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Article

Determination of Trifloxystrobin in Soy Grape Juice and Natural Water by Photo-Induced Fluorescence and High-Performance Liquid Chromatography

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A fluorescent (340/380 nm) photochemical product, identified at *m/z* 206 by mass spectrometry was obtained by photo-derivatization (15 s ultraviolet exposure) in water/acetonitrile 10/90 v/v. The photoproduct was used for indirect determination of trifloxystrobin in water and soy grape juice by high-performance liquid chromatography and fluorescence detection. Separation was made under isocratic conditions (acetonitrile/water 70/30% v/v, at 1.0 mL min^{-1} and 35 °C) with trifloxystrobin photo-derivative eluting at 3.2 min. The influence of ultraviolet exposure was evaluated in short and long terms with photoproduct showing stability up to 120 min after 15 s of ultraviolet exposure. The limit of detection was 57 μg L^{-1} in water (87-109% recoveries). For soy grape juice, dispersive liquid-liquid micro-extraction was used to clean up and for pre-concentration (limit of detection of 9.5 μg kg^{-1} and 93-101% recoveries) attending to the maximum residue limits for citrus juices established by regulatory agencies. Potential interference by triazoles was evaluated.

Keywords: fungicide, trifloxystrobin, high-performance liquid chromatography, photochemically-induced fluorescence, dispersive liquid-liquid microextraction

Introduction

There has been an increase in the quantity and variety of organic substances detected in surface waters and in food items. Cases of pesticide contamination, originating from agricultural activities, are duly documented in the literature.[1-4] In fact, the Brazilian National Cancer Institute (INCA) estimates about 20 thousand deaths *per* year related to pesticides worldwide.[5] There is no consensus about safety levels for pesticide residues since each one has a different harmful potential, also with respect to the levels at which the effect of biomagnification (progressive bioaccumulation of persistent substances) occurs.[6] The European Union (EU) established regulatory guidelines for drinking water with maximum permissible concentration of 0.1 μg L^{-1} for individual pesticides and their degradation products, and 0.5 μg L^{-1} for total pesticides (European Commission, Regulation EU 2016/486, 2020).[7] For citrus juices, the maximum residue limits (MRL) for trifloxystrobin are 0.2 to 0.6 mg kg^{-1} depending on the regulatory agency.[8]

Synthetic strobilurin class pesticides present, in the main structure, a group (*E*)-β-methoxy-acrylate,[9] which guarantees greater stability and greater anti-fungi potential with systemic curative, preventive and translaminar actions,[10] with rapid and concentrated action in the first period of the fungal life cycle.[11] Strobilurins have low solubility in water, about 0.6 mg L^{-1} at 25 °C in the case of trifloxystrobin,[12] but are promptly soluble in polar organic solvents, usually presenting insignificant or no natural fluorescence, which is also the case for trifloxystrobin (Figure S1, Supplementary Information (SI) section). Their structure contain isolated aromatic nuclei (absorbing electromagnetic radiation in the range of 200 nm) and substituent groups which, in solution at room temperature, reduce rigidity of the molecular structure, favors energy relaxation by radiantionless processes.[13] Studies[14-20] concerning strobilurin photoproducts, including trifloxystrobin have

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been reported. For instance, Almeida *et al.*[14] reported the kinetics, with half-life ($t_{1/2}$) of 1.07 min, in which after 20 min of UV exposure, only about 1.3% of the trifloxystrobin (in a thin film) remained in its original form.[14]

Dispersive liquid-liquid microextraction (DLLME) is a new extraction and pre-concentration technique that was developed in 2006 by Rezaee *et al.*[21] aiming to miniaturize sample treatment and minimize the use of excessive amounts of toxic solvents, common in conventional extraction methods.[22] In DLLME, the extraction is accomplished by an interaction between the sample and a cloud of fine droplets of extractor after injecting an appropriate mixture of extractor and dispersing solvents into an aqueous sample.[23] After the formation of a cloudy solution, the surface area between the extraction solvent and aqueous sample increases, causing a rapid equilibrium state.[24] The advantages of this approach are its technical simplicity, low cost, shorter extraction time, faster analysis and, high enrichment factor.[23] Many works[21-25] that used these new extraction techniques have already been developed.

Literature reports different analytical methods for the determination of strobilurins. In the case of trifloxystrobin, a brief review of methods is presented in Table S1 (SI section). Besides them, it can be highlighted the following works based on the use of high-performance liquid chromatography (HPLC), gas chromatography (GC) and voltammetry. Abreu *et al.*[12,20] used liquid-liquid extraction (LLE) and cleaning up on silica cartridges prior to quantification of strobilurins, including trifloxystrobin, in grapes and wine using HPLC with absorption photometric detection. Limit of detection (LOD) for trifloxystrobin was 0.1 mg kg^{-1} in grapes and 0.2 mg L^{-1} in wines. Campillo *et al.*[26,27] determined seven strobilurins, including trifloxystrobin, in fruits using stir bar micro-extraction and liquid chromatography (LC) coupled with absorptiometric detection. Recovery for trifloxystrobin varied between 83 and 102% and the LOD was 0.3 ng g^{-1}. Dost *et al.*[28] used high-resolution HPLC with absorption photometric detection (265 nm) to determine boscalid, pyraclostrobin, and trifloxystrobin. They used an ODS column with acetonitrile/water as mobile phase at 1.5 mL min^{-1} flow rate. Recoveries for trifloxystrobin were from 52 to 64%. Chen *et al.*[29] developed a method for the simultaneous determination of trifloxystrobin and its metabolite (trifloxystrobin acid) in rice and in soil using a solid phase extraction (SPE) based on QuEChERS (quick easy cheap effective rugged and safe) protocol prior to the high-resolution HPLC analysis using tandem mass spectrometry (MS) detection. The LOD was 0.22 μg kg^{-1} and recoveries from 74.2 to 107.4%. Schurek *et al.*[30] determined six strobilurins, including trifloxystrobin, in wheat using two analytical techniques: time-of-flight MS (DART-TOF MS) and HPLC-MS with desorption electrospray ionization (DESI). Extraction with ethyl acetate by DART-TOF MS method, or extraction by LLE with methanol by HPLC-DESI-MS or the use of SPE (with a QuEChERS protocol) allowed recoveries from 78 to 96%, with limits of quantification (LOQ) for trifloxystrobin as low as 2 μg kg^{-1}. More recently, Feng *et al.*[31] determined trifloxystrobin and its acid metabolite by HPLC-MS after solid phase extraction (with a QuEChERS protocol). Recoveries from 84 to 106% and LOQ of 0.01 (in wheat grains) and 0.05 mg kg^{-1} (in wheat plants and straw) were obtained.

Viñas *et al.*[32] determined seven strobilurins, including trifloxystrobin, in infant soups using solid phase micro-extraction and GC coupled to MS detection (recoveries from 88 to 104 % and LOD of 2 pg mL^{-1}) for trifloxystrobin. Mohapatra[2] assessed residue levels of trifloxystrobin and tebuconazole in mango using SPE (with a QuEChERS protocol) before GC-MS determination, achieving LOD of 0.015 μg mL^{-1} for trifloxystrobin (residue levels found up to 1.2 mg kg^{-1}). Paramasivam *et al.*[33] determined residues of trifloxystrobin, and its acid metabolite in tea by GC-MS (recoveries from 84.2 and 96.3% and LOD was 0.015 μg g^{-1}). Mohapatra[3] also determined residues of trifloxystrobin and tebuconazole on gherkin by GC-MS, finding concentrations up 0.65 mg kg^{-1}. Authors also found that trifloxystrobin residues dissipated at the half-life of 2.9-3.7 days achieving levels below the MRL of 0.2 mg kg^{-1} set by the EU.[3] Almeida *et al.*[14] studied the UV degradation of trifloxystrobin using square-wave anodic voltammetry with a boron-doped diamond electrode, at +1744 mV, with instrumental LOD of 0.058 mg L^{-1}. Orange juice samples were analyzed with recoveries about 80 % while in water samples recoveries from 92.4 to 104 %.[14]

No report in the literature has yet addressed studies aiming the fluorimetric determination of trifloxystrobin by means of photochemical derivatization. Photochemical reactions induced by UV causes breaking of structural chemical bonds of molecules and the availability of protons in the reaction medium may also induce cyclization and formation of unsaturated bonds as a result of dehydration, producing smaller fluorophore products with more rigid structures. UV radiation can be seen as a low cost means of reaction and is readily available at various intensities, depending upon source and photochemical reactor design.[34] This research group has already studied the effect of UV on other strobilurins (azoxystrobin, dimoxystrobin, fluoxastrobin, kresoxim-methyl, picoxystrobin, pyraclostrobin and trifloxystrobin) regarding the production of luminescent photo-derivatives.

Kresoxim-methyl showed a satisfactory fluorescence at 370/430 nm (excitation and emission wavelengths)[35] and trifloxystrobin at 340/380 nm. Based on results achieved in this preliminary study, this work aims to develop a HPLC based method to determine trifloxystrobin in soy grape juice and water (from a local creek) after off-line exposure of the sample to UV radiation in order to produce a fluorescent photoproduct also degrading potential fluorescent sample matrix interferent components. Potential interferences from tebuconazole and cyproconazole were evaluated.

Experimental

Apparatuses

A Milli-Q gradient A10 ultra-purifier (Milipore, Billerica, USA) was the source of the ultra-pure water. Analyses by reverse-phase HPLC were made on an Agilent 1200 series system (Agilent Technologies, Tokyo, Japan) with a fluorescence detector (set at 340/380 nm) and an automatic sampler. Separation was achieved using a column Agilent Eclipse XDB C18 (4.6 × 250 mm, 5 µm particle size) and oven temperature set at 35 °C. A 9 L volume NSC2800 model ultrasonic bath (Unique, São Paulo, Brazil) was used to aid the dissolution of analytes and for degassing of mobile phases. Steady state fluorescence measurements were made on a PerkinElmer (Norwalk, USA) LS 55 luminescence spectrophotometer. Mass spectrometry studies were made using an ultra-performance liquid chromatography (UPLC) system (Waters, Milford, USA) with MS detection on an Orbitrap XL mass spectrometer (Thermo Scientific, Bremen, Germany) utilizing electrospray ionization (ESI) in positive mode. The pH measurements (performed to obtain reference values that do not reflect exactly the acid condition of the acetonitrile/water system) were made on a Tecnopon MPA 210 model pHmeter (Tecnopon, Piracicaba, Brazil) calibrated in the pH ranges of 4, 7 and 10 (aqueous buffer solutions) at room temperature. The photochemical reactor consisted of six sterilization mercury lamps (6 W each with main line at 253 nm and secondary lines in the range of 296 to 313 nm) mounted inside a cylindrical PVC cabinet (200 mm of diameter and 290 mm of depth), which enabled uniform irradiation of samples and placed in the central part of the reactor. Quartz tubes (20 mL volume) were used to accommodate sample solutions, then placed on a support that kept rotating during the UV exposure. A small fan, placed at the back of the reactor, kept internal temperature stable and below 30 °C. A centrifuge BE 4000 Brushless (Bio-Eng, Rio de Janeiro, Brazil) was used to perform phase separation during the DLLME procedure. Statistica® Software (7.0, Statsoft, USA)[36] was used for the experimental design and for the statistical treatment of the data.

Reagents and materials

Trifloxystrobin (99.5% purity), tebuconazole and cyproconazole were obtained from Sigma-Aldrich (St. Louis, USA). HPLC grade acetonitrile was obtained from Tedia (Fairfield, USA). Sodium hydroxide, hydrochloric acid and carbon tetrachloride were purchased from Merck (Darmstadt, Germany). Ultra-pure N_2 gas was from Linde Gases (São Paulo, Brazil). Borosilicate glass microfiber membrane (0.2 µm) and polytetrafluoroethylene (PTFE) syringe filters (0.45 µm) were from Whatman (Maidstone, UK).

Preparation of standard solutions

Trifloxystrobin, tebuconazole and cyproconazole stock solutions (at 1.0×10^{-3} mol L^{-1}) were prepared in acetonitrile. Hydrochloric acid and sodium hydroxide stock solutions (1.0 mol L^{-1}) were prepared in ultrapure water. To evaluate the influence of the solvent system on the UV-induced derivatization of trifloxystrobin, different conditions were tested with trifloxystrobin solutions (at 1.0×10^{-4} mol L^{-1}) prepared using 90% acetonitrile and 10% water containing either HCl or NaOH (at different final concentrations). For other studies, diluted solutions of trifloxystrobin were prepared in acetonitrile/water (70/30% v/v). Mobile phase solvents were previously filtered through a 0.2 µm borosilicate glass microfiber membrane.

Preparation of samples

Water from the Queen creek and from the Rodrigo de Freitas Lagoon (Rio de Janeiro, Brazil) were collected and kept in the refrigerator for no longer than 24 h after collection. Prior to analysis, procedure followed the steps: (*i*) water samples were collected in amber flasks, previously cleaned with 10% v/v nitric acid and washed with ultrapure water; (*ii*) 1.0 mL volume of water sample was mixed with 0.5 mL of ultrapure water and then transferred to a 10.0 mL volumetric flask and fortified with a specific volume of analyte standard solution; (*iii*) the volume was adjusted with acetonitrile at the proper proportion (water/acetonitrile 30/70% v/v). When necessary, water samples were fortified at two concentration levels (3.0×10^{-6} and 7.0×10^{-6} mol L^{-1}).

Dispersive liquid-liquid micro-extraction (DLLME) procedure was adapted from Toloza *et al.*[35] but using 3 g of the soy grape juice sample, weighed in a 15 mL centrifuge

polypropylene tube, then fortified with microvolumes of the trifloxystrobin stock solution (1.0×10^{-4} mol L^{-1}) before homogenization. Tests with non-fortified samples were also made and used as sample blanks. A mixture of carbon tetrachloride (60.0 μL) and acetonitrile (2.0 mL) was rapidly added, with the aid of a 3 mL plastic syringe with a stainless-steel needle, directly inside the analyte fortified aqueous sample solution (and sample not fortified in the case of the blank) under constant stirring (up to 2 min). The turbid mixture was centrifuged (at 1080 relative centrifugal force, (RCF)) for 20 min and the separated organic phase was collected, using a Pasteur pipette, and transferred to 15 mL quartz tube. The organic phase was dried, with a flow of N_2, and then re-dissolved with 0.5 mL acetonitrile/water (70/30% v/v). Samples were UV irradiated and then placed in amber vial.

Photoderivatization procedure

The photoderivatization of trifloxystrobin was made by exposing solutions in acetonitrile/water 70/30% v/v at the original pH (ca. 6.5, which only a reference values measured using the pHmeter calibrated with aqueous buffers). The same procedure was performed for the pH 4 and 10 buffers. At the end, a value above 95% of confidence was obtained. Next, the assessment of the solutions to be used in the work is carried out. Other proportions such as 80/20% v/v and 90/10% v/v were also tested, and the pH also measured after addition of HCl or NaOH solution when necessary. The tests made by adding HCl or NaOH were performed aiming to provide more efficiency in photoderivatization. These solutions (3 mL) were placed in 20 mL quartz tubes, placed in the reactor, and exposed to UV (15 s) in order to produce the fluorescent photoderivative to be used for the indirect detection of the original analyte. After removed from the reactor, solutions were filtered on a 0.45 μm syringe filter and kept in the dark until analysis was performed.

Spectrofluorimetric and chromatographic analyzes

Steady state fluorescence quantitative measurements were made at 340/380 nm while fluorescence scans were made at 1500 nm min^{-1} with 10 nm emission/excitation spectral band passes. Chromatographic analyses were made under isocratic conditions and mobile phase was acetonitrile/ultrapure water 70/30% v/v at 1.0 mL min^{-1} flow rate and fluorescence monitored at 340/380 nm. Sample volume was 20.0 μL and the column was kept at 35 °C. Under such conditions, trifloxystrobin photo-derivative has a retention time (t_R) of 3.2 min.

The chromatographic study to characterize the photochemical derivative was made using high resolution HPLC-MS analysis with sample solution (2.0 μL) directly introduced in the mobile phase. To enable the separation of the photoproduct, an Acquity BEH C8 (100 mm × 2.1 mm, 1.7 μm) separation column was used. Mobile phase consisted of a mixture 0.1% formic acid aqueous solution/ acetonitrile (90/10% v/v) at a 0.2 mL min^{-1} flow rate (isocratic mode) enabled the photoderivative to elute at the retention time of 1.2 min. The spraying ionization source was kept at 5 kV, the capillary was kept at 42 V and at 275 °C with de-solvation temperature of 350 °C under sheathing gas (N_2).

Experimental design using circumscribed central composite design

Optimization of conditions were performed using a circumscribed central composite design (CCD)[37] allowing to obtain a model to adjust fluorescence intensity in function of the variables, reaching the condition closer to best efficiency of photoderivatization also obtaining information on synergistic or antagonistic effects between variables. The 2^2 CCD design can be viewed as two squares displaced relative to one another at an angle of 45°. The vertices of the original square, within the *x*-coordinate axis and *y*-coordinate axis, present coded values as ± 1, where the + and − signs are assigned as a function of the position of the vertex in relation to the central point, coded as (0,0). The vertices of the square, displaced by 45º, have a coded value $\pm\sqrt{2}$ and a 0, depending on their position. It was used two independent variables, nine experimental points, each one containing a set of specific values for the variables, being one of them the central point (0,0). Three replicates were performed at the central point. The design of experiments was carried out in two consecutive days with trifloxystrobin solutions (at 1.0×10^{-5} mol L^{-1}).

Results and Discussion

Preliminary studies

As a preliminary evaluation of the photoderivatization process, solutions of trifloxystrobin (at 1.0×10^{-5} mol L^{-1}) were prepared in acetonitrile/water (90/10% v/v), based on the work of Toloza *et al.*[35] Aliquots of NaOH (0.1 mol L^{-1}) or HCl (0.1 mol L^{-1}) solutions were added in order to evaluate its effect on the UV-induced derivatization. No fluorescence, above blank levels, was observed from any of these solutions prior to the UV exposure. A significant increase in fluorescence (at 340/380 nm) was observed after

10 min of UV exposure, which indicated the production of fluorophore(s) in solution at its original condition (without the addition of HCl or NaOH). In alkaline conditions, less intense fluorescence was observed after UV, alongside an increase of blank signal. While more acidic solutions did not produce any fluorescence upon UV exposure. Further experiments were performed (see experimental design in the next sub-section) to adjust acetonitrile proportion (acetonitrile/ultrapure water 70/30% v/v) in the solvent system and the UV exposure time without additions of neither HCl or NaOH solutions. In function of the results observed, the studies were carried out using the acetonitrile/water 70/30% v/v solvent system (measured pH between 6 and 7) without addition of neither HCl nor NaOH.

Photochemical derivatization conditions

The adjustment of conditions to perform photochemical derivatization was made aiming the two most relevant factors: the acetonitrile content in the solvent system and the UV exposure time. A 2^2 CCD was used, and the parameter monitored was the net fluorescence intensity also observing if any significant changes in $\lambda_{excitation}/\lambda_{emission}$ occurred in function of the variation of the levels of variables. The chosen experimental levels (and the coded values) of CCD are indicated in Table 1.

Table 1. Acetonitrile proportion and UV exposure time levels (and coded values) chosen to perform the 22 circumscribed Central Composite Design to adjust conditions for photochemical derivatization of trifloxystrobin

Parameter	Coded values				
	$-\sqrt{2}$	−1	0	+1	$+\sqrt{2}$
Acetonitrile proportion / %	66	70	80	90	94
UV exposure time / min	2	4	10	16	18

The Pareto chart (Figure S2a, SI section) showed that the most intense fluorescence is achieved as the UV exposure time is reduced for solution with high acetonitrile content, confirmed by the response surface (Figure S2b). It was also found that the interaction between factors is not relevant and mathematical equation, modeling response surface of the UV-induced fluorescence, is depicted in equation 1 where I is the net fluorescence intensity (in arbitrary units), t is the UV exposure time (in min) and ACN% is the proportion of acetonitrile in the solvent system. The response surface pointed out better conditions for photo-derivatization using UV irradiation time at 2 min (the minimum tested level) with maximum fluorescence using acetonitrile proportions from 70% v/v to 100%. In fact, the response surface indicates that UV exposure times below 2 min should be tested, but it seems that the acetonitrile proportion is a very robust factor at proportions starting from 70%, in volume, up to the higher proportion (94% in volume). Any further adjustment of UV time, aiming to increase analytical frequency, was made by a univariate study.

$$I = -262.8t + 40.2t^2 + 58.2\%ACN - 4.2(\%ACN)^2 - 1.8(t \times \%ACN); R^2 = 0.952 \quad (1)$$

The analysis of variance showed a lack of significant adjustment of the model ($F_{calculated} < F_{critical}$) indicating that equation 1 explains up to 99.99% of the variance of the model. Fluorescence spectrum of a standard solution of trifloxystrobin submitted to the photo-derivatization process using 120 s UV irradiation time in acetonitrile/water (90/10 v/v) is in Figure 1.

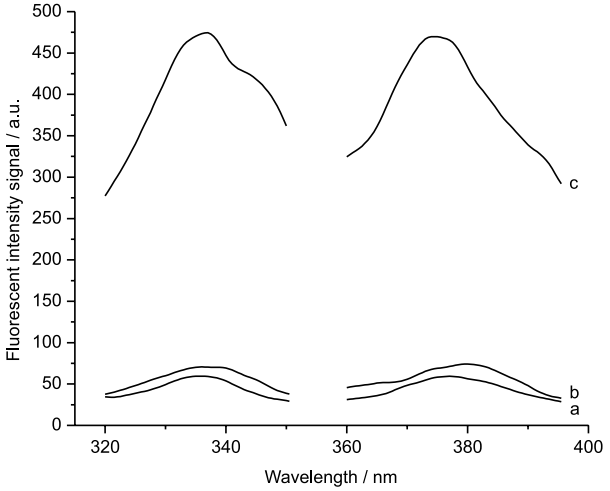

Figure 1. UV derivatized trifloxystrobin fluorescence excitation and emission spectra: (a) blank, (b) before ultraviolet exposure, and (c) after UV exposure for 2 min. Analyte solution is 1.0×10^{-6} mol L^{-1} in 90/10% (v/v) acetonitrile/water.

Optimization of chromatographic conditions

For the HPLC-fluorescence analyses, photochemical derivatization was first performed by exposing the analyte standard solution (at 1.0×10^{-5} mol L^{-1}) to UV for 2 min. Initially, separation was made on a C18 column (250 mm × 4.6 mm, 5 μm particle size) under isocratic elution with a mobile phase consisting of acetonitrile/water (60/40% v/v) at a flow rate of 1.0 mL min^{-1} and column temperature at 35 °C. Under these conditions the retention time for the derivative trifloxystrobin was 3.2 min, appearing as a single and sharp peak, which indicates that fluorescence (at 340/380 nm) is most probably due to a single fluorescent derivative.

The variation of the proportion of acetonitrile in the mobile phase was made and as the proportion of organic

solvent increased to 70%, the symmetry of the photoproduct (1.05 ± 0.05) chromatographic peak improved. Adjustments in column temperature were also made between 30 and 40 °C and it was observed no significant effect, therefore 35 °C was chosen for the continuity of the experiments. Variations in mobile phase flow (values from 0.7 to 1.0 mL min^{-1}) were made in an attempt to shift analyte peak to longer retention times but peak retention time was not affected within this range of flow rates, with 1.0 mL min^{-1} chosen for the method.

A univariate study was performed to further adjust UV exposure time ranging from 10 to 120 s (below the lowest time tested in the CCD 2^2 design) aiming to find a robust range combining analysis time and method sensitivity. Intense fluorescence was found for solutions exposed to UV between 12 and 20 s (Figure 2a), confirming the CCD results that indicated UV times shorter than 120 s would lead to more intense results. In fact, fluorescence increased abruptly in the first 20 s then promoting the degradation of the fluorescent photoderivative at higher UV exposure times. The time of 15 s was chosen.

The stability of the generated photoproduct (after 15 s of UV exposure of a standard solution of trifloxystrobin at 1.0×10^{-5} mol L^{-1}) was evaluated by short term (up to 420 min) and long term (up to 36 h) studies with fluorescence being monitored after the photochemical treatment was performed. At chosen times within these intervals, aliquots of the derivatized solution, stored in the dark at room-temperature, were introduced into the HPLC following the 0 and 420 min (short-term) interval. Two robust ranges were observed (Figure 2b) one covering the interval between the final of the UV exposure up to 120 min of storage time after derivatization and the other one after 270 min after the exposure to UV. Results indicated that the reaction still occurs, slowly at first, then leading to an improvement of fluorescence (almost increasing intensity by 3 times) from 120 up to 270 min reaching a new level of intensity but preserving spectral features with excitation/emission at 340/380 nm. In the interval between 270 and 420 min, the signal variation was random with coefficient of variation of less than 3%, indicating the photoproduct stability. The long-term study, after 24 h, degradation of the photoproduct was observed as the intensity of the fluorescence significantly decreases. Based on the results, analytical measurements were made right after UV exposure time (within in first plateau of signals) but, if required, to improve LOQ, samples should be allowed to rest at least 270 min (beginning of the second plateau of signals) in order to enable maximum signal measurement.

The conditions chosen for the indirect determination of trifloxystrobin using HPLC with fluorimetric detection of the photochemical derivative is shown in Table 2. The chromatograms were obtained by introducing the UV irradiated solvent blank and obtained from a trifloxystrobin solution (at 5.0×10^{-6} mol L^{-1}) before and after the photochemical treatment as seen in Figure 3.

Table 2. Conditions chosen for the indirect determination of trifloxystrobin by HPLC with fluorimetric detection after photochemical derivatization

Parameter	Condition chosen
Derivatization solvent system	acetonitrile/water (70:30 v/v) in original pH (6.5)
UV exposure time / s	15
Mobile phase	acetonitrile/water at 70:30% v/v
Introduced volume / μL	20
Fluorimetric detection ($\lambda_{excitation}/\lambda_{emission}$) / nm	340/380
Analyte retention time / min	3.2

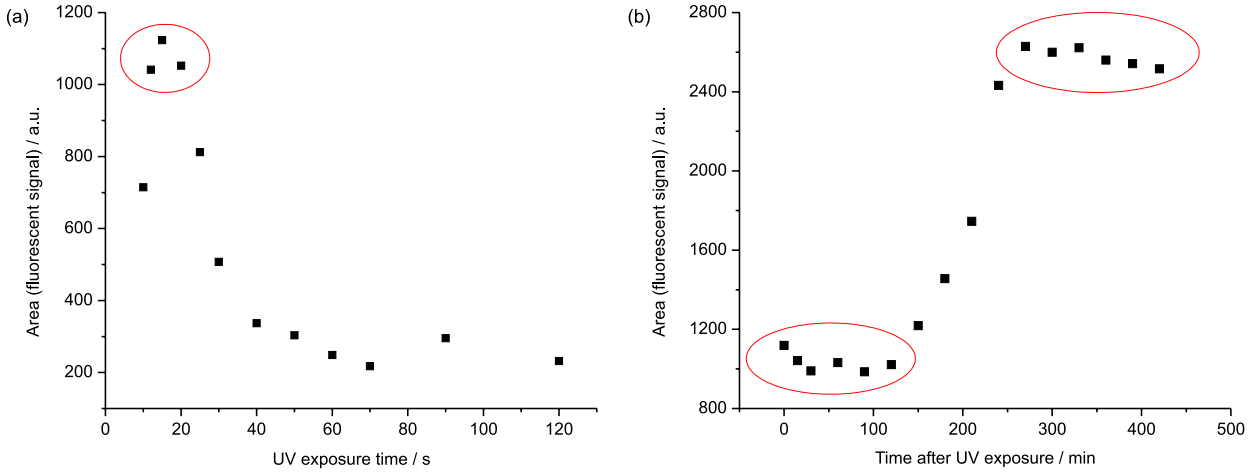

Figure 2. (a) Univariate study to adjust UV exposure time for derivatization of trifloxystrobin (at 1.0×10^{-5} mol L^{-1}). Robust range with higher fluorescence (between 12 and 20 s) highlighted. (b) Short (up to 420 min) study of the fluorescence signal of a standard solution of trifloxystrobin 1.0×10^{-5} mol L^{-1} after photochemical treatment. Robust ranges of fluorescence between 0 and 120 min and after 260 min.

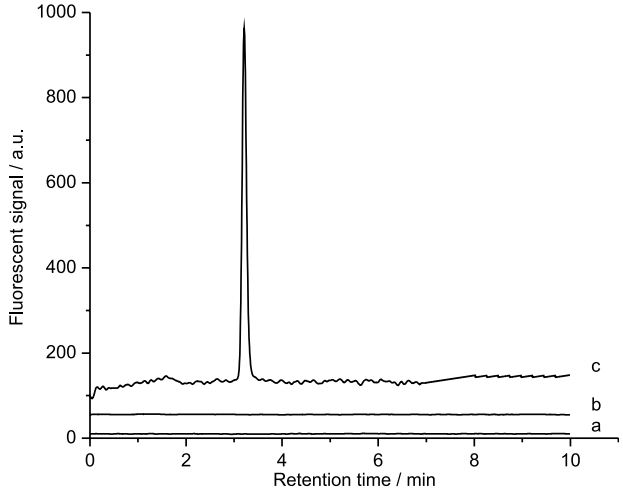

Figure 3. Chromatograms of: (a) UV irradiated solvent blank assay, (b) trifloxystrobin standard solution (at 1.0×10^{-6} mol L^{-1}) before UV exposure and (c) trifloxystrobin standard solution (at 1.0×10^{-6} mol L^{-1}) after submitted to photochemical derivatization under the optimized conditions described in Table 2.

Analytical figures of merit

The linear analytical response was modeled by the following equation: $Y = -(4.3 \pm 2.4) \times 10^{1} + (2.08 \pm 0.06)$ L $mol^{-1} \times 10^{8}X$. The limit of detection (LOD) and the limit of quantification (LOQ), in terms of the original analyte (trifloxystrobin), were respectively 0.2 μmol L^{-1} (57.1 μg L^{-1}) and 0.4 μmol L^{-1} (155.1 μg L^{-1}) for water samples. LOD and LOQ were calculated considering the concentration that produced a signal equivalent to $3s_b$ (for LOD) and $10s_b$ (for LOQ), where s_b is the standard deviation of 10 signal measurements of the lowest detectable concentration (manual integration of the chromatographic peak). For soy grape juice samples, LOD and LOQ were calculated also considering the initial amount of sample, and the resuspension volume used after DLLME procedure. In this case, the method LOD and LOQ obtained were 9.5 μg L^{-1} and 25.8 μg L^{-1}, respectively.

The linearity of the response was evaluated by the determination coefficients (R^2 and R^2_{adj}), which were superior to 99% up to the higher analyte concentration used in the curve (1.0×10^{-5} mol L^{-1}) (4.1 mg L^{-1} of trifloxystrobin). The homogeneity of variances was also used to evaluate linearity, using the Fisher-Snedocor test and the Student *t*-test, which indicated a good fit to the linear model, verified by a random distribution of residues. Besides, analysis of variance showed significant regression ($p < 0.001$) and the fit to linear model ($p > 0.05$), confirming homoscedastic behavior.

Intra-day and inter-day precisions were calculated from experiments performed in two consecutive days at three analyte concentration levels (5.0×10^{-7}; 1.0×10^{-6} and 1.0×10^{-5} mol L^{-1}). These solutions were measured in triplicate. From the *F* values obtained, it was possible to confirm that there was no significant difference among variances. Precision, in terms of coefficient of variation (CV), was below 3% and the intermediate precision was lower than 6%.

Interference study

Strobilurins (in this case, trifloxystrobin) have characteristics and functional groups (methoxy-acrylated groups) that act differently from the functional groups of triazoles (tebuconazole or cyproconazole) and they are used together to enhance the effectiveness of the pesticide products. Interferences were evaluated by combining trifloxystrobin with either tebuconazole or cyproconazole in the proportions 1:2 and 2:1 m/m. These combinations and proportions are the ones commonly found in commercial samples.[38]

According to the results, recoveries were satisfactory (Table 3) at the two concentration levels tested for the analyte in the sample solution. As an example, the chromatogram of the blank solution, along with the ones obtained after photochemical derivatization, including a trifloxystrobin solution (1.0×10^{-6} mol L^{-1}) and the one from a mixture containing trifloxystrobin and tebuconazole, equivalent to a 1:2 m/m (analyte:tebuconazole proportion), are presented in Figure 4A. In Figure 4B a similar set of chromatograms are presented but consisting, along the one of the blank solution, of the one of trifloxystrobin solution (1.0×10^{-7} mol L^{-1}) and the one of a mixture of trifloxystrobin and cyproconazole, equivalent to a 2:1 m/m analyte:cyproconazole proportion.

Table 3. Recovery test by combining trifloxystrobin with tebuconazole and cyproconazole in the proportions 1:2 and 2:1 (m/m), respectively

Analyte:potential interferent	Trifloxystrobin / (mol L^{-1})	Recovery (n = 3) / %
TRIF:CYP 2:1 (m/m)	1.0×10^{-6}	97 ± 5
	1.0×10^{-5}	99 ± 5
TRIF:TEB 1:2 (m/m)	1.0×10^{-6}	82 ± 4
	1.0×10^{-5}	78 ± 1

Analyte and potential interferents: trifloxystrobin (TRIF), tebuconazole (TEB), and cyproconazole (CYP).

Application of the method in water and soy grape juice samples

The method was applied to the analysis of analyte fortified water samples (residual from Rodrigo de Freitas Lagoon and natural from the Queen creek) and soy grape juice samples.

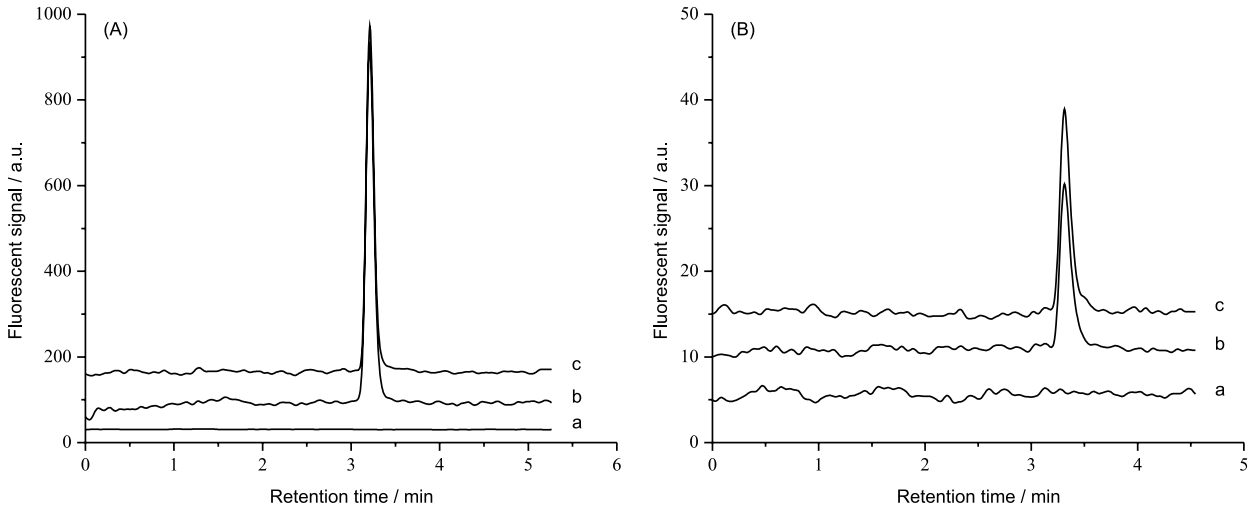

Figure 4. Chromatograms of standard solutions after photochemical treatment: (A) UV exposed blank assay (a); trifloxystrobin at 1.0×10^{-6} mol L^{-1} (b); mixture of trifloxystrobin and tebuconazole (1:2 m/m) (c). (B) UV exposed blank assay (a); trifloxystrobin solution at 1.0×10^{-7} mol L^{-1} (b); mixture of trifloxystrobin and cyproconazole (2:1 m/m) (c). Chosen experimental condition in Table 3.

The spring water from the Queen creek was collected at a point inside the university campus and residual water was collected at two points of Rodrigo de Freitas Lagoon (a mix of sweet and salty water). Water samples were collected and then fortified with trifloxystrobin before filtration. Recoveries were between 85 and 89% for the sample of Queen creek and 88 and 108% for the sample of Rodrigo de Freitas Lagoon (Table 4) considering the two different concentration levels of the analyte fortification (3.0×10^{-6} and 7.0×10^{-6} mol L^{-1}).

The method was also applied in soy grape juice sample, purchased in a local market. A previous evaluation using the proposed method indicated undetectable concentrations of trifloxystrobin in the original sample. Therefore, samples were fortified with the analyte (at 3.0×10^{-6} and at 7.0×10^{-6} mol L^{-1} concentration levels) to evaluate the method. Recoveries between 93 and 101% were achieved by fortifying the samples with standard solution of trifloxystrobin before and after microextraction (Table 4), which indicated the efficiency of DLLME and the suitability of the method for such application.

Table 4. Recovery test in water and soy grape juice fortified of trifloxystrobin in the concentration levels 3.0×10^{-6} and 7.0×10^{-6} mol L^{-1}

Trifloxystrobin / (mol L^{-1})	Recovery (n = 3) / %		
	Natural water from the Queen creek	Residual water from Rodrigo de Freitas Lagoon	Soy grape juice
3×10^{-6}	89 ± 2	88 ± 1	101 ± 7
7×10^{-6}	85 ± 3	108 ± 1	93 ± 6

The present work sought innovation from the point of view of simplicity, speed, and ease of analysis. The reduction or replacement of the use of toxic reagents (in replacing chemical derivatization for UV photo-derivatization), the method fulfilled requirements of green analytical chemistry. Photochemical derivatization of the analyte, in which a simple photochemical reactor developed in the laboratory was used, also contributed to a faster analysis, since several samples can be irradiated at the same time. Another innovation is related to sample preparation, using the DLLME technique, which was developed in 2006 aiming at the reduction of the use of toxic reagents. The analytical figures of merit are comparable to the ones of several methods reported in the literature (Table S1, SI section). Therefore, this study is a contribution that expands the possibilities for a cleaner and cost-effective analysis of samples aiming to determine pesticides.

Chromatograms of a soy grape juice sample are shown in Figure 5; Figure 5a after DLLME and photochemical derivation; Figure 5b after fortification with standard solution of trifloxystrobin, 3.0×10^{-6} mol L^{-1} before DLLME and photochemical derivatization; Figure 5c after fortification with standard solution of trifloxystrobin, 3.0×10^{-6} mol L^{-1} submitted to photochemical derivatization with fortification after DLLME.

Proposed photochemical derivatization mechanism

A solution containing an irradiated trifloxystrobin solution (exposed to UV for 15 s) was analyzed by HPLC-MS. In Figure S3 (SI section), the high-resolution chromatogram shows the peak at a retention time of 1.05 min. This peak corresponds to the photo product generated after irradiating the trifloxystrobin solution.

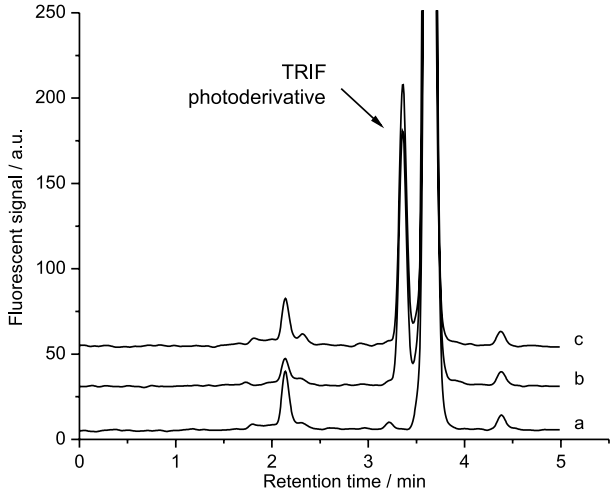

Figure 5. Chromatogram of soy grape juice sample: (a) after DLLME and photochemical derivation without trifloxystrobin fortification, (b) fortified with standard solution of trifloxystrobin, 3×10^{-6} mol L^{-1} before DLLME with photochemical derivatization; (c) fortified with standard solution of trifloxystrobin, 3×10^{-6} mol L^{-1} after DLLME with photochemical derivatization.

The mass spectrum of trifloxystrobin that corresponds to the retention time of 1.05 min after analysis by HPLC-MS shows a peak in *m/z* 206 (Figure S4, SI section), which corresponds to the deprotonation of the proposed species (207 Da) in the mechanism proposed in Figure 6.

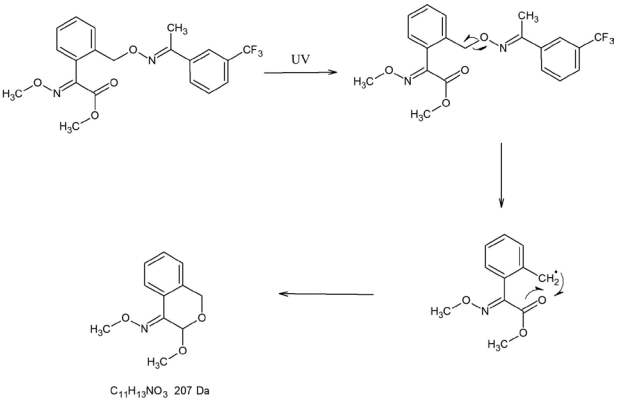

Figure 6. Proposed reaction scheme for the photoderivatization of trifloxystrobin (solution exposed to UV for 15 s) leading to fluorescent photoproduct after HPLC-MS analysis.

Conclusions

Photochemical derivatization provided a simple and efficient approach for the liquid chromatography determination of trifloxystrobin. UV exposure provided the increase of fluorescence and stable conditions to achieve appropriate metrological conditions. Besides, photoderivatization does not require the use of toxic chemical derivatization reagents and the generation of hazardous waste. The proposed method that uses HPLC and photochemical derivatization enabled to selective determine trifloxystrobin, enabling the analysis in commercial formulations combined with triazoles. The photoproduct showed stability in two robust ranges with analysis made right after UV exposure time. For soy grape juice, DLLME procedure was used aiming to pre-concentrate the analyte (in this case 6 times) before derivatization also providing simplicity, high extraction efficiency and leading to minimal residual generation as used recently for other strobilurins.[35] LOD values achieved attended the maximum residue limits (MRL) for citrus juices (0.2 to 0.6 mg kg^{-1}, depending on the regulatory agency).[8]

Supplementary Information

Supplementary data are available free of charge at http://jbcs.sbq.org.br as PDF file.

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Author Contributions

Joseany M. S. Almeida was responsible for investigation, methodology, formal analysis, writing and editing; Rosana C. Macedo for investigation, methodology, formal analysis, writing and editing; Alessandra L. M. C. da Cunha for funding, investigation, methodology, formal analysis, writing and editing; Carlos A. T. Toloza for investigation, methodology, formal analysis and writing; Ricardo Q. Aucelio for funding, methodology, writing and editing.

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