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A new validated method for determination of tembotrione and its metabolite residues in orange fruit

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ABSTRACT

A simple and inexpensive method was developed using solid-phase extraction, together with high performance liquid chromatographic method with UV detection for determination of tembotrione and its metabolite (tembotrione – dihydroxy (AE1417268)) residues. The evaluated parameters include the extracts by florisil packed column using hexane: ethyl acetate solvent mixture (2:3), methanol and acetonitrile solvents. The method was validated using fruit samples spiked with tembotrione and its metabolite (tembotrione – dihydroxy) at different fortification levels (0.03 and 0.3 µg/g). Average recoveries (using each concentration six replicates) ranged 84-94%, with relative standard deviations less than 2%, calibration solutions concentration in the range 0.03-10.0 µg/mL and limit of detection (LOD) and limit of quantification (LOQ) were 0.01µg/g and 0.03µg/g respectively. Finally the fruit residue samples were analyzed by HPLC.

KEYWORDS

HPLC;
Florisil;
Tembotrione;
Tembotrione–dihydroxy (AE1417268) and Orange fruit.

INTRODUCTION

Tembotrione is a new powerful herbicide developed for the selective post-emergence control of grass and broadleaved weeds in corn, including glyphosate, Als-inhibitor and dicamba resistant weeds[1]. The primary biochemical target site of tembbotrione is the enzyme 4- hydroxyphenyl pyruvate dioxygenase. Tembotrione is moderately to highly mobile and exhibits variable persistence in laboratory soil. Data specifically designed to investigate the extent of these properties demonstrate that it is not expected to leach significantly under field conditions in Canadian soils. Tembotrine is toxic to small mammals, terrestrial plants, fresh water vascular plants, and marine interstates. Tembotrione poses a negligible risk to earthworms, honey bees, birds, fresh water invertebrates, fresh water and marine fish, and fresh water and marine algae. In soil, tembotrione id expected to break down in the presence of oxygen; however, in soils lacking oxygen, tembotrione is expected to persist[2]. New information allowed for the calculation of revised half-lives and characterization of impartment breakdown products. Tembotrione very easily soluble in water. When it enters the aquatic environment, it

tends to settle out of the water column and ends up in the sand or sediment.

This study has been undertaken to develop an improved method for analysis of tembotrione and its metabolite tembotrione – dihydroxy (AE1417268) to determine residue retention in orange fruit.

EXPERIMENTAL

Standards, reagents and samples

The analytical standards of tembotrione (99.5%) and tembotrione – dihydroxy (98.2%) were obtained from Sigma Aldrich. Acetonitrile was purchased from rankem, New Delhi, analytical grade solvents i.e., ethyl acetate, hexane, methanol and florisil sorbet were supplied from Merck Limited and orange fruits were purchased from local market.

Standard stock solutions

The tembotrione and tembotrione–dihydroxy standard stock solutions were individually prepared in acetonitrile at a concentration level 1000 µg/mL and stored in a freezer at -18°C. The stock standard solutions were used for up to 3 months. Suitable concentrations of working standards were prepared from the stock solutions by dilution using acetonitrile, immediately prior to sample preparation.

Sample preparation

Representative 20.0 g portions of orange fruit fortified with 0.1 mL of working standard solutions. The sample was allowed to stand at room temperature for one hour, before it was kept at refrigerator condition, until analysis.

Extraction procedure

Weighed 20g of fruit sample in to a 250ml separatory funnel, added 50 mL of deionized water and shake vigorously. Added 10mL of saturated sodium chloride. Partitioned the aqueous phase with 50ml of n-hexane/ethyl acetate (2: 3 v/v). Discarded the organic layer. Partitioned the aqueous layer with 50 ml of dichloromethane. Collected the dichloromethane layer and evaporated to near dryness using a vacuum rotary evaporator and the contents were re-dissolved in 20mL of methanol.

Clean-up procedure

Prepared a pre-washed florisil chromatographic column by placing a glass wool in the bottom of the column, added 50 ml methanol. Rinsed the sides of the column with solvent methanol and placed a 15g layer of Sodium sulphate over the florisil[3,4]. Again rinsed the sides of the column with methanol and allowed the solvent to drain to the top of the column.

The sample in a small volume of methanol is adsorbed on the top of the column. Elute the column with 50 ml of methanol and then with methanol: water (7:3) and discard both eluates. Eluate the column with 100 mL of acetonitrile and concentrate in a rotary vacuum evaporator to near dryness and and then re-dissolved in 20 mL of acetonitrile. The sample was filtered through 0.45 µm filter and analyzed by HPLC.

Chromatographic separation parameters

The HPLC-UV system used, consisted shimadzu high performance liquid chromatography with LC-20AT pump and SPD-20A interfaced with LC solution software, equipped with a reversed phase C18 analytical column of 250 mm x 4.6 mm and particle size 5 µm (Phenomenex Luna-C18) Column temperature was maintained at 30°C. The injected sample volume was 10µL. Mobile Phases A and B was Acetonitrile and 0.1% formic acid in HPLC grade water (50:50 (v/v)). The flow- rate used was kept at 1.4 mL/min. A detector wavelength was 225 nm. The calibration curve method was used for determination of tembotrione and its metabolite (tembotrione–dihydroxy) residues in fruit.

Method validation

Method validation ensures analysis credibility[5, 6]. In this study, the parameters accuracy, precision, linearity and limits of detection (LOD) and quantification (LOQ) were considered. The accuracy of the method was determined by recovery tests, using samples spiked at concentration levels of 0.03 and 0.3 mg/kg. Linearity was determined by different known concentrations (0.03, 0.1, 0.5, 1.0, 5.0 and 10.0 µg/mL) were prepared by diluting the stock solution. The limit of detection (LOD, µg/mL) was

determined as the lowest concentration giving a response of 3 times the baseline noise defined from the analysis of control (untreated) sample. The limit of quantification (LOQ, µg/mL) was determined as the lowest concentration of a given fungicide giving a response of 10 times the baseline noise[7-8].

RESULTS AND DISCUSSION

Specificity

Aliquots of tembotrione and its metabolite (tembotrione–dihydroxy) standard solutions, spiking sample solution, fruit control, extracted solvents and mobile phase solvents were assayed to check the specificity. There were no matrix peaks in the chromatograms to interfere with the analysis of residues shown in (Figure 1 and 2). Furthermore, the retention times of tembotrione and tembotrione–dihydroxy (AE1417268) were constant at 5.4 ± 0.2 and 4.3 ± 0.2, minutes.

Linearity

Preparation of tembotrione standard stock solution

Accurately weighed 10.05 mg of reference standard of tembotrione (Purity 99.5%) in 10 mL volumetric flask and dissolved in acetonitrile, sonicated and made upto the mark with the same solvent. The concentration of the stock solution was 1000 µg/mL.

Preparation of tembotrione–dihydroxy Metabolite stock solution

Accurately weighed 10.18 mg of reference standard of tembotrione–dihydroxy (Purity 98.2%) in 10 mL volumetric flask and dissolved in acetonitrile, sonicated and made upto the mark with the same solvent. The concentration of the stock solution was 1000 µg/mL.

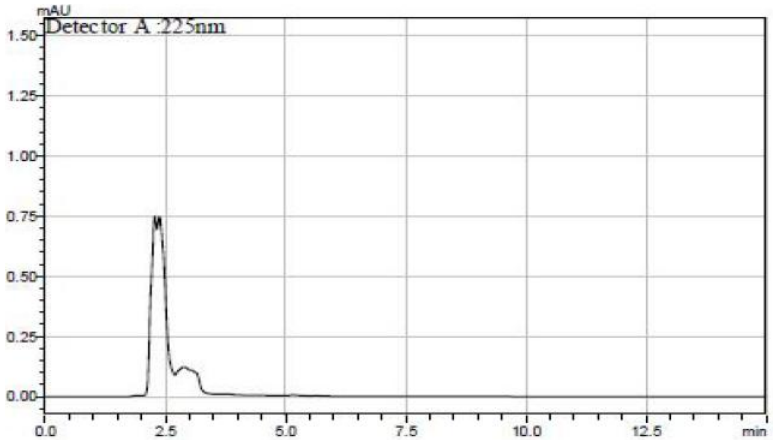

Figure 1 : Representative chromatogram of orange fruit control

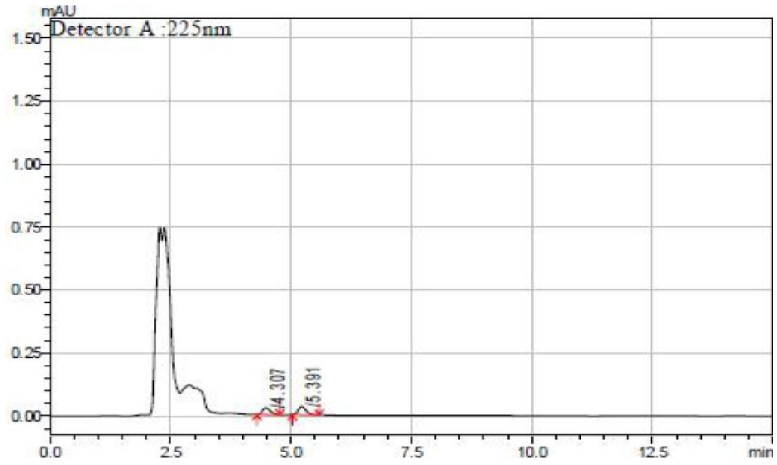

Figure 2 : Representative chromatogram at fortification level of 0.03 µg/g

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TABLE 1 : Serial dilutions of linearity standard solutions

Stock solution concentration (µg/mL)	Volume taken from stock solution (mL)	Final make up volume (mL)	Obtained concentration (µg/mL)
1000	1.000	10	100
100	1.000	10	10
100	0.500	10	5
100	0.100	10	1
10	0.5	10	0.5
10	0.1	10	0.1
1	0.3	10	0.03

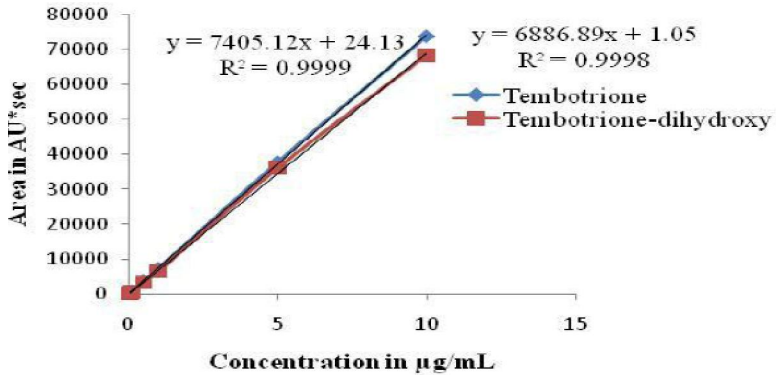

Figure 3 : Representative calibration curve of tembotrione and tembotrione–dihydroxy

Preparation of calibration solutions

Different known concentrations of standard solutions (0.03, 0.1, 0.5, 1.0, 5.0 and 10.0 µg/mL) were prepared in acetonitrile by diluting the above stock solutions. The serial dilution details were presented in TABLE 1. These standard solutions were directly injected into a HPLC. A calibration curve has been plotted of concentration of the standards injected versus area observed and the linearity of method was evaluated by analyzing six solutions[9]. The peak areas obtained from different concentrations of standards were used to calculate linear regression equations. These were Y=7405.12X + 24.13 and Y=6886.89 + 1.05 with correlation coefficients of 0.9999 and 0.9998 for tembotrione and tembotrione–dihydroxy respectively. A calibration curve showed in (Figure 3).

Accuracy and precision

Recovery studies were carried out at 0.03 and 0.3 µg/mL fortification levels for tembotrione and tembotrione–dihydroxy in fruit. The recovery data and relative standard deviation values obtained by this method are summarized in TABLE 2.

These numbers were calculated from four (6) replicate analyses of given sample (tembotrione and tembotrione–dihydroxy) made by a single analyst on one day. The repeatability of method satisfactory (RSDs<2 %).

Detection and quantification limits

The limit of quantification was determined to be 0.03 µg/mL. The quantitation limit was defined as the lowest fortification level evaluated at which acceptable average recoveries (84-94%, RSD<2%) were achieved. This quantitation limit also reflects the fortification level at which an analyte peak is consistently generated at approximately 10 times the baseline noise in the chromatogram. The limit of detection was determined to be 0.03 µg/mL at a level of approximately three times the back ground of control injection around the retention time of the peak of interest.

Storage stability

A storage stability study was conducted at refrigerator condition (5 ± 3°C) and Ambient temperature (25 ± 5°C) of 0.1 µg/g level fortified fruit samples were stored for a period of 30 days.

TABLE 2 : Recoveries of the tembotrione and tembotrione–dihydroxy from fortified orange fruit sample (n=6)

Fortification Concentration in µg/mL	Replication	Recovery (%)	
		Tembotrione	Tembotrione–dihydroxy (AE1417268)
0.03	R1	83.26	81.23
	R2	82.17	82.96
	R3	83.98	84.28
	R4	84.63	84.01
	R5	83.26	82.98
	R6	85.96	83.41
	Mean	83.88	83.15
	RSD	1.56	1.30
0.3	R1	92.39	90.89
	R2	94.12	91.76
	R3	93.41	92.36
	R4	92.52	93.85
	R5	93.74	93.62
	R6	94.89	92.95
	Mean	93.51	92.57
	RSD	1.02	1.22

TABLE 3 : Storage stability details at refrigerator condition (5 ± 3°C)

Fortification Concentration in µg/mL	Storage Period in Days	Recovery in %	
		Tembotrione	Tembotrione–dihydroxy (AE1417268)
0.1	0	95.23	93.26
		93.96	94.21
		94.51	93.66
		94.05	93.88
		93.57	90.74
		94.89	92.78
	Average	94.4	93.1
	STDEV	0.62	1.25
	RSD in %	0.66	1.35
	30	92.11	90.27
		91.56	91.41
		92.04	92.09
		91.74	90.87
		91.22	90.19
		90.96	89.56
	Average	91.6	90.7
	STDEV	0.45	0.92
	RSD in %	0.50	1.01

Analysed for the contents of tembotrione and tembotrione–dihydroxy before storing and at the end of storage period[10]. The percentage dissipation observed for the above storage period was only less than 4% for tembotrione and tembotrione–dihydroxy showing no significant loss of residues

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TABLE 4 : Storage stability details at ambient temperature (25 ± 2°C)

Fortification Concentration in µg/mL	Storage Period in Days	Recovery in %	
		Tembotrione	Tembotrione–dihydroxy (AE1417268)
0.1	0	93.55	92.33
		93.20	92.78
		93.17	93.26
		94.56	90.69
		92.98	92.85
		91.47	93.21
	Average	93.20	92.5
	STDEV	1.00	0.96
	RSD in %	1.07	1.04
	30	91.45	89.63
		91.24	90.58
		90.56	91.03
		91.22	90.14
		89.74	89.57
		90.96	89.74
	Average	90.9	90.1
	STDEV	0.63	0.59
	RSD in %	0.69	0.65

on storage. The results are presented in TABLE 3 and 4.

CALCULATIONS

The concentration of acetaminophen in the samples analyzed by HPLC was determined directly from the standard curve.

Y = mx + c

Where; Y = peak area of standard (mAU*sec); m = the slope of the line from the calibration curve; x = concentration of injected sample (mg/L); c = 'y' intercept of the calibration curve

The recovered concentration or Dose concentration was calculated by using the formula:

Recovered concentration or Dose concentration

$$\text{Recovered concentration or Dose concentration} = \frac{(x\text{-}c)\ X\ D\ X\ 100}{m\ X\ P}$$

Where; m = the slope of the line from the calibration curve; x = sample area of injected sample (mAU*sec); c = 'y' intercept of the calibration curve; D = Dilution Factor; P = Purity of Test item

$$\text{Recovery} = \frac{\text{Recovered Concentration}}{\text{Fortified Concentration}} \times 100$$

CONCLUSIONS

This paper describes a fast, simple sensitive analytical method based on SPE-HPLC-UV simultaneous determination of tembotrione and tembotrione–dihydroxy residues in orange fruit The SPE extraction procedure is very simple and inexpensive method for simultaneous determination of tembotrione and tembotrione–dihydroxy residues in orange fruit. The mobile phase Acetonitrile and HPLC grade water showed good separation and resolution and the analysis time required for the chromatographic determination of the tembotrione and tembotrione–dihydroxy were very short (around 15 min for a chromatographic run). Satisfactory validation parameters such as linearity, recovery, precision and very low limits were obtained and according to the SANCO guidelines[14]. Therefore, the proposed analytical procedure could satisfactorily be useful for regular monitoring of tembotrione and tembotrione–dihydroxy residues on a large number of leaf, seed, oil, fruit, water and soil samples[11-13].

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