DETERMINATION OF THE HERBICIDE MESOTRIONE USING HPLC-DAD: AN EVALUATION OF DEGRADATION

DETERMINAÇÃO DO HERBICIDA MESOTRIONE POR HPLC-DAD: UMA AVALIAÇÃO DE DEGRADAÇÃO

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RESUMO

A quantificação de moléculas tóxicas em ambientes naturais tem se tornado determinante para estudos envolvendo manejo e conservação de áreas contaminadas. O mesotrione é um herbicida seletivo utilizado em culturas de milho para o controle de ervas daninhas de folhas largas. Desta forma, o objetivo deste trabalho foi desenvolver um método cromatográfico rápido e eficiente por CLAE-DAD para determinação da cinética de degradação e quantificação do mesotrione. A técnica de cromatografia líquida de alta eficiência (CLAE) associada a uma coluna C18 de fase reversa (Eclipse XDB C18) foi usada para desenvolver e validar um método para se determinar a degradação do mesotrione [2-(4-methilsulfonil-2-nitrobenzoil)-1,3-ciclohexanodiona], a molécula ativa do herbicida Callisto® em meio de cultura contendo a bactéria *Escherichiacoli* DH5-α. O método foi validado de acordo com a resolução da ANVISA (RE 899/03). A curva de calibração mostrou bom coeficiente de correlação (r) entre 9.0-196.5 $\mu mol.L^{-1}$ para a concentração do herbicida no meio de cultura. Precisão (intra e inter dia com coeficiente de variação abixo de 4%), exatidão (entre 99,6% e 108,4%) e outros parâmetros avaliados mostraram também de acordo com a resolução brasileira. O método validado foi aplicado com sucesso para a quantificação do herbicida mesotrione no meio de cultura contendo *E. coli* DH5-α. Permitiu ainda determinar a concentração de mesotrione em diferentes tempos de avaliação experimental demostrando ser uma ferramenta adequada para a quantificação do herbicida em estudos de degradação e bioremediação com outros modelos biológicos, principalmente para agricultura e ambiente.

Palavras-chave: separação cromatográfica, contaminação, análise ambiental, microorganismos, contaminantes orgânicos.

ABSTRACT

The quantification of toxic molecules in natural environments has become an important factor for studies involving the management and conservation of contaminated areas. Mesotrione is a selective herbicide that is used in corn crops to control broad-leaved weeds. The objective of this study was to develop a fast and efficient chromatographic method using HPLC-DAD to determine the kinetics of degradation and quantification of mesotrione. High-performance liquid chromatography (HPLC), utilizing a C18 reverse-phase column (Eclipse XDB C18), was used to develop and validate a method to determine the degradation of mesotrione [2-(4-methylsulfonyl-2-nitrobenzoyl)-1,3-cyclohexanedione], the active molecule in Callisto® herbicide, in culture medium with *Escherichia coli* DH5-α bacteria. The method was validated according to

Brazilian regulations (ANVISA Resolution 899/03). The calibration curve showed a good correlation coefficient (r) from 9.0-196.5 μmol.L^{-1} for the concentration of herbicide in the medium. Precision (intra and inter-day, coefficient of variation below 4%), accuracy (from 99.6%-108.4%) and the other evaluated parameters were also in accordance with the aforementioned resolution. The validated method was successfully applied to quantifiy the herbicide in the culture medium containing *E. coli* DH5-α. The developed method also made it possible to measure the herbicide at different times under experimental conditions and was shown to be a good tool to quantify mesotrione in degradation and bioremediation studies with other biological models, mainly for agricultural and environmental purposes.
Keywords: chromatographic separation; contamination; environmental analysis; microorganisms; organic contaminants.

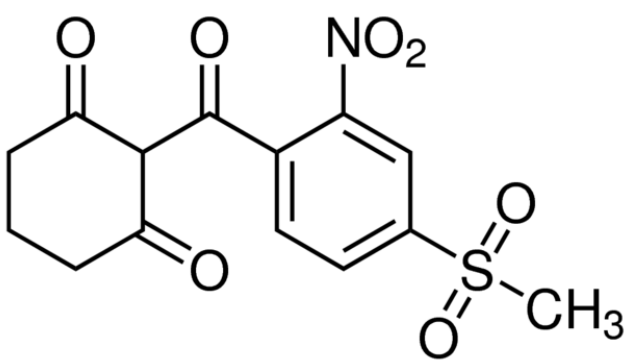
INTRODUCTION

Analytical methods are efficient techniques to qualify and quantify xenobiotics (Marques, Oehmen, Carvalho, & Reis, 2015). High-performance liquid chromatography (HPLC) and gas chromatography (GC) enable the use of different stationary phases and they are able to couple different detectors. Therefore, these techniques have been commonly used as alternatives among the analytical techniques that are currently available to determine herbicides in complex matrices, such as vegetables, soil, water and others (Rodriguez, Ferreira, Cardoso, Ferreira, & Benoliel, 2007; Yan, Shao, Liang, Zhang, & Yu, 2013). Moreover, the phases used in HPLC and GC allow analytes of interest to be separated from other compounds that occur during metabolism, or those which might be present in the complex structure of the analytical matrix (Rodriguez et al., 2007).

According to the United States Environmental Protection Agency (EPA), liquid chromatography is becoming the most frequently used analytical technique to evaluate biodegradable herbicides and their metabolites in agricultural settings, especially if these compounds have high polarity and heat-sensitivity, as well as being non-volatile (Grosser, Ryan, & Dong, 1993).

It is estimated that approximately 2.27 million tons of pesticides are released annually into the environment (Kiely, Donaldson, & Grube, 2004). Currently, herbicides comprise 35% of the pesticides available on the market (Vercraene-Eairmal et al., 2010). Mesotrione [2-(4-methylsulfonyl-2-nitrobenzoyl)-1,3-cyclohexanedione] (Figure 1) is the active ingredient in the commercial product known as Callisto® (Syngenta), which is a systemic application herbicide that is widely used in corn crops because of its selectivity. Callisto® is recommended for pre and post-emergence control of broad-leaved weeds (Batisson et al., 2009).

Figure 1: Molecule of the herbicide mesotrione (C14H13NO7S)



This herbicide is derived from a phytotoxin that is produced by *Callistemon citrinus*. This phytotoxin inhibits the enzyme 4-hydroxyphenylpyruvate dioxygenase (HPPD) in the target organisms and interferes in the synthesis of carotenoids (Mitchell et al., 2001).

Despite the contribution of herbicides to the productivity of different crops, there are still concerns related to the negative effects of these compounds. These effects include health problems in humans and animals; the contamination of ground and surface water; and damage to soil microorganisms as a result of the persistence of these compounds in high concentrations in this matrix (Lopez-Perez et al., 2006; Martins et al., 2007; Jiang, Huang, Liang, & Zheng, 2008; Sharma, Nagpal, Pakade, & Katnoria, 2010). For these reasons, recent studies have evaluated the presence

of the herbicides in these different matrices and the degradation of the herbicides by microorganisms.

Durand et al. (2006) evaluated the capacity of the degradation of mesotrione using a strain of the genus *Bacillus*. In that study, the mesotrione degradation kinetics were monitored using a technique that coupled high-performance liquid chromatography with a diode array detector and (H^1) nuclear magnetic resonance (HPLC-DAD-NMR): it was determined that the herbicide was completely biotransformed and identified as 2-amino-4-methylsulphonylbenzoic acid (AMBA).

Corroborating that study, Bonnet, Bonnemoy, Dusser and Bohatier (2008) reported that the metabolite AMBA, which is derived from the oxidative metabolism of mesotrione, was more toxic than the original molecule; this finding provided important information that can be used to predict the environmental impact of these metabolites on different ecosystems.

On the other hand, Pileggi et al. (2012) evaluated the degradation of mesotrione by a strain of the *Pantoea ananatis* bacteria using liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) and reported that the metabolic products were less toxic than the original molecule in the reaction medium.

The importance of evaluating the quality of chemical measurements through their comparability, traceability and reliability has become increasingly recognized and required. To ensure that these measurements are correct, analytical methods need to generate reliable and interpretable information about the sample and the quantification. To reach this goal a validation process needs to be performed (Oskar et al., 2004).

The validation of chromatographic methods has facilitated the use of such methods to quantify xenobiotics in the environment, especially herbicides (Rodriguez et al., 2007; Beceiro-González et al., 2007). Thus, there is increased interest among research groups regarding the development of chromatographic methodologies that enable the qualification and quantification of herbicides present in different matrices. There are no published articles about the validation of HPLC to quantify the herbicide mesotrione.

Perez-Ruiz, Martinez-Lozano, Tomas and Martín (2005) analyzed some herbicides in potatoes, peppers and tomatoes by applying HPLC. They provided a validation procedure before performing the analysis. Good validation parameters were determined as linearity (the relationship between peak and compound concentration was in the range 0.01-7.00 $g.mL^{-1}$ with a correlation coefficient greater than 0.9995) and detection limits (between 4 $ng.mL^{-1}$ and 12 $ng.mL^{-1}$).

Thus, the objective of this study was to evaluate the degradation kinetics of mesotrione, which is an important environmental pollutant, in culture medium containing the *Escherichia coli* DH5-α bacteria and to apply a validated HPLC-DAD method.

MATERIALS AND METHODS

CHEMICALS AND INSTRUMENTATION

Acetonitrile (ACN) was HPLC grade (J.T. Baker, PA, USA); phosphoric acid was analytical grade (Synth, SP, Brazil); water was purified with a Millipore Milli-Q system (Millipore, SP, Brazil) and used for the experiments. Mesotrione (93% purity) was purchased from Syngenta. The bacterial culture media was prepared using analytical grade of several salts (3 $g.L^{-1}$ NaNO3, 0.5 $g.L^{-1}$ MgSO4, 0.5 $g.L^{-1}$KCl, 0.01 $g.L^{-1}$ FeSO4, 0.04 $g.L^{-1}$ CaCl2, 0.001 $g.L^{-1}$ MnSO4; 0.4 $g.L^{-1}$glucose, 10 mmol potassium phosphate pH 7.0). The high-performance liquid chromatograph system consisted of a Waters 2695 Alliance HPLC system (Milford, MA, USA), which was composed of a quaternary pump, an on-line degasser and an auto-injector. The detector was a diode array detector, model 2998 Diode Array Detector (DAD) (Waters™ Corporation, MA, United States of America).

QUANTITATIVE CHROMATOGRAPHIC CONDITIONS

The HPLC analysis was carried out using an Eclipse XDB C_{18} column (150 mm × 4.6 mm, 3.5 μm) and the mobile phase consisted of water acidified with 0.1% phosphoric acid (solvent A, pH 3.0) and acetonitrile (solvent B). The gradient elution conditions started at 30% B, 30 % to 55 % B at 15 min and 55 to 100% B at 17 min, isocratic until 18 min. The separations were performed at 20 ˚C and the flow-rate was 1 $mL.min^{-1}$. Compound detection was recorded at 254 nm.

SAMPLE PREPARATION

The bacterial strain of *E. coli* DH5-α was grown in 100 mL of Luria broth (LB; 10 $g.L^{-1}$tryptone, 5 $g.L^{-1}$ yeast extract and 10 $g.L^{-1}$NaCl) in 250 mL flasks and incubated at 37 ˚C at 200 rpm. After 10 h of incubation

the material was centrifuged at 800 rpm at 4 ˚C for 5 min. The precipitate was washed twice with phosphate buffered saline (PBS; 8 $g.L^{-1}$NaCl, 0.2 $g.L^{-1}$KCl, 1.44 $g.L^{-1}$ Na_2HPO_4 and 0.24 $g.L^{-1}$ KH_2PO_4) and the cells were then re-suspended in 10 mL of mineral medium with an added 2 µL of a solution of mesotrione (39.3 $\mu mol.L^{-1}$). This mixture was then incubated at 37 ˚C at 200 rpm for 7 h. Aliquots (1 mL) were collected, using calibrated micropipettes, from the culture medium for every hour of incubation (0 to 7 h) and centrifuged at 400 rpm for 10 min, filtered in a 0.22 µm membrane (syringe filters - K18-230, Kasvi, PR, Brazil) and transferred to a vial. Aliquots of 50 µL were injected into the chromatographic system (Sabin, Prestes, Adaime, & Zanella, 2009).

METHOD VALIDATION

The validation was conducted according to Resolution (RE) 899/03 (Agência Nacional de Vigilância Sanitária - ANVISA), and the following parameters were evaluated: selectivity; linearity; precision; accuracy; recovery; limits of detection (LOD); limits of quantification (LOQ); robustness and sample stability.

SELECTIVITY

A DAD detector was used to identify the mesotrione and to evaluate the purity of mesotrione at the analytical run. The retention factor of the mesotrione standard was also used to provide identification at the analytical run.

LINEARITY

Two curves were prepared. The first was an external standard curve that was evaluated using adequate standard solutions of mesotrione to provide the following calibration points: 9.0 µmol L^{-1}19.6 µmol L^{-1}; 29.4 $\mu mol.L^{-1}$; 39.3 $\mu mol.L^{-1}$; 78.6 $\mu mol.L^{-1}$; 176.4 $\mu mol.L^{-1}$ and 196.5 $\mu mol.L^{-1}$. All the samples were solubilized in acetonitrile (1 mL), centrifuged at 400 rpm for 10 min, filtered in a 0.22 µm membrane, and added to vials. The samples were prepared in triplicate and the linearity was determined by plotting the peak area of the herbicide versus the concentration of the compound. For the second external standard curve, the standard solutions of mesotrione were solubilized in culture medium at the same concentration points as the first curve (in triplicate) and the same treatment was applied as in the sample preparation. The calibration curve was drawn by plotting the peak area against the concentration of the analyzed compound. For all the points, 50 µL were injected into the chromatographic system.

INTERMEDIATE PRECISION, ACCURACY AND RECOVERY

The intra and inter-day variability (precision) were determined by using three different quality control solutions of mesotrione, which were prepared in acetonitrile (58.9 $\mu mol.L^{-1}$, 98.2 $\mu mol.L^{-1}$ and 176.8 $\mu mol.L^{-1}$) and processed as described above. These solutions (triplicate) were prepared and evaluated on three non-consecutive days. The accuracy of the method was determined by back calculation (comparing the theoretical and practical values of the quality control) and tested by using blind unknowns at two different concentrations that were prepared by a different analyst. The relative recovery was evaluated by calculating the peak area ratios of three quality control samples that were prepared and processed as described in the sample preparation (used in the precision assay). These were compared with the peak area ratios of three samples prepared in acetonitrile and non-processed to give the percent recovery.

LIMITS OF DETECTION AND QUANTIFICATION

The herbicide concentration with a peak area that reached the value of a signal-to-noise ratio of three times was accepted as the limit of detection (LOD). The limit of quantification (LOQ) was the signal-to-noise ratio of ten.

ROBUSTNESS

Three standard solutions (quality control) were prepared in triplicate and analyzed by varying the pH and temperature values. The peak areas and retention times were assessed (Oskar et al., 2004).

SAMPLE STABILITY AT ROOM TEMPERATURE

The 39.3 $\mu mol.L^{-1}$ solution was maintained in the auto-sampler for seven days and read on days 1, 3, 5 and 7. The area of the peak was compared and the amount of the herbicide was determined.

STATISTICAL ANALYSES

The data were obtained, analyzed and reported using Empower™ software (Milford, MA, USA): the experimental results were presented as the mean ± standard deviation.

RESULTS AND DISCUSSION

Several methods of the qualification and quantification of pesticides in different matrices have been proposed (Sabin et al., 2009; Lehotay, Mastovská, & Lightfield, 2005; Saadati et al., 2013). The main criteria for choosing an analytical method to evaluate these compounds are the speed, cost effectiveness, and applicability to different matrices (Rodriguez et al., 2007; Yan et al., 2013).

To determine an analytical technique that meets all of these criteria, HPLC has been widely used in the chemical, medical and pharmaceutical industries, as well as in various scientific fields and even in government agencies, because it is a method that meets the majority of specified requirements (Sharma et al., 2010).

Freitas, Götz, Ruff, Singer & Müller (2004) studied mesotrione in surface water samples using HPLC; they applied gradient elution conditions that consisted of water acidified with 0.1% formic acid (solvent A) and methanol acidified with 0.1% formic acid (solvent B).The analysis was performed using an isocratic condition (50% A:50% B) for 10 min, followed by a linear gradient of solvent B in solvent A: 80% at 12 min and then returned to 50% B for 3 min with a flow rate of 0.2 $mL.min^{-1}$.

Similarly, Durand et al. (2006) developed a chromatographic condition using HPLC, through which the presence of mesotrione was determined in cultures of *Bacillus* sp. 3B6 by applying a 40 min run (gradient: 0-5 min, 5% B; 5-25 min, 5-95% B; 25-30 min, 95% B; 30-35 min, 95-5% B; and 35-40 min, 5% B). The flow rate was 0.3 $mLmin^{-1}$ with acidified water (phosphoric acid, 0.1% v/v; pH 2.6, solvent A) and acetonitrile (solvent B) as solvents. The aforementioned authors found that, compared with other studies in the literature, this method exhibited an optimized time for the analysis of the selected herbicide and provided a good resolution relative to other matrix components when using this chromatographic condition.

In the present study, to start the development of the method an exploratory chromatographic analysis was conducted using water (solvent A) and acetonitrile (solvent B) and 5 to 100% of B for 60 min in a C_{18} column to obtain information about the matrix and chromatographic conditions in order to determine the concentration of mesotrione present in the culture medium containing *E. coli* DH5-α, which was previously characterized as a mesotrione-degrading strain (Olchanheski et al., 2014; Snyder & Dolan, 1996).

After several injections, as well as the evaluation of different approaches to determine the best chemical conditions of the analyzed matrix and the presence of the chromatographic band of mesotrione, a gradient elution condition was optimized using a mobile phase that consisted of water acidified with 0.1% phosphoric acid (solvent A, pH 3.0) and acetonitrile (solvent B) - 30% B initially, 30% to 55% B for 15 min, 55 to 100% B for 2 min, and held isocratic for 1 min. An adequate analysis time was obtained, and the mesotrione chromatographic band was determined in 8.88 min (k = 4.44).

Having developed the method, the validation protocol based on RE 899/03 was performed using the optimized chromatographic conditions.

The peak purity of the mesotrione between 200 and 400 nm was evaluated using a diode array detector. The selectivity was established by comparing the ultraviolet (UV) spectra, the retention factor of the standard solution, and the peak of mesotrione in the analytical run.

The recoveries of the herbicide were determined by analyzing the extracted and non-extracted quality controls, which expressed the peak area ratios of the samples, and the evaluation between these two kind of samples (extracted and non-extracted) showed that the recoveries presented values of 37.5 and 37.3 $\mu mol.L^{-1}$ with CV% of 3.88 and 3.54 (n = 3, for each analysis).

The analytical curve (external standard) at 254 nm was linear and with a good fit of the model from 9.0 $\mu mol.L^{-1}$ to 196.5 $\mu mol\ L^{-1}$ of mesotrione. The regression equation that was determined was $y= 3.10^{7}x + 110496$, with a correlation coefficient of r = 0.9998 and a variation coefficient lower than 5% for the triplicates.

The precision (intra and inter-day) and accuracy of the method were determined by evaluating the triplicate of quality control samples on three non-consecutive days. The precision results were expressed as coefficients of variation (CV%) and the accuracy was calculated. The accuracy was evaluated by back calculation of the quality control samples and the two samples prepared

by the blind analyst. The results showed adequate precision and accuracy values for the evaluated quality controls (Table 1). The blind samples containing the unknown concentration prepared by another analyst presented accuracy in the range of 106.9-101.9.

Table 1: Precision and accuracy for the mesotrione assay.

Q.C.(mmol L^{-1})	1st day		2nd day		3rd day	
Mesotrione	CV (%)	Accuracy (%)	CV (%)	Accuracy (%)	CV (%)	Accuracy (%)
0.0589	0.83	106.9 ± 0.98	0.27	106.7 ± 0.00	3.97	104.1 ± 4.38
0.0982	2.76	101.9 ± 2.91	2.90	102.0 ± 3.06	2.71	102.2 ± 2.87
0.1768	1.97	106.9 ± 2.14	2.26	105.7 ± 2.44	3.70	106.9 ± 4.04

Q.C. = quality control samples.

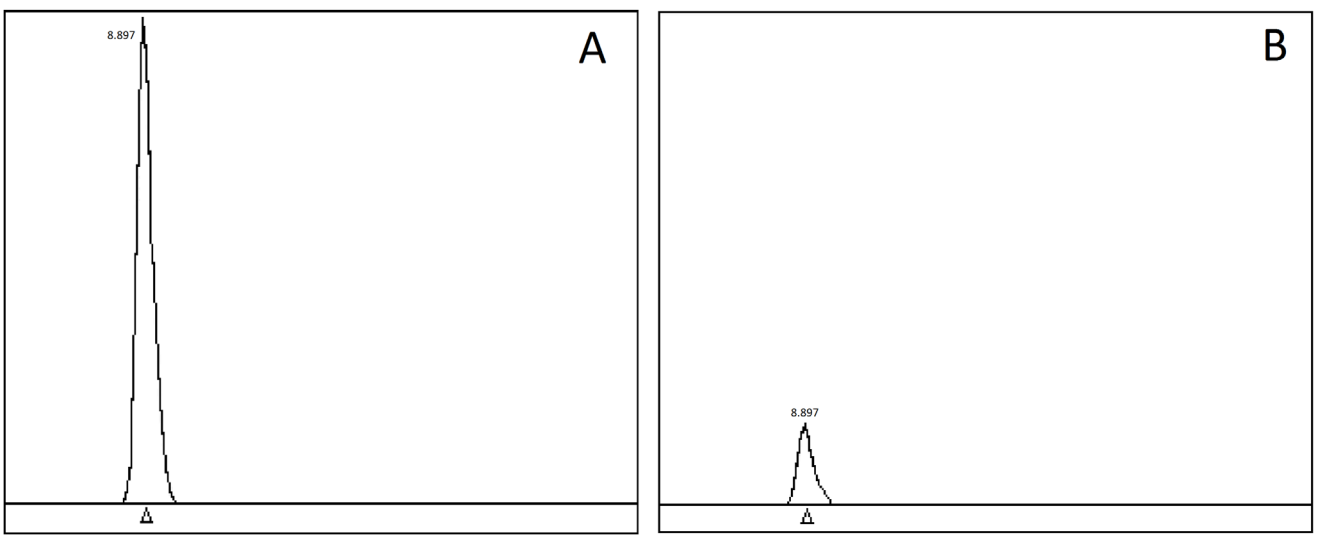
The limit of quantification was 9.0 µmol.L^{-1} while the limit of detection was 0.3 µmol.L^{-1}.

According to Oskar et al. (2004), robustness can be evaluated using HPLC by varying the quantity of organic solvent in the mobile phase at ± 2%, the pH of the mobile phase at 0.1 unit of pH and the temperature of the column at ± 5%. If these changes do not interfere with the analysis, and the results present accuracy, precision and acceptable selectivity, then the method is robust and these variations can be added to the method and procedure. To determine if the proposed method presented adequate robustness, two factors were assessed; temperature (20 to 25°C) and pH (3.0 to 3.4). The characteristics and retention time of the mesotrione bands did not vary significantly, and they exhibited acceptable accuracy, precision and selectivity, thereby demonstrating that the technique was robust under the evaluated conditions.

ANVISA Resolution 899/03 states that in order to guarantee the stability of a herbicide during validation it is recommended that the stability of a quality control sample is evaluated at one concentration. For this reason, the sample stability at room temperature was determined by maintaining the vial with sample (quality control at a concentration of 39.3 µmol.L^{-1}) in the auto-injector carrousel for seven days. Triplicate injections of this sample during those seven days were performed; the area of the peak was compared and the amount of the herbicide was determined for each analysis. The results showed good coefficients of variation (CV = 1.42%) according to the relevant legislation, which indicated good stability of the mesotrione at room temperature throughout seven days.

Finally, to estimate the interference of the culture medium in the mesotrione analysis, a second calibration curve was carried out. However, this time, the mesotrione was solubilized in medium used for bacteria development. The mesotrione was dissolved in culture medium to give the calibration solutions (19.6 µmol.L^{-1}; 29.4 µmol.L^{-1}; 39.3 µmol.L^{-1}; 78.6 µmol.L^{-1};176.4 µmol.L^{-1} and 196.5 µmol.L^{-1}). The calibration curve plotted in culture medium also presented good linearity ($y = 3.10^{07} x + 111056$). A correlation coefficient of 0.9998 and good correlation values were obtained when analyzing the two calibration curves, indicating that the matrix (culture medium) did not affect the mesotrione analysis (Figure 2).

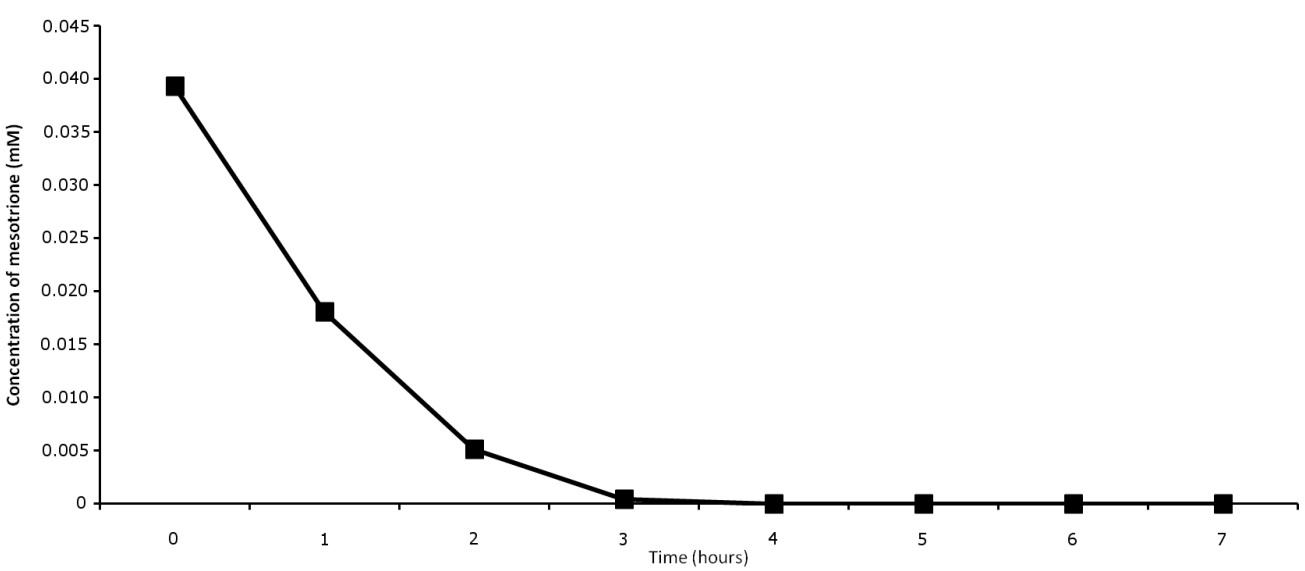
Figure 2: Typical chromatograms of the analysis of mesotrione obtained during the validation method. A: chromatogram of 0.1965 mmol L^{-1} calibration solution in acetonitrile, B: chromatogram of 0.029475 mmol L^{-1} calibration solution in culture medium. Column: Eclipse XDB C18, injection volume of 50 µL. Mobile phase consisted of (A) water (acidified with 0.1% phosphoric acid - pH 3.0) and (B) acetonitrile. Gradient elution condition: 30% B initially, 30% to 55% B for 15 min, 55 to 100% B for 2 min (17 min), held isocratic for 1 min (18 min), returned to the initial gradient of 30% B for 1 min (19 min) and then held in this isocratic position for 10 min (29 min) (conditioning of the column for new injections). The separations were performed at 20 °C with a flow of 1 mL min^{-1}. Compound detection was monitored



The *E. coli* DH5-α strain was previously characterized as having potential to degrade the herbicide mesotrione (Olchanheski et al., 2014) so the validated method obtained was used to determine mesotrione in samples with the *E. coli* DH5-α bacteria.

The measurement at different times of mesotrione in culture medium with *E. coli* DH5-α made it possible to determine the presence of 17.0 µmol.L^{-1} of mesotrione after one hour of incubation, characterizing a degradation of 57.5% of the herbicide by the bacteria (Figure 3).

Figure 3: HPLC measurement of the herbicide mesotrione mediated by *E. coli* DH5-α.at 254 nm.



After two hours of exposure, the concentration of mesotrione present in the medium was lower that the limit of quantification of the proposed method. Consequently, it can be inferred that after this time it was not possible to measure the sample in the medium with precision and accuracy, although traces of the compound could be determined.

Certain bacteria have the ability to completely degrade mesotrione in different times (e.g., *Bacillus* sp. 3B6 within 24 hours (Durand et al., 2006) and *P. ananatis* CCT 7673 within 17 hours (Pileggi et al., 2012)). Another study (Olchanheski et al., 2014) showed that *E. coli* DH5-α has the capacity to degrade mesotrione but this process becomes shorter under certain conditions for this bacteria, as observed in the herbicide measurement by HPLC in the present study.

CONCLUSIONS

A new, rapid, simple and efficient HPLC method to determine mesotrione in culture medium containing *E. coli* DH5-α was developed and validated. The application of the validated method made it possible to measure the herbicide at different times and to propose degradation kinetics for mesotrione under the experimental conditions.

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