








TECHNICAL NOTE

Development of Paraquat Pesticide Determination Methodology in Urine Samples by UHPLC-MS/MS

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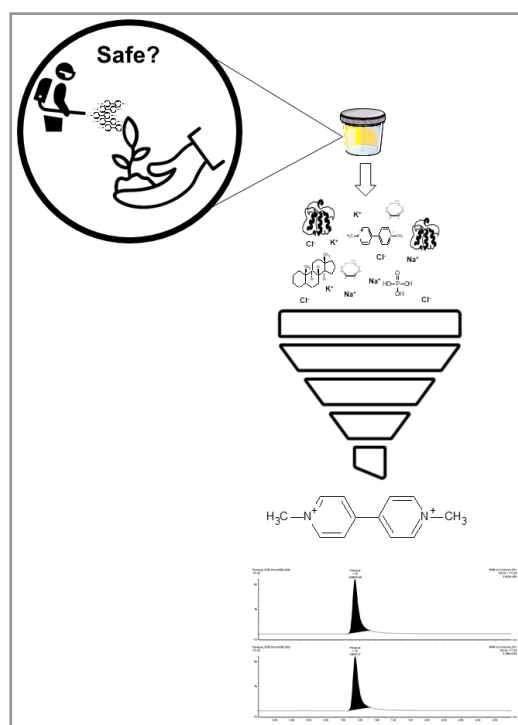
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In technical-scientific development, mankind has developed different ways of managing pests, diseases and weeds, increasing agricultural production impacting social, economic and environmental-aspects. As major grains producer (such as corn, soybeans and beans), the Brazilian State, through the Brazilian agencies as Institute for the Environment and Renewable Natural Resources (IBAMA), the National Health Surveillance Agency (ANVISA), and the Ministry of Agriculture, Livestock and Supply (MAPA) authorize and regulate the use of different pesticides, including Paraquat, to improve and guarantee the production. However, the direct contact and long-term exposure to these substances offer environmental and occupational risks, impacting negatively on the workers, population of neighbor cultures, and environmental health. Considering the potential damages, it is of utmost importance to develop effective analytical methodologies for the biomonitoring and assessment of the level of exposure of farmers and ranchers in direct contact with pesticides. Thus, this work proposed to develop a sensitive and selective analytical method, using the ultra-high efficiency liquid chromatography technique, coupled to mass spectrometry

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seeking a fast and simple sample preparation for the Paraquat quantification in urine samples. To achieve this, two sample preparations were compared in terms of speed and practicality. The first method (A) used C18 silica as adsorbent to remove the non-polar interferences. The second preparation (B) consisted in direct sample dilution in acetonitrile. Both methodologies used centrifugation under refrigeration to precipitate suspended artifacts. The linearity of the Paraquat detection by the analytical methodology, developed in a HILIC column, was evaluated between 10 to 70 $\mu\text{g L}^{-1}$ ($r^2=0.9911$; $y=19427x+479868$), enabling the matrix evaluation after applying the best sample preparation. The method developed was simple, fast, which makes it useful and efficient for toxicology laboratories routine to monitor the exposure levels of farmers dealing with Paraquat daily.

Keywords: Paraquat, farmers, biomonitoring, urine, UPLC-MS/MS.

INTRODUCTION

It is known that the use of pesticides of different chemical classes, such as herbicides, insecticides and fungicides, aims to increase in productivity and to deal with the life forms that directly affect agricultural production [1,2].

Brazil is recognized by the large-scale agricultural production also in the use of pesticides, and this using is increasing year by year. If the large-scale use of pesticides favors the development of agriculture, on the other hand, its indiscriminate or irregular use, can lead to problems: in the environment, soil, water, food, human and animal health. In addition, rural workers who have direct and chronic contact with the paraquat pesticide, may suffer serious damage to health, this kind of substance can influence the human body functioning, since this substance has a wide distribution in the tissues [3–5].

The Paraquat (PQ) is an herbicide, widely used in crops as a desiccator in soybean plantations, and the poisoning is a very serious situation that unfortunately almost all physicians in the countryside have already encountered. The poor prognosis, with mortality of almost 100% for ingestion above 40/45 mg kg^{-1} , is associated with the lack of reliable therapeutic protocols [6].

Paraquat, 1,1'-dimethyl-4,4'-dipyridyl cation (Figure 1), has low, but rapid gastrointestinal absorption (5-10%), plasma peak concentrations appear in less than 2 h after ingestion. It is transported to all major organs, especially to the lung, where it is reduced to form highly reactive free radicals, and is slowly excreted unchanged in urine and faeces [6–8].

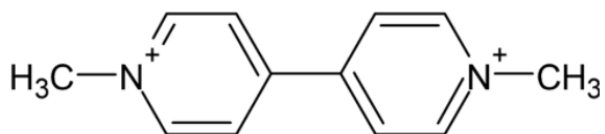


Figure 1. Structural formula of paraquat ion pesticide.

Studies indicate that chronic exposure to the PQ promotes an imbalance in the antioxidant system and behavioral changes. This exposure is also considered a risk factor to neurodegenerative diseases development, such as Parkinson's disease. Up until now, it was observed that PQ acts on dopaminergic neurons inducing the state of oxidative stress through its redox cycle on an adenine nicotinamide dinucleotide (NADPH) and also on mitochondria, inducing mitochondrial dysfunction and death of neurons [6,9–11].

Determining the level of PQ in the urine is a useful way to measure the degree of absorption for evaluating the exposure. It is suspected that direct and daily contact with this pesticide may compromise the farmer's health by affecting the kidneys, lungs and liver (due to the reactive oxygen species). Given the severity of the chronic exposure consequences, biomonitoring is necessary to evaluate the exposure levels of rural workers, who have greater chances of poisoning by these agents due to chronic and/or acute risk [12,13].

This exposure follow-up can be made by evaluating levels of PQ in urine, and different analytical methodologies are used: radioimmunoassay, colorimetric methods, gas chromatography coupled with mass spectrometry detection (CG-MS), capillary electrophoresis coupled with mass spectrometry detection (CE-MS), and high efficiency liquid chromatography coupled with mass spectrometry (HPLC-MS) [7,12–14].

For the urine preparation to chromatographic analysis, different strategies can be adopted: the use of acid or base, followed or not by centrifugation, use of an ion exchange or C18 cartridge, direct dilution, and other methodologies that make possible the matrix effect dealing. As Paraquat is insoluble in organic solvents, and hydrophilic, the most common extraction method is SPE with weak cation exchange SPE cartridges. In these methodologies ion-pairing reagents are used, such as heptafluorobutyric acids (HFBA) and trifluoroacetic acids (TFA), used to promote the separation, which can retain analyte on LC columns used in some HPLC–MS methods. But the presence of these ions can suppress the PQ signal decreasing the MS detection [15–19].

These limitations associated with high costs or many steps always reinforce the need for methods of sample preparation for investigation. Thus, to enrich the sample preparation alternatives and to help in the diagnosis of rural workers exposed to Paraquat, this paper proposed to develop a sensitive and rapid methodology for the PQ quantification in human urine samples by ultra-high efficiency liquid chromatography technique coupled with mass spectrometry (UHPLC-MS/MS), with fast and simple sample preparation.

MATERIALS AND METHODS

Materials

In this project development the ultra-performance liquid chromatography (UPLC) equipment used was in the following configuration: automatic sampler, coupled to a triple quadrupole detector (MS/MS) (Waters® Acquity Class I). The data were analyzed and processed using Mass Lynx software version 4.2. Chromatographic columns tested: ACQUITY UPLC BEH C18 12.1 x 30 mm, 1.7 µm; Agilent Poroshell UPLC BEH C8 12.1 x 30 mm, 1.7 µm; the Acquity type UPLC BEH HILIC 2.1 x 50 mm, 1.7 µm.

For the sample preparation a centrifuge (NT 805, Nova Técnica, Brazil), sample concentrator (LV Concentration TurboVap® Workstation, Biotage), water purification system (EPOD IQ 7003 - Merck Millipore®) were used. Sample solutions and 99.9% HPLC acetonitrile mobile phase (Sigma Aldrich®); Ammonium acetate (Merck®), Formic Acid (Merck®), Paraquat standard 99.9% (Sigma Aldrich®), Modified C18 silica adsorbent (Merck®).

Standard solution

The standard paraquat mother solution, as well as other dilutions for the linearity test was performed in methanol, with ultrasound bath homogenization.

Urine sample preparation

The urine standard sample was prepared with a known paraquat concentration. To avoid analytical signal suppression and loss of sensitivity of detection of the analyte, two methods of sample preparation were evaluated A and B, Figure 2.

The A method consisted of adding 100 µL of sample and 500 µL of methanol, followed by the addition of a modified C18 silica spatula tip. The system was shaken for 1 minute in a vortex mixer, and centrifuged at 9000 rpm for 10 minutes under refrigeration, then the supernatant was transferred to the injection vial.

The B preparation consisted of diluting 100 µL of urine in 900 µL of methanol. The flask was homogenized for 1 minute in a vortex mixer and centrifuged at 9000 rpm for 10 minutes under refrigeration, then the supernatant was analyzed.

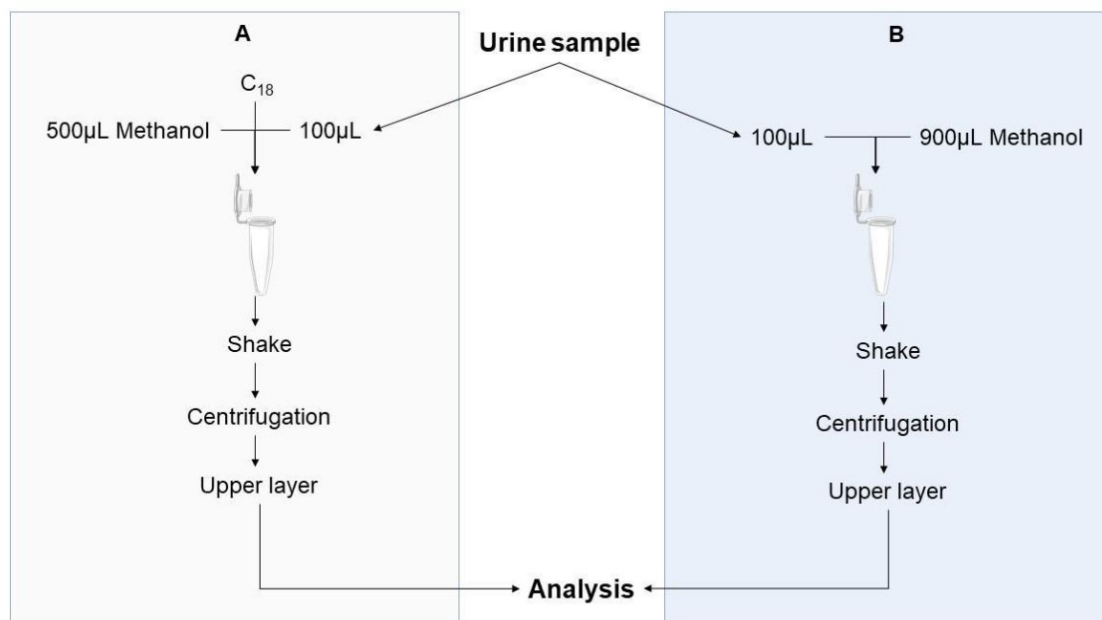


Figure 2. Sample preparation strategies: A with C18 adsorbent addition, and B only the sample and solvent.

Chromatographic parameters evaluated by UPLC-MS / MS

To develop the chromatographic system, the following parameters were evaluated: signal intensity, peak symmetry, retention time, peak resolution, and sensitivity. In this study C-8, C-18, and HILIC stationary phases were tested. In the mobile phase selection, as organic phase methanol and acetonitrile were tested; and as aqueous phase, buffer solutions containing formic acid, acetic acid and the ammonium acetate, ammonium phosphate and ammonium formate salts were tested. After comparing the developed methods, the one that best favored the sensitivity (improving the Paraquat ionization), symmetry and the analyte detection was chosen.

Method evaluation

The analytical methodology evaluation was based on some criteria of the Brazilian National Health Surveillance Agency (ANVISA by Resolution No. 27, of May 17, 2012, for bioanalytical methods validation, and National Institute of Metrology, Standardization and Instrumental Quality (INMETRO). The evaluated parameters were selectivity, linearity and matrix effect.

Specificity / Selectivity

Selectivity is the property of a procedure of providing measured values for one or more substances, so that the values of each measure are independent of each other. For this, each solution was injected independently, with visual assessment.

Linearity

Linearity is the ability of an analytical method to produce results that are directly proportional to the concentration of the analyte, in a given concentration range. The linearity was evaluated in three replicates at concentrations of 10, 20, 30, 40, 50, 60, and 70 µg L⁻¹. One of the mathematical models used to assess linearity is to describe this dependency and the adjustment of this equation by the method of ordinary least squares. The equation of the line that relates the regression parameters a and b is given by:

$$y = ax + b$$

where y = Peak area; x = Concentration; a = Slope of the calibration curve (sensitivity) and b = Intersection with the y axis, when x = 0.

The determination coefficient (r^2) is often used to indicate how adequate the straight line can be considered as the model obtained. The working range of a method is the interval between the lower and upper levels of analyte concentration where it was possible to be demonstrated the required precision, accuracy and linearity, under the conditions specified in rehearsal [20,21].

Matrix Effect

The matrix effect is a selectivity study that aims to ascertain possible interferences caused by the substances that make up the sample matrix, basically generating phenomena of decrease or enlargement of the instrumental signal. This parameter was evaluated comparing two standard curves with and without urine. Then, the two curves were plotted and compared, also variance values were compared by F test with 0.05 significance.

RESULTS AND DISCUSSION

After a sequence of tests, the stationary phase that best met the requirements was the column with separation based on hydrophilic interaction liquid chromatography (HILIC), since the paraquat molecule (1,1'-dimethyl-4,4'-bipyridinium) is an ionized bicyclic system with two protonated nitrogen with a high-water solubility. Hilic separation showed a good efficiency in different kinds of polar molecules, showing adequate retention that provides the analyte separation from the matrix. The results evaluation leads to the analytical method adopted in this work: 5 mM ammonium formate, in 0.3% formic acid (mobile phase A), and acetonitrile (mobile phase B), to facilitate system stabilization the isocratic elution mode at 60% of FM B was chosen, at a flow rate of 0.3 mL min^{-1} , column oven at $35 \text{ }^\circ\text{C}$ and samples kept at $10 \text{ }^\circ\text{C}$ [14,22,23].

The mass spectrometer condition that best favored the detection of the analyte and the diagnostic ions were: capillary 0.5 kV, 13 V cone, dissolution, temperature $550 \text{ }^\circ\text{C}$, at a gas flow of 100 L h^{-1} . The ions of m/z 186, and their fragments m/z 171 and m/z 77.9 were monitored in the MSⁿ mode.

A fragile point of analytical quantification is the sample preparation: a crucial step, and more susceptible to errors. Pre-analytical errors compromise the reliability of the results, so the simpler and more efficient this step, the better. In the sample preparation, an extraction was tested with the modified silica C-18, added directly to the microcentrifuge. After chromatographic analysis (Figure 3), it was not possible to observe the presence of the analyte.

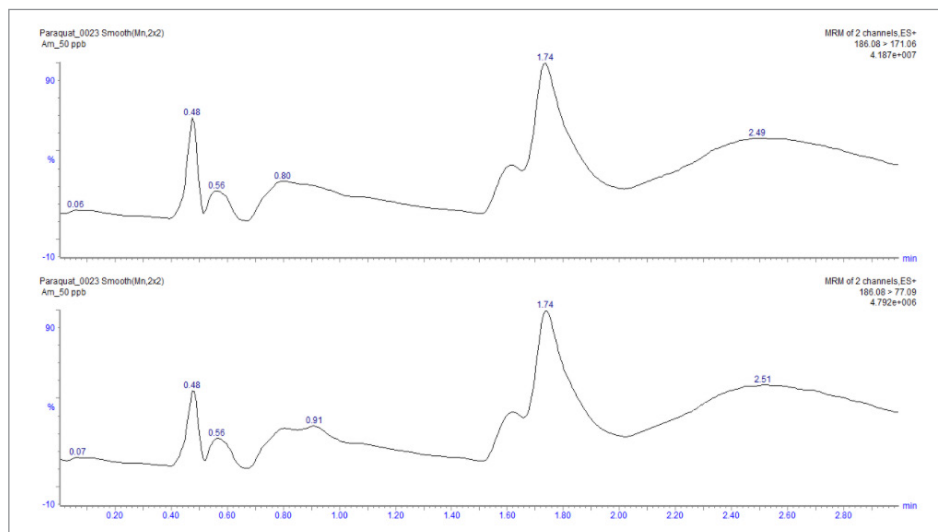


Figure 3. Chromatogram resulting from sample preparation with C-18.

The choice of sorbent C18 in sample preparation was based on reviews in the literature. In environmental analysis, particularly in the isolation and pre-concentration of pesticides in water, non-selective hydrophobic sorbents, such as C18, are the most used. These phases can differentiate and isolate the analyte of interest from the interfering compounds present in the sample, and it is common to remove non-polar interfering compounds in the matrix before chromatographic analysis. According to Carvalho et al. (2008) the most used sorbents in SPE for the extraction of pesticides in aqueous matrices are: C18, used in extraction of organochlorine insecticidal herbicides and in methods with different particle classes. Many studies used for fungicide and pesticide determinations already dissipate between 70 to 110% demonstrating the suitability of the C18 sorbent for these compounds, which makes it a good option in the extraction of interferers, but it was not applicable to this work [17,24–26].

In view of the obtained results, and still envisioning simplicity in this preparation step, it was decided to test the direct dilution of the sample and later centrifugation. The methanol has the capacity of precipitating urine protein content, and the chromatogram represented by Figure 4 shows the presence of the interested analyte. For evaluation of this preparation method, the recovery was calculated revealing almost 100% [18].

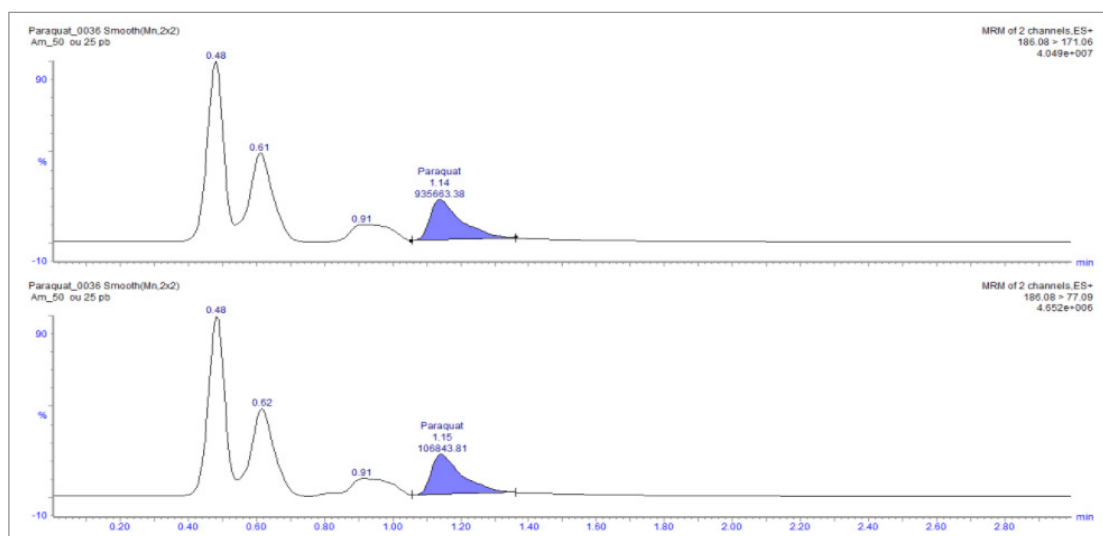
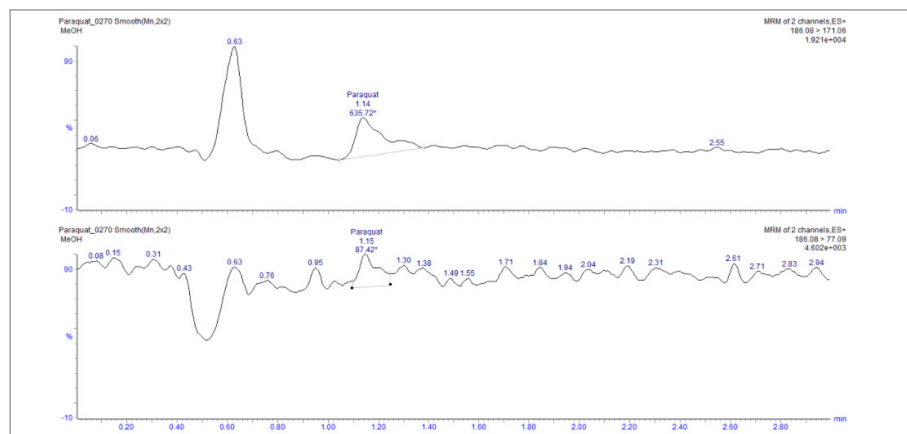


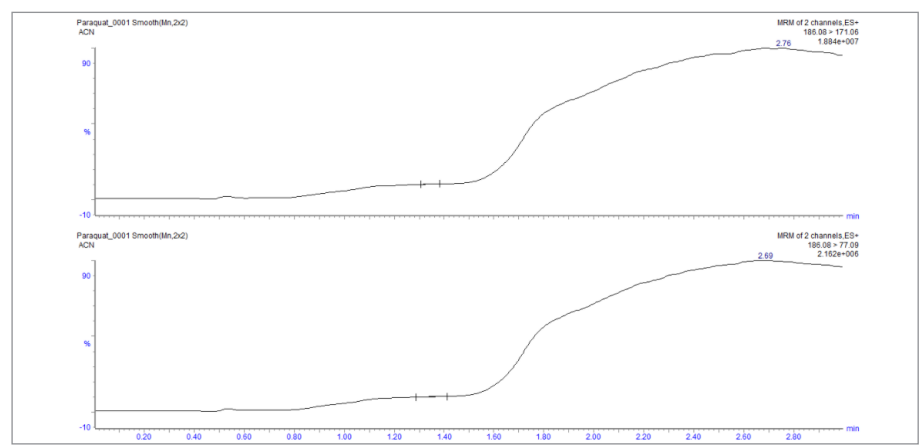
Figure 4. Chromatogram of the sample preparation by centrifugation.

Selectivity was determined by comparing the chromatogram, after the injection of the diluent (methanol), mobile phases A and B, urine without the analyte, and urine with the analyte (illustrated by Figure 5). Note the absence of signs of the pesticide ions studied in the retention time of 1.14 minutes.

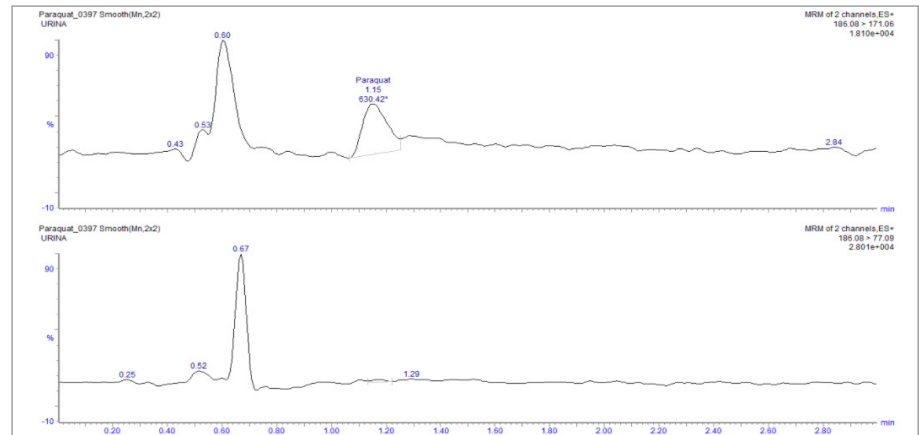


(A)

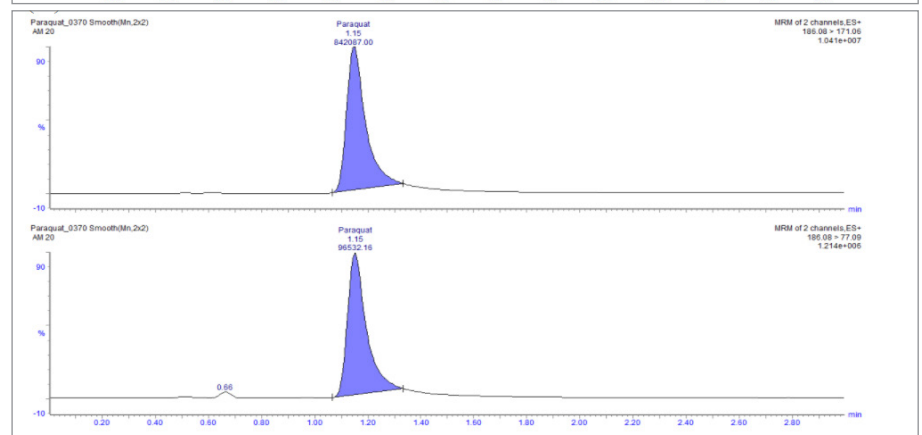
Figure 5. (A) Chromatograms of the diluent; (B) mobile phase; (C) Urine without the analyte (D) urine with the standard (E) analyte in diluent.



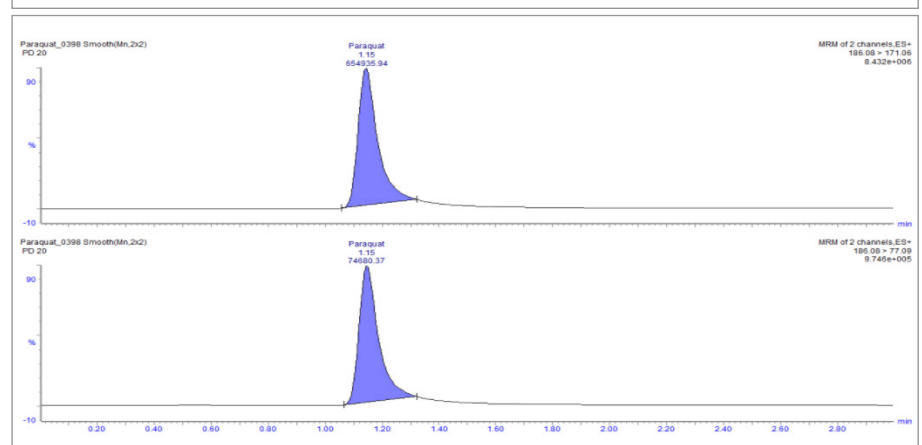
(B)



(C)



(D)



(E)

Figure 5 continuation. (A) Chromatograms of the diluent; (B) mobile phase; (C) Urine without the analyte (D) urine with the standard (E) analyte in diluent.

The Figure 6 (A) shows the calibration curve of the analyte studied in the diluent (methanol). It is observed that the method developed was linear with a determination coefficient of 0.99, thus confirming the linearity of the method. In the standardized residuals analysis (Figure 6 B), it reveals that the data obtained shows independence and randomness.

The matrix effect is an important analysis to be performed, specially to attest the sample preparation, the possible sources of potential errors in the chromatographic response need to be verified. In addition, the matrix effect allows it to generate recoveries above 100% of the studied analyte, leading to mistakes and still determine possible changes during the ionization process such as ion suppression, low response of the studied analyte, and other interferences [10].

The analysis of this effect through the mass spectrometer showed that there were no interferences that influenced the analysis of paraquat in urine samples. For this, an analytical curve was performed on the matrix (urine) (Figure 6A). The analysis interval was linear, obtaining a coefficient of determination of 0.99. Furthermore, in the standardized residual analysis (Figure 6C), it also showed that the data obtained were independence and randomness. Comparing the curves obtained in the matrix and in the solvent, it was possible to perform the F test ($\alpha = 0.05$), revealing that the sample preparation minimized the effect of the matrix in the studied analytical range.

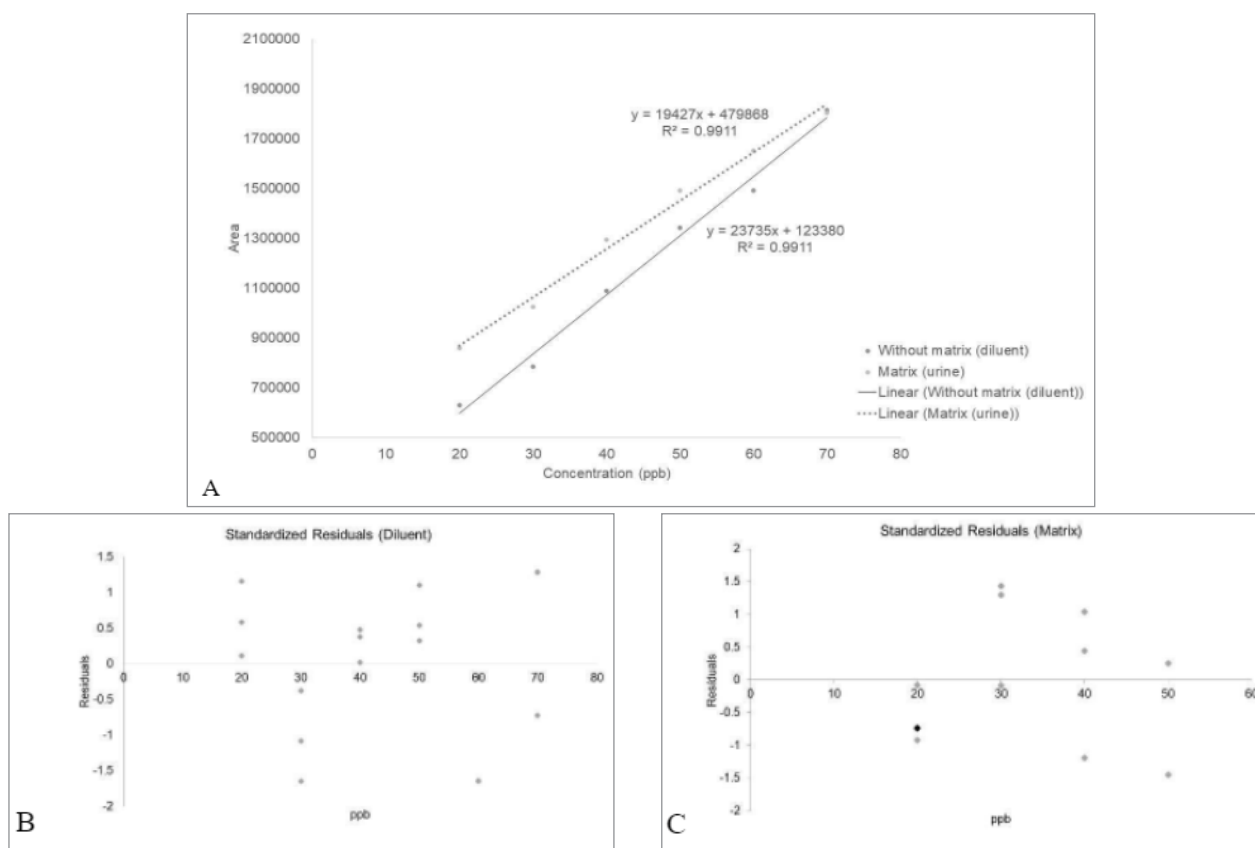


Figure 6. (A) Paraquat analytical curve in urine (matrix), and in the diluent (without matrix). (B) Standardized Residual analysis of paraquat samples on diluent. (C) Residue analysis of paraquat samples in urine (matrix).

CONCLUSIONS

Based on the results obtained, it can be concluded that the direct dilution and centrifugation to prepare the sample makes the paraquat detection possible. This strategy minimized the matrix influence. The chromatographic elution using HILIC column was possible, and it demonstrated to be able of quantifying paraquat in urine samples using UHPLC-MS/MS.

Conflicts of interest

The authors declare no conflict of interest.

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