Anais da Academia Brasileira de Ciências (2016) 88(3): 1235-1241
(Annals of the Brazilian Academy of Sciences)
Printed version ISSN 0001-3765 / Online version ISSN 1678-2690
http://dx.doi.org/10.1590/0001-3765201620150234
www.scielo.br/aabc

Validation of a liquid chromatography ultraviolet method for determination of herbicide diuron and its metabolites in soil samples

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Manuscript received on March 27, 2015; accepted for publication on March 18, 2016

ABSTRACT

Diuron is one of the most widely herbicide used worldwide, which can undergo degradation producing three primary metabolites: 3,4-dichlorophenylurea, 3-(3,4-dichlorophenyl)-1-methylurea, and 3,4-dichloroaniline. Since the persistence of diuron and its by-products in ecosystems involves risk of toxicity to environment and human health, a reliable quantitative method for simultaneous monitoring of these compounds is required. Hence, a simple method without preconcentration step was validated for quantitation of diuron and its main metabolites by high performance liquid chromatography with ultraviolet detection. Separation was achieved in less than 11 minutes using a C_{18} column, mobile phase composed of acetonitrile and water (45:55 v/v) at 0.86 mL min^{-1} and detection at 254 nm. The validated method using solid-liquid extraction followed by an isocratic chromatographic elution proved to be specific, precise and linear ($R^2 > 0.99$), presenting more than 90% of recovery. The method was successfully applied to quantify diuron and their by-products in soil samples collected in a sugarcane cultivation area, focusing on the environmental control.

Key words: pesticides, degradation products, HPLC-UV, soil, environmental control.

INTRODUCTION

Demand for quality as many as quantity of food in general requires the use of chemicals for fighting invasive weeds and pests. However, there is a concern that these toxic pesticides once introduced into environment, affect ecosystem conservation, as well as human and animal health (Stasinakis et al. 2009).

Among different types of pesticides, diuron (3-(3,4-dichlorophenyl)-1,1-dimethylurea) is one of the most widely used herbicide in Brazil. It is extensively employed for pre- and post-emergent weed control in cotton, sugarcane, citrus and pineapple cultivation areas, also being applied in non-cultivation areas as roads and railways (Gondim-Tomaz et al. 2005, Giacomazzi and Cochet 2004). As a consequence, it can be found in groundwater, surface water and sediments, originating from soil leaching (Blanchoud et al. 2004). Due to its low water solubility (42 mg L^{-1} at 25°C) and high sorption capacity to organic matter, this herbicide is relatively persistent in soil with

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half-life of 1 month to 1 year (Giacomazzi and Cochet 2004, Field et al. 2003).

According to toxicity degree and environmental hazard, diuron is considered moderately toxic and dangerous to environment. U.S. Environmental Protection Agency (EPA) has classified this herbicide as a "known/likely" carcinogen since 1997. Furthermore, diuron may be a precursor of nitrosodimethylamine, a compound belonging to N-nitrosamine family with high carcinogenic potential (Chen and Young 2008, Di Bernardo-Dantas et al. 2011). Although most of the toxicity studies focuses on herbicide, degradation products can represent an environmental risk. According to Tixier et al. (2001), diuron can undergo transformations forming by-products with even greater toxicity. Its degradation in environment mainly occurs through microorganism biological activity, producing three primary metabolites: 3,4-dichlorophenylurea (DCPU), 3-(3,4-dichlorophenyl)-1-methylurea (DCPMU), and 3,4-dichloroaniline (DCA) (Dalton et al. 1996, Cox et al. 2007). These by-products can persist longer in aquatic and terrestrial ecosystems due to its great stability (Tixier et al. 2000, Sorensen et al. 2003). Since main breakdown product (DCA) is considered more toxic and persistent in the environment than diuron itself (Giacomazzi and Cochet 2004), analytical methods able to detect diuron and its metabolites are extremely important.

High performance liquid chromatography (HPLC) has been widely used to analyze pesticides, including diuron determination in water, soil and sediment (Martinez and Barceló 2001, Lourencetti et al. 2008, Chaplain et al. 2001, Balakrishnan et al. 2012, Tworkoski et al. 2000). Due to analyte low concentration levels comprising interfering diversity, pesticides in water determined by HPLC usually requires the use of very sensitive detectors (such as mass spec's) and involves preconcentration steps which can take over half of the total analysis time (Stork et al. 2008, Brondi and Lanças 2005, Azevedo et al. 2001, Gallitzendorfer et al. 2011). However, diuron and its by-products can be monitored in soil from cropping areas by using sensitivity and reasonable cost equipment as ultraviolet detectors (UV). The evaluation of these compounds under field conditions, such as farming areas, is useful to assist in studies of sorption, desorption, degradation and transport of this analytes in different types of soil. Thus, the aim of this study was to validate a simple and reliably HPLC-UV method using solid-liquid extraction followed by an isocratic chromatographic elution for direct simultaneous quantitation of diuron and its three main metabolites.

EXPERIMENTAL

CHEMICALS AND REAGENTS

DCPU, DCPMU, diuron and DCA analytical standards were purchased from Dr. Ehrenstorfer-Schafers Laboratory (Augsburg, Germany) with purity around 99%. Acetonitrile and methanol (HPLC grade) were obtained from J.T. Baker (Phillipsburg, NJ, USA). High purity water was obtained from a *Millipore MilliQ*® purification system (Billerica, MA, USA).

SAMPLING SITE AND SAMPLE PREPARATION

Soil samples were collected in a sugarcane cropping area located in the countryside of Porecatu municipality, Paraná State, Southern Brazil. In addition, a soil sample from a nearby preserved forest was used as a blank sample.

Each soil sample was milled in porcelain mortar applying pestle and sieved through two mesh of 9 (2.0 mm) and 250 (63 µm) (Bertel Ind. Metalúrgica, Caieiras, SP, Brazil). For extraction of diuron and its metabolites, 10 mL of methanol were added to 5.00 g of sample and kept overnight in an end-over-end shaker at 24°C. The soil extracts were centrifuged at 1200 rpm for 30 min and 5.0

mL of each supernatant was filtered through a 0.22 µm polytetrafluoroethylene (PTFE) membrane (Millipore Corporation, Cork, Ireland) directly into glass vials, and storage at 4°C until further use.

INSTRUMENTATION

Herbicide diuron and its metabolites were analyzed by HPLC using a Waters Alliance® e2695 Separations Module (Milford, MA, USA) equipped with degasser, quaternary solvent organizer pump, autosampler and a Waters 2998 photodiode array detector. Separations were performed using a XTerra C_{18} column (4.6 x 250 mm, 5 µm) from Waters Corporation. Data acquisition and treatment were performed using an *Empower* 2.0® software package.

VALIDATION AND QUANTITATION

The proposed HPLC-UV method was based on a procedure for determination of diuron and its metabolites in water (Field et al. 1997). In order to ensure reliable results, a method was validated in terms of specificity, selectivity, linearity, accuracy, repeatability, intermediate precision, limit of detection (LOD), and limit of quantitation (LOQ).

Diuron and its metabolites quantitation was performed by external standard method. Analytical curves were acquired by solutions standards injection prepared with methanol in concentration range from 0.05 to 35.1 mg L^{-1} (DCPU, DCPMU and diuron) and 1.60 to 35.1 mg L^{-1} (DCA). All determinations in soil samples were performed in triplicate.

RESULTS AND DISCUSSION

METHOD VALIDATION

For the proposed HPLC-UV method in isocratic elution mode each standard of interest (DCPU, DCPMU, diuron, and DCA) was injected observing suitable resolution, without soil matrix interference and co-elution in retention times. Characteristic spectra were checked for each compound. Ideal analytical conditions able to promote separation for four analyte of interest were obtained using a mobile phase composed by water and acetonitrile (55:45 v/v) at 0.86 mL min^{-1}. Sample injection volume was 20 µL with detection done at 254 nm. Column and injector temperature were maintained at 30°C and 20°C, respectively. In these conditions, analyte separations were archived in less than 11 min.

Compared to the method developed by Field et al. (1997) it was not necessary to use gradient elution to separate compounds of interest and analysis time was reduced considerably. An isocratic HPLC-UV method to analyze diuron and its metabolites in calcareous soil was presented by Gooddy et al. (2002). However, according to those authors, the sample preparation was more laborious, including two types of extractions, requiring even methanol evaporation and suspension with water; separation was performed in about 20 minutes. Moreover, post-run backflushing due to the amount of injected sample (1 mL) was demanded to prevent column carry-over.

Specificity and selectivity of the proposed method was investigated using soil sampled in preserved area (reference). Chromatograms obtained by injections of reference sample (Fig. 1a) and standard solution (Fig. 1b) showed absence of analytical response, regarding reagents or interfering species in retention time of the peaks of compounds of interest.

To assess the linearity, random injections of standard mixtures in triplicate were performed to allow analytical curves construction. Working range and information concerning to linear data regression analysis are presented in Table I. Coefficients of determination (R^2) were higher than 0.99, which indicate that regression line perfectly fits the experimental data, showing a linear response over concentration range tested.

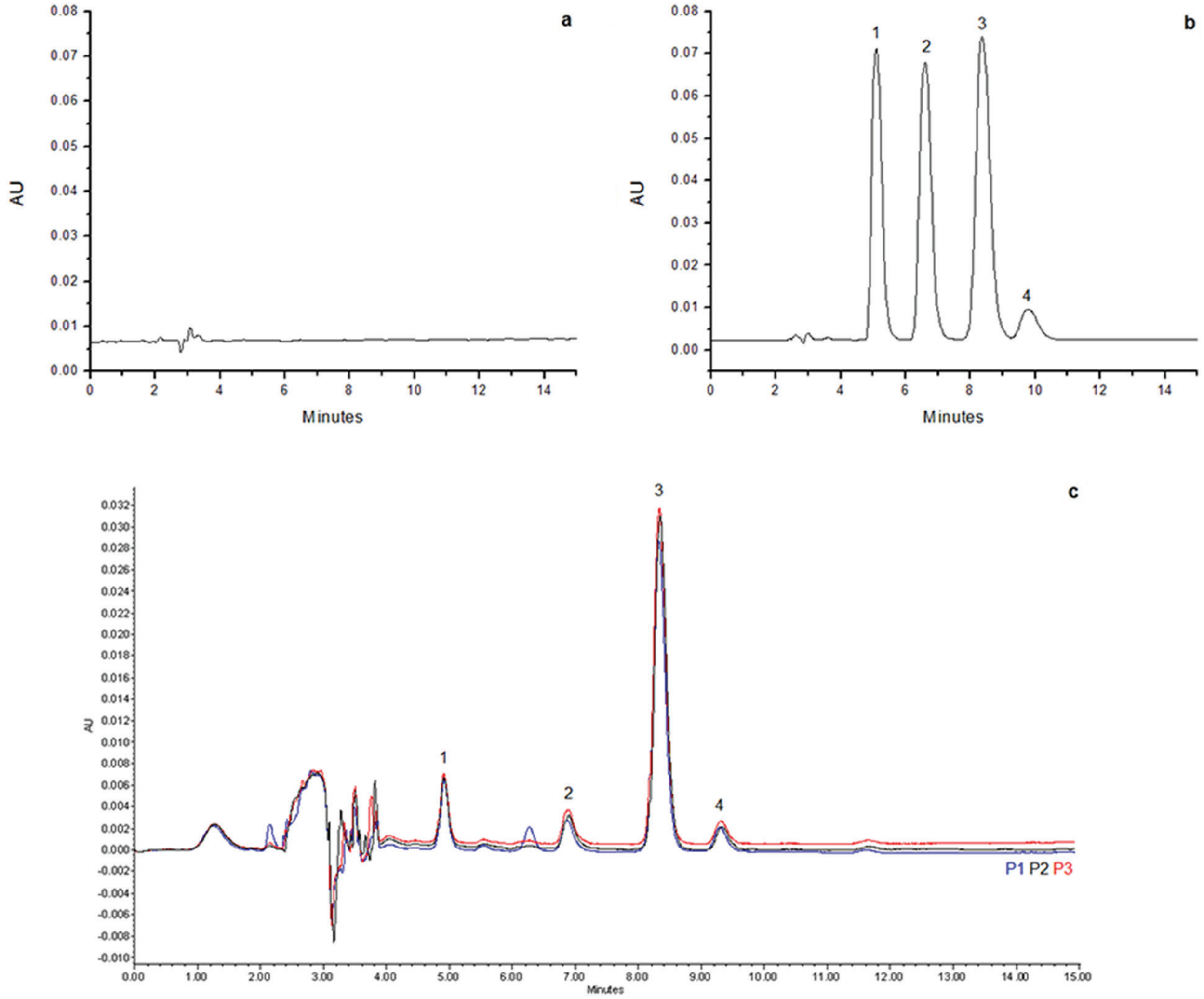

Figure 1 - (**a**) Chromatogram of preserved area soil sample used as a reference. (**b**) Chromatogram of analytical standards mixture at an individual concentration of 9.6 mg L^{-1}. (**c**) Chromatograms of three soil samples collected in a sugarcane cultivation area. Analytes: DCPU (1), DCPMU (2); diuron (3), and DCA (4). Mobile phase: water:acetonitrile (55:45 v/v); flow rate: 0.86 mL min^{-1}; injection volume: 20 µL; column: XTerra C_{18} (4.6 × 250 mm, 5 µm); λ: 254 nm.

The limits of detection (LOD) and quantification (LOQ) of diuron and its metabolites were estimated considering a signal-to-noise (S/N) ratio of 3 and 10, respectively, according to the International Committee on Harmonization Q2(R1) for HPLC methods validation (ICH 2005). The values of LOD and LOQ obtained from dilutions of a standard mixture are shown in Table I. In addition, soil samples from a preserved area (blank) were spiked with known amounts of analytical standards and subjected to the solid-liquid extraction procedures. The extract was evaluated by HPLC-UV and successively diluted until the peaks of the compounds were barely observed in the chromatogram. In this condition, the lowest detectable and quantifiable concentrations for the analytes in the soil were 0.02, 0.01, 0.04 and 0.05 mg kg^{-1} and 0.07, 0.04, 0.13 and 0.13 mg kg^{-1} for DCPU, DCPMU, diuron, and DCA, respectively.

TABLE I
Parameters of linear regression, limits of detection (LOD) and quantitation (LOQ) for diuron and the metabolites (n=3).

Analyte	Working Range (mg L^{-1})	Linear Regression Equation	Coefficient of Determination (R^2)	LOD (mg L^{-1})	LOQ (mg L^{-1})
DCPU	0.05 - 35.1	150992 x + 20592	0.99993	0.01	0.03
DCPMU	0.05 - 35.1	129016 x – 509	0.99996	0.01	0.02
Diuron	0.05 - 35.1	196603 x + 12985	0.99993	0.02	0.05
DCA	1.60 - 35.1	35848 x – 50263	0.99020	0.02	0.07

DCPU: 3,4-dichlorophenylurea; DCPMU: 3-(3,4-dichlorophenyl)-1-methylurea; DCA: 3,4-dichloroaniline.

Analytical method accuracy, indicating nearness between expected value and experimental value found, was assessed using blank soil samples spiked with standard solutions at low, medium and high concentration levels (Table II). The fortified samples were subjected to the solid-liquid extraction procedures. After extraction, aliquots were analyzed and the experimental data were statistically evaluated by analyte recovery rates. Average percentages of recovery performed in triplicate were 91.9, 92.1, 94.4 and 90.1% for DCPU, DCPMU, diuron, and DCA, respectively.

TABLE II
Accuracy given by recovery of the proposed method for different levels of analyte concentration.

Compound	Concentration (mg L^{-1})	Mean ± SD, n=3
DCPU	0.4	84.2 ± 2.8
	9.6	91.0 ± 6.6
	20.0	100.5 ± 4.4
DCPMU	0.4	82.8 ± 4.3
	9.6	92.8 ± 1.5
	20.0	100.6 ± 2.9
Diuron	0.4	86.6 ± 2.1
	9.6	91.4 ± 1.7
	20.0	105.3 ± 3.6
DCA	1.6	78.1 ± 7.3
	9.6	94.6 ± 6.1
	20.0	97.7 ± 3.5

DCPU: 3,4-dichlorophenylurea; DCPMU: 3-(3,4-dichlorophenyl)-1-methylurea; DCA: 3,4-dichloroaniline.

Precision of the developed method was assessed with respect to repeatability (intraday precision) and intermediate precision (interday precision) by standard solutions replicate injections (n=6). Coefficients of variation (CV) were determinate through data statistical treatment. In both precision tests, CV was greater for low concentration solutions. The CV values obtained for repeatability test were 1.89, 1.15 and 1.85 %, respectively, for DCPU, DCPMU and diuron at 0.4 mg L^{-1} and 1.43% for DCA at 1.6 mg L^{-1}. The intermediate precision assay showed CV values of 3.51, 3.34 and 3.76 %, respectively, for DCPU, DCPMU and diuron at 0.4 mg L^{-1} and 4.29% for DCA at 1.6 mg L^{-1}. Thus, the method can be considered precise, since CV for all measures were below 5%.

QUANTITATIVE ANALYSIS IN SOIL SAMPLES

The validated method was applied to real soil samples from sugarcane cropping area. Figure 1c shows chromatograms corresponding to the samples collected in three different points around 20 meters far from each other, without the addition of analytical standards. The identification of diuron and its metabolites was based on the retention times (Fig. 1b) in addition to the comparison of its UV-VIS spectrum profiles in the same chromatographic conditions.

Quantitative analyses were performed using external standard method, by comparing peak areas of samples with standard solutions. Concentration of each compound of interest in the sample is shown in Table III.

Analyzed compounds presented low variation regarding to concentration in the soil samples (Table III). It is worth to emphasize that sample preparation was conducted without any pre-concentration or dilution step, since the studied analytes were within validated working range. Moreover, interfering compounds were not observed in the sample matrix, which ensures the applicability of the proposed method in real soil samples.

TABLE III
Concentration of diuron and its metabolites in sugarcane soil samples.

Sample	**Concentration (mg kg^{-1}) (Mean ± SD, n=3)**			
	DCPU	**DCPMU**	**Diuron**	**DCA**
P1	0.88±0.04	0.20±0.02	3.91±0.19	3.98±0.02
P2	0.86±0.02	0.23±0.03	4.16±0.16	4.12±0.61
P3	0.82±0.01	0.22±0.01	3.89±0.09	4.15±0.14

DCPU: 3,4-dichlorophenylurea; DCPMU: 3-(3,4-dichlorophenyl)-1-methylurea; DCA: 3,4-dichloroaniline. SD: standard deviation; P: sampling point.

CONCLUSIONS

This paper reports a validated simple HPLC-UV method for simultaneous determination of herbicide diuron and its metabolites (DCPU, DCPMU, DCA) in soil. Chromatographic separation was performed in less than 11 minutes using isocratic mode after an extraction that does not require pre-concentration steps. Validated parameters as specificity, selectivity, linearity, sensitivity, accuracy and precision, proved that this method can be useful for diuron and its main by-products quantitation being applicable to soil samples in field condition. Therefore, the proposed method represents a reliable and feasible potential tool to monitoring, in an easy way, the persistence of diuron and its metabolites in environment.

ACKNOWLEDGMENTS

The authors would like to thank the Conselho Nacional de Desenvolvimento Cientifico e Tecnológico (CNPq, Brasília, Brazil) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES, Brasília, Brazil) for their financial support and fellowships.

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