Analytical Methods

Determination of paraquat and diquat: LC-MS method optimization and validation

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ABSTRACT

This study describes the optimization and single-laboratory validation of a single residue method for determination of two bipyridylium herbicides, paraquat and diquat, in cowpeas by UPLC-MS/MS in a total run time of 9.3 min. The method is based on extraction with an acidified methanol-water mixture. Different extraction parameters (extraction solvent composition, temperature, sample extract filtration, and pre-treatment of the laboratory sample) were evaluated in order to optimize the extraction method efficiency. Isotopically labeled internal standards, Paraquat-D6 and Diquat-D4, were used and added to the test portions prior to extraction.

The method validation was performed by analyzing spiked samples at three concentrations (10, 20 and 50 μg kg^{-1}), with seven replicates (n = 7) for each concentration. Linearity (r^2) of analytical curves, accuracy (trueness as recovery % and precision as RSD%), instrument and method limits of detection and quantification (LOD and LOQ) and matrix effects were determined. Average recoveries obtained for diquat were between 77 and 85% with RSD values ⩽20%, for all spike levels studied. On the other hand, paraquat showed average recoveries between 68 and 103% with RSDs in the range 14.4–25.4%. The method LOQ was 10 and 20 μg kg^{-1} for diquat and paraquat, respectively. The matrix effect was significant for both pesticides. Consequently, matrix-matched calibration standards and using isotopically labeled (IL) analogues as internal standards for the target analytes are required for application in routine analysis.

The validated method was successfully applied for cowpea samples obtained from various field studies.

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1. Introduction

Paraquat (1,1'-dimethyl-4,4'-bipyridinium dichloride) and diquat (1,1'-ethylene-2,2'-bipyridyldiylium dibromide) are bipyridylium herbicides widely used in the world (Paraquat Information Center, 2015; The Pesticide Manual, 2012). Both are very polar substances, having a high solubility in water, 620 g L and 700 g L, for paraquat and diquat, respectively (Paraquat Information Center, 2015).

Paraquat is a banned substance in the European Union (Maya, Estela, & Cerdà, 2011; Yao et al., 2013), however, it is still applied in nearly 90 countries, such as China, Thailand and Brazil (ANVISA (Brazilian Sanitary Surveillance Agency), 2015; Paraquat Information Center, 2015; Yao et al., 2013), which can cause problems through importation/exportation procedures over the whole world. It is used either to prepare the land for planting or for controlling weeds in more than 100 crops, including major crops as corn, rice, soya, wheat, potatoes, apples, oranges, bananas, coffee, tea, cocoa, cotton, palm oil and sugarcane (Paraquat Information Center, 2015).

Diquat is a non-selective herbicide, contact desiccant and it is also used for weed control. Among many other crops, it is recommended to be used on potato, coffee, beans, soya and citrus fruits (The Pesticide Manual, 2012).

The group of quaternary ammonium salts, also known as "quats", is a particular difficult type of herbicides (Shawish, Ghalwa, Hamada, & Basheer, 2012) due their physico-chemical properties, which preclude their determination via multi-residue methods. Several analytical methods and techniques have been

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reported for paraquat and/or diquat determination. During the last few years, many voltammetric methods were described (Farahi, Achak, Gaini, Mhammedi, & Bakasse, 2014; Harmoudi et al., 2012, 2013; Tyszczuk-Rotko, Beczkowska, & Nosal-Wiercinska, 2014), as well as spectrophotometric (Maya et al., 2011), potentiometric (Shawish et al., 2012), spectrofluorimetric (Yao et al., 2013) and immunoassay methods (Garcia-Febrero, Salvador, Sanchez-Baeza, & Marco, 2014). Some methods were developed using GC with solid-phase extraction (SPE) (Almeida & Yonamine, 2007) and solid-phase microextraction (SPME) (Gao et al., 2014). Most of the cited studies were concerning environmental matrices, such as water (Maya et al., 2011; Shawish et al., 2012; Tyszczuk-Rotko et al., 2014; Yao et al., 2013) and biological matrices, such as urine and human plasma (Shawish et al., 2012; Almeida & Yonamine, 2007; Gao et al., 2014). Nowadays, LC coupled with tandem mass spectrometry (LC-MS/MS) and ESI ionization, is becoming the most preferable approach, due to parameters as sensitivity and selectivity (Hao et al., 2013; Ruan et al., 2014).

However, often obtained inconsistent results for the paraquat determination suggested problems with the chromatographic and/or mass spectrometric behavior of mainly paraquat (Hao et al., 2013). Three types of quasi-molecular ions, radical cations $M^{+\cdot}$ (m/z 184 for DQ and m/z 186 for PQ), singly charged cations $[M+H]^+$ (m/z 183 for DQ and m/z 185 for PQ) and dications M^{2+} (m/z 92 for DQ and m/z 93 for PQ), were observed in the ESI mass spectra of DQ and PQ (Castro, Moyano, & Galceran, 2001; Grey, Nguyen, & Yang, 2002). Neither the MRM transitions nor the ESI capillary voltages described in the literature for the quantitation of DQ and PQ were consistent (Hao et al., 2013).

In 2012, Kolberg et al. (2012) developed and validated a method using isotope dilution mass spectrometry (IDMS) to compensate for paraquat and diquat losses during the whole analytical procedure in order to improve accuracy and precision. Then, based on the method (QuPPe-Method) of the European Union Reference Laboratory for Pesticides Requiring Single Residue Methods (EURL-SRM) (Anastassiades et al., 2013), our group started further optimizing the sample extraction parameters, such as extraction solvent composition, temperature, sample extract filtration and pre-treatment of the laboratory sample, also applying IDMS, in order to obtain acceptable results, mainly for paraquat. The optimized method was finally validated for cowpea beans, according to document No. SANCO/12571/2013 (SANCO, 2013). The method developed for this study was successfully applied for samples from an EMBRAPA field study.

2. Experimental

2.1. Chemical and reagents

Acetonitrile, pesticide grade (99.9%) and formic acid, analytical grade (99.8%), were purchased from J.T. Baker (Phillipsburg, NJ, USA); ammonium formate (99.0%) from Fluka Analytical (Seelze, Germany). Methanol, pesticide grade (99.5%), was obtained from Mallinckrodt (Phillipsburg, NJ, USA) and fuming hydrochloric acid (HCl, p.a., 37%) from Casa da Química (Diadema, SP, Brazil). Reference standards of diquat dibromide (98%), paraquat dichloride (97%), diquat dibromide-D4 (98.5%) and paraquat diiodide-D6 (98%) were obtained from Dr. Ehrenstorfer (Augsburg, Germany).

2.2. Standard solutions

Standard stock solutions were prepared at 1 mg mL^{-1} in methanol, acidified with 1% formic acid. Paraquat diiodide-D6 was dissolved in water due to it's insolubility in organic solvents. Each compound was individually weighed directly into the 20 mL

polypropylene tubes and immediately dissolved in the appropriate solvent. Finally, the solutions were placed for 5 min in an ultrasonic bath for complete dissolution.

From the individual stock solutions, a mixture of paraquat and diquat was prepared at 100 μg mL^{-1}, which was further diluted with methanol to a concentration of 10 μg mL^{-1}. For the preparation of analytical solutions, the 10 μg mL^{-1} mixture solution was diluted with methanol to concentrations of 0.1, 1 and 5 μg mL^{-1}, which were then used for preparation of working standard solutions in solvent and in blank matrix extract.

Deuterated internal standards, paraquat-D6 and diquat-D4, were diluted with methanol from their individual stock solutions to a mixture with a concentration of 40 μg mL^{-1}, which was used to spike samples for recovery studies and quantitation of the analytes in real samples. In order to use the deuterated standards in calibration standard solutions, at a fixed concentration, a solution was prepared at 2 μg mL^{-1} from the solution of 40 μg mL^{-1}.

Analytical solutions were stored in a freezer at −20 °C.

Before use, all solutions were taken out from the freezer and allowed to stand until room temperature was reached. Subsequently, the solutions were placed in an ultrasonic bath for 5 min to obtain complete dissolution.

2.3. UPLC-MS/MS experimental conditions

The chromatographic system consisted of an ACQUITY UPLC (ultra-performance liquid chromatograph) coupled to a XEVO TQ-S tandem mass spectrometer (Waters, Milford, MA, USA).

Chromatographic separations were carried out using a Sielc® Obelisc R column (5 μm, 2.1 mm i.d × 150 mm), maintained at 40 °C. The LC mobile phases consisted of water containing 20 mM ammonium formate, acidified to pH 3 with formic acid (solvent A) and acetonitrile (solvent B). The following gradient elution (0.4 mL min^{-1} flow rate) was performed: 20% A at the time of injection, increasing linearly to 80% A over 5.2 min. This eluent composition was maintained for 2.6 min and then decreased linearly to 20% A. This condition was maintained for 1.5 min.

The mass spectrometer was operated in the positive electrospray ionization mode (ESI$^+$). Two MRM transitions per analyte were monitored in the multiple reaction monitoring (MRM) mode for diquat (183.1 → 157.0 for quantification; 183.1 → 168.0 for confirmation) and paraquat (186.1 → 171.1 for quantification; 186.1 → 77.0 for confirmation). For isotopic labeled internal standards, only one transition (quantification) was monitored: 192.1 → 174.1 for paraquat-D6 and 186.1 → 158.0 for diquat-D4

Preliminary tuning experiments were carried out via direct infusion of diluted standard solutions (200 ng mL^{-1}) into the mass spectrometer, in order to establish the optimal detection conditions. This procedure was done for labeled and unlabeled standards. The infusion flow rate was set at 5 μL min^{-1}.

2.4. Optimization of sample preparation

Cowpea samples were milled and grinded until a fine powder. Then, in order to obtain a completely homogeneous sample, a slurry of cowpea and water was prepared. To this end, the ground cowpea was homogenized with water using an Ultra-Turrax for 5 min.

To optimize the homogeneity of the slurry preparation, different ratios of matrix/water were tested: 1:1, 1:1.5, 1:3, and 1:4 (milled cowpea:water, w:w). Respectively, 5, 4, 2.5 and 2 g of ground cowpeas were weighed, with an analytical balance, in 4 different Teflon (PTFE) bottles. To each bottle, respectively, 5, 6, 7.5 and 8 g of water (ultrapure quality) was added, and the mixture was homogenized using an Ultra-Turrax for 5 min. The

homogeneity of the slurry was determined visually and the most suitable ratio of cowpea/water was determined.

For this study, blank samples of cowpea were provided by Embrapa.

2.5. Optimization of extraction parameters

All optimization experiments were performed in triplicate and the recoveries presented in the results and discussion paragraph are the average (n = 3).

2.5.1. Extraction solvent composition
Methanol/acidified water was used as extraction solvent and various ratios of methanol/0.5 mol L^{-1} HCl (1:1, 6:4, 7:3, 8:2, 9:1) were tested. The mixtures were prepared in 500 mL volumetric flasks. The use of HCl solution 0.1 mol L^{-1} instead of 0.5 mol L^{-1} was also studied.

2.5.2. Temperature
The influence of temperature on the extraction was tested as follows. After the addition of the extraction solvent, the tube containing the matrix and extraction solvent was left at room temperature or directly transferred to a water bath which was kept at 80 °C for 15 min.

The influence of the temperature during centrifugation was compared by taking the sample extract tubes directly from the water bath (at 80 °C), without cooling down, or after prior cooling down to room temperature, before centrifugation.

2.5.3. Filtration
The effect on recoveries of omitting or using a filtration step (0.45 μm) after centrifugation, before injecting the final extract into the UPLC-MS/MS system, was tested.

2.5.4. Optimized extraction method
The final optimized method is as follows. From a homogenized slurry (matrix/water ratio of 1/1.5), 12.5 g analytical test portion (corresponding to 5 g original cowpea) is taken and the isotopically labeled internal standards are added. This portion is transferred to a 50 mL polypropylene centrifuge tube. Then, 10 mL extraction solvent, MeOH/0.5 mol L^{-1} HCl (6:4, v/v) is added and the tube is shaken vigorously, manually for 2 min. The tubes are thereafter transferred to a water bath (at 80 °C) and kept there for 15 min. The tubes are allowed to cool down to room temperature and then centrifuged for 15 min at 4000 rpm. From the final extract, 10 μL is injected in the UPLC-MS/MS system.

It is very important to mention that throughout the whole procedure and for the autosampler vials, use of glass ware must be avoided and only polypropylene tubes and vials must be used in order to not lose the analytes.

2.6. Validation experiments

2.6.1. Calibration curves, linearity, estimated LOD and LOQ
The linearity of the analytical curves was established based on injections of the standard solutions at concentrations of 1, 2, 2.5, 5, 10, 50 and 100 ng mL^{-1}. Each of these solutions was injected seven times (n = 7) in the chromatographic system. Average peak responses (areas), relative standard deviations (RSDs) and analytical curve equations were calculated. Also the determination coefficients (r^2) and linear dynamic ranges were determined for each compound.

Using both the analytical curves equation and the repeatability (RSD) data at the lowest concentration of each individual herbicide studied, the instrument limits of detection (LOD$_i$) and limits of

quantification (LOQ$_i$) and also the estimated method LOD and LOQ (LOD$_m$ and LOQ$_m$, respectively) were calculated.

The LOD$_i$ was calculated from the RSD% of the average peak areas of seven replicate injections at the lowest detectable concentration, by the formula: LOD$_i$ (ng mL^{-1}) = 3 × RSD × concentration. From these calculated values, a best estimated, rounded LOD$_i$ value was established. Consequently, this concentration is the lowest injected standard concentration (ng mL^{-1}) that could be detected all seven times, and for which the RSD should not exceed 33%.

The LOQ$_i$ was calculated via the formula: LOQ$_i$ = 10 × RSD × concentration, which gives LOQ$_i$ = 3.3 × LOD$_i$. The real LOQ$_m$ was based on the accuracy and precision data obtained via the recovery studies and was defined as the lowest validated spike concentration meeting the requirements of a recovery within the range of 70–120% and an RSD ⩽ 20%.

2.6.2. Matrix effect evaluation
The matrix effect (%) was calculated in two different ways using data from the analytical solutions in blank cowpea extract and in organic solvent (methanol) via the following formulas:

$$\text{Matrix effect}(\%) = \left(\frac{\text{peak area std in matrix} - \text{peak area std in solvent}}{\text{peak area std in solvent}} \right) \times 100$$

and

$$\text{Matrix effect}(\%) = \left(\frac{\text{slope analytical curve std in matrix} - \text{slope analytical curve std in solvent}}{\text{slope analytical curve std in solvent}} \right) \times 100$$

2.6.3. Accuracy (trueness and precision)
The accuracy (trueness and precision) of the method was evaluated through recovery experiments by spiking the mentioned herbicides to blank cowpea at three concentrations (10, 20 and 50 μg kg^{-1}), seven replicates at each level (n = 7). The spiking procedure was performed by adding the standard mixture solution, containing diquat, paraquat and the isotopically labeled internal standards, to the homogenized cowpea slurry before applying the extraction solvent. A blank cowpea, with just the internal standards added, was also extracted and analyzed concurrently.

2.7. Validation field experiments

2.7.1. Field trials
Field trial validation studies were conducted in three experimental fields of the Brazilian Agricultural Research Corporation – Embrapa, in Dourados (Mato Grosso do Sul), Teresina (Piauí) and Sinop (Mato Grosso), in three Brazilian states, from December 2011 to February 2014.

Each field trial consisted of 4 treatments, two sprayings (at stage of 50 and 75% of dry pods) with those two herbicides (at 300 g of active ingredient ha^{-1} and 1.5 L of commercial product ha^{-1}) plus the control treatment (without herbicide). The cultivation type used was the "Nova Era", sown with 0.5 m spacing between lines and 16 plants/square meter. Crop management followed the good agricultural practices for cowpea. The fertilizers were added according to the soil analysis (Raij, Andrade, Cantarella, & Quaggio, 2001) and recommendations common for beans (Raij, Cantarella, Quaggio, & Furlani, 1996). The experimental design was a randomized block with four replications in plots measuring 3.0 m × 7.0 m.

The crop was sprayed considering the pods into two distinct phases, 50 and 75% of dried pods, whereas most of the seeds had reached physiological maturity. The herbicide spraying was

performed with a knapsack sprayer pressurized with CO_2 and the volume was calibrated in order to spray 150 L h^{-1}.

2.7.2. Gathering samples

The sampling of the seeds was done on the harvest day of each experiment, in Dourados – MS, Teresina – PI and Sinop – MT, corresponding to 9, 7 and 13 days after the last herbicides were sprayed, respectively. The seeds sampling was made at an useful plot area and also on the control plot without herbicide application. Cowpea samples (200 g) were taken separately from each plot to compose a sample of each treatment. In total, 800 g of each treatment were fractioned and submitted to extraction of each herbicide studied.

The seed harvest was performed manually after herbicides desiccation. Cowpea seed samples were dried in the shade on aluminum foil and stored in plastic pots coated with the same material, and immediately sent to the laboratory, while keeping them under ambient temperature and humidity conditions during transport to the final destination. In addition to the herbicide residues, the percentage of defoliation, green stems, germination and seed vigor were evaluated (data not shown here).

3. Results and discussion

3.1. Optimization of sample preparation

It was considered not to use the cowpea powder directly as test portion for analysis, as indicated in the method of the EURL (QuPPe method version 7.1, 2013), but to use a sample slurry. With the slurry technique, better homogeneous samples can be obtained, especially for large sized laboratory samples. It also assists in the wetting process of dry samples, in order to make the pesticides better available at the time of extraction (Pizzutti et al., 2012). This is especially important in the case of incurred residues in samples after pesticides application in the field.

During the slurry optimization experiments, it appeared that the most appropriate matrix/water ratio was 1:1.5 (w:w). The visual inspection of the slurry with 1:1 ratio demonstrated that this proportion was too dense and slurries with ratios of 1:3 and 1:4 were too much diluted, which could easily cause segregation.

3.2. Optimization of extraction solvent composition

At first, the extraction conditions of the EURL method were tested. At 1:1 ratio methanol/HCl 0.1 mol L^{-1}, the recoveries for diquat and paraquat at the three spike concentrations showed too much variation and were completely out of the acceptable recovery range (70–120%) for paraquat (Table 2).

In this study, other proportions of methanol/water and a different concentration of HCl for extraction optimization of cowpea samples were investigated. In Table 1, it can be seen that an increase in the percentage of methanol causes a slight decrease of diquat recovery and a major decrease of paraquat recovery values. At the other hand, an increase in HCl concentration from 0.1 to 0.5 mol L^{-1} caused an increase of the recovery for mainly paraquat. When the methanol proportion is higher than 60%, recoveries for paraquat started to be outside of the acceptable range. A methanol/HCl 0.5 mol L^{-1} composition 60:40 (v/v) showed the optimal recoveries for the three different spike concentrations. No further adjustment of the concentration of HCl was thus necessary.

It is very striking that, in general, diquat recoveries are good and consistent at all 3 spike concentrations, but paraquat recoveries are many times outside of the 70–120% range, on both sides. Apparently, even the use of isotopically labeled internal standards could not fully compensate for the changes in absolute recoveries.

3.3. Effect of extraction temperature and filtration on recovery

3.3.1. Temperature

In the first experiment, the influence of the extraction temperature was investigated by performing the extractions at room temperature and in a water bath at 80 °C, for 15 min. Recoveries were tested at the highest spike concentration (50 µg kg^{-1}). The results showed that the recovery seems to be slightly higher at the extraction temperature of 80 °C for paraquat only (Table 1).

In the second experiment, the effect on recoveries (spike concentration of 50 µg kg^{-1}) of cooling down or not cooling down the extract to room temperature before centrifugation was evaluated. The results are shown in Table 1. Differences in recovery are negligible for paraquat, but diquat recovery improved after cooling down the extract to room temperature.
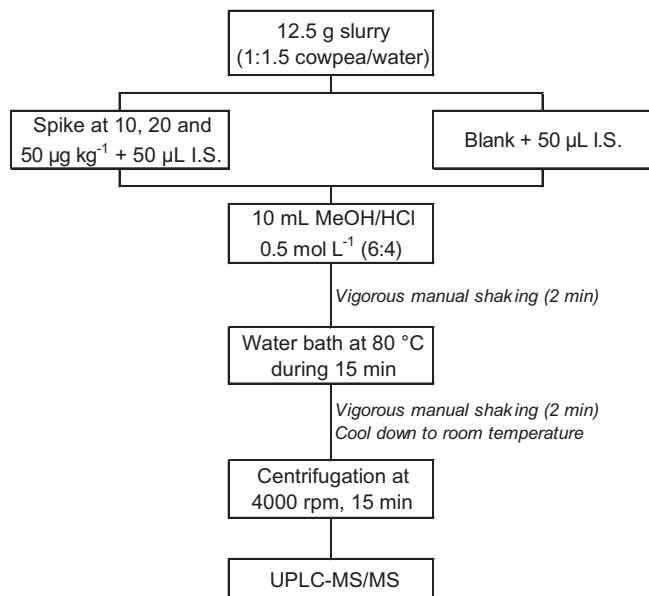
3.3.2. Filtration

The effect on recoveries of omitting or using a filtration step after centrifugation, before injecting the final extract into the UPLC-MS/MS system, was tested for the 3 spike concentrations (10, 20 and 50 µg kg^{-1}).

The results in Table 1 show that filtration did not significantly change the recovery value for diquat. Without filtering, the

Table 1
Recoveries obtained from extraction of cowpea samples, spiked with diquat and paraquat using different compositions of extraction solvent (MeOH/HCl), and the ones obtained from filtration and temperature study.

Studied parameter	Studied condition	Diquat						Paraquat					
		10 µg kg^{-1}		20 µg kg^{-1}		50 µg kg^{-1}		10 µg kg^{-1}		20 µg kg^{-1}		50 µg kg^{-1}	
		Rec (%)	RSD (%)	Rec (%)	RSD (%)	Rec (%)	RSD (%)	Rec (%)	RSD (%)	Rec (%)	RSD (%)	Rec (%)	RSD (%)
Extraction solvent	Methanol/HCl, 0.1 mol L^{-1} (1:1)	90	2.5	86	8.5	91	12.6	0	–	4	2.4	7	7.9
	Methanol/HCl, 0.5 mol L^{-1} (1:1)	104	6.9	99	10.6	93	6.7	268	22.9	100	27.9	182	12.4
	Methanol/HCl, 0.5 mol L^{-1} (6:4)	94	2.1	94	1.7	90	2.4	134	21.4	103	19.7	135	17.4
	Methanol/HCl, 0.5 mol L^{-1} (7:3)	79	9.1	84	4.5	89	12.2	11	30.2	50	15.6	36	16.8
	Methanol/HCl, 0.5 mol L^{-1} (8:2)	83	4.7	94	5.9	83	14.5	23	14.4	24	17.3	96	26.9
	Methanol/HCl, 0.5 mol L^{-1} (9:1)	84	2.2	90	9.8	92	11.1	0	–	0	–	17	14.1
Filtration	Without filtration	95	5.8	94	8.7	90	4.6	134	15.4	103	20.1	135	12.8
	With filtration	97	2.7	91	4.9	94	9.9	76	19.7	52	17.4	53	18.1
Temperature	Room temperature	–	–	–	–	64	10.6	–	–	–	–	39	15.7
	At 80 °C	–	–	–	–	66	5.4	–	–	–	–	43	12.8
	Without extract cooling	–	–	–	–	60	2.9	–	–	–	–	41	13.0
	With extract cooling	–	–	–	–	73	3.1	–	–	–	–	42	11.5

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┌─────────────────────────────────┐
│      12.5 g slurry              │
│  (1:1.5 cowpea/water)           │
└─────────────────────────────────┘
         │
   ┌─────┴──────────────────────┐
   │                            │
┌──────────────────────┐  ┌──────────────────────┐
│  Spike at 10, 20 and │  │                      │
│ 50 µg kg⁻¹ + 50 µL I.S.│  │  Blank + 50 µL I.S.  │
└──────────────────────┘  └──────────────────────┘
   │                            │
   └─────┬──────────────────────┘
         │
┌─────────────────────────────────┐
│    10 mL MeOH/HCl               │
│    0.5 mol L⁻¹ (6:4)            │
└─────────────────────────────────┘
         │  Vigorous manual shaking (2 min)
┌─────────────────────────────────┐
│   Water bath at 80 °C           │
│   during 15 min                 │
└─────────────────────────────────┘
         │  Vigorous manual shaking (2 min)
         │  Cool down to room temperature
┌─────────────────────────────────┐
│   Centrifugation at             │
│   4000 rpm, 15 min              │
└─────────────────────────────────┘
         │
┌─────────────────────────────────┐
│        UPLC-MS/MS               │
└─────────────────────────────────┘
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I.S. = Internal standard (diquat D4 and paraquat D6) at 40 mg L⁻¹.

Fig. 1. Validated, optimized procedure for the determination of paraquat and diquat in cowpea, applying UPLC-MS/MS.

recoveries percentages at the three spike concentrations are close to 100%. For paraquat inconsistent results were obtained.

3.4. Validation

The final extraction method, after all optimization steps, as shown schematically in Fig. 1, has been validated. The expanded measurement uncertainty (U) for diquat is ±0.002 and for paraquat is ±0.003.

3.4.1. Linearity of analytical curves

The linearity of the analytical curve of each herbicide was established by plotting the detector response area ratio versus the concentration of the analytical solutions. Both analytes showed

linear behavior in the studied concentration range of $1–100$ ng mL^{-1}. The determination coefficient (r^2) was 0.9675 for diquat and 0.9753 for paraquat for analytical solutions prepared in blank matrix extract. For standards solutions prepared in methanol, values of $r^2 = 0.9995$ for diquat and $r^2 = 0.9620$ for paraquat were obtained.

The range of mean (n = 7) residuals for the individual pesticide concentrations, from 1 to 100 ng mL^{-1}, was $−1.2$ to 0.9% and $−16.7$ to 3.2%, for diquat and paraquat in solvent, respectively. For the standard solutions prepared in blank matrix extract, the range of mean (n = 7) residuals was 1.2–2.1% and $−26.9$ to 2.8%, for diquat and paraquat, respectively. Only the standard solution of paraquat in blank matrix extract at 2 ng mL^{-1} showed a mean residual of $−26.9$%. Otherwise, the range would be from $−16.7$ to 2.8%.

#### 3.4.2. Detection and quantification limits (LOD and LOQ)

The instrument LOD was estimated as the lowest concentration of herbicide injected that yielded a repeatability RSD of <33%. This appeared to be the 1 ng mL^{-1} concentration for both diquat and paraquat. From this, an estimated, rounded instrument and method LOQ of 3 ng mL^{-1} and 5 µg kg^{-1}, respectively, could be calculated. Thus, a lowest spike concentration and target method LOQ of 10 µg kg^{-1} should be realistic.

3.4.3. Matrix effect for cowpeas

Matrix components, especially from a difficult matrix as cowpea, can reduce the detector response of the analytes due to ion suppression, that can occur when LC-MS/MS is used. This effect originates in the interface (ion source) when the matrix components co-elute with the target analytes and compete in the ionization process, thereby causing target analyte ion suppression and a reduced analyte signal (Cassiano, Barreiro, Martins, Oliveira, & Cass, 2009; Kebarle & Tang, 1993). The sample treatment procedure was designed, aiming at minimizing the potential matrix effects. The matrix effect was evaluated by comparing the slopes of analytical curves obtained from matrix-matched standards and from standards in organic solvent (Fig. 2). Diquat and paraquat both showed a negative matrix effect of $−37$% and $−85$%, respectively. Because these matrix effects were very significant, quantification of the pesticides was performed with matrix-matched
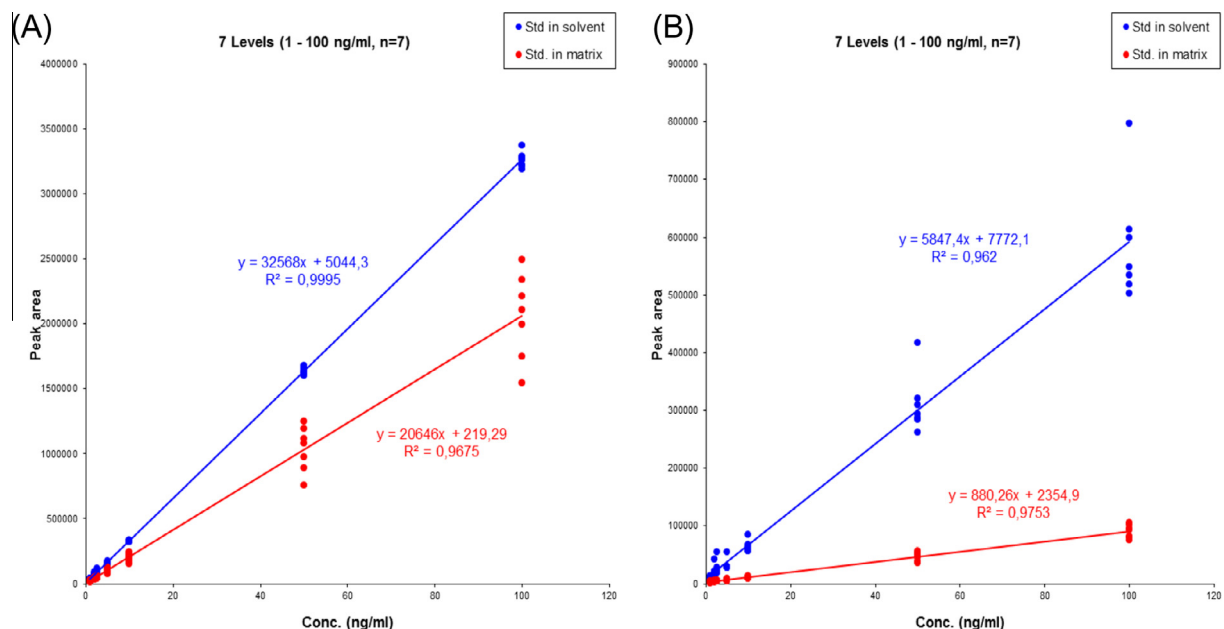
Fig. 2. Analytical curves showing the matrix effect by comparing the slopes of the curves obtained from standards in solvent and those in matrix blank extract; (A) Diquat and (B) Paraquat.
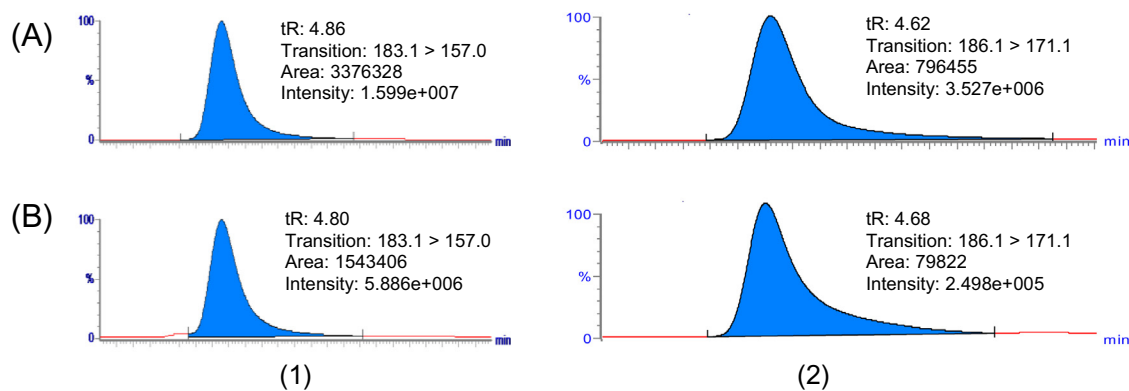
Fig. 3. Reconstructed ion chromatograms for the quantification transitions of (1) Diquat and (2) Paraquat, from standards (at 100 ng mL^{-1}) prepared in (A) solvent (methanol) and (B) blank matrix extract. Responses are normalized to 100%; absolute responses given as peak areas and intensities in the figures.

Table 2
Average recoveries and RSDs obtained after extraction of cowpea spiked with paraquat and diquat, at three different levels using the original and the optimized method.

Method	Pesticide	Spike level ($\mu g\ kg^{-1}$)					
		10		20		50	
		Rec (%)	RSD (%)	Rec (%)	RSD (%)	Rec (%)	RSD (%)
Optimized method	Diquat	77	1.9	78	1.1	85	1.4
	Paraquat	103	25.4	82	14.4	68	20.2
EURL-method	Diquat	90	5.9	86	10.6	91	14.8
	Paraquat	0	2.0	4	20.7	7	22.8

calibration, besides the use of isotopically labeled internal standards. Fig. 3 shows the reconstructed ion chromatograms of the quantification transitions for diquat and paraquat, showing the reduction of signal intensity when these compounds were analyzed in matrix extract compared to standards in solvent (methanol).

3.4.4. Accuracy (trueness and precision) and selectivity

The optimization of the extraction method for determination of paraquat and diquat in cowpea finally resulted in satisfactory recovery data, as can be seen in Table 2. The average recoveries for diquat ranged from 77% to 85% for the 3 spike concentrations (10, 20 and 50 $\mu g\ kg^{-1}$), with a very good precision (RSDs < 2%). Paraquat proved to be a very difficult pesticide to analyze in the also difficult cowpea matrix. At the 3 spike levels studied significantly different average recoveries were obtained of 103, 82 and 68%, respectively. Also the RSDs were much higher than for diquat, although still ⩽20%, except for the lowest spike concentration. Thus the data are in agreement with the quantitative method performance criteria of document No. SANCO/12571/2013 (SANCO, 2013), with an average recovery of 70–120% and a within-laboratory repeatability $RSD_r \leqslant 20\%$. Therefore, the developed method can be considered sufficiently accurate and precise, and fit for purpose.

The validated method LOQs are 10 $\mu g\ kg^{-1}$ for diquat and 20 $\mu g\ kg^{-1}$ for paraquat. These values are below the maximum residue limits (MRLs) according to EU (0.05 and 0.02 mg kg^{-1} for diquat and paraquat, respectively) and ANVISA (0.5 and 0.05 mg kg^{-1} for diquat and paraquat, respectively).

3.4.5. Method comparison

In order to show the improvement of the developed and optimized method relative to the EURL method, a comparison of both was executed as recoveries at the three different spike concentrations. Table 2 demonstrates that the optimized method has similar recoveries for diquat, but is much more efficient for paraquat at all three spike concentrations for the cowpea matrix studied.

3.5. Determination of diquat and paraquat in cowpea samples from a field study (EMBRAPA)

The optimized method was applied to analyze cowpea samples from a field study. The samples originated from a study performed by EMBRAPA, evaluating the effectiveness of different desiccant herbicides, as to the active ingredient, formulation and implementation period in the cultivation of cowpea. The optimized and validated procedure was applied to 15 samples, which were analyzed in triplicate by UPLC-MS/MS. The results for the cowpea samples are presented in Table 3.

Results for samples from Piauí state (Teresina city) showed that for both diquat and paraquat the residue concentration is ∼10 to 15 times higher for the samples from application of the herbicides at the stage of 50% dry pods compared to the samples from application at the stage of 75% dry pods.

For the samples from Mato Grosso do Sul state (Dourados city) with sample numbers 6–10, no residues could be detected above the method LOQ for both diquat and paraquat. Samples 12–15 from Mato Grosso state (Sinop city) showed detectable residues (>LOQ) in the range of 0.02–0.06 and 0.01–0.02 mg kg^{-1} for paraquat and diquat, respectively. The concentrations for the samples from application of the herbicides at the stage of 50% dry pods were slightly higher compared to the samples from application at the stage of 75% dry pods. The average residues for paraquat taken at the stage of 50% dry pods were slightly exceeding the Brazilian MRL (0.05 mg kg^{-1}). In none of the samples analyzed, the Brazilian MRL for diquat (0.5 mg kg^{-1}) was exceeded.

Differences in residue concentrations between the states are correlated to weather conditions during the cowpea growing. Periods of water stress for plants or high temperatures can accelerate the process of drying the pods or decrease absorption rates of

Table 3
Concentrations of paraquat and diquat detected in cowpea samples from the EMBRAPA field study.

Sample number	Origin	Field treatment	Replicate number	Diquat (mg kg^{-1})	Paraquat (mg kg^{-1})
1	Piauí state (Teresina City)	Blank sample	1	nd	nd
2		Paraquat applied at stage of 50% dry pods	1	nd	0.267
			2	nd	0.261
			3	nd	0.240
3		Paraquat applied at stage of 75% dry pods	1	nd	0.020
			2	nd	<LOQ
			3	nd	<LOQ
4		Diquat applied at stage of 50% dry pods	1	0.313	nd
			2	0.321	nd
			3	0.318	nd
5		Diquat applied at stage of 75% dry pods	1	0.022	nd
			2	0.021	nd
			3	0.020	nd
6	Mato Grosso do Sul state (Dourados City)	Blank sample	1	nd	nd
7		Paraquat applied at stage of 50% dry pods	1	nd	<LOQ
			2	nd	<LOQ
			3	nd	<LOQ
8		Paraquat applied at stage of 75% dry pods	1	nd	<LOQ
			2	nd	<LOQ
			3	nd	<LOQ
9		Diquat applied at stage of 50% dry pods	1	<LOQ	nd
			2	<LOQ	nd
			3	<LOQ	nd
10		Diquat applied at stage of 75% dry pods	1	<LOQ	nd
			2	<LOQ	nd
			3	<LOQ	nd
11	Mato Grosso state (Sinop City)	Blank sample	1	nd	nd
12		Paraquat applied at stage of 50% dry pods	1	nd	0.055
			2	nd	0.050
			3	nd	0.049
13		Paraquat applied at stage of 75% dry pods	1	nd	0.023
			2	nd	0.025
			3	nd	0.022
14		Diquat applied at stage of 50% dry pods	1	0.017	nd
			2	0.018	nd
			3	0.017	nd
15		Diquat applied at stage of 75% dry pods	1	0.014	nd
			2	0.014	nd
			3	0.013	nd

nd: not detected.
LOQ$_m$ for Diquat/Paraquat: 0.01/0.02 mg kg^{-1}.

herbicides, resulting in residue accumulation. In general, considering the results of samples from different regions, a lower residue level effect of both herbicides can be observed, when desiccant herbicide applications were performed in a more advanced stage of cowpea growth. Samples 3, 5, 13 and 15, corresponding to the treatments at the stage of 75% of dry pods illustrate this. The effect can be explained by the lower absorption of herbicides in the dry pods and lower translocation to the grains.

From the field study, it can be concluded that the optimum stage of applying the herbicides paraquat or diquat and harvesting the cowpeas is when the pods have a water content of 50% or less, which results in residue concentrations below the MRL. Residue monitoring before and/or at harvest time is thus highly recommended. The method developed and validated in our study has proven to be fit for purpose of residue monitoring in cowpeas.

4. Conclusions

The optimized procedure for diquat and paraquat extraction from cowpea samples involves extraction with methanol/HCl 0.5 M (6:4) at 80 °C for 15 min. The analyses were performed by UPLC-MS/MS using isotopically labeled analogues of the target pesticides as internal standards, in order to effectively eliminate errors caused due to losses during sample preparation or to compensate for matrix effect in UPLC-MS/MS. The validation studies,

using cowpea spiked with diquat and paraquat at three concentrations, revealed that the optimized method is robust and suitable for cost effective routine analysis of these herbicides, achieving good recoveries for all spike levels tested. Good recoveries (within the acceptable range of 70–120%), good precision (with RSDs < 20%) were achieved during method validation. The low method LOQs of 0.01 and 0.02 mg kg^{-1} for diquat and paraquat, respectively, illustrate the good sensitivity for this difficult matrix of cowpeas. The method was successfully applied in a survey to analyze samples from a field study performed by EMBRAPA. Positive residue findings were observed in cowpea samples, emphasizing the need for residue monitoring at the relevant stages of growing.

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