

## Special Article - Herbicides

# Optimization and Validation of an Analytical Method for Determination of Herbicides Residues in Elephant Grass

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## Abstract

Elephant grass (*Pennisetum purpureum* Schum) is a traditional forage used to feed dairy herds and due to the ability to produce high biomass, it has been also used for biofuel production. The productivity and quality of the forage are directly affected by weed interference and the application of the herbicides is one of the strategies used to control. As it is used for animal feed, there is a need to assess the presence of herbicide residues in order to avoid contamination in the production of food derived from livestock. Thus, this study aims to optimize, validate and apply a reliable multi-residue method for the determination of herbicides (atrazine, chlorimuron-ethyl, halosulfuron-methyl, metsulfuron-methyl, nicosulfuron, and metolachlor) in elephant grass by LC-QTOF/MS. Most of the compounds studied presented satisfactory recoveries within the range 70.6 and 106.6 %, and intraday and interday precision less than 15% for all spiked levels, except metsulfuron-methyl in the lowest spiking level (0.01mg Kg<sup>-1</sup>) which resulted in recovery below 70%. However, according to SANTE/11813/2017 guideline, recoveries in wider range are accepted (i.e., 30-140%) when the RSDs are consistent and (lower than 20%). Thus, this method is important to fulfill the demand for analytical methods for quantifying herbicides residues in elephant grass, contributing to research on selective weed management in this crop.

**Keywords:** Pesticides; Residues; Sulfonylurea; QuEChERS; *Pennisetum purpureum* Schum, QTOF/MS

## Introduction

Herbicides play a key role in weed control in agriculture activities. They are extensively used for ensuring the productivity of agriculture given the high demand for food and biofuels [1,2]. Herbicides have different modes and sites of action. Sulfonylurea herbicides (amino acid synthesis inhibitors group) belong to the family of acetolactate synthase inhibitor herbicide. Triazine (photosynthesis inhibitors group) is an inhibitor of photosynthesis, with blocking of electron transport in photosystem II [3]. Chloroacetamide (seedling shoot growth inhibitors group) is an inhibitor of long-chain fatty acid. Although vital for agriculture, inappropriate use of agrochemicals can result in a potential threat to the environment, and non-target organisms [4]. Besides that, people can also be exposed indirectly to the herbicides through residues on food [5,6].

Elephant grass (*Pennisetum purpureum* Schum.), a member of the Poaceae family, is a perennial, rhizomatous grass native to tropical Africa [7,8]. It is mainly used as an animal feed [9]. Elephant grass can produce high biomass yield, especially in tropical countries such as Brazil. Due to its high bioenergy production, research has been directed to using elephant grass biomass for biofuel production [7]. Though, weed interference is a major factor limiting elephant grass production [7,10].

As it is used for animal feed, there is a need to assess the presence of herbicide residues to avoid contamination in the production of food derived from livestock, such as milk and beef.

Thus, it is necessary to develop analytical methods for the

determination and quantification of herbicides residues in elephant grass. The analysis of samples of elephant grass is important to control herbicides residues in food and feed products and to contribute to researches about chemical control of weed management in this crop.

Sample preparation is one critical step of an analytical procedure. Optimized sample preparation is necessary to reduce the time taken and additional steps that can potentially be a source of error [11]. Besides that, the pretreatment of the sample can improve the quantitative analysis and reduce the number of matrix components (co-extractives) that are introduced into the analytical system [12].

The original method, QuEChERS (Quick, Easy, Cheap, Robust and Safe) was developed in 2003, and it was based on acetonitrile extraction/partitioning, and clean-up step by dispersive solid-phase extraction (d-SPE) for determination of pesticide residues in fruits and vegetables [13]. Nowadays QuEChERS approach has been used in the determination of contaminants in innumerable matrices [14-16]. Several modifications have been introduced in QuEChERS method for pesticide analyses. Acidified QuEChERS (EN 15662 procedure), uses the acetonitrile containing 1% formic acid instead of pure acetonitrile, and no citrate buffer salts are used [17,18]. Accepted recovery values (70.2-119.8) of sulfonylurea herbicides (ethoxysulfuron, halosulfuron-methyl, mesosulfuron-methyl, and orthosulfamuron) in rice, maize, wheat, and soybean were obtained by Ni et al., (2018) in acid conditions (acetonitrile 1% formic acid). Clean up step effectiveness by dispersive-Solid-Phase Extraction (d-SPE) depends on the type of matrix, the nature of target compounds, and the amounts and types of used sorbents [4].

Primary-Secondary Amine (PSA) is the most commonly used sorbent because of its chelating ability. This sorbent is used to remove many polar compounds, including fatty acids, organic acids, and sugars [19-21]. Graphitized Carbon Black (GCB) and florisil are used to remove pigments from sample extracts, such as chlorophyll, a problematic interference in pesticide analysis. All adsorbents have their advantages and disadvantages, therefore the selection of sorbents and their amounts used in the clean up step are challenges of the sample preparation [22].

The main goal of the present study was to optimize, to validate, and to apply a reliable multi-residue method of herbicides in grass elephant (*Pennisetum purpureum* Schum) by LC-QTOF/MS determination. Acidified QuEChERS sample preparation approach was evaluable to extract the atrazine, chlorimuron-ethyl, halosulfuron-methyl, metsulfuron-methyl, nicosulfuron, and metolachlor from elephant grass samples. The clean up step was optimized for satisfactory recoveries criteria of target herbicides in the lowest spiking level ( $10\text{mg Kg}^{-1}$ ). The optimized method was validated and was applied to samples from field experiment, made for choosing the most efficient and selective herbicide, and also to provide a basis for monitoring these herbicides in grass elephant pastures.

## Methods and Materials

### Herbicides

As elephant grass is considered a crop (pasture) and there are no herbicides registered for selective weed control in this crop. In this study were evaluable herbicides from three chemical classes, triazine (atrazine), sulfonyleurea (chlorimuron-ethyl, halosulfuron-methyl, metsulfuron-methyl, nicosulfuron) and cloroacetylilnide (metolachlor) for potential selective weed control in elephant grass pastures. The analytical standards of pesticides were purchased from Dr. Ehrenstorfer (Germany), ChemService and Accustandard (USA) with purity greater than 98%. Individual stock solutions ( $1000\mu\text{g mL}^{-1}$ ) of each herbicide were prepared in Methanol (HPLC grade) and stored at  $-18^\circ\text{C}$ . The working standard containing all herbicides ( $10\mu\text{g mL}^{-1}$ , methanol) was used to obtain the analytical curve at concentration set between  $0.01$  and  $0.3\mu\text{g mL}^{-1}$  in the mobile phase.

### Reagents

The reagents used in the QuEChERS extraction method were Anhydrous magnesium sulphate, sodium chloride, supplied by Sigma-Aldrich Company Ltd. (St. Louis, MO, USA). The reagents used in the clean-up step were Primary Secondary Amine (PSA)-bonded silica supplied by Agilent (USA), Octadecyl (C18) and Graphitized Carbon Black (GCB) purchased from Sigma-Aldrich Company Ltd. (St. Louis, MO, USA), florisil<sup>®</sup> (synthetic amorphous magnesium silicate, Merck Millipore). Fresh ultra-pure water was produced by a Milli-QElix-Rios/Advantage A10 system (Millipore, Molsheim, France). Methanol (MeOH) and Acetonitrile (ACN) were obtained from Tedia (Fairfield, USA). All solvents were of HPLC grade, and all reagents were of analytical grade.

### Preparation of standards

Individual stock solutions ( $1000\mu\text{g mL}^{-1}$ ) of each herbicide were prepared in Methanol (HPLC grade) and stored at  $-18^\circ\text{C}$ . The working standard containing all herbicides ( $10\mu\text{g mL}^{-1}$ , methanol) was used to obtain the analytical curve at concentration set between  $0.01$  and

$0.3\mu\text{g mL}^{-1}$  in the mobile phase.

### Instrumentation

The identification and quantitation of herbicides were carried out using a UPLC-Q-TOF system comprising an Acquity UPLC system coupled to a hybrid Quadrupole orthogonal Time-of-Flight (Q-TOF) mass spectrometer (SYNAPT HDMS Q-TOF mass spectrometer) with Electrospray Source Ionization (ESI) in positive mode (Waters Corp., Milford, MA, USA). Data acquisition employed Mass Lynx software (version 4.1). The analytes separation was performed in a BEH C18 ( $100\text{mm}\times, 2.1\text{mm}, 1.7\mu\text{m}$ ) analytical column (Waters), flow rate  $0.15\text{mL min}^{-1}$ , injection with a run time of 15 min and injection volume of  $10\mu\text{L}$ . The chromatographic separation was performed at  $40^\circ\text{C}$  and gradient elution mode. The initial gradient was 50 % B (Methanol) and 50% A (formic acid  $0.1\%$ ), maintained for 1 minute, increased to 90% (B) in 10 minutes, maintained for another minute, and then returned to initial conditions. The optimized parameters established for the ESI-QTOF/MS system were positive ionization mode, capillary voltage:  $3\text{kV}$ , detector voltage:  $2000\text{kV}$ , sample cone voltage:  $18.0\text{V}$ , extraction cone voltage:  $4.0\text{V}$ , source temperature:  $100^\circ\text{C}$ , desolvation gas temperature:  $400^\circ\text{C}$ , nitrogen gas flow in the cone:  $20\text{L}\cdot\text{h}^{-1}$ , and desolvation flow:  $500\text{L}\cdot\text{h}^{-1}$ . The herbicides were quantified by monitoring the signal related to the protonated molecular ion  $m/z$  ( $M+H^+$ ) or ion  $m/z$  ( $M+Na^+$ ). The herbicides were confirmed by the accurate mass of the protonated molecular ion, as well as by the consideration of fragment ions in order to obtain the Identification Points (IPs) according to Commission Decision 2002/657/EC [23].

### Sample preparation

Elephant grass samples were homogenized in a Robot CoupeBlixer 3. Initially, 5g of sample was added to a polypropylene tube (50mL) and 15mL of acidified acetonitrile (1% formic acid) were added for the extraction of the analytes. The mixture was shaken vigorously by vortexing for 1 min to assist extraction. Then, 4g  $\text{MgSO}_4$  and 1g NaCl were added for partitioning and were shaken (2 min). Then the mixture was centrifuged at  $10,000\text{rpm}$ , for 10 min, at  $10^\circ\text{C}$  to separate the organic phase from the aqueous phase and the sample solids. The clean-up step was performed by dispersive solid-phase extraction (d-SPE) using 7mL of supernatant from the extraction stage and 750 mg of  $\text{MgSO}_4$ , 150mg of PSA, 50mg C18, and 25mg GCB. The extract was shaken (2 min) and centrifuged at  $10,000\text{ rpm}$ , for 10 min, at  $10^\circ\text{C}$ . A 5.0mL aliquot of the supernatant was pipetted and transferred to another tube, and the solvent was completely evaporated under a nitrogen stream and reconstituted in 1.0mL of the mobile phase (MeOH :  $0.1\%$  aqueous formic acid, 50:50 v/v). The resulting extracts were filtered through a syringe membrane filter ( $0.22\mu\text{m}$ -PVDF) directly into the vial and injected in the LC-QTOF/MS system.

### Method validation

The blank samples of elephant grass (*Pennisetum purpureum* Schum) with no detectable analyte concentration were used for the development and validation of the analytical method. The Validation procedure has been conducted to determine, selectivity, linearity, limits of Quantification (LOQs), accuracy (recovery of the pesticides), intraday precision (repeatability, RSDr), interday precision (intermediate precision, RSDR) based on parameters and criteria established in SANTE 11813/2017 document [24] and National

**Table 1:** Recovery efficiency for clean up procedures using Florisil® and CGB adsorbents.

Herbicides	Recovery Efficiency (%) Florisil® (n=5)		Recovery Efficiency (%) CGB (n=5)	
	0.02mg Kg <sup>-1</sup>	0.1mg Kg <sup>-1</sup>	0.02mg Kg <sup>-1</sup>	0.1mg Kg <sup>-1</sup>
Atrazine	95.0-100.5	83.9-118.2	102.0-110.1	88.7-93.8
Chlorimuron-ethyl	101.9-107.7	82.0-109.4	110.4-99.6	95.4-106.6
Halosulfuron-methyl	76.8-92.1	69.1-97.3	71.1-95.4	73.4-85.2
Metolachlor	92.5-103.2	82.4-110.9	85.5-102	83.391.6
Metsulfuron-methyl	93.1-128.4	87.9-124.8	56.4-90.0	74.2-85.9
Nicosulfuron	No quantified	93.3-120.9	78.3-93.6	65.8-79.8

**Table 2:** Elemental composition, retention time and the m/z experimental ions for the studied analytes.

Compound	Molecular Formula	Retention Time (min)	Monoisotopic mass (Da)	m/z experimental
				[M+H] <sup>+</sup>
Atrazine	C <sub>8</sub> H <sub>14</sub> ClN <sub>5</sub>	3.25	215.093781	216.1016
Chlorimuron-ethyl	C <sub>15</sub> H <sub>15</sub> ClN <sub>4</sub> O <sub>6</sub> S	4.39	414.04007	415.0479
				437.0299
Halosulfuron-methyl	C <sub>13</sub> H <sub>15</sub> ClN <sub>6</sub> O <sub>7</sub> S	5.34	434.041138	435.049
				457.0309
Metolachlor	C <sub>15</sub> H <sub>22</sub> ClNO <sub>2</sub>	5.29	283.133911	284.1339
				306.1237
Metsulfuron-methyl	C <sub>14</sub> H <sub>15</sub> N <sub>5</sub> O <sub>6</sub> S	2.17	381.07431	382.0281
				404.0641
Nicosulfuron	C <sub>15</sub> H <sub>18</sub> N <sub>6</sub> O <sub>6</sub> S	2.17	410.100861	411.1087
				433.0906

Institute of Metrology, Quality and Technology (INMETRO)/2018 [25]. The selectivity of the method was evaluated by comparing the chromatograms obtained from blank samples (n=10) and the samples spiked with herbicides standard solutions (n=10). The analytical curves were made in matrix-matched using seven different concentrations (0.01-0.3 µg mL<sup>-1</sup>) for each pesticide, which was added to the extracts after the extraction procedure of the elephant grass sample, with three replicates for each concentration point, considering relative standard deviation (RSD%) lower than 20%. Linearity was estimated using linear regression analysis by the least square regression method and the linear ranges were established for each pesticide and the coefficient of determination ( $r^2 > 0.99$ ). The limit of Quantification (LOQ) was established as the lowest concentration that was validated with acceptable accuracy and precision for each pesticide.

The accuracy was determined as percent recovery at five replicates of the three spiking levels (1×, 2×, and 10×LOQ) and was considered adequate those between 70-120%. The intraday precision was obtained with five replicates at three different concentrations (1×, 2×, and 10×LOQ) analyzed on the same day by the same analyst. The intermediate precision was obtained with five replicates at three different concentrations (1×, 2×, and 10×LOQ) analyzed on two different days by the same analyst. For the precision, pesticides with RSD<sub>r</sub> and RSD<sub>R</sub> < 20% were accepted.

## Results and Discussion

In this study, the method Acidified QuEChERS was evaluable

to extract the herbicides from elephant grass samples. This method corresponds to the EN 15662 procedure, using the acetonitrile containing 1% formic acid instead of pure acetonitrile, and the same way, no citrate buffer salts are used. The sulfonylureas herbicides are weak acids, with pKa values in the range 3.4 - 4.6, while metolachlor is a nonionic herbicide, and atrazine is a weak base (pKa=1.7). Acetonitrile containing 1% of formic acid results in pH value around 3. In this acid condition, all target herbicide is in a non-ionized state that improves the extraction efficiency by organic solvent. Accepted recovery values (70.2-119.8) of sulfonylurea herbicides (ethoxysulfuron, halosulfuron-methyl, mesosulfuron-methyl and orthosulfamuron) in rice, maize, wheat, and soybean were obtained by Ni et al., (2018) in acid conditions (acetonitrile 1% formic acid). Different d-SPE sorbents (PSA, C18, florisil<sup>®</sup>, and GCB) and mass were evaluable in the clean-up step to reduce the effect of co-eluting components on the ionization efficiency of the target analytes. The recovery efficiency using matrix-matched calibration curves for target herbicides at the same spiked level was selected for comparison to the effect of d-SPE sorbents. These adsorbents have been generally used in the d-SPE cleanup step for removal polar compounds some sugar, non-polar co-extracts such as lipids and waxes and pigments [22,4,26]. Elephant grass is constituted by water, carbohydrates, proteins, lipids, and pigments (chlorophyll). Chlorophyll is a problematic interference in pesticide analysis because of its nonvolatile characteristics [27]. Recovery efficiency obtained by procedures using florisil<sup>®</sup> (125mg) and GCB (25mg) were compared (Table 1).

**Table 3:** Validation results of the methodology.

Pesticides	LOD (mg Kg <sup>-1</sup> )	LOQ (mg Kg <sup>-1</sup> )	Linearity (R <sup>2</sup> )	Working ranging (mg·mL <sup>-1</sup> )
Atrazine	0.006	0.01	0.998	0.01-0.3
Chlorimuron-ethyl	0.006	0.01	0.996	0.01-0.3
Halosulfuron-methyl	0.006	0.01	0.998	0.01-0.3
Metolachlor	0.006	0.01	0.998	0.01-0.3
Metsulfuron-methyl	0.006	0.02	0.995	0.01-0.3
Nicosulfuron	0.006	0.01	0.997	0.01-0.3

**Table 4:** Accuracy (% recovery), intraday and interday precision.

ValidationParameter	Herbicides					
	Atrazine	Chlorimuron-ethyl	Halosulfuron-methyl	Metolachlor	Metsulfuron-methyl	Nicosulfuron
Intraday precision (CV %)						
0.01 mg Kg <sup>-1</sup>	1.93	8.91	10.57	4.88	4.72	12.9
0.02 mg Kg <sup>-1</sup>	2.68	4	8.5	6.18	19.43	6.8
0.1 mg Kg <sup>-1</sup>	2.7	4.45	4.59	3.46	5.66	13.46
Interdays precision (CV %)						
0.01 mg Kg <sup>-1</sup>	10.55	11.34	14.56	5.86	21.18	27.01
0.02 mg Kg <sup>-1</sup>	9.68	10.66	11.48	14.16	18.89	10.03
0.1 mg Kg <sup>-1</sup>	5.5	11.31	10.66	11.51	5.29	6.2
Accuracy Recovery (n=5) (%)						
0.01 mg Kg <sup>-1</sup>	82.1	82.8	73.9	73.6	46.9	74.6
0.02 mg Kg <sup>-1</sup>	106.6	106.6	85.1	94.5	72.5	86.8
0.1 mg Kg <sup>-1</sup>	90.7	90.7	79.6	86.7	78	70.6

In both procedures were used the same amount of PSA and C18 adsorbents. Kaczynski (2017) reported that florisol has not exhibited satisfactory recoveries for polar herbicides in onion, wheat, and pea [22]. However, GCB could adsorb polar and planar pesticides and induce a hydrophobic interaction with the aromatic structures of some pesticides, the amount used in this study results in a good performance for all of the analytes [28].

### Method validation

In this study, a high-resolution mass spectrometer (SYNAPT HDMS Q-TOF) (resolution $\geq$ 10,000) was used to quantify and to confirm the identity of target herbicides. For the quantitative purpose, the molecular ion was monitored. The identity confirmation was performed according to Commission Decision 2002/657/EC by the system of Identification Points (IPs). The mass accuracy of a high-resolution mass spectrometer acquires 2 IPs for the precursor ion and 2.5 for each transition product, reaching the requirements to confirm their identity. The protonated molecule, the fragmented ion, and retention time for target analytes are showed in Table 2.

The selectivity of the method was evaluated by comparing the chromatograms obtained from blank samples (n=10) and the samples spiked with herbicides standard solutions (n=10). No peaks were observed in chromatograms of the blank sample at the same retention time of the analytical signal of analytes of interest. Table 3 shows the LOD, LOQ, linearity, and working range. The linearity of analytical curves prepared in solvent and matrix-matched curves (0.01-0.3 $\mu$ g mL<sup>-1</sup>) the slope (angular coefficient) was much different. Therefore, matrix-matched standard calibration was employed in

the quantification of herbicides. Accuracy (% recovery), intraday and interday precision of herbicides obtained during the validation method are showed in Table 3.

The most compounds studied presented satisfactory recoveries within the range 70.6 and 106.6, %, and intraday and interday precision less than 15% for all spiked levels. Only the metsulfuron-methyl in the lowest spiking level (0.01 mg kg<sup>-1</sup>) resulted in recovery below the acceptance value (70%). SANTE/11813/2017 guideline allows the application of a wider recovery range (i.e., 30-140 %) when the recovery rate is consistent and RSDs obtained are lower than 20%.

### Applications to field samples

To assess the method developed, the analyses were performed on genuinely contaminated (incurred) elephant grass samples obtained from an experiment conducted at Brazilian Agricultural Research Corporation (Embrapa) experimental fields. This study aimed to evaluate herbicides for selective weed control in elephant grass pastures. This method was used to identify and quantify residues of herbicides after the application of different doses of the commercial formulations of herbicides in the field. A total of 12 samples were analyzed. The residue of atrazine was found in two samples in the concentration of 0.0657 and 0.0509 mg Kg<sup>-1</sup>. The residue of metolachlor (0.0116 mg Kg<sup>-1</sup>) was found in a unique sample. Halosulfuron-methyl was found in two samples at concentrations 0.0321 and 0.0108 mg Kg<sup>-1</sup>. The residues of other herbicides were lower than the LODs. In Brazil, only metsulfuron-methyl has been registered for application in pasture and its Maximum Residue Limit (MRL) established by ANVISA is 0.1mg Kg<sup>-1</sup>.

## Conclusions

A multiresidue method for determination four sulfonyleurea herbicide (chlorimuron-ethyl, halosulfuron-methyl metsulfuron-methyl and nicosulfuron), atrazine, and metolachlor were optimized and validated. The sample preparation process, based on Acidified QuEChERS was suitable to perform the extraction of all herbicides from elephant grass samples. The clean-up step performed using PSA, C18, and GCB provided lesser LOQ value for nicosulfuron than the procedure using florisil instead of GCB. Quantitation by liquid chromatography-quadrupole time-of-flight mass spectrometry (LC-ESI-QTOF/MS) showed to be a selective and low detectability method. The parameters of validation showed that the method is adequate for the analysis of these herbicides residues in elephant grass. This method is important to fulfill the demand for analytical methods for quantification of herbicides in elephant grass samples due to limited research on selective weed management in this crop.

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