Preconcentration and post-column fluorescent derivatization for the environmental water monitoring of an antihelmintic macrocyclic drug used in livestock

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ABSTRACT

In this paper, a green analytical methodology based on fluorescence derivatization is proposed for the antihelminthic drug monitoring ivermectin as environmental emergent contaminant. After sample clean-up, ivermectin was converted into a highly fluorescent derivative through a catalytic oxidation process followed by dehydration and tautomerization. Under optimal experimental conditions, a linear response was obtained for ivermectin within the range 0.38–600 µg L^{-1}, with detection and quantification limits of 0.11 and 0.38 µg L^{-1}, both values are lower than other previously reported. This method has been applied for ivermectin determination in environmental water samples at trace levels, showing its potential for contamination monitoring.

1. Introduction

Macrocyclic lactones are a group of compounds used for their efficacy against a broad spectrum of parasites in human and veterinary medicine [1]. Ivermectin (IVM) is one of the most representative compounds belonging to this family and is extensively used in a wide range of animals, including livestock [2, 3].

High doses levels of IVM are frequently administered to farm animals with the aim of ensuring the treatment success, even though that recommended IVM doses for anti-parasitic treatment are low (normally 0.2 mg Kg^{-1}). Commercial IVM formulations are available for systemic and topical administration, being the pour-on products the preferred for cattle prophylaxis and treatment. In case of systemic administration, IVM is mostly excreted unaltered and deposited in soil [4, 5], and then through rainfalls, certain amount of accumulated IVM in soil is swept along to aquifers and rivers, becoming a potential threatening for aquatic organisms [6]. Some possible consequences of environmental antibiotic accumulation are the microbiological equilibrium alteration and the occurrence of antibiotics resistant strains. Recently, some results have highlighted that the effects of IVM in the ecosystem must be investigated from a global perspective [7]. In addition, other results show that concerns of both acute and long-term risks of IVM to the aquatic environment cannot be dismissed and thus its use has to be monitored and controlled following a precautionary principle [8].

The main challenge for environmental monitoring is that the pollutants are usually found at low concentration and therefore, it is necessary the development of sensitive and selective analytical methodologies. Solid phase extraction (SPE) is one of the most commonly used techniques for this purpose in nowadays due to its efficiency to separate analytes from a complex matrix [9, 10, 11, 12, 13, 14]. Nevertheless, the use of SPE technique for sample treatment often is a tedious and time-consuming step that could be amended introducing a flow extraction system. Besides, additional advantages in the analytical performance are usually obtained such as the improvement of method precision and accuracy by reducing human errors.

Chemically, IVM is a mixture of 22,23-dihydroavermectin B_{1a} and 22,23-dihydroavermectin B_{1b} and its analysis commonly involves UV-Vis detection, since it absorbs strongly at UV spectrum range [15, 16, 17]. Analytical strategies for enhancing IVM detection sensitivity were reported including fluorescence derivatization of the target molecule,
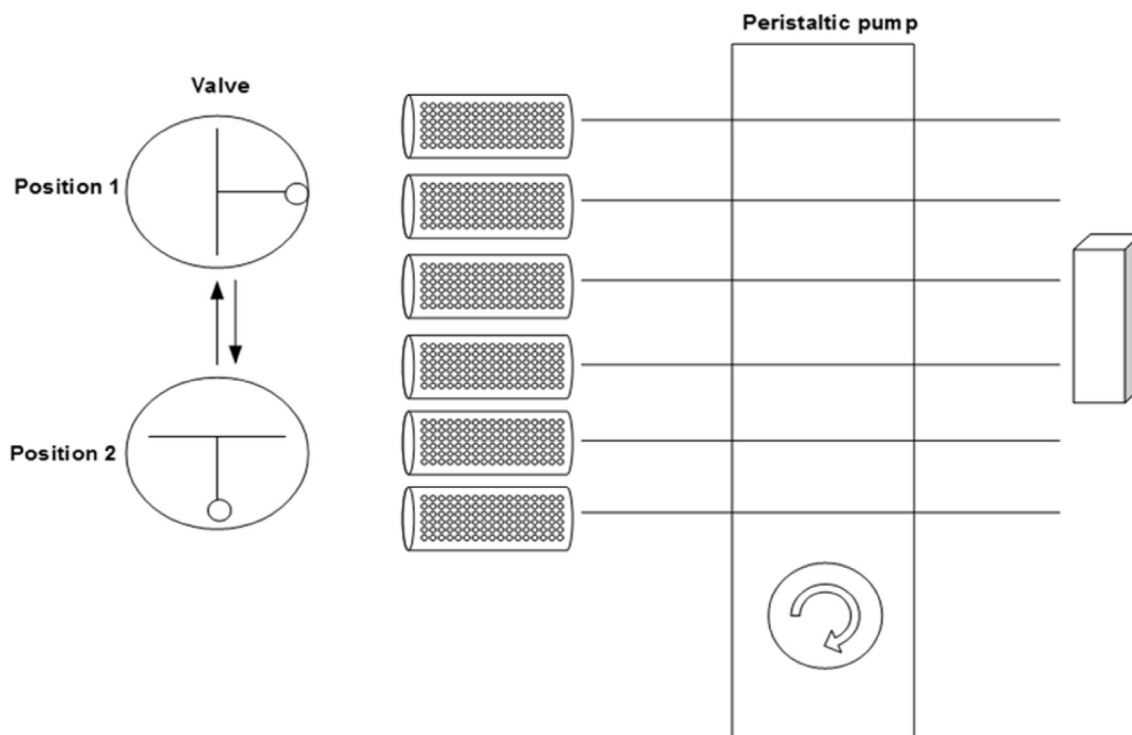

Fig. 1. Scheme of online pre-concentration device.

either by coupling a fluorophore or by chemical modification of the molecule structure. The last approach was considered since while the molecule of IVM lacks of native fluorescence, its deoxy-derivative is strongly fluorescent when aromatization of C2–C7 ring is achieved.

The purpose of this paper is the study of the spectral behavior of derivatized products obtained from the heterogeneous catalytic oxidation of IVM combined the extraction efficiency of SPE with sensitivity inherent to molecular fluorescence in environmental water samples. Furthermore, non-toxic and environmentally friendly solvents were used in the whole procedure including the extractive stage. The purpose methodology was validated by HPLC according to the International Conference on Harmonization (ICH) guidelines. Also, the combination of low reagents consumption techniques SPE, make this methodology, a green chemistry alternative to the conventional analysis.

2. Experimental

2.1. Reagents and assay solutions

Amberlite XAD-4 resin was purchased from Rhom-Haas (Philadelphia, USA) and was used as adsorbant after activation with HNO_3 (c)/ ethanol (1:4) for about 4 h.

Standard of IVM (>80% 22,23-dihydroavermectin B_{1a} (H_2B_{1a}) and <20% 22,23-dihydroavermectin B_{lb} (H_2B_{1b})) was kindly provided by Andrómaco S.A. (Buenos Aires, Argentine). NaOH and HCl were purchased from Merck (Darmstadt, Germany); Ammonium acetate was purchased from Sigma-Aldrich.

All chemicals used throughout the experiment were of analytical reagent grade. All solutions were prepared in ultrapure water (18 MΩ cm, Milli-Q system, Millipore). Ethanol (HPLC grade) was used as the eluent in SPE step.

Stock solution of IVM (1.0 mg mL^{-1}) was prepared by dissolving 100 mg of standard in a 100 mL flask with absolute ethanol. Standard working solutions were freshly prepared before each assay by diluting stock solution of IVM.

2.2. Instrumentals

A Shimadzu RF-5301PC spectrofluorimeter (Shimadzu Corporation, Analytical Instrument Division, Kyoto, Japan), equipped with a Xenon discharge lamp and quartz cells were used for molecular fluorescence measurements.

Solutions were propelled by Gilson Minipuls 3 peristaltic pump with PVC pumping tubes and 3 valves were used for FIA configuration.

All HPLC experiments were carried out on a Gilson HPLC system equipped with a Gilson 322 controller pump operating at 1.0 mL min^{-1}, a Rheodyne 7725i injector with a 50 μL sample loop, and a variable-wavelength UV-Vis 156 Gilson detector measuring at 245 nm. The analytical column used was a Phenomenex Luna RP Si C18 column (5 μm, 100 Å, 250 × 3 mm) (Torrance, CA, USA), the separation was carried out at room temperature. All calculations were performed using Matlab 6.0 software. The surface responses were graphed using Statistica 6.0 software.

A pH meter (Orion Expandable Ion Analyzer, Orion Research, Cambridge, MA, USA) Model EA940 with combined glass electrode was used for monitoring pH adjustment.

2.3. Preparation of water samples y preconcentration columns

Water samples were collected from a waterhole of a rural zone (San Luis, Argentina). Each aliquot of 1000 mL was filtered through filter paper to eliminating solid remains, and stored in polyethylene flasks under refrigeration at 4 °C. Bi-destiled water free of IVM was used as sample blank.

Each preconcentration column was a modified 1.0 mL syringe barrel, with 1 cm of inner diameter, filled with XAD-4resin (1.0 g), previously activated with diluted HCl and washed with bi-destiled water, until getting a transparent color in the washings.

2.4. Manifold of the IVM preconcentration in flow system

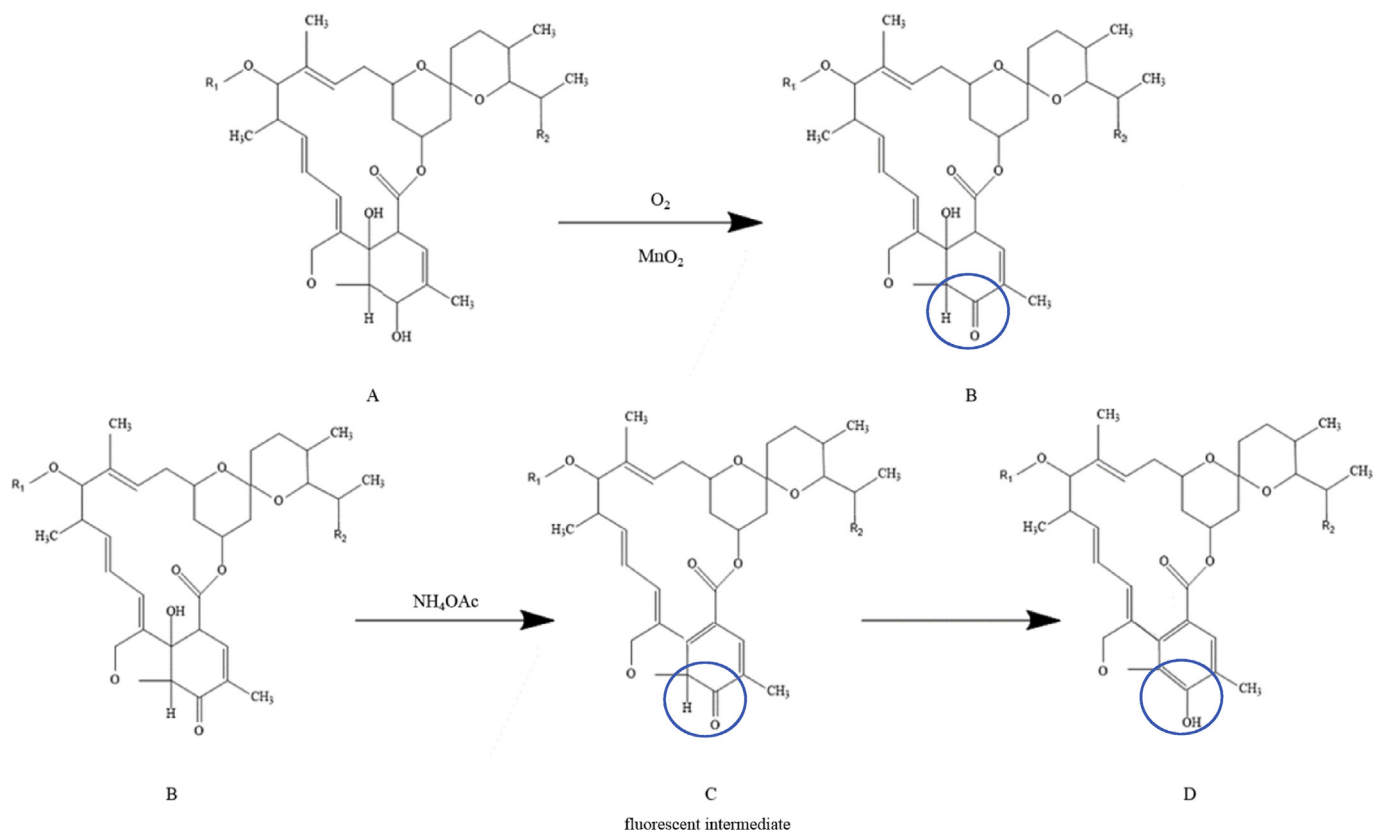The online preconcentration protocol was as follows: the standard or

Fig. 2. (A) IVM, (B) H_2B_1a -5-one, (C) fluorescent intermediate, (D) final fluorescent product; R_1: a -L-oleandrosyl-a-L-oleandrosyloxy, R_2: C_2H_5.

sample solution was flow through the homemade column to waste (3.0 mL min^{-1}) with the valve in load position as shown in Fig. 1. The adsorbed analyte was then eluted with ethanol by switching the valve to eluting position, allowing the eluent stream flow through the column in the opposite direction. After discharging the first mL, the following 15 mL of eluate were collected for further analysis and the eluent stream continued for 15 min in order to regenerate the resin for the next sample preconcentration.

2.5. Fluorescence derivatization process

The obtained eluate after preconcentration was transferred to a centrifuge tube and 0.10 g of MnO_2 were added. The mixture was shaken for 30 min and then centrifuged for 15 min at 3500 rpm. The MnO_2 was removed by filtration, obtaining a clean and transparent solution. Afterward, the solution was poured off into a rotary bottle and dried using rotary evaporator. Then, 3 mL of ethanol solution saturated with ammonium acetate was vigorously agitated for 30 seconds and the content was transferred to 1 mL Pierce Reacti-vials. The vial was placed in a water bath at 60 °C for 30 min.

2.6. Fluorescent quantification analysis

The total content of the vial was quickly transferred to a 1 cm quartz cell for spectrofluorescent measurement for avoiding evaporation of the solvent. The experimental conditions were established for performing off-line assays at $\lambda_{em} = 440$ nm ($\lambda_{ex} = 340$ nm). Calibration curves of ergotamine were achieved and data were fitted by standard least-squares treatment.

2.7. Recovery study by HPLC analysis

The quantification of IVM in water samples was carried out by HPLC-DAD (Chromatographic separations were carried out by UHPLC focused Thermo Scientific Dionex Ultimate 3000 Series equipped with Dionex Ultimate 3000 autosampler, Quaternary separation system Dionex Ultimate 3000 Pump and Diode Array detector DAD-3000 (RS). An Zorbax Eclipse XDB-C18 (4.6 × 150mm; 5 μm; Agilent) column was employed for all chromatographic analysis) and the obtained results were statistically compared against those found with the proposed methodology. Standards solutions of IVM were added over water samples within a concentration range of 0.01–1.00 mg L^{-1} and the flow preconcentration procedure was applied. After preconcentration step, the obtained eluate was then evaporated until dried, re-suspended in 1.0 mL of methanol and injected in an HPLC instrument using as a mobile phase a solution composed of acetonitrile-methanol-H_2O (53:27:20). Sample and standard (50 μL) were successively injected to HPLC, with a flow rate of 1.0 mL min^{-1}.

3. Results and discussion

Due to its non-polar nature, IVM was efficiently adsorbed onto the non-polar resin bead, while the sample stream washed out all polar and ionic solutes. The robustness of preconcentration process was evidenced by showing no variations in its performance when the pH range was varied between 2.0-11.0. Once IVM was desorbed from XAD-4 resin using ethanol, the eluate was collected for further fluorescence derivatization step.

3.1. Fluorescent derivatization

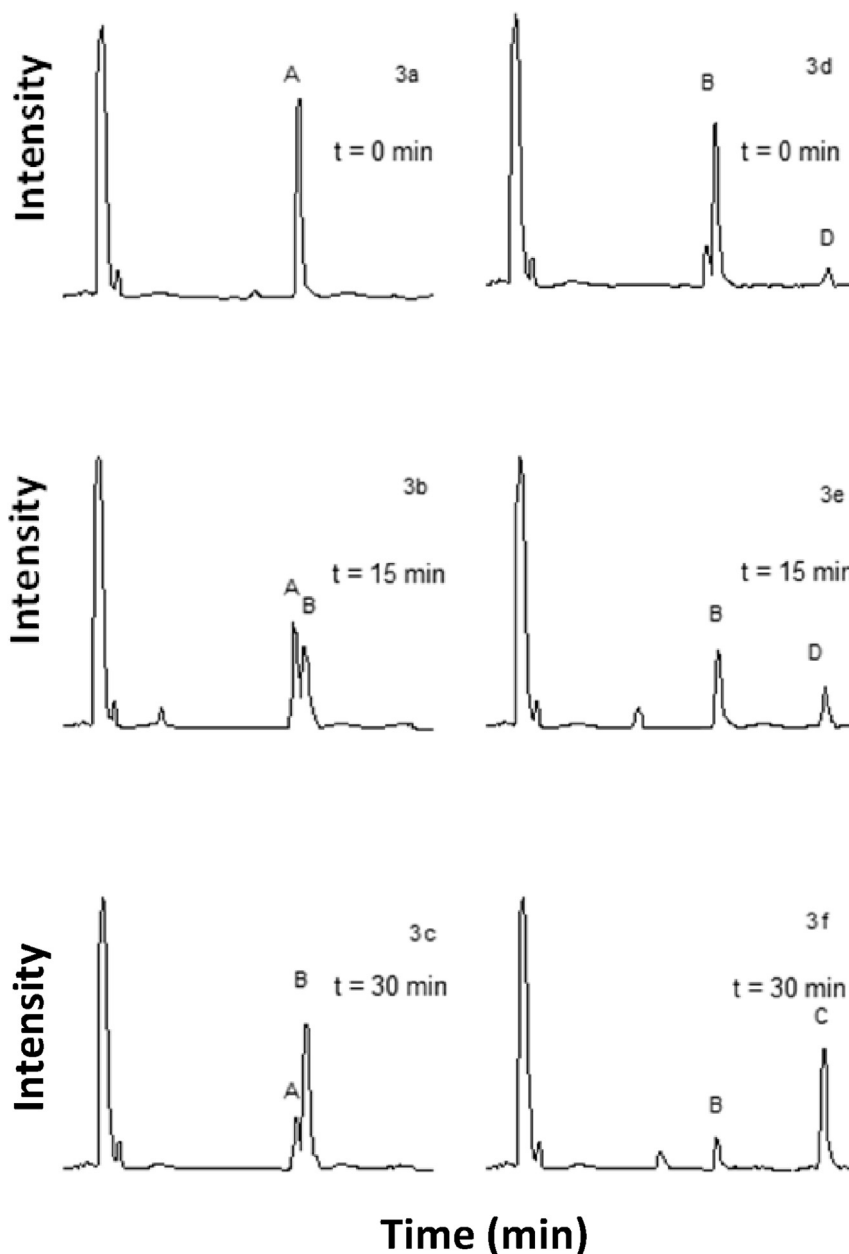The UV-Vis spectrum of IVM molecule exhibits a strong maximum

Fig. 3. Derivatization reaction progress monitored by HPLC. Step 1: oxidation after MnO$_2$ addition at 0, 15 and 30 min (Fig. 3a, b and c, respectively). IVM peak (A) was gradually decreasing while a second peak corresponding to H$_2$B$_{1a}$ -5-one (B) increased. Step 2: dehydration and tautomerization at 0, 15 and 30 min (Fig. 3d, e and f, respectively).

absorption at 262 nm, but it has null native fluorescence due to its rapid non-radiant deactivation process. We have achieved a modification of the Stong method [14] for IVM fluorescence derivatization which consisted in two distinct steps (Fig. 2). Firstly, MnO$_2$ was used as catalyst for IVM oxidation leading to obtain the 5-keto derivative (H$_2$B$_{1a}$ -5-one) (Fig. 2a). In second place, ammonium acetate produced the ketone dehydration followed by a tautomerization to form the fluorescent phenol (Fig. 2b).

The progress of the formation of the fluorescent derivative from H$_2$B$_{1a}$ -5-one proceeds through an intermediate that is shown in Fig. 2b [14]. The conversion of H$_2$B$_{1a}$-5-one (B) to the fluorescent derivative (D) proceeds through a fluorescent intermediate (C) after a dehydration resulting in the formation of a C$_2$-C$_7$ double bond as was proposed by Stong [14]. This is consistent with the known acidity of the C$_2$ proton in H$_2$B$_{1a}$ and the other avermectins due to the charge delocalization by resonance. The formation of the phenolic hydroxyl of the final fluorescent product (D) could happen by tautomerization between C$_6$-H and the

5-ketone.

The progress of both steps was monitored by HPLC. As can be seen in Fig. 3a and b y c, after MnO$_2$ addition (at 0, 15 and 30 min), the IVM signal (A) was gradually decreasing while a second signal (B, corresponding to H$_2$B$_{1a}$ -5-one) was appearing.

Several experimental conditions were studied to achieve the molecular dehydration by ammonium acetate, such as temperature, reaction time and reagent concentration. The reaction rate with ammonium acetate increases exponentially with temperature up to the boiling point of ethanol. Fig. 3d, e and f shows monitored by HPLC the relative extent of the reaction of H$_2$B$_{1a}$ -5-one in ethanol saturated with ammonium acetate after 30 min. Based on this result, a temperature of 60 °C was selected for analytical applications. The reaction reaches a maximum concentration of the oxidized derivative after 30 min at 60 °C and remains essentially constant thereafter for at least 100 min.

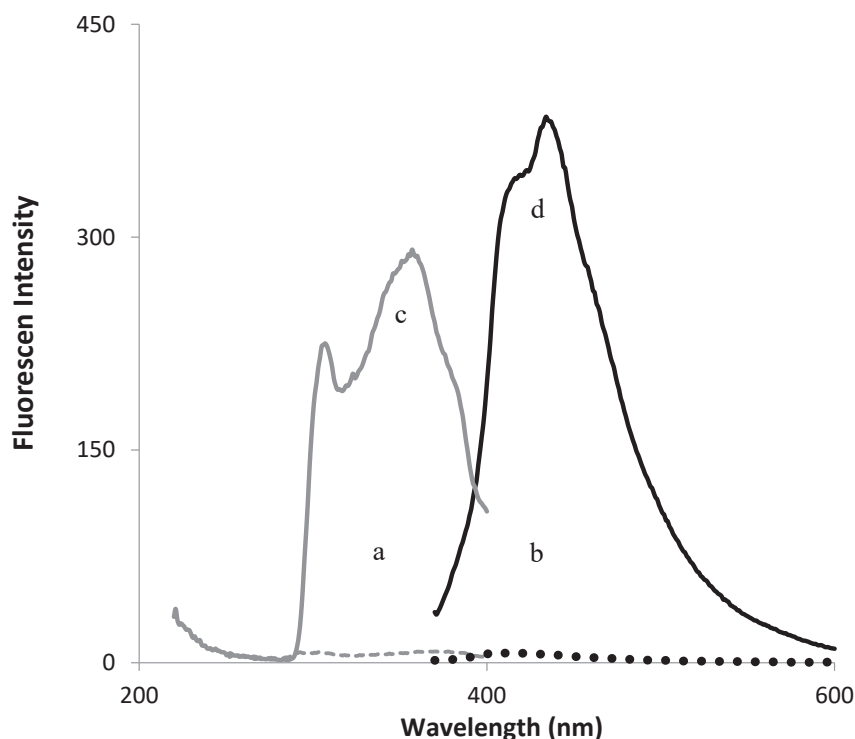The proposed methodology shows several advantages when is

Fig. 4. Fluorescence spectra of IVM, previous and after derivatization procedure, IVM initial concentration 0.6 μg/mL. Where: a) and b) are the excitation and emission spectra of the derivatized product of IVM, respectively; c) and d) are the excitation and emission spectra of IVM, respectively. Derivatized product exhibit maximum λ_{em} = 433 nm (λ_{ex} = 350 nm).

Table 1
Analytical parameters of the proposed methodology.

Parameters	Values
SD[a]	0.05
Slope (m)[a]	1.44
R^2	0.9993
LOD (μg L^{-1})[a]	0.11
LOQ (μg L^{-1})[a]	0.38
Linearity range (μg L^{-1})[b]	0.38–600

[a] Excitation and emission slit width of 5-5 nm.
[b] Varying excitation and emission slit width from 1.5-1.5 nm to 5-5 nm.

compared against the reported by Stong. The evaporation under a stream of pure N_2 was not needed and the use of acetone was avoided.

3.2. Fluorescent quantification

The obtained derivatized product exhibited a maximum fluorescence emission at 433 nm when it was excited at 350 nm (Fig. 4).

The stability of the fluorescent derivate was studied by monitoring its emission intensity. After 2 h of synthesis, it was observed a decreasing emission intensity and the red-shifting of the maximum emission wavelength.

Under optimal conditions, the IVM calibration curve was obtained by plotting fluorescence intensity against the concentration (reactive) and was linear within the range 20–500 μg L^{-1} for IVM, with a correlation coefficient of 0.9989. The obtained results when applying the proposed SPE-spectrofluorescence were compared against those obtained by SPE-HPLC-DAD method. The solvent volume consumption is lower in the developed SPE-FD methodology and is also less time consuming and simpler than the chromatographic method.

3.3. Method validation

3.3.1. Linearity, precision and accuracy

The analytical validation of this methodology was performed according to the Guidelines of the International Conference on Harmonization (ICH) [18]. For the evaluation of the linearity of the SPE-spectrofluorescence methodology, IVM was studied within the range of 0.3–600 μg L^{-1}. The obtained fluorescence intensities were used to plot the calibration curve and the corresponding equation was obtained by the least-squares linear regression method. Calibration curve was linear within the range 0.38–600 μg L^{-1}, with the regression coefficient (R^2) of 0.9993 for determination of IVM (Table 1). This linear concentration range is greater than the obtained when performing the SPE-HPLC-DAD method.

The calculated F value was higher than tabulated critical value for a significant level of 0.05 showing that the regression adjust found has a slope significantly different from zero [19].

3.3.2. LOD and LOQ

Concentration level of analyte, which could be detected with a signal-to-noise ratio ≥3 was considered to be the limit of detection (LOD). Limit of quantification (LOQ) was defined as the analyte concentration that can be accurately and reliably determined with a signal-to-noise ratio ≥10. For the experimental LOD and LOQ values determination, a signal blank was obtained after the fluorescent measurement. The LOD was calculated as three times of the standard deviation (3 SD; n = 6) of the blank and LOQ as ten times of SD (10 SD n = 6) of the blank. The obtained LOD and LOQ for this methodology were 0.11 μg L^{-1} and 0.38 μg L^{-1} respectively. The reported values were obtained by applying the developed procedure after preconcentration and derivatization steps and were more sensitive than those recently reported by Raich-Montiu and Park et al for tap and lake water in the first case and stream water in the second case [20, 21]. In the first work, when the authors used hollow fiber-supported liquid membrane (HF-SLM) extraction and LC-MS/MS for quantification, LOD

Table 2
Recovery study of the proposed methodology in real samples.

Aliquots[a]	Base value (µg L^{-1})	Added (µg L^{-1})	Found ± RSD (%) (µg L^{-1})	Recovery[b] (%) ± RSD (%)
I	110.01	–	110.01 ± 4.5	–
II	–	50	157.55 ± 1.1	100.8 ± 1.5
III	–	100	213.41 ± 2.2	100.7 ± 1.9
IV	–	150	245.72 ± 2.6	101.5 ± 2.1
V	–	300	409.72 ± 3.0	99.8 ± 2.6
VI	–	500	609.95 ± 1.2	99.9 ± 0.9

Excitation and emission slit width of 5-5 nm.
[a] 1000 mL of environment water obtained from waterhole of a rural zone.
[b] Recovery = (Found-Base value)/Added × 100.

and LOQ were 0.20 µg/L and 0.75 µg/L respectively. Park et al obtained LOD and LOQ values of 0.15 and 0.5 µg/L respectively, by using hollow fiber-assisted liquid-phase microextraction (HF-LPME) coupled with LC–MS/MS.

3.3.3. Accuracy, precision and matrix interference study

The accuracy of the present methodology was evaluated by means of a recovery test by using six aliquots of the water sample spiked with IVM by triplicate. The precision of the methodology was evaluated by calculating the respective RSD% after applying the proposed method by triplicate and using it for quantification. The results of the recovery test are shown in Table 2 demonstrating an adequate accuracy with a reliable precision for IVM determination in the analyzed samples. No significant differences were found in the calculated slop of the calibration plot for the standard and the spiked sample, which indicates no matrix effect on IVM determination.

Furthermore, the calibration plot of this methodology was checked against those obtained by the HPLC method. Using paired t-test analysis at 95% confidence interval of the mean, SPE-spectrofluorometry and SPE-HPLC methods gave no significant difference in the determined amounts of analytes in water samples (P-value = 0.19; T-value = 1.547; 5 degrees of freedom).

4. Conclusions

This study demonstrates the feasibility of employing a spectrofluorescence methodology combined to a flow preconcentration step using XAD-4 resin for the monitoring of ivermectin in real samples. A rapid and simple fluorescence derivatization of IVM in the final eluate method was developed. The reported methodology enhanced greatly the parameter of sensitivity in comparison to traditional separation techniques. The obtained LOD and LOQ values were lower than others recently reported for environmental and stream water that require expensive and complex equipment.

The two-step derivatization only involved a rapid catalytic oxidation followed by a tautomerization with ammonium acetate. Neither for preconcentration nor derivatization steps was involved the use of long-chain polluting solvents, in order to offer a green alternative for IVM determination.

The proposed methodology involves the study of the spectral behavior of the derivatization products obtained from heterogeneous catalytic oxidation of IVM. The simplicity, versatility and high sensitivity achieved for the present methodology has demonstrated its potential for the analysis of IVM in environmental water samples.

Declarations

Author contribution statement

Roxana Gomez, Emiliano Felici, Chien Wang, Cristian Casado, Ana Vicario, Víctor Pereyra: Conceived and designed the experiments;

Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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