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Determination of Kresoxim-Methyl in Water and in Grapes by High-Performance Liquid Chromatography (HPLC) Using Photochemical-Induced Fluorescence and Dispersive Liquid-Liquid Microextraction (DLLME)

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ABSTRACT
A high-performance chromatographic method was developed to determine the fungicide kresoxim-methyl. Off-line photochemical derivatization was used to induce the formation of a stable and fluorescent product since the fungicide does not present natural fluorescence. Intense fluorescence at 370/430 nm was achieved by treating the analyte in solution at pH 6 to ultraviolet light for 45 s. The chromatographic conditions included isocratic elution with 50/50\% (v/v) acetonitrile/water and the photochemical product appeared at a retention time of 7.2 min. The short and long term stabilities of the photoproduce were evaluated and variation of less than 5\% was achieved. The limits of detection in water samples and in grapes samples were 0.019 mg kg\textsuperscript{-1} and 0.065 mg kg\textsuperscript{-1} of kresoxim-methyl residue, respectively. The linear response covered three orders of magnitude up to 10.6 mg kg\textsuperscript{-1} of kresoxim-methyl. The robustness was evaluated through a Box–Behnken experimental design showing the insignificance of all factors and their interactions. The potential interference of tebuconazole for the determination of kresoxim-methyl was studied. The use of the dispersive liquid-liquid microextraction (DLLME) allowed recoveries between 80\% and 101\% depending on concentration with the minimum generation of waste products.

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Introduction
Occupational exposure to pesticides affects farmers, in particular those responsible for the direct manipulation of pesticides. Inadequate storage, contaminated clothing, and wastewater from cleaning procedures are also important sources for direct contamination (ANVISA 2018). Studies have detected the presence of a myriad of pesticides in human blood, breast milk and in food and water consumed by the general population,

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increasing the possibility of congenital anomalies, cancer, and many other dysfunctions (Siqueira and Kruse 2008). In this context, environmental and public health agencies have established very strict parameters for the disposal of pesticides and also for the monitoring of contaminated areas aiming to control the use of pesticides and the establishment of the maximum residue limits in different agricultural products and environmental compartments.

Fungicides from the strobilurin class are widely used in several crops. In addition to controlling a wide spectrum of plagues, strobilurin-based fungicides are the only compounds that increase production of cereals due to the greening effect, increasing grain weight and yield (Vincelli 2012). Kresoxim-methyl (molecular structure in Figure 1), a Class III fungicide in the toxicological classification, is authorized for use in Brazil by the National Agency for Sanitary Vigilance (ANVISA). It can be applied in conjunction with other pesticides of different classes, such as triazoles, e.g., tebuconazole (AGROFIT 2019). According to European (EU 2016/486) and the Brazilian (SINITOX/ICICT/ FIOCRUZ 2015) legislation, the acceptable values for kresoxim-methyl in fruit are up to 1.5 mg kg$^{-1}$.

There are several methods described in the literature for the determination of kresoxim-methyl in different samples (Table 1). Because of its proven efficiency, extraction methods based on QuEChERS are largely used as a sample preparation procedure in the analysis of food samples for pesticide residues (Malhat et al. 2013; Zhu et al. 2013; Martínez et al. 2015; Cermeno et al. 2016; Oliva et al. 2018). However, liquid-liquid microextraction has found relevance for sample preparation, especially if the analytical technique to be used for determination of pesticide residues provides great selectivity such as gas chromatography (GC) and high-performance liquid chromatography (HPLC).

Kresoxim-methyl has been determined by HPLC with absorption photometric detection at 220 nm in different samples (water, fruits and vegetables) after liquid-liquid extraction (LLE) leading to limits of detection (LOD) down to the ng mL$^{-1}$ level. HPLC has also being associated with solid-phase extraction (SPE) to preconcentrate kresoxim-methyl along other analytes (Abreu et al. 2005, 2006; Zhu et al. 2013) with ionic liquid-based ultrasound-assisted emulsification microextraction (UA-LLME) (Liang, Wang, and Wan 2013; Liang et al. 2013) and with pre-concentration on adsorbents dispersed in the sample solution (Fróés, Santos, and Navickiene 2013). Micellar electrokinetic chromatography with photometric detection was used to determine strobilurins (including kresoxim-methyl) in fortified samples of fruits and vegetables achieving a limit of detection of 1 µg kg$^{-1}$ after samples had been submitted to a complex clean-up

Figure 1. Structure of kresoxim-methyl (methyl(E)-2-methoxyimino [2-(o-tolyloxymethyl) phenyl] acetate).
Table 1. Comparison of figures of merit for methods reported in the literature for the determination of kresoxim-methyl.

| Sample                  | Technique       | Analyte                          | Derivatization/extraction | Range                | Limit of detection         | Limit of quantification    | Reference                     |
|-------------------------|-----------------|----------------------------------|---------------------------|-----------------------|-----------------------------|-----------------------------|-------------------------------|
| Apple                   | GC-ECD          | Kresoxim-methyl and others pesticides | –                        | 15–200 pg mL⁻¹       | 5–30 pg mL⁻¹                 | 0.02 mg kg⁻¹                | Bempelou and Liapis (2009)   |
| Baby food               | GC-MS           | Kresoxim-methyl and others pesticides | DI-SPME                  | 0.05–5.0 mg kg⁻¹     | 0.02–0.05 mg kg⁻¹            | 0.05–0.1 mg kg⁻¹            | Vinas et al. (2009)          |
| Tropical fruit          | HPLC            | Kresoxim-methyl and others pesticides | MSPD                     | 0.05–50 µg mL⁻¹      | 0.005 mg kg⁻¹                 | 0.01 mg kg⁻¹                | Fröes, Santos, and Navickiene (2013) |
| Citric juices           | HPLC            | Kresoxim-methyl and others pesticides | QuEChERS                 | 3–5000 ng mL⁻¹       | 0.73–137 ng mL⁻¹              | 0.01 mg kg⁻¹                | Zhu et al. (2013)           |
| Water                   | HPLC            | Kresoxim-methyl and Others pesticides | IL-USAEME               | –                     | –                           | –                           | Liang et al. (2013)         |
| Fruit juice             | HPLC            | Kresoxim-methyl and others pesticides | –                        | 0.01–5.0 mg L⁻¹      | 0.001–0.05 mg kg⁻¹           | 0.01 mg kg⁻¹                | Liang, Wang, and Wan (2013)  |
| Fruits and vegetables   | MEKC            | Kresoxim-methyl and others pesticides | –                        | –                     | –                           | –                           | Wang et al. (2014)          |
| Grape and wine          | HPLC-triple quadrupole MS/MS | Kresoxim-methyl and others pesticides | QuEChERS                | 5–100 µg L⁻¹         | 0.003 mg kg⁻¹                 | 0.01 mg kg⁻¹                | Martinez et al. (2015)       |
| Wine grapes and red wine| HPLC-MS/MS QqQ | Kresoxim-methyl and others pesticides | QuEChERS                | 5–100 µg L⁻¹         | 0.003 mg kg⁻¹                 | 0.01 mg kg⁻¹                | Oliva et al. (2018)         |
| Wines                   | HPLC-MS/MS QqQ  | Kresoxim-methyl and others pesticides | QuEChERS                | –                     | –                           | –                           | Cermeno et al. (2016)       |
| Apple                   | HPLC            | Kresoxim-methyl and others pesticides | QuEChERS                | 0.01–1.0 mg L⁻¹      | 0.01 mg kg⁻¹                 | 0.05 mg kg⁻¹                | Malhat et al. (2013)        |
| Chrysanthemum           | CG-ECD          | Kresoxim-methyl and others pesticides | ME-VADLLME              | 0.001–0.5 mg kg⁻¹    | (0.005–0.05) x 10⁻⁷ mg kg⁻¹  | (0.02–0.2) x 10⁻⁷ mg kg⁻¹    | Xue et al. (2015)           |
| Soya-based drinks       | HPLC-DAD-ESI-IT-MS/MS | Kresoxim-methyl and others pesticides | DLLME                   | 0.2–500 ng mL⁻¹     | 4–130 ng g⁻¹                  | 15–450 ng g⁻¹              | Campillo et al. (2015)       |
| Cotton seed             | CG-ECD          | Kresoxim-methyl and others pesticides | DLLME                   | 0.001–1 mg L⁻¹      | 0.1 x 10⁻⁷–2                 | –                           | Xue et al. (2014)           |
| Apple juice             | HPLC            | Kresoxim-methyl and others pesticides | EADLLME                 | 0.1–4 mg L⁻¹        | 0.05–0.01 mg L⁻¹              | –                           | Jiang et al. (2014)         |
| Grape                   | HPLC            | Kresoxim-methyl and others pesticides | DLLME-UA-SFO + QuECHERS | 0.3–6 mg L⁻¹       | 0.2–2 mg kg⁻¹                 | 0.5–5 mg kg⁻¹               | You et al. (2013)           |
| Tomato                  | HPLC-MS         | Kresoxim-methyl and others pesticides | DLLME                   | 0.075–1 mg kg⁻¹     | 2.7 x 10⁻³ and 2.5           | 8.9 x 10⁻³ and 8.4 x 10⁻³ mg kg⁻¹ | Melo et al. (2012)         |
| Red wine                | HPLC            | Kresoxim-methyl and others pesticides | DLLME-UA                | 0.05–2 mg L⁻¹       | 2.8–16.8 pg L⁻¹              | –                           | Wang et al. (2011)         |

GC-ECD: gas chromatography with electron capture detector; GC-MS: gas chromatography with mass spectrometry; HPLC: high-performance liquid chromatography; MEKC: micellar electrokinetic capillary chromatography; HPLC-triple quadrupole MS/MS: high-performance liquid chromatography triple quadrupole mass spectrometer; fHPLC-MS/MS QqQ: high-performance liquid chromatography with mass spectrometry with a triple quadrupole analyzer detection; HPLC-DAD-ESI-IT-MS/MS: liquid chromatography with dual detection using DAD and electrospray ionization with an ion trap analyzer and tandem mass spectrometry; HPLC-MS: high-performance liquid chromatography mass spectrometer; DI-SPME: direct immersion solid-phase microextraction; QuEChERS: multi-residue extraction method; IL-USAEME: ultrasound-assisted emulsification microextraction; UASEME-SFOD: ultrasound-assisted dispersive liquid–liquid microextraction; DLLME: dispersive liquid–liquid microextraction; EADLLME: effervescence-assisted dispersive liquid–liquid microextraction; DLLME-UA-SFO + QuECHERS: ultrasound-assisted dispersive liquid–liquid microextraction based on solidification of floating organic droplet; ME-VADLLME: matrix extraction-vortex-assisted dispersive liquid–liquid microextraction; DLLME-UA-SFO + QuECHERS: ultrasound-assisted dispersive liquid–liquid microextraction.
procedure to make the analyzed solution suitable for the introduction into theelectrophoretic system (Wang et al. 2014).

GC with electron capture detection (Bempelou and Liapis 2006), mass spectrometry (MS) detection (Bo, Hai-Yan, and Ming-Hua 2008; Viñas et al. 2009, 2010) and tandem mass spectrometry (MS/MS) (Sanino, Bolzoni, and Bandini 2004) have been used for kresoxim-methyl determination in a myriad of samples with limits of detection as low as 0.005 mg kg\(^{-1}\). As in HPLC, sample preparation procedures, such as liquid-liquid extraction, ultrasound-assisted liquid-liquid microextraction, solid phase extraction, immersion solid-phase microextraction, and gel permeation chromatography were required to enable sampling into the instrument. The determination of kresoxim-methyl has been also performed by square-wave voltammetry in fortified samples of grape juice providing a limit of detection equal to 270 µg L\(^{-1}\). According to EU 2016/486 and SINITOX/ICICT/FIOCRUZ (2015), the maximum residue limit (MRL) in grapes is equivalent to 0.5 mg kg\(^{-1}\). Solid phase extraction was used to preconcentrate the analyte and eliminate potential interferents (Dornellas et al. 2013).

Despite the variety of existing methods, the miniaturized procedures for sample preparation are gaining relevance because of their cost-effectiveness, high efficiency, reduced need for consumables and low generation of waste. Dispersive liquid-liquid microextraction (DLLME) is based on the partition of an analyte between two immiscible liquid phases, one being the aqueous phase containing a miscible organic solvent used to improve solubility of analyte and to allow the interaction with the other phase consisting of an extractive organic solvent(s) that is dispersed as microdroplets to improve efficiency of the extraction. It is usually performed by injecting a suitable microvolume of an extractive mixture of solvents, dispersing it into the aqueous sample solution containing the analytes and then centrifuging the turbid mixture to separate the analyte-enriched extracting phase from the sample solution (Rezaee et al. 2006; Rezaee, Yamin, and Faraji 2010). For the application of the DLLME technique, some parameters must be optimized and subsequently validated, such as the type and volume of extractor and dispersant solvents, the requirements for agitation and the extraction time (Rezaee et al. 2006; Caldas, Costa, and Primel 2010).

Photochemical derivatization involves treating a sample with ultraviolet light aiming to transform the original analyte into new chemical species that present properties that are useful for analytical application. Ultraviolet photons induce bonding disruptions, leading to the formation of products with a more rigid molecular structure, thus with higher fluorescent quantum efficiencies (Garcia et al. 2017). Photochemical derivatization, when performed under robust conditions, produces reliable results, eliminating the need for toxic and expensive derivatization agents. Photochemical derivatization to induce fluorescence from pesticides has been studied. Relevant references were studied including a review with photo-induced chemiluminescence or fluorescence by flow injection analysis (FIA) in different matrices (Icardo and Calatayud 2008). Other reported works analyzed water samples for \(\alpha\)-cypermethrin (Mbaye et al. 2009), benzoylurea and phenylurea (Diaw et al. 2014) and fenvalerate (Thiaré et al. 2015). However, so far, there is no reported procedure for the photochemical generation of fluorophores aiming analytical applications concerning strobilurins class.
In this work, the determination of kresoxim-methyl was achieved by high-performance liquid chromatography (HPLC) with fluorimetric detection of a photochemical derivative (off-line photo-derivatization) produced under robust experimental conditions. Initially, the method was characterized for the analysis of water samples due to the simplicity of the matrix. Next, the method was applied in grapes after extraction of the pesticide by DLLME. Grapes have been selected as a widely consumed fruit presenting high maximum residue value of 0.5 mg kg\(^{-1}\) based on Brazilian legislation. The fast and reliable photochemical derivatization method does not require the use of toxic chemical derivatization reagents and the sensitivity, allied to the use of a microextraction technique, simplified sample treatment and minimized production of waste.

**Experimental**

**Reagents and chemicals**

Ultrapure water was obtained from a Milli-Q gradient A10 ultra-purifier (Milipore, Massachusetts, USA). The kresoxim-methyl and tebuconazole standards were from Sigma-Aldrich (Missouri, USA). HPLC grade acetonitrile was obtained from Tedia (Ohio, USA). Hydrochloric acid, sodium hydroxide and carbon tetrachloride were purchased from Merck (Darmstadt, Germany). Ultra-pure nitrogen was from Linde Gases (Rio de Janeiro, Brazil).

Whatman 41 filter paper was obtained from Whatman (Massachusetts, USA). The 0.45 \(\mu\)m PTFE syringe filter and 0.2 \(\mu\)m borosilicate glass microfiber membrane were from Sigma-Aldrich (Missouri, USA).

**Instruments and apparatuses**

Chromatographic analyzes were made on a HPLC system (Agilent Technologies 1200 series, California, EUA) with a fluorescence detector and an automatic sampler. Chromatograms were treated using the Agilent ChemStation software. The separation was performed using a Agilent Eclipse XDB C18 (4.6 \(\times\) 250 mm and 5 \(\mu\)m particle size) column.

In order to identify photochemical derivatives, a liquid chromatography system AccelaLC (Thermo Fisher Scientific, Bremen, Germany) coupled to a Q-Exactive Orbitrap mass spectrometer (MS) (Thermo Fisher Scientific, Bremen, Germany) was used. The chromatographic separation was performed in the same reversed-phase column at 40 \(^\circ\)C. A 9 L volume NSC2800 model ultrasonic bath (Unique, Brazil) was used to solubilize analytes and for the degassing of mobile phases. Fluorescence measurements were made on a Perkin-Elmer (USA) LS 55 luminescence spectrometer.

Statistica Software (7.0, Statsoft, USA) was used for the experimental design and for the statistical treatment of the data.

The pH measurements were made on a Tecnopon MPA 210 model pH meter (Tecnopon, Brazil).

The laboratory made photochemical reactor consisted of six sterilization mercury lamps (6 W each) placed inside a cylindrical polyvinyl chloride cabinet (200 mm diameter and 290 mm depth). This device allowed uniform irradiation of samples.
accommodated in 20 mL quartz tubes placed in the center part of the reactor, with the
distance between the tube and the lamp approximately 2.5 cm. A small fan was placed
at the back of the reactor in order to keep its internal temperature stable at less
than 30 °C.

A commercial mixer (Cuisinart, USA) was used to macerate the grape fruits and a
centrifuge BE 4000 Brushless (Bio-Eng, Rio de Janeiro) was used to perform phase sep-
aration during the DLLME procedure.

**Procedures**

**Preparation of standard solutions**

Kresoxim-methyl and tebuconazole stock solutions (1 × 10^{-4} mol L^{-1}) were prepared by
dissolving proper masses of the standard in acetonitrile. The solutions submitted to
photoderivatization were prepared by dilution using 50/50% (v/v) acetonitrile:water.

**Preparation of water samples and purple grape samples**

Water sample from a local source (Queen Creek) was collected inside the Campus of
Pontifical Catholic University of Rio de Janeiro in amber flasks previously cleaned with
10% v/v nitric acid and ultrapure water. Samples were stored in the refrigerator no lon-
ger than 24 h after collection. A volume of 5 mL of water sample was transferred to a
10 mL flask and fortified with kresoxim-methyl at three concentration levels: 7.0 × 10^{-8};
5.0 × 10^{-7}; 5.0 × 10^{-6} mol L^{-1}. The final volume was adjusted with acetonitrile.

In order to perform the analysis of grapes, approximately 500 g of purple grapes were
macerated in a commercial mixer. In a Falcon tube, approximately 1.5 g of the macer-
ated sample was fortified with kresoxim-methyl (at 10^{-6} and 10^{-7} mol L^{-1} concentra-
tion levels), mixed with 400 µL of acetonitrile and 1.1 g of ultrapure water. The mixture
was agitated by a vortex mixer for 10 s and centrifuged at 3000 rpm for 20 min. The
supernatant was transferred to 15 mL Falcon tube.

**Microextraction procedure**

The DLLME procedure, adapted from Caldas, Costa, and Primel (2010), was made by
rapidly introducing through a syringe a mixture of carbon tetrachloride (60 µL) and
acetonitrile (2000 µL) into the sample solution. The turbid mixture was centrifuged at
3000 rpm for 20 min and the organic phase was collected, using a Pasteur pipette, and
transferred to 10 mL volumetric flask. The carbon tetrachloride phase was dried with a
flow of nitrogen and then re-dissolved in 50/50% (v/v) acetonitrile:water.

For the application of DLLME technique, some parameters must be optimized and
subsequently validated. The initial tests, besides employing a small solvent mixture vol-
ume (2000 µL of acetonitrile and 60 µL carbon tetrachloride) presented a good extrac-
tion efficiency. However, in order to minimize the total analysis time and improve the
procedure, the optimization of some steps was necessary: the sample clean-up, the pre-
vious addition of acetonitrile to the sample to improve analyte solubility, the require-
ment for agitation after the extraction process, the centrifugation time obtain phase
separation and the evaluation of extractor and dispersant solvent proportions.
**Photo-derivatization procedure**

The photo-derivatization of kresoxim-methyl was made by exposing standard solutions or sample extract solutions prepared in 50/50% (v/v) acetonitrile/water. The solution (approximately 3 mL) was placed in 20 mL quartz tubes, stoppered and placed into the photochemical reactor to be exposed to the ultraviolet light for 45 s. After photo-treatment, solutions were kept in the dark until passage through a 0.45 μm syringe filter before introduction into the HPLC system.

**Spectrofluorimetric batch analyzes**

Stationary spectrofluorimetric analyzes were made during optimization of the photochemical treatment. Fluorescence spectra were acquired using a scan rate of 1500 nm min⁻¹ and 15 nm emission and excitation spectral band passes. The analytical signal was measured at excitation at 370 nm and emission at 430 nm. When required, neutral density filters (Newport, USA) were used to attenuate the fluorescence when saturation of the detector occurred.

**Chromatographic analysis**

Chromatographic analyzes were made with an isocratic regime using a mobile phase consisting of 50/50% (v/v) acetonitrile/water. For studies with solutions containing both kresoxim-methyl and tebuconazole, a mobile phase gradient was applied by using 50/50% (v/v) acetonitrile/water up to 4.40 min at 1 mL min⁻¹ and then using 70/30% (v/v) acetonitrile/water to the end of the chromatographic run at 1.4 mL min⁻¹. The sample volume introduced was 20 μL and the chromatographic column was kept at 40°C.

Using the optimized photochemical treatment conditions, the kresoxim-methyl photoderivative appeared at a retention time (tR) of 7.2 min while the tR of tebuconazole was 4.0 min. Fluorescence was monitored at 226/325 nm up to 4.0 min to monitor the tebuconazole photo-product and at 370 nm and 430 nm to monitor the run up to 8.0 min in order to detect the kresoxim-methyl photodervative.

The chromatographic study to identify photochemical derivative(s) was made using the mobile phases: (A) ultrapure water with 5.0 mmol L⁻¹ ammonium formate and (B) acetonitrile, both with 0.1% formic acid. The flow rate was set to 1 mL min⁻¹ and the elution profile was isocratic with a solvent A: solvent B proportion of 50/50%. The overall run time was 20 min and sample injection volume was 8.0 μL.

The LC mobile phase output was introduced into a Q-Exactive mass spectrometer, operating by switching positive and negative ionization modes, equipped with an electrospray ionization source. The spray voltage was set to 4.1 kV and to 3.6 kV in respectively for the positive and negative ionization modes. The capillary temperature was 320°C and the S-lens RF level was set to 50 (arbitrary units). The nitrogen sheath gas flow rate and auxiliary gas were set at 10 and 5 (arbitrary units), respectively.

In order to ensure mass accuracies were below 6 mg L⁻¹, the instrument was calibrated using the manufacturer’s calibration solutions (Thermo Fisher Scientific, Germany). The mass spectrometer acquired full scan data in both ionization modes at a resolution of 70,000 full width at half maximum (FWHM) each and with automatic gain control of 10⁶. In addition, an all-ion fragmentation full scan was acquired in both
ionization modes with a normalized collision energy of 40 at a resolution of 17.500 FWHM.

Results and discussion

Photochemical derivatization

Kresoxim-methyl presents no significant natural fluorescence when dissolved in aqueous solvent systems at room temperature and at $1.0 \times 10^{-5}$ mol L$^{-1}$. Preliminary studies using kresoxim-methyl dissolved in 10/90% (v/v) ultrapure acetonitrile/water showed that a brief exposure to ultraviolet light for 15 s induced intense fluorescence with excitation and emission maxima at 370 nm and 430 nm as shown in Figure 2. A 50% neutral density filter was used to attenuate the measured fluorescence after the saturation of the detector occurred.

Conditions to produce intense photochemical-induced fluorescence were adjusted by a series of univariate studies. First, the proportion of acetonitrile in the $1.0 \times 10^{-6}$ mol L$^{-1}$ kresoxim-methyl solution was varied from 30 to 100% by volume and the most intense fluorescence was found with 50% acetonitrile by volume in the aqueous solution (Figure 3a).
The pH of the solution was varied across the range between 2 and 12, adjusted with addition of either hydrochloric acid (0.01 mol L\(^{-1}\)) or sodium hydroxide (0.01 mol L\(^{-1}\)). It was found that fluorescence intensity linearly increased as the pH was varied from 2 to 8, then decreasing as the medium became more alkaline (Figure 3b). Since the result observed from the 50/50% (v/v) acetonitrile/water at the original pH of approximately 6 was only about 15% less intense than the one observed at a solution adjusted at pH 8, it was decided not to perform any adjustment for the sake of simplicity of the experimental procedure, especially considering the HPLC conditions.

In terms of the ultraviolet exposure, 1.0 \(\times\) 10\(^{-6}\) mol L\(^{-1}\) kresoxim-methyl solutions were irradiated inside the photochemical reactor for a time interval up to 300 s (Figure 3c). The signal sharply increased after 30 s and remained constant up to 50 s with a random variation of less than 5%. The signal sharply decreased after 60 s of ultraviolet treatment, until fluorescence totally disappears at 300 s because of degradation of the initially
produced fluorophore(s). The selected irradiation time was 45 s, enabling a very short off-line photo-derivatization step.

The irradiated solution was then removed from the photo-reactor and its fluorescence was measured at specific times, firstly every 5–30 min (immediate stability), every 30–360 min, and lastly every 120–720 min (short-term experiment), with the solution stored in the dark between measurements. During this time frame, no significant variation of fluorescence was observed, with a random variation of less than 2%.

In a long-term experiment, the fluorescence from solutions was measured once a day from day 1 up to day 7. During this time period, the fluorescence slightly decreased reaching a final intensity measured at the seventh day that was only 11% less intense than the value obtained during the short-term interval.

**Chromatography of the photo-derivatized kresoxim-methyl**

The conditions to perform chromatography of the ultraviolet exposed 1.0 × 10⁻⁶ mol L⁻¹ kresoxim-methyl solution were studied. The influence of the solvent proportion (acetonitrile proportions from 40% to 70%, in volume) and column temperature (between 30 and 40 °C) were the two parameters evaluated. Concerning acetonitrile in the mobile phase at column temperature of 40 °C, the results showed that using a proportion of 40%, a single isolated peak with a retention time of 9.8 min detected by fluorescence was present but was significantly broadened when compared to those obtained with higher acetonitrile proportions. As the proportion of acetonitrile increased, the peak became significantly sharper and more symmetric (1.05 ± 0.05) with a shorter retention time of 7.2 min. The increase of the acetonitrile proportion up to 70% did not cause a significant improvement in terms in peak characteristics. Therefore, the selected mobile phase contained 50% acetonitrile in order to match the composition of the ultraviolet irradiated sample solution.

These results also indicated the formation of a single fluorophore under the conditions chosen for the photo-derivatization. In terms of column temperature, no significant differences were found in the range studied, and therefore 40 °C was deemed to be the optimum. In Figure 4, the chromatograms from an ultraviolet derivatized kresoxim-methyl standard solution (10⁻⁶ mol L⁻¹ and 20 μL introduced volume) is shown using 50% and 40% acetonitrile in the aqueous mobile phase under isocratic conditions. The blank of the irradiated solvent mixture is shown in Figure 4a.

**Evaluating robustness of the photoderivatization conditions**

The optimization of analytical methods has often been performed by multivariate techniques and several statistical approaches have been employed for robustness assessments. The number of factors can influence directly in the choice of the ideal design and the critical conditions of the factors have been chosen using Box–Behnken surface response (Candioti et al. 2014; Novaes, Bezerra, et al. 2016; Novaes, Ferreira, et al. 2016; Ferreira et al. 2017).

Robustness is the capacity of an analytical procedure to remain unaffected by small but deliberately introduced variations in parameters, providing an indication of the
reliability of the method (ICH 2005). The robustness was evaluated with $1.0 \times 10^{-6}$ mol L$^{-1}$ kresoxim-methyl solutions using the two-level full factorial design that allows a preliminary evaluation of the factors by the development of linear model. The factors and the selected range were (i) the pH of the solution from 5.5 to 6.5; (ii) the ultraviolet exposure time from 40 to 50 min, and (iii) the proportion of acetonitrile in the aqueous mobile phase under isocratic conditions. The excitation and emission wavelengths are 370 nm and 430 nm.

Table 2. Factors and levels (real values and coded in parenthesis) for the evaluation of robustness.

| Experiment | pH     | Ultraviolet exposure time (s) | Proportion of acetonitrile (%) |
|------------|--------|-------------------------------|-------------------------------|
| 1          | 5.5 (-1) | 40 (-1)                      | 45 (-1)                      |
| 2          | 6.5 (1)  | 40 (-1)                      | 45 (1)                       |
| 3          | 5.5 (-1) | 50 (1)                       | 45 (-1)                      |
| 4          | 6.5 (1)  | 50 (1)                       | 45 (-1)                      |
| 5          | 5.5 (-1) | 40 (-1)                      | 55 (1)                       |
| 6          | 6.5 (1)  | 40 (-1)                      | 55 (1)                       |
| 7          | 5.5 (-1) | 50 (1)                       | 55 (1)                       |
| 8          | 6.5 (1)  | 50 (1)                       | 55 (1)                       |
| Pareto chart | 6.0 (0)   | 45 (0)                       | 50 (0)                       |
| Pareto chart | 6.0 (0)   | 45 (0)                       | 50 (0)                       |
| Pareto chart | 6.0 (0)   | 45 (0)                       | 50 (0)                       |

Analyte solution: $1.0 \times 10^{-6}$ mol L$^{-1}$ kresoxim-methyl in ultrapure acetonitrile/water.

Figure 4. Chromatograms from a $10^{-6}$ mol L$^{-1}$ kresoxim-methyl standard solution ultraviolet derivatized for 45 s with a 20 µL introduced volume: (a) blank (solvent only), (b) 40% acetonitrile, and (c) 50% acetonitrile in the aqueous mobile phase under isocratic conditions. The excitation and emission wavelengths are 370 nm and 430 nm.
experiments, a total of 11 experiments, eight combinations of extreme levels and three experiments using levels at the center of the design \((n = 3)\). The significance of the factors and their possible interactions were evaluated using a Pareto chart (Figure 5) that shows that the standardized effects (SE), calculated by the ratio between the effect values and the standard deviation estimated at the central point, were insignificant for all of the selected factors and their interactions (SE below the critical values established for the test). Therefore, the experimental conditions are robust for the experimental dominions established.

**Analytical figures of merit**

The analytical response considering the original concentration of kresoxim-methyl in function of the signal measured from the photo-chemically produced fluorophore at a retention time of 7.2 min was linear and described by the following equation: 

\[
Y = (2.1 \pm 0.079) \times 10^8 X + (32 \pm 5.5),
\]

where \(Y\) is the measured signal, in arbitrary units, and \(X\) is the original concentration of kresoxim-methyl in mol L\(^{-1}\). The limit of detection and the limit of quantification values were 0.065 mg kg\(^{-1}\) (equivalent to \(3.1 \times 10^{-8}\) mol L\(^{-1}\)) and 0.106 mg kg\(^{-1}\) (equivalent to \(5.1 \times 10^{-8}\) mol L\(^{-1}\)) for grape samples and 0.019 mg kg\(^{-1}\) and 0.032 mg kg\(^{-1}\) for water samples, respectively, calculated using the concentration equivalent to a signal equal to \(3\, s_b\) for the former and \(10\, s_b\) for the latter where \(s_b\) is the standard deviation for 10 signal measurements of the lowest concentrated standard of the curve that required manual integration. The values for the limits of detection and quantification are below the Brazilian and European specifications in grapes with a maximum residue limit of 0.5 mg kg\(^{-1}\).

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 (10.6 mg kg\(^{-1}\), equivalent to \(1.0 \times 10^{-5}\) mol L\(^{-1}\) kresoxim-methyl). The homogeneity of variances was also used to evaluate the linearity, using the Fisher–Snedecor and the \(t\)-Student statistical tests, which indicated the goodness of fit to the linear model.
verified by a random distribution of residues. The analysis of variance showed significant regression ($p < 0.001$) and fit to the linear model ($p > 0.05$), confirming the homoscedastic behavior.

The intra-day and inter-day precision were calculated from measurements performed on two consecutive days. The results confirmed that there were no significant differences among the variances. The average results of the measurement were compared to those performed in single days and no significant differences ($t_{\text{calculated}} < t_{\text{critical}}$) were found. The precision, in terms of coefficient of variation (CV), was less than 3% and the intermediate precision was less than 5%.

**Interference study**

The interference study was evaluated by combining kresoxim-methyl with tebuconazole in the proportion up to 1:1.25 (m/m) which is the characteristic proportion of these pesticides found in commercial formulations (AGROFIT 2019). A specific gradient of fluorescence wavelength (excitation/emission) was used since photoproducts generated from these two pesticides have very distinct fluorescence (226 nm/325 nm for tebuconazole and 370 nm/430 nm for kresoxim-methyl). The chromatographic peaks appeared at different retention times under the selected chromatographic conditions. These results show that tebuconazole produces one single fluorophore with only 5% of the intensity observed for the photoderivative obtained from an equivalent concentration of kresoxim-methyl. In addition, selectivity is not only guaranteed by the difference in excitation but also by the different retention times as the photo-product from tebuconazole appeared at a retention time of 3.7 min as shown in the chromatogram in Figure 6.

The recoveries for kresoxim-methyl in mixtures containing tebuconazole (maintaining the 1:1.25 (m/m) ratio) were up to 97% in synthetic mixtures at concentration levels of kresoxim-methyl equal to $7.0 \times 10^{-8}$, $5.0 \times 10^{-7}$, and $5.0 \times 10^{-6}$ mol L$^{-1}$ using the reported chromatographic method with photo-derivatization of sample solutions.
Table 3. Recoveries in natural water and grape samples fortified with kresoxim-methyl.

| Sample        | Kresoxim-methyl (mol L⁻¹) | Recovery (%) |
|---------------|---------------------------|--------------|
| Natural water | 7.0 × 10⁻⁸                | 96 ± 6       |
| (n = 3)       | 5.0 × 10⁻⁷                | 85 ± 5       |
|               | 5.0 × 10⁻⁶                | 99 ± 1       |
| Grape         | 1.0 × 10⁻⁷                | 80 ± 2       |
| (n = 5)       | 1.0 × 10⁻⁶                | 96 ± 5       |

Evaluation of dispersive liquid-liquid microextraction in grape samples

For sample clean-up, a study was performed comparing the sieving followed by filtration with centrifugation. The centrifugation at 3000 rpm was evaluated at time intervals equal to 5, 10, 15, 20, and 25 min. The results were assessed by visual observation. A time of 20 min was selected, as the phases were well defined facilitating the separation of lumps and shells. The replacement performed in addition to minimizing losses considerably reduced the analysis time.

As the strobilurins do not present good solubilization in aqueous phase, the previous addition of different acetonitrile volumes to the sample (0, 100, 200, 300, 400, and 500 µL) was also evaluated. For the fortified samples, 100 µL of 1 × 10⁻⁶ mol L⁻¹ kresoxim-methyl standard solution was added. In order to perform DLLME, a mixture of 2000 µL of acetonitrile and 60 µL of carbon tetrachloride was introduced (Caldas, Costa, and Primel 2010) into the sample solution with a syringe. The aqueous phase is sedimented in the bottom of the Falcon tube. The previous addition of 300 µL of acetonitrile presented the greatest gain in terms of fluorescent signal efficiency due to the low solubility of the analyte in water. Higher volumes impaired the separation of the phases in the microextraction.

Different agitation processes, including manual and vortex mixing, were investigated and the results demonstrated no improvement in the efficiency of the extraction.

In the univariate studies performed to evaluate the centrifugation time following DLLME, the cloudy mixture was centrifuged for 5, 10, 15, 20, and 25 min. The optimum centrifugation time was 20 min, producing well separated phases. Two volumes of carbon tetrachloride were tested in the extractor mixture (80 and 100 µL). The extraction efficiency was identical for both volumes.

The best experimental conditions after univariate studies were an initial sample centrifugation for 20 min at 3000 rpm, the addition of 300 µL of acetonitrile to ensure analyte dissolution and final centrifugation for 20 min at 3000 rpm for the phase separation after DLLME.

Application of the method

The method was applied for the analysis of natural water from a local source (Queen Creek). The recoveries were between 85 and 99% (Table 3) using three concentration levels of analyte fortification equal to 7.0 × 10⁻⁸, 5.0 × 10⁻⁷, and 5.0 × 10⁻⁶ mol L⁻¹. The method was also applied in three types of grape samples purchased in a local market after the microextraction procedure. A previous analysis using the proposed method indicated undetectable concentrations of kresoxim-methyl in the extracts from the original samples. Therefore, sample extracts were fortified with the analyte at 10⁻⁶ and
10^{-7} \text{ mol L}^{-1} \text{ concentration levels in order to evaluate the method. After DLLME, evaporation, the reconstitution of residue and photochemical derivatization, the resulting solution was introduced into the HPLC system that produced the chromatogram in Figure 7, which is compared to the blank chromatogram and with the results achieved from a standard calibration solution. Recoveries of 80\% at the 10^{-7} \text{ mol L}^{-1} \text{ concentration level and of 96\% at the 10^{-6} \text{ mol L}^{-1} \text{ concentration level were achieved (Table 3), which indicates the suitability of the method for this application.}
In addition, in order to evaluate the efficiency of DLLME extraction, measurements were performed by fortifying the samples with standard solution of kresoxim-methyl at $10^{-7}$ mol L$^{-1}$ before and after microextraction. According to the results, the recoveries obtained were satisfactory with values between 96% and 101% for $n = 3$, corroborated by the use of the proposed technique. Figure 8 shows the injection of a purple grape sample: (a) after DLLME and photochemical derivation, (b) fortified with standard solution of kresoxim-methyl before DLLME and after photochemical derivatization of 45 s, and (c) fortified with standard solution of kresoxim-methyl after DLLME and photochemical derivatization of 45 s.

Figure 9. (a) Proposed mechanism for the photo-derivatization of kresoxim-methyl in 50/50% (v/v) acetonitrile/water treated with ultraviolet radiation for 45 s. (b) Mass spectrum of $1.0 \times 10^{-6}$ mol L$^{-1}$ kresoxim-methyl in 50/50% (v/v) acetonitrile/water after 45 s of ultraviolet irradiation.


Proposed photochemical derivatization mechanism

The inducing of fluorescence from kresoxim-methyl (1.0 $\times$ 10^{-6} mol L^{-1}) by ultraviolet light indicates that there was a significant change in molecular structure. The strong observed fluorescence intensity may be explained by a formation of a photo-product with better fluorescence quantum yield due to a greater structural rigidity when compared to the original kresoxim-methyl molecule. In Figure 9a, a mechanism for the photochemical reaction of kresoxim-methyl is proposed, showing the formation of a fluorescent and stable product.

Since the irradiation source does not have sufficient energy to break the bonds in the aromatic rings, it is assumed the photolysis of the C–O bond occurs that connects the two aromatic rings. A further cyclization through the carbonyl group forms a system with two rings, increasing both rigidity and conjugation of double bond system, increasing the electron delocalization. Analysis by mass spectrometry was carried out to obtain a qualitative evidence of the formation of the proposed photoproduct. For this, a solution of kresoxim-methyl was treated with ultraviolet radiation for 45 s before introduction into the LC-MS system. The experiment revealed the presence of a peak with m/z 176 Da (Figure 9b), which corresponds to the proposed photo-product after the loss of a hydrogen (M-1).

Conclusions

The photochemical derivatization associated with the fluorimetric detection excitation at 370 nm and emission at 430 nm enabled the innovative determination of kresoxim-methyl by HPLC, leading to limits of detection equal to 0.019 mg kg^{-1} and 0.065 mg kg^{-1} for the kresoxim-methyl residue in water and grapes samples, respectively. These values are very competitive when compared to the results reported in the literature shown in Table 1. The proposed method presents a very simple photo-derivatization procedure, avoiding the use of expensive and toxic chemical derivatization reagents, eliminating the possibility of problems with contamination from the reaction with derivatization reagents, and with reduced toxic chemical waste.

Box–Behnken experimental design was used to evaluate the robustness of the method. The proposed method allowed the adequate quantification of kresoxim-methyl in water and grape samples. The use of DLLME was found to be appropriate as a preparation procedure for grape samples, demonstrated by the satisfactory results for the recovery values between 80% and 101%. The main advantages of DLLME are the miniaturization, simplicity, high extraction efficiency, generation minimal residual generation and the possibility for pre-concentration of the analyte.

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Disclosure statement

No potential conflict of interest was reported by the author(s).
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