A SIMPLE METHOD FOR DETERMINATION AND CHARACTERIZATION OF IMIDAZOLINONE HERBICIDE (IMAZAPYR/IMAZAPIC) RESIDUES IN CLEARFIELD® RICE SOIL

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Abstract. A study was conducted to evaluate residues of imidazolinone (IMI) in soil. Samples were taken from three Clearfield® rice fields as IMI which have been used for six years. IMI herbicides (imazapic/imazapyr) were widely used in Clearfield® rice soils. To date, few studies are available on the residues of these herbicides, especially in the context of Malaysian soil. Therefore, for this purpose, high performance liquid chromatography (HPLC) with UV detection was performed using a Zorbax stable bond C_{18} (4.6 × 250 mm, 5 μm) column, with two mobile phases. The average percentage recovery for imazapyr and imazapic varied from 76%-107% and 71-77%, with 0.1-5 μg/ml fortification level, respectively. The limit of detection (LOD) and limit of quantification (LOQ) were found to be 1.05 and 4.09 for imazapic and 0.171 and 0.511 μg/ml for imazapyr respectively, in the top 15 cm. In the extracted soil sample, it was 0.19 μg/ml for imazapic and 0.04 μg/ml for imazapyr, respectively. Based on this study, a pre-harvest period of 40-60 day is suggested for rice crops after IMI application.
Keywords: *recovery, acetonitrile, Zorbax stable bond C_{18}, HPLC, terminal residues, Clearfield® rice*

Introduction

Weedy rice (WR) is a notorious weed associated with rice paddy crops. WR in Malaysia (locally known as Padi Angin) was first observed and reported in 1988 (Watanabe et al., 1996). WR also always acted as dominant competitor in terms of inter- and intra- varietal competitions among rice species (Baki and Shakirin, 2010). The application of pesticides in the agricultural system becomes unavoidable in the present day, as it increases production and decreases yield loss caused by pests (Schreiber et al., 2017). In 2010, the introduction of a new type of imidazolinone herbicide (IMI) known as OnDuty® with its main ingredient being imazapyr/imazapic, was used in Malaysia's agricultural system (Azmi et al., 2011). Because sometimes mixing two or more herbicides into one spray solution can offer producers multiple benefits (Fish et al.,

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2015). This resulted in an overnight success in rice cultivation in terms of yields and WR control. It also helps control a broad spectrum of weeds, encompassing grasses, and cyperaceous and broad-leaved plants, and those that WR are closely associated to (Neto et al., 2017).

IMI inhibits the enzyme function in the plant known as acetohydroxyacid synthase (AHAS) (Bailey and Wilcut, 2003), which starves the plant and lead to its death. Upon the introduction of IMI – resistant cultivated rice (Clearfield®), most difficulties faced by farmers that are related to WR have almost been completely solved. OnDuty® herbicide formulation containing imazapyr is a generic name for [2-(4-isopropyl- 4-methyl-5-oxo-2-imidazoline-2-yl) nicotinic acid]; trade names of Arsenal and Chopper), while imazapic is the generic name of [2-(4,5dihydro-4-methy-4-(1-methylethyl)-5-oxo-1Himidazol-2-yl)5-methyl-3-pyridinecarboxylic acid]; trade names of Cadre and Plateau (Azmi et al., 2012). Both are widely used in WR, and its efficiency has been proven. IMI is relatively persistent in soil, with half-lives ranging from 30 - 150 days (Kemmerich et al., 2015). Shorter half-lives of imazapyr (34–65 days) detected in forest soils (Michael and Neary, 1993).

IMI have two enantiomers (Krieger, 2001) derived from the chiral center, which consists of imazapyr, imazapic, imazaquin, imazamox, imazethapyr and imazamethabenz-methyl, as per *Table 1*. IMI detection and research separation from water and soil are limited compared to other types of herbicides due to the low application rate (100-200g/ha) and co-extraction of other substances that interfere with chromatogram and low rates of application (Ramezani et al., 2009; Andreu and Picó, 2004). However, In the last decade, diverse studies were done on these types of herbicides to assess the risk assessment to the environments as water and soil (Silva et al., 2009; Anastassiades et al., 2003; Andreu and Picó, 2004; Börjesson et al., 2004; Bajrai et al., 2017). Also, the massive use of IMI herbicides might have contributed to the increase in resistant weedy rice, also, in most cases, herbicides-resistant weeds go undetected until they represent about 30% of the total population (Burgos et al., 2014). Many reports showed the imidazolinone carryover affected many non-rice crops in rotational systems (Alister and Kogan, 2005).

Studies on how much of these herbicides run-off into soil and water are important due to their potentially harmful influence on the environment (Schreiber et al., 2017). Developing methods for extraction in water and soil are very important, because some studies revealed that the residues of these herbicides remain present in Swedish soil after 8 hours (Börjesson et al., 2004). Herbicidal residue is defined as the remaining herbicides on or in the soil after its application in agricultural soil. Its persistence in soil sometimes causes injuries in the next crop (Assalin et al., 2014). Most previous studies on this type of herbicides used alkaline/acidified water, methanol, acetonitrile, and diverse techniques for the extraction process (Helling and Doherty, 1995; Ramezani et al., 2009). The extraction of IMI herbicides and determination using HPLC-UV is popular in literature, and it was used by many researchers because it provides clearer and more realistic results (Pace et al., 1999; Helling and Doherty, 1995; Laganà et al., 1998). Generally, herbicidal residues are usually concentrated in the top of 10 cm, although it could leach deeper. These residues are injurious to non-tolerant rotational crops, such as wheat and corn (Alister and Kogan, 2005). Areas such as Tanjung Karang sees intensive use of IMI herbicides by farmers. The rice farmers have been applying IMI herbicides for ~6 years. We present a simple HPLC method that can be used to detect, analyze, and evaluate the residues of the imazapic and imazapyr in soils

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cultivated in Clearfield system. Reversed – phase liquid chromatography (RP-LC) with ultraviolet (UV) detection is widely used because it can separate high or medium polarity pesticides and detect them at low levels, making it one of the most powerful technique in separation methods (Hogendoorn, 2006). The farmers in this area have been applying IMI herbicides repeatedly (Mazlan et al., 2016). Therefore, this study was undertaken in the Clearfield fields to determine residues of IMI in the soil.

Material and methods

Study site description

Sawah Sempadan-Tanjung Karang district is located on (N 3°25'35.0724", E 101°10'36.1704") in Kuala Selangor, Malaysia as shown in (*Fig. 1*). The soils samples were collected on November 2016/2017 from these fields, because the farmers there have been using IMI herbicides since its introduction in Malaysia in 2010 (Azmi et al., 2012). This area is the most prosperous agricultural district in Malaysia, and has many hectares of paddy rice (Mazlan et al., 2016).To determine the final IMI residue, the soil samples were collected at harvest time, which was ~90 days after IMI was sprayed on Clearfield rice crop.

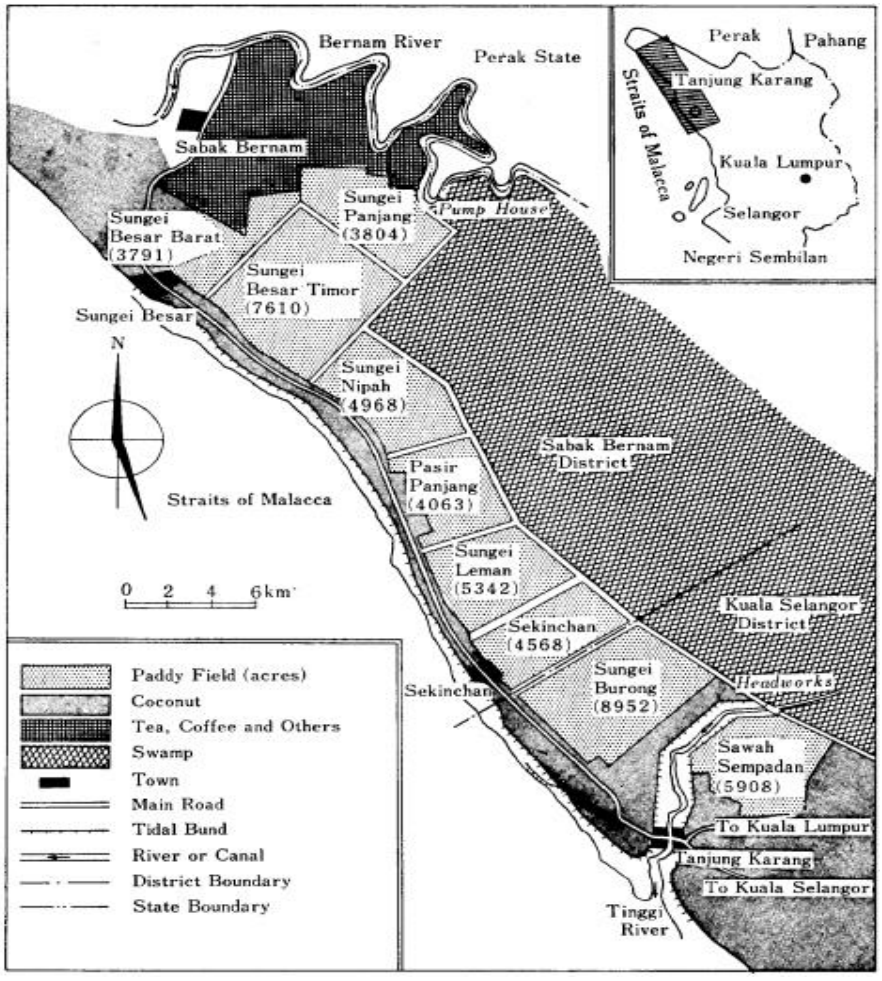

Figure 1. *The site of soils sample from Sawah Sempadan- Tanjung Karang district(Fredericks, 1981)*

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Chemicals, reagents, and apparatus

Standards of imazapyr (99.5% purity) and imazapic (99.9% purity) were purchased from Sigma-Aldrich (USA), while formic acid (85%), methanol 99.9 % (HPLC grade) and acetonitrile 99.9 % (HPLC grade), acetic acid-ACS reagent (Fisher), formic acid-98% (EM Science), Sodium phosphate (Fisher), hydrochloric acid 6N (Fisher), phosphorous acid dichloromethane (DCM) 99.9 % (HPLC grade), and Rotary evaporator were purchased from Sigma Aldrich (Germany). Ultrapure water was obtained from a Milli-Q Direct UV3® system (Millipore, USA), and was further purified by passing it through a 0.2µm Whatman filter paper. The HPLC 1100 series fitted with a UV detector was used. The HPLC column used in this work was a Zorbax RX–C_{18} (4.6 × 250 mm, 5 µm). Its temperature was maintained at 30 °C. Centrifuge-Dupont Sorvall Model RC-5C, centrifuge bottles with cap 45 ml polypropylene (Kontes Scientific), vortex mixer (Labmart 3000), Thermo-ultra-sonic, analytical balances (AUW-220D and UX-420H from Shimadzu, Japan), 0.22 µm nylon filters, glass vials with capacity of 2 mL (Agilent, USA), and screw-capped polypropylene tubes (45 ml, Germany), DSC-18 6 ml tubes 500 mg (6 cm × 3cm) SPE cartridges (supelco), anhydrous sodium sulfate, and a vacuum pump were all used in this work as well.

Stock solution and working standards preparation

Standards stock solutions of the herbicides imazapyr and imazapic were individually prepared in methanol at concentrations of (100 µg/ml), respectively, from (1000 µg/ml). Different fresh diluted solutions were prepared as 0.1, 0.5, 1, 5, 10, and 20 µg /ml, and diluted in methanol. All stock and working solutions were stored at -18 ºC in the dark (Marcia, 2014). Then, each of these solutions was injected (17 µL) into the HPLC system, at 251 nm, and peak areas were recorded and plotted versus the concentration of the herbicides.

Soil collection and preparation

The sample preparation process which involves extracting the analyte is very important and crucial. To determine the herbicidal residues from the soil samples, the samples were taken systemically from a randomly chosen area from three Clearfield® rice fields that were exposed to the herbicides. The basic approach is to analyze the depth intervals of the soils samples for each field. Each sample was within 0 - 20 cm and 20 – 40 cm, about 30-m distance between each two samples was taken with the helical shape method. A 20 soil samples were taken, and ~500-gram (gm) soil samples were collected using special auger for collection of the soil samples for increased control, and were stored in sterile zip lock polyethylene bags and coded with special code water proof stickers.

Three random samples were selected (two from each field), then one sample was selected randomly for examination, while the rest were stored in a refrigerator at a suitable temperature for subsequent analyses. The samples were air-dried in the special room at 35 C° for up to 5 days, grounded with a mortar and electronic machine, sieved through stainless steel sieve (2.0 mm) and stored at 4 °C. A100 g of homogenized soil samples was stored in polyethylene bag at a temperature of ~15 C° until it was analyzed for herbicidal residues. The soil physico-chemical characteristics were analyzed and the basic properties of these soils are shown in (*Table 1*).

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Table 1. *soil texture characteristics of three locations soil*

Location	Depth (cm)	PH	Moisture%	Sand%	Silt%	Clay%	OM%	Soil type
Field A	0-20	6.21	38	39	29	30	2	Clay loamy
	20-40	6.7	33					
Field B	0-20	6.81	44	24.6	35.7	39.2	1.3	Clay loamy
	20-40	6.61	57					
Field C	0-20	7.1	38	25	35	38	1.9	Clay loamy
	20-40	6.94	59					

Soil extraction procedure for IMI Residue level

Analyses of the samples of soil were carried out using the modified extracted published methods proposed by (Ramezani et al., 2009; Krynitsky et al., 1999). About 5 ±0.001 g of a randomly homogenized soil sample was weighted, which provides appropriate and representative amount as some authors have used (Martins et al., 2014). The portion soil was placed in 250 ml centrifuge tube (polypropylene), 150 ml of extracted 0.5 N NaOH. Then sample was kept 45 minutes in an end-over-end shaker at 30°C to assess the homogeneity of the sample. A 10 ml methanol was added to precipitate humic acids and sonicated for 10 minutes, then centrifuge the sample for 10 minutes at 7000 rpm to remove particulates.

The sample solution was filtered and adjusted to pH 2 by addition of 6N hydrochloric acid. Clean-up is necessary to shift down the detection limits of methods and to avoid interferences from the matrix. The suspension was left at room temperature for 10 minutes until analysis, then transferred into a 500 ml separatory funnel and extracted with two 50 ml portions of dichloromethane (DCM). The extracts were combined and the DCM was dried using anhydrous Na_2SO_4, then passed through smooth activated charcoal. The resulting solution was then transferred into a 250-ml round bottom flask and solvent was evaporated at 65 °C using a rotary evaporator at a low speed to near dryness. The residue was diluted with about 2 ml of a 1:1 solution of methanol:0.1% formic acid, then loaded (under vacuum) into the 6 ml DSC-18 (Supelco) solid phase extraction cartridge containing 500 mg of polymeric of adsorbing material conditioned with 3ml of each of the solvents methanol, acetonitrile, and H_2O.

The vacuum was slowly reduced and the analytes were washed with 9 ml H_2O and 6 ml (60:40) (H_2O: acetonitrile). Finally, the vials were placed in the vacuum apparatus and the cartridge eluted with 3 ml of the methanol:0.1% formic acid solution. The resulting extract was filtered through a 0.22μm polytetrafluoroethylene (PTFE) membrane, transferred to a 1.5 ml HPLC auto sampler vial, and stored at 4°C until separation by HPLC.

Method application

In this study, 20 samples were taken from the same three sites of Sawah Sempadan-Tanjung Karang district, and different chemical compositions and pH values were analyzed.

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Accuracy (%Recovery), limit of detection (LOD), and limit of Quantitation (LOQ)

LOD is the lowest concentration that can be detected, and it could be determined by a statistical method. This could be achieved by measuring the more dilute concentrations of analyte. These concentrations are expected to produce a response of ~3 times the background noise. LOD should be between 3 - 10. The LOQ is expected to behave similarly, but with a ratio of 10 times the background noise. Recovery studies in soils samples were conducted using the standard calibration curves equation.

These herbicides were spiked to blank soils (clean soils free from herbicides), taken from the land around University Malaya (N 3°7'8.9328" E101°39'28.494"). This soil was selected due to its similar characteristics with the tested soil samples. Acetone was added to 5 g of dried homogenized soil at different concentrations, and left to dry for 48h at room temperature to activate the introgression and equilibrium while slowly evaporating the solvent (Rebelo et al., 2016; Laganà et al., 2000), followed by extraction and analysis using HPLC-UV.

Results and discussion

Contamination of environmental resources by herbicides is an increasing environmental concern. Undoubtedly, soil plays a significant role in an agro-ecosystem, but information for analysis of these types of herbicide residues in the soil can be very difficult to achieve. HPLC with UV detection was chosen due to it being a fast and effective separation method. This study involves trying different columns and mobile phases for the HPLC technique. Finally, in this method a proper separation was achieved using the gradient mobile phase and C_{18} column (4.6 × 250 mm, 5 μm) was used for stationary phase separation.

Purified water was used as one of the mobile phases, due to its low cost, lack of toxicity to the environment (Laganà et al., 2000). The mobile phase acetonitrile (100%), as one of mobile phase, is the best mobile phase (Martins et al., 2014; Demoliner et al., 2010), along with purified water acidified with 10% acetic acid (pH to 2.8), due to the pH's effect on the peak shape (Singh, 2013).

Therefore, acetonitrile was chosen due to it is great solubility and higher elution strength than dichloromethane for fractionating the analytes. Acetonitrile is the best choice for the mobile phase (Singh, 2013). However, analysis was carried out using gradient solvent program using mobile phase A (acetonitrile (100%)) and mobile B (purified water acidified with 10% acetic acid (pH adjusted to 2.8). The initial gradient program was 35% A, maintained for a minute, then increased to 45% for 3 min, then decreased to 35% at 8 mins. The column temperature was set to 30C°. The flow rate was 1 ml/min, injection volume was set to 17 μL, and UV detection was set to a wavelength of 251 nm.

Simultaneously, methanol was evaporated before the sample is injected into the HPLC apparatus. Standard curve linearity and calibration was determined at six concentrations (0.1, 0.5, 1, 5, 10, and 20 μg /ml), and were prepared in the laboratory by diluting the stock solution and plotting the analytes' concentration against peak area. Each level of the concentration was analyzed repeatedly. The equation of analytical calibration was obtained by plotting the peak areas on y-axis and the concentration on the x-axis within the previous calibration levels for both imazapic and imazapyr. The concentration of both herbicides was calculated by comparing the peak values in the calibration, using the regression equation. The linearity of the method was determined from the correlation coefficient, as per *Fig. 2*.

The matrix effect has been mentioned in literature and is explained via multiple perspectives, with some reporting a shift of over 10% on the analytical results (Kemmerich et

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al., 2015). However, some that are less than 20% does not affect the matrix (Ferrer et al., 2011). The chemical analysis of these herbicides in soil are often problematic due to the low detection limits required and the pH adjustment during the extraction process. IMI is a weak acid, as per (*Table 2*), therefore their presence in soil is influenced by pH (Schreiber et al., 2017). Soil particles were fine-grinded to increase the interaction between the solvents and soil particles, which lead to increased herbicides extraction.

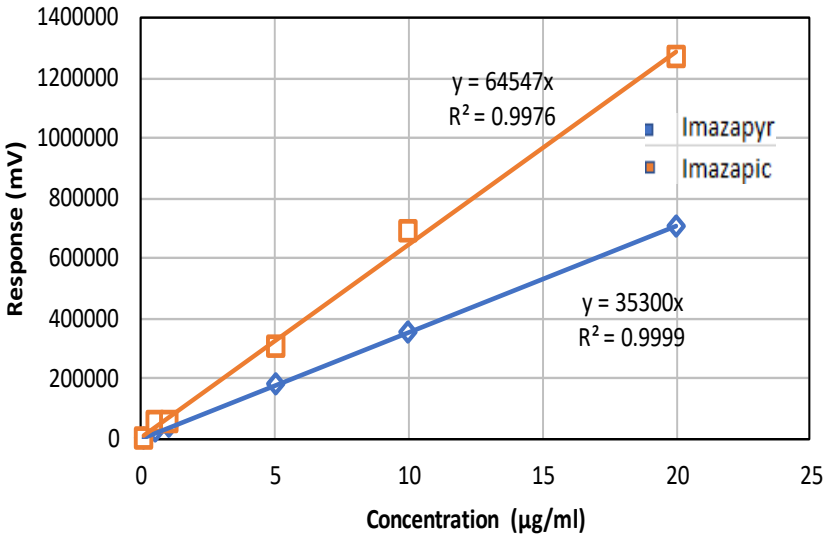
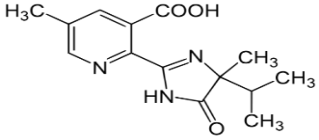
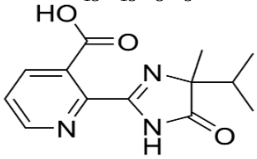


Figure 2. *Representative calibration curve for IMI was obtained by the determination of six levels in duplicate at ranged from 0.1-20µg/ml*

Table 2. *The characteristics (Molecular and physicochemical) of imazapic and imazapyr*

Name	[a] **Imazapic**	[a] **Imazapyr**
Family/chemical class	Imidazolinone	Imidazolinone
Trade name	Cadre, panoramic, plateou	Arsenal, Chopper, Habitat, Stalker
Chemical name	[2-(4,5dihydro-4-methy-4-(1-methylethyl)-5-oxo-1Himidazol-2-yl) 5-methyl-3-pyridinecarboxylic acid]	[2-(4-isopropyl- 4- methyl-5-oxo-2-imidazoline-2-yl) nicotinic acid]
Molecular weight	275.30308g/mol	261.2765g/mol
Molecular formula	$C_{14}H_{17}N_3O_3$	$C_{13}H_{15}N_3O_3$
Structural formula		
Water solubility	2200mg/L	9740 mg/L
Life time in soil	Around 120days	90-120 days
[b] pKa	2.1, 3.9	1.9, 3.6
[c] Goss	High potential	High potential

[a] Data quoted from (Senseman, 2007, Schreiber et al., 2017).
[b] Indicates the pH value at which 50% of total molecules are associated in soil and 50% of total molecules are dissociated.
[c] Method of classification of potential surface water contamination.

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The traditional types of extractions ordinarily use the chemical compound PSA (primary secondary amine), and due to the fact that the IMI family are present in multiple forms, it acts as a weak acid/base, which allows PSA to hold over acidic herbicides (Marcia, 2014). One of the important effects occurs when the types of herbicides have pK_a values in the range 1.3-3.9 (Krieger, 2001), which includes the weak acid IMI herbicides. Based on this, the shape of the peak area during analysis was expected to be affected by the value of the pH of the mobile phase. Soil pH and the microbial activity are the main factors in the degradation process of IMI herbicides in the soil (Sondhia et al., 2015). For example, when the pH increases, the adsorption and persistence decreases.

Also, another important factor that control the residues' concentration is the depth and type of soil. IMI sorption is correlated and increased with clay content, due to increased binding of the herbicide to soil particles, where (Gianelli et al., 2014). (Burnside et al., 1963) show that some herbicides can leach deep into the soil. For example, some studies revealed that the sorption of these types of IMI as imazapyr to sandy soils is very weak compared to its sorption to clay and humic soils (Lode and Meyer, 1999). The agricultural soils contain numerous impurities and old chemicals, which can persist for a long time, which would cause separation problems in the column, especially if the soil contained only very low concentrations of imazapic or imazapyr. Imazapyr and imazapic have the potential to leach into groundwater due to its persistence and mobility in soils, and very low volatility (Gianelli et al., 2014). Certified imazapic and imazapyr (USA) were used for calibration (*Fig. 3* and *Fig. 4*).

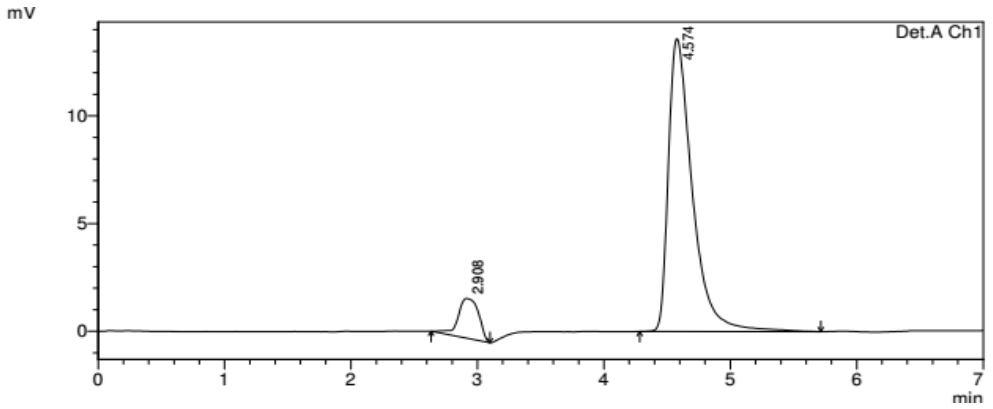

Figure 3. *Imazapyr standard, 10ppm*

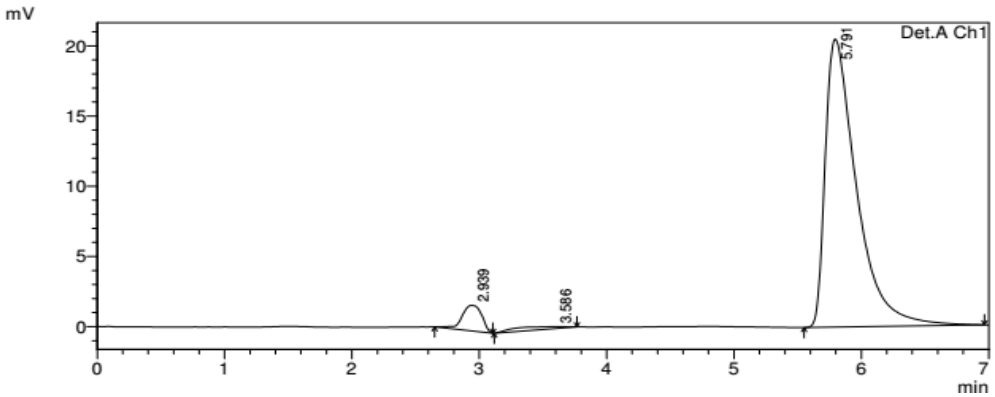

Figure 4. *Imazapic standard, 10ppm*

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The adsorption of IMI herbicides decreases by increasing heavy rain and temperatures. The higher solubility of these types in water, high temperatures, and great rainfall in Malaysia are main factors that play important roles in the transition of residual particles of herbicides via its pores or movement to other places and shift up the degradation mechanism, as per (Grey et al., 2012; Fish et al., 2015). Malaysia has almost daily high intensity rain fall and temperatures. Studies revealed that temperatures between 35C°- 45C° and increased soil moisture enhance both the chemical and microbial degradation for herbicides (Neto et al., 2017). Different methods are applicable for extraction of IMI herbicides from soil samples, but most are not satisfactory (de Oliveira Arias et al., 2014). Despite the fact that imazapyr and imazapic were applied in low doses, both can remain for long periods of time in the soil , which can cause agronomic and environmental problems (Kraemer et al., 2009). However, leaching is influenced by the environment, which means that when the water content decrease from the upper surface, it leads to increased pH. Also, some chemical herbicides move to the upper surface of the soil due to capillary action, which causes it to evaporate (Mangels, 1991).

Selectivity

Selectivity is defined as the evaluation or detection of the analyte from others analytes and different compounds that could be present at the same moment in the matrix or the sample (Ahuja, 1989). There were no matrix peaks in the chromatogram analysis that interfere with analysis of the residues as shown in *Fig. 5* and *Fig. 6*.

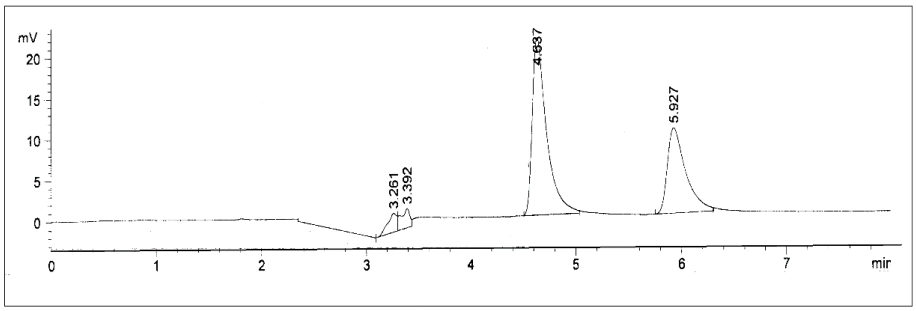

Figure 5. *Extraction imazapic and imazapyr with good resolution*

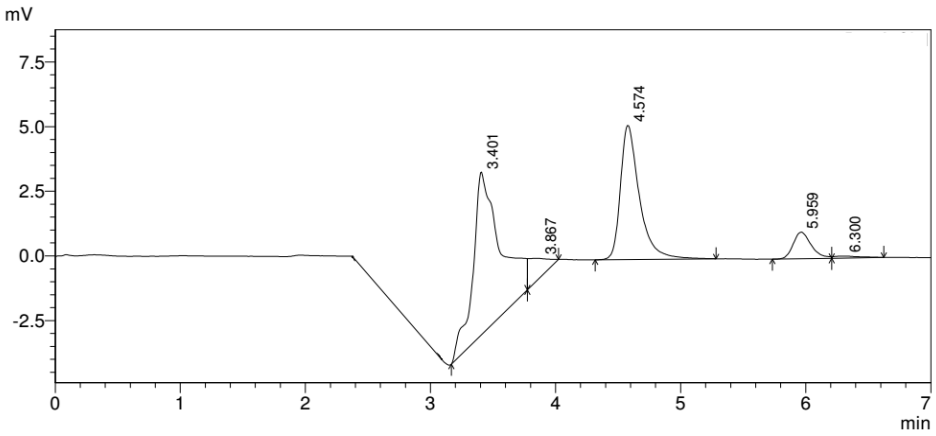

Figure 6. *Extraction imazapic and imazapyr with good resolution*

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Accuracy (%Recovery)-Limit of detection (LOD) and limit of Quantitation (LOQ)

The achieved results revealed an excellent linearity at different concentrations of imazapyr and imazapic standards in the range from 0.1 to 5 µg/mL. These herbicides' concentrations are spiked to blank soils as described in the experimental section.

Due to the spiking of the extracts, the final comparison between the two systems is expected to be valid. The precision and recovery for the two herbicides was calculated through the injection of freshly prepared six standards. The proportion of the area of the peak of herbicide resulting from the spiked solution to the area of the herbicide peak resulting from a standard solution prepared previously was calculated. The average percentage recoveries for imazapyr and for imazapic varied from 76%-107% and 71-79% with 0.1-5 µg/ml fortification level, and 0.1-10 µg/ml at fortification level, respectively, are shown in (*Table 3*). The LOD and LOQ were found to be 1.04 and 3.15 µg/ml for imazapic, and 0.135 and 0.411 µg/ml for imazapyr, respectively, in the top 15 cm. In the extracted soil sample, it was 0.19 µg/ml for imazapic and 0.04 µg/ml for imazapyr. This proves the slow degradation process of these residues in the soils under environmental conditions. The soil samples were taken during rice crop cultivation of about 90 days and the residues are evidently still present.

The Koc for the two herbicides were 137 and 100 ml g^{-1}, respectively, which means low adsorption and high mobility, and eventually high levels of leaching. Nevertheless, both herbicide residues are still present after ~90 days, especially imazapic with 0.2 µg/ml, which was proven by a previous study stating that these types of herbicides are highly persistent (Souza et al., 2016). Simultaneously, persistence of residues in the soil does not necessarily mean that it injures sensitive crops, as persistence differs from bioavailability.

Table 3. *Recovery of imazapic from soil*

Con. (µg/ml)	Recovered peak imazapic	Recovered peak imazapyr	Average % imazapic	Recovery % imazapyr
0.1	2475	3943.66	71.18	107.165
0.5	43513	17263.6	79.19	80.74
5	236358	137529.6	77.27	76.768
10	521185	341438.3	75.75	96.253

Repeatability and stability

The repeatability of this method was determined by calculating the RSD of the peak areas of the six duplicate injections of fortified samples which is < 15. It represents the closeness of the results from similar methods, laboratories, and tools. This is achieved via six concentrations, each replicated trice to a total of eighteen times, encompassing the specified range of the procedure. Accuracy = mean ±SD, for imazapic 75.85 ± 3.4, and for imazapyr, it was 90.232±14.

Conclusions

A simple analytical method based on HPLC-UV was developed and validated to determine the IMI residues in the Clearfield® rice soils. It is necessary to monitor the

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presence of herbicides residues in soils and waters and develop methods for reliable analysis, as important tools of regulatory programs to protect the environment. A gradient of mobile phase A (acetonitrile (100%)) and mobile B (purified water acidified with 10% acetic acid (pH adjusted to 2.8)) yields excellent separation and resolution, in a short analysis time, for the two herbicides (less than 7 min), with retention time for imazapyr and imazapic at ~4.6 and 5.9 min respectively. Excellent linearity in the range of injected standard concentrations with a high degree of precision and accuracy could be achieved. Therefore, the proposed analytical method could be useful for detecting the imidazolinone family in agricultural soil and water in the future. Results of this study suggests the need for an extensive research to determine factors affecting the half-life of these herbicides and their contribution to their persistence. Also, further studies are needed on the laboratory level and plant bioassay to evaluate if these residues can indeed cause injuries to other crops.

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