborate and costly controls for ensuring that pesticide residues are below the safety standards (MRL) and, in many cases, below detection[3].

In summary, the society is convinced that only those commodities with healthy and safety benefits will undergo a high consumption increment in future years. In this sense, a complete collaboration of all social statements and the development of powerful analytical techniques to accomplish these wishes are desirable.

Current pesticide detection methods are mainly performed by high-performance liquid chromatography (HPLC)[4] and gas chromatography-mass spectrometry (GC-MS)[5]. All these instrumental methods are very robust and well-established, but in most cases extensive sample pre-treatment including extraction, clean-up and preconcentration is necessary in order to eliminate matrix interferences and reach the required sensitivity. These factors limit the number of samples that can be analysed. In this sense, there is a real need for developing fast, easy to use, robust, sensitive, cost-effective and field-analytical techniques. Immunoassays meet these requirements and many pesticides can be analysed and monitored at regulatory levels without any or minimal sample preparation[6].

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Immunoassays used in residue analysis are mostly based on microtiter plates as the solid support. They allow the quantification of analytes using sophisticated photometers, often combined with computer-automated calculation[7]. The main disadvantage of this immunoassay methodology is the need of long incubation times, which implies several hours from the sample collection to the results generation. So, this technique is normally restricted to laboratories. Therefore, our objective was to provide a valuable field test system for the screening of food samples towards a potential atrazine contamination on-site. A dipstick immunoassay format can achieve this goal.

In addition, there is a clear and important difference between laboratory-based techniques and techniques for on-site assays. For laboratory-based systems, speed is less important than throughput while this is vice versa for field assays[8]. Rapid tests can be used in the field by non-skilled staff, since they are fast, require little or none sample processing, generate yes/no answers without using an instrument and are often cheaper than microplate assays. However, rapid immunoassays are usually neither very sensitive nor can they be used to accurately quantitate an analyte.

The main goal was the application of a previously optimised dipstick assay for the quantitative determination of atrazine in vegetables. For this purpose, a study for the development of rapid extraction protocols compatible with the dipstick assay format was carried out. The analytical method consisted in a fast extraction procedure followed by a strip test immunoassay. As a consequence, assay time was significantly shorter[9]. The feasibility of the developed dipstick for atrazine determination in vegetables under the $0.1\,mg\,kg^{-1}$ EU established MRL would be carried out. On the other hand, the use of a spectrophotometer for atrazine quantitation and the availability of automatic immobilisation of the reagents, was considered. Since immunochemistry technologies develop assays with the end-user in mind, we tried to eliminate as many dilution, mixing and measuring steps as possible. The developed dipstick could be used as a routine-check tool into the farmers schedule process control.

EXPERIMENTAL

Chemicals

Analytical standards of atrazine, propazine, simazine, terbuthylazine, prometrin and ametrin were purchased from Ciba-Geigy (Barcelona, Spain), Dr. Ehrenstorfer (Augsburg, Germany) and Riedel de Häen (Seelze-Hannover, Germany). Stock solutions – 1000 mg L^{-1} – of atrazine were prepared in methanol (MeOH). From stock solutions kept at −20°C, working standard solutions were daily prepared. Alkaline Phosphatase (AP), poly(oxiethylensorbitan)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 polyvinylidene difluoride (0.45 μm pore-sized) membrane (Immobilon-P) was purchased from Millipore Corp. (Bedford, MA). Before the immobilisation step, the membrane was preconditioned as suggested by the manufacturer. Basically, a piece of the Immobilon-P (12 × 7 cm) was introduced in 100% MeOH for 15 s, after in Milli-Q water for 2 min, and last in coating buffer for 5 min to equilibrate the membrane. All other reagents used were analytical or biochemical grade.

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Buffers and Solutions

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

Immunoreagents

Anti-atrazine polyclonal serum R10 was obtained from female New Zealand white rabbit immunised with hapten 2d *N*-(4-Chloro-6-isopropylamino-[1,3,5]triazin-2-yl)-6-aminohexanoic acid) attached covalently to KLH. The serum as freeze-dried powder containing 0.02% NaN_3 and 2d hapten (used for enzymatic hapten conjugate preparation), were kindly provided and characterised by Gascón *et al.*[2]. The serum (12 mg) was reconstituted in TBS/$(NH_4)_2$ SO_4 (v/v) (12 mL).

Goat Anti-Rabbit linked to AP enzyme (GAR-AP) was from Sigma Chemical Co, (St. Louis, MO). The conjugate was provided as a clear light yellow solution in 0.05 M Tris buffer, pH 8.0, containing 1% BSA, 1 mM $MgCl_2$ and 0.1% sodium azide. Protein content (0.9 mg mL^{-1}) and enzyme activity (250–500 units mL^{-1}) were certified by the manufacturer.

Preparation of Enzymatic Hapten Conjugate

Hapten 2d was covalently attached to AP using the NHS ester method as described in a previous work[10]. Tracer was stored frozen at −20°C on small aliquots (50 μL, 255 mg L^{-1} in TBS). Working solutions were daily prepared and stored at 4°C.

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Instrumentation

An immunofiltration system ELIFA from Pierce Chemical Co. (Rockford, IL), was used for work conditions optimisation and immunoreagents immobilisation[11]. The device consists of a 96 well 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. The 96 individual cannula emptied the filtrated solutions into a waste chamber. A silicone tube connected the vacuum valve of the device to a single-channel peristaltic pump, Minipuls-3 (Gilson, Villiers LeBel, France), which controlled the flow rate and provided the vacuum for pulling the reagents through the membrane.

For GC analysis a 6890 Hewlett-Packard device – automatic sampler – provided with a 5% phenyl-methyl siloxane capillary column (HP-5MS) model 19091S-433 (30 m length $\times$ 250 μm diameter $\times$ 0.25 μm film thickness) and flame-ion detector (FID) was used. Also, a 5973 mass selective detector (MSD) operated in a selected ion monitoring (SIM) mode was employed for peak identification. Helium was used as the carrier gas with a flow rate of 1.2 mL min^{-1} and the samples were injected in the splitless mode by an autoinjector. The column temperature was held at 60°C for 1 min, then increased at 30°C min^{-1} to 110°C, 10°C min^{-1} to 240°C, 30°C min^{-1} to 285°C and held at this temperature for 10 min. Injector temperature was 250°C.

Dipstick Immunoassay

For routine use, strips included only two test-points: one as negative control and the other for analyte determination. Briefly, 200 μL/well of 1/10,000 GAR-PA solution in CB was added to ELIFA pair rows (negative controls) and 200 μL/well of 1/200 R10 serum solution in CB was added to ELIFA impair rows and filtered through the membrane. The remaining binding sites were blocked with BSA 1% (wt/v) in TBS buffer (200 μl/well). Then, the membrane was taken away and dried. The reactive zone was determined by the ELIFA well size (4 mm diameter). Dried membrane (12×7 cm) was cut in 48 pieces (0.5×1.5 cm). These were stuck on a Nunc plastic self-adhesive sealing tape for easy handling. The ready to use strip tests could be stored in plastic vacuum-sealed bags at room temperature in the dark, for several weeks.

Semi-Quantitative Assays

The atrazine assay conditions were optimised in a previous work[10]: R10 1/200, 2d-PA 0.1 mg L^{-1} as Ab-tracer combination, 2 min as competition time, 2 min for washing step with TBS-T and 5 min for colour development by immersion into undiluted BCIP/NBT substrate.

The test results were visually interpreted taking into account the reference spot was intense purple and independent of atrazine concentration, while the colour of the test spot was inversely related to atrazine concentration (Fig. 1). For visual determination, colour disappearance was interpreted as a positive result (+). Thus, qualitative analyses were done on the basis of previously established sensitivity criterion (10 μg L^{-1}).

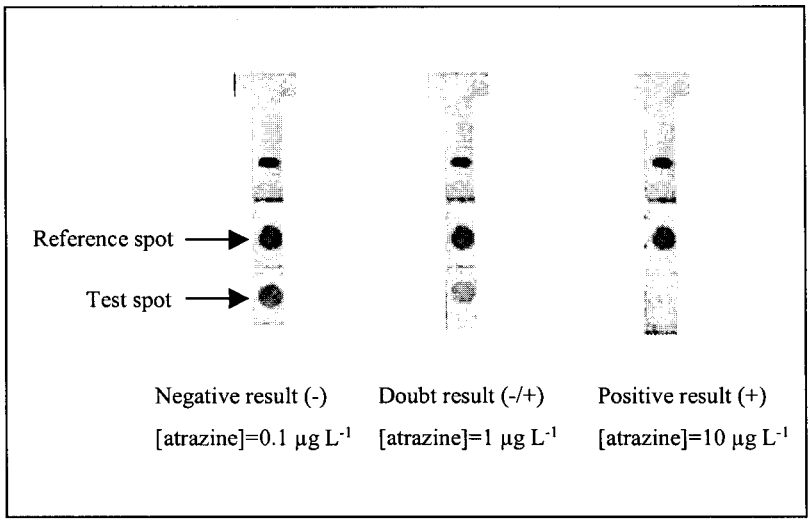

FIGURE 1 Comparison of results obtained by dipstick immunoassay for different atrazine concentrations.

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Quantitative Assays

For increasing assay sensitivity, instrumental colour measurements were made considering the colour space CIELab proposed in 1974 by TC-1.3 colorimetric committee from the Commission Internationale de l'Eclairage (CIE).

In order to quantify the differences in colour intensities between negative and positive samples, as well as among the different concentrations, a spectrophotometer CM-3500 *d*-photometer with Croma Control S v 1.41B package software (Minolta, Japan) was used. This was done measuring – from 400 to 700 nm reflectance values ± 10 nm intervals – the colour intensity differences between the witness spot (X_0, Y_0, Z_0) and the dot colour intensity of the test membrane (X, Y, Z). This colour difference was expressed as a single numerical value (a^*)[12].

Reflectance values were calculated as the sum of medium reflectance product [$T(\lambda)$], standard illuminate relative concentrations D_{65} [$S(\lambda)$] and colour functions for standard observer UNE 3 [$x(\lambda)$, $y(\lambda)$, $z(\lambda)$]. Average values were calculated from triplicate runs for each atrazine concentration using three measurements for each determination and quantified according to the equation: $a^* = 500\,\{(X/X_0)^{1/3} - (Y/Y_0)^{1/3}\}$.

Competitive curves were carried out fitting experimental results to a four-parameter logistic equation [13] using the Sigmaplot software package (Jandel Scientific, Erkrath, Germany).

Automatic Immunoreagents Immobilisation

An inkjet micro-dispenser controlled by a personal computer (PC) provided of a polypropylene sterile syringe was used to print R10 polyclonal sera onto a membrane. These devices are able to dispense micro drops of different sizes (1–100 μm).

General assay procedure was as follows: the membrane (21 × 28 cm) was previously conditioned as suggested by the manufacturer and placed into the device. The syringe

was filled with 1/100 (v/v) R10 solution in CB and straight lines printed in parallel. Since 1/200 dilution gave a weak dye line, a 1/100 dilution of R10 sera was established as optimum. Unoccupied sites were blocked by membrane immersion in a BSA 1% (wt/v) solution for 30 min. Finally, the membrane was dried and cut in 5×1 cm portions. In this sense, 105 strips could be obtained from each membrane. The steps of competition, washing and colour development were carried out as mentioned for semi-quantitative assays.

Due to size and shape of immobilisation surface being essential, several straight lines ranging from 100 μm to 3 mm of thickness were applied. For visual detection a big surface is required, whereas a smaller assay surface improves competition step increasing sensitivity. In this sense, both parameters were checked. For competition step, strips were immersed in a plastic box divided into eight compartments. Each compartment contained 1 mL of 2d-PA 0.1 mg L^{-1} and 1 mL of atrazine serial solution (from 0 to 10 mg L^{-1}), both in TBS buffer. The further assay was carried out as described before.

Different amounts of R10 sera were achieved by injecting several lines of antibody diluted to 1/100 in the same place. The protein quantity depended on the number of lines printed one over the previous one, and varied from 10 to 1. As strip size is 5 cm large, we could print a maximum of 10 lines in each strip.

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In addition, optimised conditions (size and number of lines) were used for atrazine determination in fortified water samples. For this purpose, serial atrazine solutions (from 0 – as control – to 10 mg L^{-1}) were prepared in distilled water from a 1000 mg L^{-1} atrazine stock solution. One millilitre of each concentration was mixed in a 2.5 mL reaction tube with 1 mL of enzymatic tracer in TBS 2X for conditioning purposes and strips were immersed for competition step. The following steps were carried out as described in the general assay procedure, and results were visually interpreted. In order to accomplish this goal, strip tests – by triplicate – were numerically identified from 1 to 7 corresponding to different atrazine concentrations (0 – as control – 0.1, 1, 10, 100, 1000, and 10,000 μg L^{-1}). With a slight modification of the basic program we could immobilise different immunoreagents. Thus, one position was occupied for a sterile syringe filled with 1/10,000 GAR-PA solution in CB and the other position for a sterile syringe containing 1/100 R10 solution in CB. In this sense, lines were alternatively printed starting with GAR-PA, acting as control line. Therefore, each strip test alternatively contained five control lines and five test lines (5×1 cm) with a distance between lines of 2 mm.

Application to Food Samples

Fresh and processed vegetables (asparagus, bean, broccoli, bonnet pepper, celery, cucumber, lettuce, melon, pea, red pepper, tomato and watermelon) were collected from a local market of Valencia (Spain) and checked for incurred atrazine residues by GC-MS. Before extraction procedure, samples were chopped and homogenised in a blender Osterizer (Milwaukee, WI). Atrazine-free samples were fortified at different levels (0 – as control – 50, 120 and 1000 μg L^{-1}) with a 1000 mg L^{-1} atrazine standard, mixed, homogenised overnight and extracted according to the following procedure. Vegetable samples (5 g) and 10 mL of MeOH were blended for 10 min and the supernatant vacuum filtered through 0.45 μm filters (Durapore® membrane filters) using a Millipore extraction device. After, the extracts were conditioned diluting one millilitre in a 2.5 mL reaction tube containing 1 mL of enzymatic tracer in TBS 2X. Finally, food

samples were checked for atrazine with dipsticks. In order to assess assay reproducibility, triplicates of each fortification level were done. Semi-quantitative and quantitative results were obtained by visual and instrumental determinations, respectively.

In order to assess the developed methodology (rapid extraction coupled to dipstick assay), the results were compared with the data obtained by GC-MS. On the other hand, samples were also extracted using the method described by Luke *et al.*[14] with slight modifications. Briefly, fortified samples (15 g) were homogenised with 30 mL of acetone in a centrifuge bottle for 30 s with an Ultra-turrax T-25 apparatus from Hanke and Junkle. Dichloromethane (30 mL) and 30 mL of light petroleum were subsequently added, and the mixture was homogenised for another 60 s. After centrifugation for 5 min at 4000 rpm, the organic layer was transferred to a graduated cylinder for recovered volume estimation. A portion of 25 mL was transferred to a conical-bottom flask. The solvent was evaporated to dryness and reconstituted with 5 mL of a ethyl acetate–hexane mixture (1/1, v/v). Finally, 2 μL of each sample were injected, by pulsed splitless, on GC column for atrazine quantification.

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RESULTS AND DISCUSSION

ELIFA Immunoreagents Immobilisation

Quantitative Assays

In order to ascertain instrumental sensitivity of the proposed methodology, dipsticks were prepared by using optimised conditions and applied following general assay protocol. For this purpose, seven reaction tubes of 2.5 mL were used for competition step containing 1 mL of 2d-PA 0.1 mg L^{-1} and 1 mL of atrazine serial solution (from 0 to 10 mg L^{-1}). To assess assay reproducibility ten strips were daily applied for each reaction tube obtaining ten calibrates per day. Process was repeated through six different days. For each strip, instrumental measurements were run by triplicate and a mean a^* value was assumed to build the mean dose-response curve for atrazine ($n = 60$ runs). As can be seen in Fig. 2, the error bars illustrate the good reproducibility of the standard curve (RSD < 19.3%) for atrazine values between 0 and 100 μg L^{-1}. Under these conditions, the working range (colour inhibition between 20 and 80%) was from 0.16 to 475.0 μg L^{-1} with an I_{50} value of 2.04 μg L^{-1}. Summary statistics of a^* colour instrumental measures, corresponding to the overall concentrations used to attain standard curve, are gathered in Table I.

Also sensory analysis was carried out by a panel of ten trained referees and the obtained sensitivity visual value (10 μg L^{-1}) was in concordance with a previous established one[10]. Results indicate that using instrumental colour measurements instead of visual determinations, the sensitivity of the assay could be five fold improved.

Effect of MeOH in Assay Performance

Previous to dipstick application in food samples, tolerance of the dipsticks to MeOH solvent was studied. For this purpose, serial atrazine solutions (from 0 to 10,000 μg L^{-1}) in distilled water were prepared with different percentages of MeOH (0, 2.5, 5, 10, 25, 50 and 95%). One millilitre of these solutions was conditioned with 1 mL of 2d-PA in TBS 2X and used according to assay protocol.

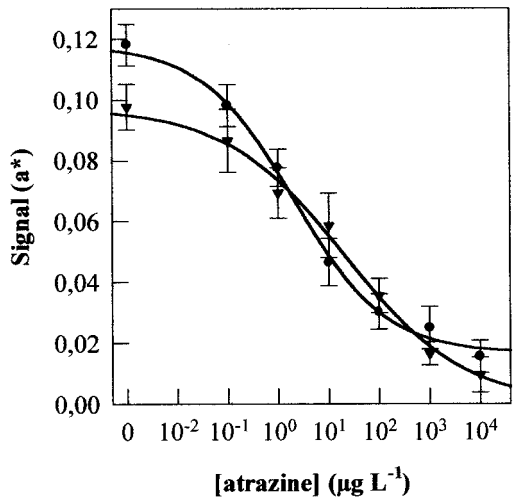

FIGURE 2 Calibration curves obtained by dipstick immunoassay: (●) using TBS buffer; (▼) using 50% MeOH – 50% TBS buffer.

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TABLE I Summary statistics of colour instrumental measurements corresponding to the overall concentrations used to accomplish the competition curve

	Atrazine concentration ($\mu g\,L^{-1}$)						
	0	*0.1*	*1*	*10*	*100*	*1000*	*10,000*
a^* (mean value)	0.118	0.098	0.078	0.047	0.030	0.025	0.016
SD	6.8×10^{-3}	6.8×10^{-3}	6.0×10^{-3}	7.8×10^{-3}	5.8×10^{-3}	7.0×10^{-3}	5.3×10^{-3}
Minimum	0.107	0.085	0.066	0.034	0.020	0.015	4.4×10^{-3}
Maximum	0.134	0.112	0.091	0.063	0.043	0.041	24.3×10^{-3}

SD. Standard deviation ($n = 60$). a^* Tendency to red colour (chromatic parameter of the CIELab system).

The results obtained indicated that even percentages of 50% MeOH could be used without loss of assay performances. Only a small attenuation of colour intensity was observed when MeOH percentage was increased. Concentrations of MeOH higher than 50% gave colour inhibition and creasing of the membrane. This result was very interesting since we could carry out the dipstick assay in an extract of the sample just diluting 1/1 (v/v) with enzymatic tracer in TBS 2X for conditioning purposes.

Dose Response Curve

In order to ascertain if the assay sensitivity was influenced by MeOH concentration, a calibration curve for atrazine using standards containing 50% MeOH was obtained as described before. As shown in Fig. 2, a^* values were lower than in aqueous media but a typical competition curve is drawn. In this sense, I_{50} value – corresponding to a colour inhibition (a^*) of 50% – was $21.09\,\mu g\,L^{-1}$. Therefore, a standard curve for atrazine containing 50% MeOH was used for food samples quantification in order to avoid erroneous atrazine results.

TABLE II Results of the analysis of spiked vegetable samples by GC-MS and dipstick test

Matrix	*Fortification level* ($\mu g\,kg^{-1}$) *(50)*		*(120)*		*(1000)*	
	DD[a]	*GC-MS*	*DD*	*GC-MS*	*DD*	*GC-MS*
Asparagus[b]	44.5 ± 1.2	47.0 ± 3.3	107.6 ± 12.4	97.3 ± 10.6	945.5 ± 15.0	994.2 ± 13.8
Bean[b]	49.3 ± 2.8	43.3 ± 1.2	102.3 ± 8.0	99.5 ± 5.3	931.1 ± 22.2	960.0 ± 11.1
Broccoli[c]	41.8 ± 2.3	39.9 ± 1.9	108.6 ± 13.7	96.3 ± 9.6	954.7 ± 13.6	973.3 ± 8.0
Pepper[b]	51.0 ± 2.1	46.1 ± 1.9	90.4 ± 5.6	105.3 ± 7.1	982.7 ± 11.1	1004.4 ± 21.1
Celery[d]	47.6 ± 1.5	47.8 ± 4.5	98.2 ± 3.0	115.0 ± 3.4	977.8 ± 9.7	940.2 ± 15.9
Cucumber[d]	44.3 ± 1.1	50.3 ± 1.8	110.0 ± 2.0	94.4 ± 4.0	997.5 ± 6.6	999.0 ± 18.4
Lettuce[d]	39.6 ± 6.1	41.0 ± 2.1	114.2 ± 4.0	94.4 ± 4.8	1007.6 ± 10.1	985.8 ± 20.4
Melon[d]	50.0 ± 2.0	42.6 ± 1.0	98.2 ± 2.9	84.5 ± 3.1	968.3 ± 13.4	977.2 ± 6.8
Pea[b]	51.5 ± 4.0	49.4 ± 3.3	104.6 ± 5.7	123.4 ± 3.0	857.9 ± 30.6	945.4 ± 11.5
Red pepper[d]	52.7 ± 2.5	51.1 ± 13.7	113.0 ± 12.1	120.0 ± 1.5	1026.5 ± 18.5	1042.4 ± 11.0
Tomato[b]	48.0 ± 2.0	44.7 ± 5.9	115.0 ± 4.0	109.2 ± 4.5	978.0 ± 16.0	1092.0 ± 10.8
Watermelon[d]	44.5 ± 0.7	44.8 ± 6.8	123.3 ± 2.5	103.2 ± 4.2	1013.5 ± 7.8	1039.9 ± 15.0

[a]Dipstick Determination. [b]Tinned, [c]Freezed and [d]Natural foods. Values are mean ± standard deviation of three determinations.

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Analysis of Food Samples

Vegetable samples were fortified and extracted as described in "Materials and Methods" section. The dipsticks were applied to the extracts and quantified as explained above.

As a preliminary validation study, the results obtained using dipsticks were compared with those obtained by coupling multiresidue extraction and GC-MS quantification. As can be seen in Table II, the effectiveness of the rapid MeOH extraction procedure was evidenced by the good recoveries generally obtained (75–105%) similar to those obtained by multiresidue extraction (70–109%).

Lettuce and broccoli samples fortified at $50\,\mu g\,kg^{-1}$ gave recoveries of 79% for both proposed and established methods. In addition, recoveries of 75% for tinned pepper and 86% for pea samples, corresponding at atrazine concentrations of 120 and $1000\,\mu g\,kg^{-1}$ respectively, were obtained by dipstick determinations (DD). Also, a value of 70% was obtained by GC-MS for $120\,\mu g\,kg^{-1}$ fortified melon samples. The recoveries were 93% (mean value) and similar to those obtained by the reference method 92% (mean value). Generally, the coefficient of variation (CV) was lower than 15.4% for both methods, with the exception of a 26.8 value for red pepper in GC-MS corresponding at an atrazine fortification level of $50\,\mu g\,kg^{-1}$.

Automatic Immunoreagents Immobilisation

Related to automatic immobilisation, two factors should be considered. First, the application system has to be thoroughly cleaned with distilled water avoiding the damage of the piezoelectric zone. Also, air bubbles remaining inside the syringe – after being filled with the immunoreactive solution – should be removed.

Related to line sizes, results obtained were in agreement with our earliest hypothesis. In this sense, 100 μm lines gave a visual sensitivity – colour disappearance – of $10\,\mu g\,L^{-1}$. This was in accordance with the value obtained in a previous work[10]. However, 3 mm lines gave more intense precipitates facilitating visual evaluation although assay sensitivity was ten fold decreased.

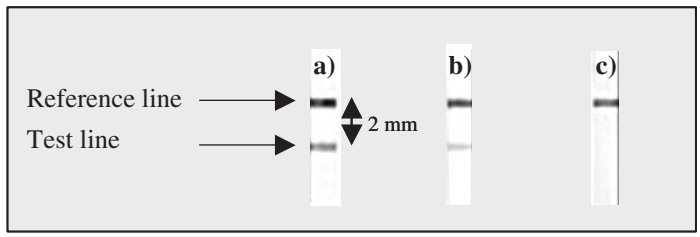

FIGURE 3 Representation of three dipsticks obtained after automatic immobilisation corresponding to atrazine concentrations of 0.1 $\mu g\,L^{-1}$ (a), 1.0 $\mu g\,L^{-1}$ (b) and 10 $\mu g\,L^{-1}$ (c). Assay Run: No coloured band may be present into the white central area of the strip. Negative Result (a, b): Two coloured blue-purple bands (more intense for the control line) appear across with the central area of the strip. Positive Result (c): Only the blue-purple control line appears across the white central area of the strip.

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Concerning to the number of lines printed one over the previous one (from 10 to 1), negative results were obtained when this number was below eight. The colour intensity was improved – without loss of assay sensitivity – when ten lines printed one over the one were applied, so this number was selected for coating purposes.

Consequently, strips were developed taking into account these considerations (lines of 100 μm and ten lines printed one over the other). The general assay procedure was performed for atrazine determination in fortified distilled water. As can be seen in Fig. 3, corresponding to atrazine concentrations of 0.1 (a), 1.0 (b) and 10 (c) $\mu g\,L^{-1}$, colour intensity of test line decreased in relation to colour of the control line. Colour disappearance was observed at an atrazine concentration of 10 $\mu g\,L^{-1}$ and it was established, the same as for dipsticks using ELIFA system, as visual assay sensitivity value.

Hence, we recommend the use of automated protocol instead of ELIFA for the immobilisation procedure, due to its high throughput, low-cost equipment, reduction of potential errors, speed and the possibility to develop multi-analyte test.

CONCLUSIONS

Strip test could be defined as powerful analytical tool. For extraction process, reference analytical methods use higher amounts of organic solvents than the proposed method. Only sample extracts dilution – with saline buffer – is required for immunoassays, decreasing the environmental impact caused by dangerous waste-solvents.

Based on the assay sensitivity, I_{50} of 2.04 $\mu g\,L^{-1}$ in aqueous buffer or 21.09 $\mu g\,L^{-1}$ in 50% MeOH, the proposed methodology will enable producers to assure that their products are free from atrazine residues, or their level is lower than the EU established MRL (0.1 $mg\,Kg^{-1}$).

The use of the proposed methodology (rapid extraction coupled to test strip determination) for the analysis of atrazine residues in vegetable samples guarantees data achievement in a fast way (about 20 min). Results could be interpreted by the user as semi-quantitative – visual determination – based on the strip cut-off value or quantitative using a portable colorimeter for colour measurements.

In essence, based on the performances and portability of the dipstick assay, producers and farmers would check for chemicals as a routine into their schedule process

control to ensure quality and safety of their products with enormous benefit for consumers and themselves.

Reproducibility and correlation between assays was improved using automatic systems for the immobilisation of immunoreagents.

Future developments include the use of fluorescent tracers (time resolved) combined with adequate-portable detection systems allowing assay quantification in a real time.

In addition, the ability for developing multianalyte strips could be very useful for multiresidue screening purposes.

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