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Technical Report

A simple and sensitive assay using GC-MS for determination of chlorfluazuron in termites

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Chlorfluazuron (CFZ) is a benzoylphenylurea insecticide that is commonly used in baits for management of subterranean termites. In this study, a new method using GC-MS for the determination and quantification of CFZ in termites was developed and validated. Since a small volume of organic solvent (250 μL) was used in the sample preparation procedure, the extract was analyzed without any evaporation. The assay was simple and rapid, with a short GC run time (10.0 min). The calibration curve was linear over the range of 0.1–2.5 μg/g, and the correlation coefficient was >0.998. This method was sensitive, as demonstrated by the detection and quantification limits of 0.003 μg/g and 0.1 μg/g, respectively. The mean recovery of CFZ from spiked samples was 95.6%. The within-day and between-day precision and accuracy of the assays ranged between 1.19 and 6.43%. This method was used to screen for CFZ transfer between nestmates of a treated *Macrotermes gilvus* mound. The highest amount of CFZ was detected in workers from the royal chamber, followed by workers in the peripheral zone, workers in the nursery zone, larvae in the nursery zone, and larvae in the peripheral zone.

Keywords: chlorfluazuron, termite bait, GC-MS, *Macrotermes gilvus*, pesticide analysis.

Introduction

Chlorfluazuron (CFZ) [1-(3,5-dichloro-4-(3-chloro-5-trifluoromethyl-2-pyridyloxy)phenyl)-3-(2,6-difluorobenzyl)-urea] is a benzoylphenylurea (BPU) insecticide that acts by inhibiting the formation of chitin in the insect cuticle, thereby leading to mortality of molting insects due to incomplete exoskeleton formation.[1,2] BPUs are widely used in the management of insect pests, especially in agriculture. Their extensive usage is largely due to their excellent properties, including high selectivity, high biological activity, and low mammalian toxicity.[3]

CFZ, like several other BPUs (*e.g.*, hexaflumuron, bistrifluron, and noviflumuron), is effective against termites. Termite baits normally consist of palatable food that has been impregnated with a BPU as the active ingredient (or bait toxicant). Use of bait is a popular method of termite management.[4,5] After termites feed on the bait, the toxicant will subsequently be shared among colony members through trophallaxis, a food-sharing behavior exhibited by social insects.[6]

Macrotermes gilvus (Hagen) is a common mound-building fungus-growing termite that is widely distributed in Southeast Asia.[7] A termite colony consists of workers, soldiers, larvae, nymphs, and a royal pair (king and queen). Figure 1 shows the mound structure of *M. gilvus*. The thick outer wall of the mound covers the inner part of the nest, which is called the hive.[8] A narrow free space (peripheral zone) exists between the thick outer wall and the hive. The hive is the main living area, consisting mainly of a nursery zone, fungus combs, and the royal chamber. The nursery zone, where eggs and larvae are kept, is located deep within the nest near ground level and is usually placed on the fungus combs. The royal chamber is roughly oval in shape and normally is located in the center of the hive, also near ground level.[9] Along with the royal pair, it contains several small access openings for workers.

Assessment of the efficacy of termite bait treatment is generally based on the absence of termite foragers or the cessation of feeding activity in monitoring stations.[10,11] However, these indicators may also be due to the death of mainly termite foragers while the remaining termite colony remains healthy.[12] Therefore, the ability to measure the amount of toxicant transferred between termite individuals is crucial in order to assess successful colony elimination through termite baiting. A number of analytical methods have been reported for determination of

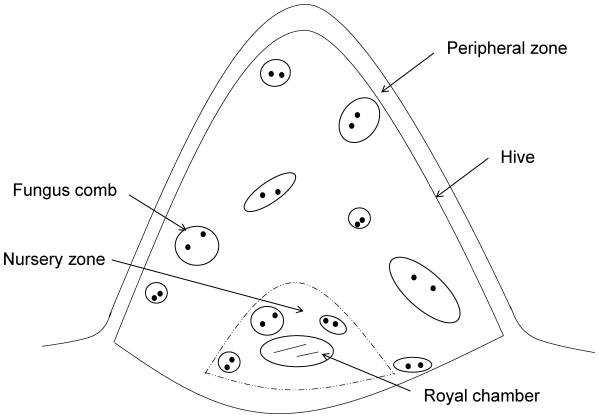

Fig. 1. Diagrammatic vertical section of a *Macrotermes gilvus* mound.

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BPU concentration, mostly in fruits and vegetables. Some of the published studies dealt with measurement of a single BPU,[13–16] whereas others involved measurements of residues of multiple BPUs.[17–22] Generally, analyses of BPUs have been accomplished by high performance liquid chromatography (HPLC) in combination with ultraviolet,[14] diode array detection (DAD),[23] mass spectrometry (MS),[13] and tandem mass spectrometry[24] detection systems. Alternative methods, such as gas chromatography (GC) coupled with electron-capture detection (ECD)[15] or mass spectrometry (MS),[16] have also been applied to screen BPUs.

Information about how BPU toxicants are distributed or transferred among termite colony members is scarce, partly due to the lack of an effective method to analyze BPUs in termites. To date, only two studies have evaluated termite samples using HPLC. Kubota *et al.*[25] and Peppuy *et al.*[26] quantified the amount of bistrifluron and hexaflumuron, respectively, in termites. No analytical method has ever been reported for quantification of CFZ in termites by a GC-MS assay. The objective of this study was to develop a novel GC-MS method for detection and quantification of CFZ in termites. The present method was validated and applied to evaluate CFZ transfer among *M. gilvus* individuals.

Materials and Methods

1. Termites

Termite samples from an untreated termite mound in Bayan Lepas, Penang in northern Peninsular Malaysia (5°32′N, 100°29′E) were collected. These blank samples were known to be free from CFZ exposure and were used to prepare calibrators and validation samples.

2. Insecticide and sample treatment

Cellulose powder containing 0.1% CFZ (Ensystex Sdn. Bhd., Kuala Lumpur, Malaysia) was tested in this study. Another *M. gilvus* mound from Bayan Lepas, Penang, Malaysia was chosen and baited with CFZ. The baiting period lasted approximately 4 months (March 2012–July 2012), and 760 mg CFZ were removed by the termites during the baiting period. After 4 months, the mound was excavated, and the termite samples were collected. During excavation, workers and larvae, if present, were collected from different parts of the mound (*i.e.*, peripheral zone, nursery zone, and royal chamber). Three replicates of samples of each termite caste and mound part were chemically analyzed using the assay developed in this study (refer to section 3). Concentrations of CFZ in each termite caste and mound part were analyzed using one-way analysis of variance (ANOVA), and means were separated using Tukey's honestly significant difference (HSD) at $\alpha = 0.05$.

Before the extraction process, the termites were rinsed with acetonitrile to remove external CFZ contamination. A preliminary chemical assay demonstrated no peak of CFZ found in the solvent (acetonitrile) that was applied to clean the termites a second time (Fig. 2). The termites had been previously spiked with a 2.5 μg CFZ/mL solution. We have proved that washing 1 time

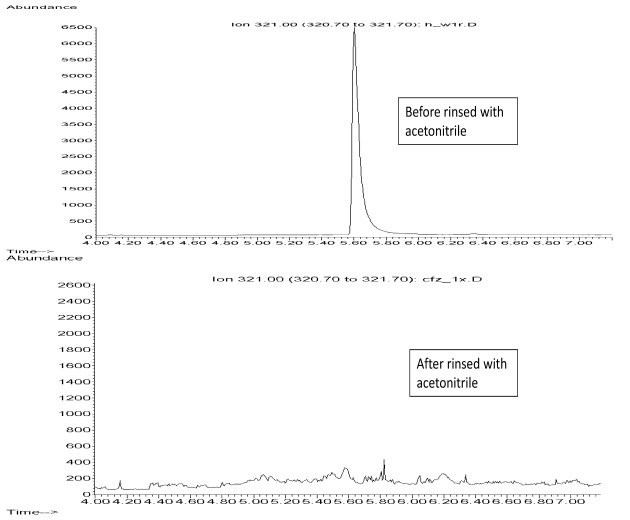

Fig. 2. Extracted ion chromatograms of chlorfluazuron at *m*/*z* 321 applied to clean termites before and after being washed with acetonitrile one time.

with 1 mL of acetonitrile was enough to eliminate this external contamination. We were interested in determining whether CFZ had been ingested and transferred among the colony members, and washing ensured that the CFZ residue detected during the analysis was due to ingestion and not to CFZ adhering to the body surface. To determine the quantity of CFZ in termite workers from the royal chamber, the weight of the termite tested was reduced to 0.1 g since a prescreening result showed that CFZ exceeded the upper limit of quantification.

3. Experimental methods

3.1. Reagents

All purchased chemicals were of analytical grade and consisted of CFZ, hexaflumuron (Sigma-Aldrich, Kuala Lumpur, Malaysia), sulfuric acid, anhydrous sodium sulfate (Merck, Shah Alam, Selangor, Malaysia), methanol, isopropanol, and acetonitrile (J.T. Baker, Simpang Ampat, Pulau Pinang, Malaysia).

3.2. Blanks, standards, and calibrators

A stock solution containing 1 mg/mL CFZ in methanol was prepared and further diluted to a working solution (10 μg/mL). A set of seven calibration solutions consisted of 0.1, 0.25, 0.5, 1.0, 1.5, 2.0, and 2.5 μg CFZ in 1 mL methanol was freshly prepared from the working solution. Next, 0.2 mL of each calibration solution was spiked into 0.2 g of fresh termite samples (~30 termite workers). These amounts were similar to detection of 0.1, 0.25, 0.5, 1.0, 1.5, 2.0, and 2.5 μg CFZ in 1 g of termite samples. Three validation samples containing 0.1, 1.5, and 2.5 μg CFZ in 1 g of termites were also prepared. The internal standard (IS), a hexaflumuron working solution, was prepared by dissolving 5 mg hexaflumuron in 10 mL methanol to give a 0.5 mg/mL concentration.

3.3. Sample extraction

A sample extraction was made by crushing 0.2 g termites sample to a fine powder in a test tube using a spatula. Fifty microliters

each of the IS and 0.1 M sulfuric acid were then pipetted into the tube, followed by 200 μL isopropanol. The mixture was vortexed for 1 min and then centrifuged for 5 min at 2500 rpm. The clear supernatant was dried over anhydrous sodium sulfate and subsequently transferred into an autosampler vial for GC-MS analysis. The blank, calibrator, and validation samples were all treated following this protocol.

3.4. GC-MS conditions

GC-MS analyses were carried out using an Agilent 7890A gas chromatograph equipped with an Agilent 5975C mass spectrometer detector and an Agilent 7693 autosampler (Agilent Technologies, Bayan Lepas, Penang, Malaysia). One microliter of injection volume using the splitless mode in a deactivated glass liner (without glass wool) was used. A fused-silica capillary column (30 m×0.25 mm i.d., 0.25 μm film thickness) coated with 5% phenyl methyl siloxane was used, and helium was the carrier gas. The injector and interface temperatures were set at 250°C and 280°C, respectively. The oven temperature was programmed from 90 to 280°C (5-min hold) at a rate of 38°C/min. The retention times and mass spectra of CFZ and IS were established using a 1 mg/mL standard solution. Two peaks representing the aniline and difluorobenzamide degradation products were observed in both CFZ and IS. No intact molecules of CFZ and IS were detected. As such, the monitoring of CFZ and IS was done using these specific aniline derivatives under a selected ion-monitoring mode. Six characteristic fragment ions (*m*/*z* 148, 176, 321, 322, 333, and 356) and three characteristic fragment ions (*m*/*z* 148, 176, and 178) were used for monitoring of CFZ and IS, respectively. The quantifying ions were *m*/*z* 321 (CFZ) and *m*/*z* 176 (IS). Other ions served as qualifying ions. Under these conditions, the retention time of CFZ was approximately 5.14 min. The total run time was 10 min.

3.5. Validation of the GC-MS method

A calibration curve was constructed for the average peak area ratio of CFZ/IS *versus* different concentrations for the range of 0.1 to 2.5 μg/g. Linearity was calculated based on the regression line and expressed as the correlation coefficient (r^2>0.995). Sensitivity of the assay was measured by limit of quantitation (LOQ) and limit of detection (LOD). LOQ was the lowest concentration or lowest validation point on the calibration curve. Both accuracy and precision of the LOQ could not exceed 20%. Six replicates of LOQ were analyzed in each run, with a total of 18 replicates processed in three runs. LOD was defined as the concentration that had a signal-to-noise ratio of at least 3 : 1. The extraction recovery was calculated by comparing the mean peak areas of CFZ in the extracted termite samples with the standard solution.

Within-day and between-day precision and accuracy were measured using validation samples by comparing data from within one run and between three runs, respectively. Precision was expressed as the percent coefficient of variation and relative accuracy was expressed as the percent difference from the nominal value.

Results and Discussion

1. Quantity of sample

Previous studies used a large sample amount (*e.g.*, 15–50 g) when employing the HPLC method to analyze BPUs.[13,17,23] In the current study, the collection of a large quantity of sample was not possible. Termite samples were collected from mounds that had been subjected to bait treatment, and it was impractical to collect 10 g termites (30 g termite workers equal approximately 4,000 termite workers) for each replicate of the analysis. In the method developed herein, only 0.2 g termites were employed for each replicate of the analysis. While this amount is much lower than those previously reported, the required sensitivity was not compromised.

2. Sample preparation

In several previously reported analytical studies on BPUs in fruits and vegetables, the sample preparation involved liquid-liquid extraction (LLE) and required further cleanup with solid-phase extraction (SPE) or gel permeation chromatography (GPC).[13,15,17,23,27] LLE, SPE, and GPC used a large volume of organic solvent (30–125 mL).[13,15,17,23,27] Therefore, an evaporation step was necessary to remove the excess solvent, which subsequently increased the sensitivity of the assays. However, the evaporation step leads to longer sample preparation time and may incur loss of the target compound if overheated. In our newly developed assay, the extraction procedure was simplified (no cleanup step) and was performed in a single test tube. Only a small volume of organic solvent (*i.e.*, 200 μL isopropanol and 50 μL methanol) was used during sample preparation. Therefore, a lengthy evaporation procedure was omitted in this assay, which subsequently enhanced the recovery rate.

3. Characteristic mass fragmentation pattern of BPUs

BPUs are thermally labile compounds. In GC system, cleavage of the urea moiety in BPUs yields two main products: 2,6-difluorobenzamide (MW 157) and a specific aniline compound, such as 3,5-dichloro-4-(3-chloro-5-trifluoromethyl-2-pyridyloxy) aniline in CFZ (MW 356) or 3,5-dichloro-4-(1,1,2,2-tetrafluoroethoxy) aniline in hexaflumuron (IS) (MW 277). Except in their work with triflumuron, Hiemstra *et al.*[23] showed similar results in which 2,6-difluorobenzamide was the main degradation fragment formed during GC-MS analysis of BPUs. Due to their specificity, only aniline compounds were monitored and quantified. The intact molecular ions of each of these aniline products are shown in Fig. 3. The based ion (*m*/*z* 321) of CFZ was derived from the loss of a chlorine atom from the specific aniline (MW 356) compound. The ion was chosen as a quantifying ion because of its highest abundance and low background noise (Fig. 4). The based ion (*m*/*z* 176) of IS was derived from the loss of a $-CF_2CHF_2$ group from the specific aniline (MW 277) compound.

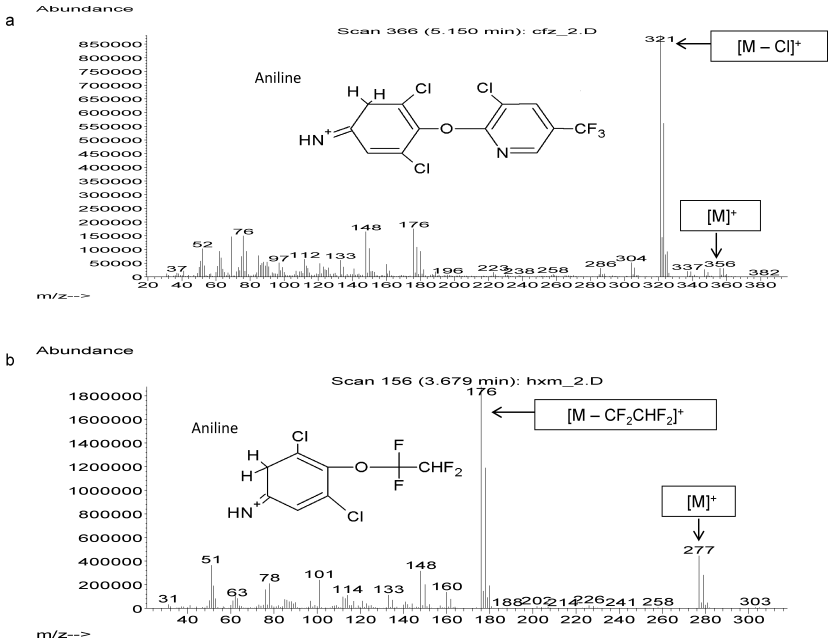

Fig. 3. Mass spectra of major degradation product of (a) chlorfluazuron and (b) hexaflumuron.

4. *Validation test*

4.1. *Sensitivity and recovery*

High sensitivity in an assay is important for screening trace amounts of CFZ inside a termite colony. Sensitivity of the analytical method described in this study was high, as indicated by the LOD (0.003 μg/g or 0.02 ng/termite) and LOQ (0.1 μg/g or 0.74 ng/termite) with an average recovery of 95.6% (Table 1). Compared with our GC-MS method in which degradation products were used for indirect determination, LC methods allow direct analysis. Determination of CFZ by HPLC-MS/MS assay in processed fruits and vegetables was found to be more sensitive, with an LOQ of 0.001–0.02 μg/g but with recovery at only 69–88%.[24] However, the sensitivity in our study was better than or comparable to the reported detection of CFZ using GC-ECD in green tea samples with an LOD of 0.008–0.015 μg/g and recovery of 75–89%.[18]

The developed GC-MS assay was also found to be more sensitive than the HPLC-DAD or UV assays using apple and pear specimens. These assays had LODs at 0.03 and 0.008 μg/g, respectively, with recovery of CFZ at 71–94%.[14,27] In addition, the developed GC-MS assay has very good chromatographic separation, and relative retention time of CFZ was 1.40. The total run time was shortened to 10 min (Fig. 4) as compared to other methods by HPLC and GC-ECD (12–20 min).[14,18,23]

There is no other reported GC-MS method for detection of CFZ in termites. The only available method was by HPLC, which described detection of bistrifluron and hexaflumuron (other BPUs) in termites with a lower sensitivity at 1.0 ng/termite.[25,26]

4.2. *Linearity and precision*

The validated assay, which was performed over 3 days, showed excellent linearity (average r^2>0.998). The working range

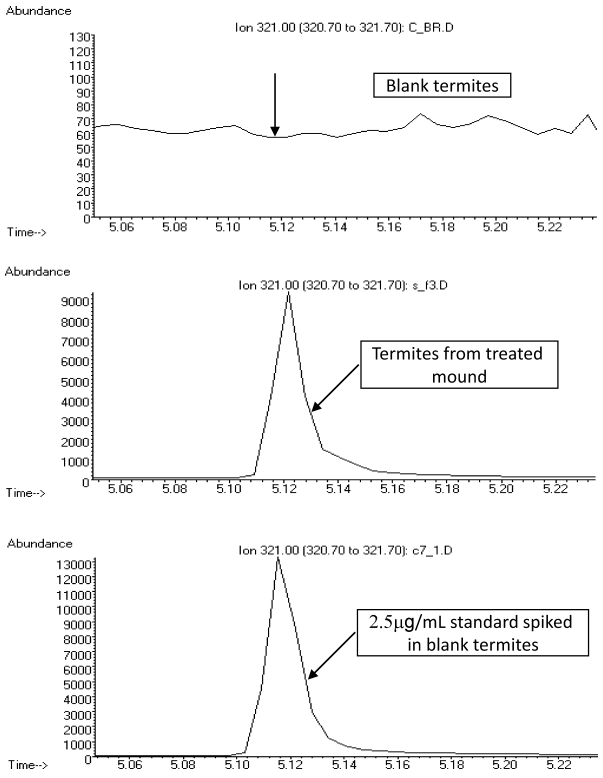

Fig. 4. Extracted ion chromatograms of chlorfluazuron at *m*/*z* 321 in blank termites, termites from the treated mound, and when blank termites were spiked with 2.5 μg/mL standard chlorfluazuron.

(0.1–2.5 μg/g) is suitable for screening trace amounts of CFZ transferred among colony members inside a termite mound. The repeatability and reproducibility of this assay were satisfactory, as within-assay precision and relative accuracy were <6.50%

Table 1. Recovery of chlorfluazuron in termites and within- and between-assay precision and relative accuracy of chlorfluazuron measurements

Concentration (μg/g)	Within-assay				Between-assay				Recovery (%)
	n	Observed conc. (mean±S.D., μg/g)	Precision (%)	Relative accuracy (%)	*n*	Observed conc. (mean±S.D., μg/g)	Precision (%)	Relative accuracy (%)	
0.1	6	0.097±0.006	6.26	2.68	18	0.104±0.005	4.96	6.35	97.9
1.5	6	1.404±0.020	1.43	6.43	18	1.461±0.042	2.88	2.98	91.3
2.5	6	2.564±0.153	5.98	2.57	18	2.519±0.110	4.38	1.19	97.5
Average			4.56	3.89			4.07	3.51	95.6

Table 2. Concentration of chlorfluazuron in termite workers and larvae from different parts of the mound

Termite caste; mound part	CFZ per gram termite[a)] (mean±S.D., μg/g)	CFZ per termite individual[a,b)] (mean±S.D., ng/termite)
Worker; Peripheral zone	2.26±0.07	16.72±0.50b
Worker; Nursery zone	2.15±0.46	15.9±3.38b
Worker; Royal chamber	4.81±0.12	35.63±0.86c
Larva; Peripheral zone	0.30±0.02	0.93±0.06a
Larva; Nursery zone	0.33±0.05	1.02±0.14a

[a)] $n=3$. [b)] Means followed by different letters are significantly different by the Tukey's HSD test at $p<0.05$.

and between-assay precision and relative accuracy ranged from 1.19 to 6.35% (Table 1). The use of hexaflumuron as the IS, which is equally extractable under the same conditions as CFZ, also contributed to the favorable accuracy and precision of this method.

5. Transmission of CFZ within termite colonies

Concentrations of CFZ detected in workers from the royal chamber was significantly highest among quantity of CFZ detected in workers and larvae from different parts of the mound ($F=246.58$; df=4,10; $p<0.05$). In general, the highest amount of CFZ was detected in workers from the royal chamber, followed by workers in the peripheral zone, workers in the nursery zone, larvae in the nursery zone, and larvae in the peripheral zone (Table 2). Peppuy *et al.*[26)] showed that 155 ng hexaflumuron was detected in a large termite worker that has been exposed to treated wood for 15 days. The lower residue level of CFZ detected in our study compared to that of Peppuy *et al.* could be due to different termite species and different feeding behaviors. Termite workers (22.75±9.83 ng/termite) transferred approximately 5% of the ingested toxicant to termite larvae (0.98±0.11 ng/termite). Lower transfer efficiency from worker to dependent caste was also found in other termite species. Under laboratory conditions, workers of *Odontotermes formosanus* transferred approximately 1.2% of initially acquired food to larvae caste by trophallaxis.[28)] Approximately 1 to 35 ng CFZ/termite individual was detected in different termite castes from different parts of the mound. This study showed that the active ingredient of bait was successfully distributed among termite colony members. However, further studies need to be carried out to determine whether the level of CFZ concentration varies among different sizes of termite colonies. This information will be helpful to evaluate the effectiveness of baiting systems.

Conclusion

A simple and sensitive methodology was developed for the detection and quantification of CFZ concentration in termites. The sample preparation is simple, uses a minimal amount of organic solvent, and does not require a further cleanup step. The GC-MS assay proved to be robust, with satisfactory accuracy and precision. The GC run time is short (10 min). The method is applicable for routine analysis of CFZ in termites. The success of this assay was demonstrated by measuring the amount of CFZ transferred among colony members in a *M. gilvus* termite mound.

Acknowledgements

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