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Development and application of a method for analysis of lufenuron in wheat flour by gas chromatography–mass spectrometry and confirmation of bio-efficacy against *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae)

Kedar C. Ahire [b], Manjit S. Arora [a], Samindra N. Mukherjee [a,*]

[a] *Entomology Laboratory, National Chemical Laboratory, Pune 411008, India*
[b] *Department of Microbiology, University of Pune, Pune 411007, India*

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Abstract

A new analytical method using gas chromatography with mass spectrometry (GC–MS) for the quantitative determination of lufenuron, a benzoylphenylurea (BPU) class of insecticide, from wheat flour has been developed and applied for time-dependant residue monitoring in treated wheat flour. The analyte was extracted from wheat flour by a single step solid–liquid extraction by using ethyl acetate and subsequently cleaned up using the Primary Secondary Amine as a sorbent prior to GC–MS analysis. The present method provides sufficient sensitivity as reflected by the values of limit of detection (LOD) and limit of quantification (LOQ), 5 ng/mL (S/N ∼3) and 50 ng/mL (the lowest validation point on the calibration curve), respectively. The calibration curve showed an excellent linearity in the concentration range of 50–1000 ng/mL ($r^2 = 0.998$). The average recovery for spiked samples at three concentrations (150, 300, and 450 ng/g) was 98.23 ± 2.52% R.S.D. The method was applied for the determination of lufenuron residues in treated wheat flour samples. Simultaneous determination of bio-efficacy of lufenuron residues was also carried out against the red flour beetle, *Tribolium castaneum* to correlate the actual residual effect of lufenuron as detected by the analytical method, over a period of 3 months. The findings revealed that the residual concentration of lufenuron were neither uniform nor in descending order over a period of 3 months in wheat flour, possibly because of an uneven dispersal in the treated wheat which was subsequently milled into flour, as confirmed by GC–MS analysis. However, the residues of lufenuron were sufficient to produce 100% mortality of *T. castaneum* larvae up to 3 months. The results have been discussed in view of the potential of lufenuron as a candidate molecule for the control of stored product pests.

Keywords: Lufenuron; GC–MS; Method development; Residue analysis; *Tribolium castaneum*; Wheat; Bio-efficacy

1. Introduction

Lufenuron ((RS)-1-[2,5-dichloro-4-(1,1,2,3,3,3-hexafluoropropoxyl)phenyl]3-(2,6-difluorobenzoyl) urea CAS: 103055-07-8) is a benzoylphenylurea (BPU) class of insecticide, which acts as a chitin synthesis inhibitor (CSI). Its efficacy has been proven in the treatment of fleas feeding on dogs and cats by oral or parenteral administration [1,2]. It was also shown that the dose higher than recommended for anti-flea treatment, was very effective in the therapy of dermatomycoses in dogs and cats [3,4]. Recently, CSIs have gained significant popularity due to their low mammalian toxicity and absence of mutagenic and teratogenic effects on warm-blooded animals. [5].

Monitoring of residues in food is mandatory for proper assessment of human exposure to pesticides in foods. In practice it is important to decide the concentration of pesticide in the food item to control the pest, keeping in mind the Maximum Residue Limit (MRL) set by the regulatory authorities. In recent years, the development of sophisticated analytical methods which are

Abbreviations: CSI, chitin synthesis inhibitor; IGR, Insect Growth Regulator; BPU, benzoylphenylurea compound; JHA, Juvenile Hormone Analogs; MRL, Maximum Residue Limit; GC–MS, gas chromatography–mass spectrometry; PTVI-LV, programmable temperature vaporization injector, large volume; LOD, limit of detection; LOQ, limit of quantification; RT, retention time; PSA, Primary Secondary Amine; EC, emulsified concentrate; LC_{50}, concentration of analyte giving 50% mortality; S/N, signal to noise ratio; % R.S.D., % relative standard deviation; *m/z*, mass to charge ratio; ANOVA, analysis of variance.

* Corresponding author. Tel.: +91 20 25902624; fax: +91 20 25902624.
E-mail address: s.mukherjee@ncl.res.in (S.N. Mukherjee).

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capable of detecting trace quantities of pesticides has become increasingly important in the field of pesticide chemistry. Previous attempts have been made for analysis of BPU's residues by HPLC with fluorescent detector [6] and LC–MS [7,8]. Alternative methods such as ultraviolet (UV) determinations have also been used for the detection of BPU residues. However, low sensitivity and specificity due to the lack of sufficient discrimination of a signal and the matrix noise was the serious drawback of these methods [9–11]. These methods do not directly appear to be applicable to the analysis of lufenuron in a complex matrix like wheat flour.

In this paper we present a novel gas chromatography–mass spectrometry (GC–MS) method for sensitive detection and quantification of lufenuron from a complex matrix like wheat flour. The present method was applied for the time-dependant monitoring of lufenuron residues in wheat flour. Simultaneously, the residual effect of lufenuron on the mortality of *Tribolium castaneum* larvae, a serious pest on stored product commodities worldwide [12] was carried out.

2. Materials and methods

2.1. Chemicals and biological materials

Lufenuron (99% technical) and its 5% emulsified concentrate (EC) formulation (Match 5®) were obtained from Syngenta India Ltd., Mumbai, India, as a kind gift. Ethyl acetate (+99%) and anhydrous sodium sulfate (99%) were obtained from Qualigens, India and the Primary Secondary Amine (Bondesil-PSA, 40 μm) was obtained from Varian, USA. All the solvents were of HPLC grade and were distilled and subsequently filtered through 0.45 μm nylon filter paper prior to use. *T. castaneum* used in the present work was maintained in the laboratory at 30 ± 1 °C and 60% relative humidity on wheat flour with 5% Brewer's yeast. Eggs and larvae were harvested as described by Salokhe et al. [13].

2.2. Preparation of lufenuron standards for GC–MS analysis

To make 1000 ppm stock solution, 10.1 mg of lufenuron (99%), was weighed accurately using an analytical electronic balance (Mettler, AX-205) in 10 mL volumetric flask and dissolved in the ethyl acetate. Fresh working solutions were prepared by appropriate serial dilution with ethyl acetate to make desired concentrations of lufenuron. All stock solutions were kept at −20 °C.

2.3. Extraction and clean up lufenuron from wheat flour

Wheat flour (25 g) was weighed in centrifugation bottle and 10 × *g* sodium sulfate and 50 mL of ethyl acetate were added in it. The mixture was homogenized in a motor driven homogenizer (Heidolf, Germany) at 15,000 rpm for 3 min, followed by centrifugation at 5000 × *g* at 4 °C for 5 min. Two milliliters of this extract supernatant was removed in an eppendorff tube prefilled with 50 mg PSA and centrifuged at 12,000 × *g* for 5 min at 4 °C. Supernatant, thus obtained was filled in the vial for GC–MS analysis.

2.4. Gas chromatography–mass spectrometry

Analysis was carried out using the GC–MS system, consisting of Thermo Finnigan Trace GC Ultra with TriPlus autosampler interfaced with Polaris*Q* Ion Trap MS/MS detector controlled by Xcalibur software (Thermo Electron Corporation, Italy). The following conditions were optimized and used for present analysis: the injections of 15 μL were done by using Triplus autosampler with each four pre-injection and post-injection washes with make up solvent, i.e. ethyl acetate. The samples were injected into a programmable temperature vaporization injector, large volume (PTVI-LV) with base temperature 60 °C, split flow 100 mL/min, splitless time 1 min, solvent valve temperature 100 °C, surge pressure 3 kPa; inject pressure 70 kPa, inject time 0.1 min, vent flow 30 mL/min; evaporation pressure 140 kPa, evaporation rate 14.5 °/s, evaporation temperature 85 °C, evaporation time 1 min; transfer pressure 210 kPa, transfer rate 14.5 °/s, transfer temperature 280 °C, transfer time 3 min; clean rate 14.5 °/s, clean temperature 285 °C, clean time 10 min, clean flow 20 mL/min. The chromatographic separation of lufenuron was performed on a Mega 5 MS column (Mega Capillary Columns Laboratory, Italy.) with 30 m × 0.32 mm i.d. and 0.5 μ film thickness. Carrier gas was helium with 99.999% purity at flow rate of 1 mL/min. Oven was programmed at 50 °C initially, ramped at 15 °C/min to 130 °C held for 0 min, further ramped at 30 °C/min to 182 °C and then ramped at 0.3 °C/min to 184 °C and thereafter held for 1 min (total run time 14.76 min). Electron Impact (EI) ionization was achieved by 70 eV ionization energy with source temperature 230 °C and auxiliary temperature 285 °C. Positive mode full scan was performed in the mass range *m/z* 50–450 with solvent delay of 6 min.

2.5. Linearity, accuracy, precision, limit of detection (LOQ) and limit of quantification (LOD) studies

The linearity was tested by preparing the working standards from stock solution in the range of 50–1000 ng/mL in ethyl acetate to prepare calibration curve. Each standard was injected in triplicate for GC–MS analysis. Accuracy and precision of the method was determined by spiking known amount of lufenuron in the wheat flour (25 g) at the levels of 50% (150 ng/g), 100% (300 ng/g) and 150% (450 ng/g) of the target level (300 ng/g) in three independent sets. The recovery of lufenuron was determined by extrapolating the observed peak area on the standard calibration curve prepared with the same batch.

The LOD was determined based on the signal to noise ratio (S/N ∼3) and the LOQ was determined as the lowest validation point of the calibration curve [14].

2.6. Effect of residual lufenuron on T. castaneum larvae

Various concentrations of lufenuron in wheat flour were fed to the 2-day-old larvae of *T. castaneum* and the data was subjected to probit analysis in order to determine the LC_{50} value.

The dose of lufenuron selected for the treatment of wheat was $LC_{50} \times 5$ (300 ng/g) and was thoroughly incorporated into wheat using water as carrier solvent. The treated wheat was kept for 24 h at 30 °C for complete evaporation of the solvent before milling. To study the residual action of lufenuron on the survival of *T. castaneum* larvae, 20 larvae (2-days old) were periodically released at every 10 days interval on 5 gm of the treated and milled wheat for 90 days. All the experiments were carried out in triplicates at 30 ± 1 °C and average 60% relative humidity.

2.7. Residual life of lufenuron in wheat flour

Lufenuron residues in the above-mentioned wheat samples were examined at an interval of 15 days initially and 30 days subsequently for a period of 90 days by using the present GC–MS method. Every time, the samples were simultaneously run with the spiked samples to estimate the residual concentration of lufenuron in the treated and milled wheat.

3. Results and discussion

3.1. Extraction and clean up of lufenuron from wheat flour

The complex wheat flour samples analyzed in the present study, made it necessary to develop a method for selective extraction and clean up of lufenuron. The simple steps were applied for the extraction of lufenuron from wheat flour avoiding complex derivatization and column packing. In order to avoid the absorption of the sugary substances and pigments in the extract, it was further treated with the Primary Secondary Amine. Ethyl acetate was used for the extraction of lufenuron as it is a very common practice to extract slightly nonpolar compounds from the biological matrices with ethyl acetate [15] and therefore was used for further experiments. To remove moisture, anhydrous sodium sulfate was added in the ethyl acetate extract.

3.2. GC–MS assay of lufenuron

In order to avoid thermal degradation of lufenuron due to oven temperature and to obtain better separation in GC–MS

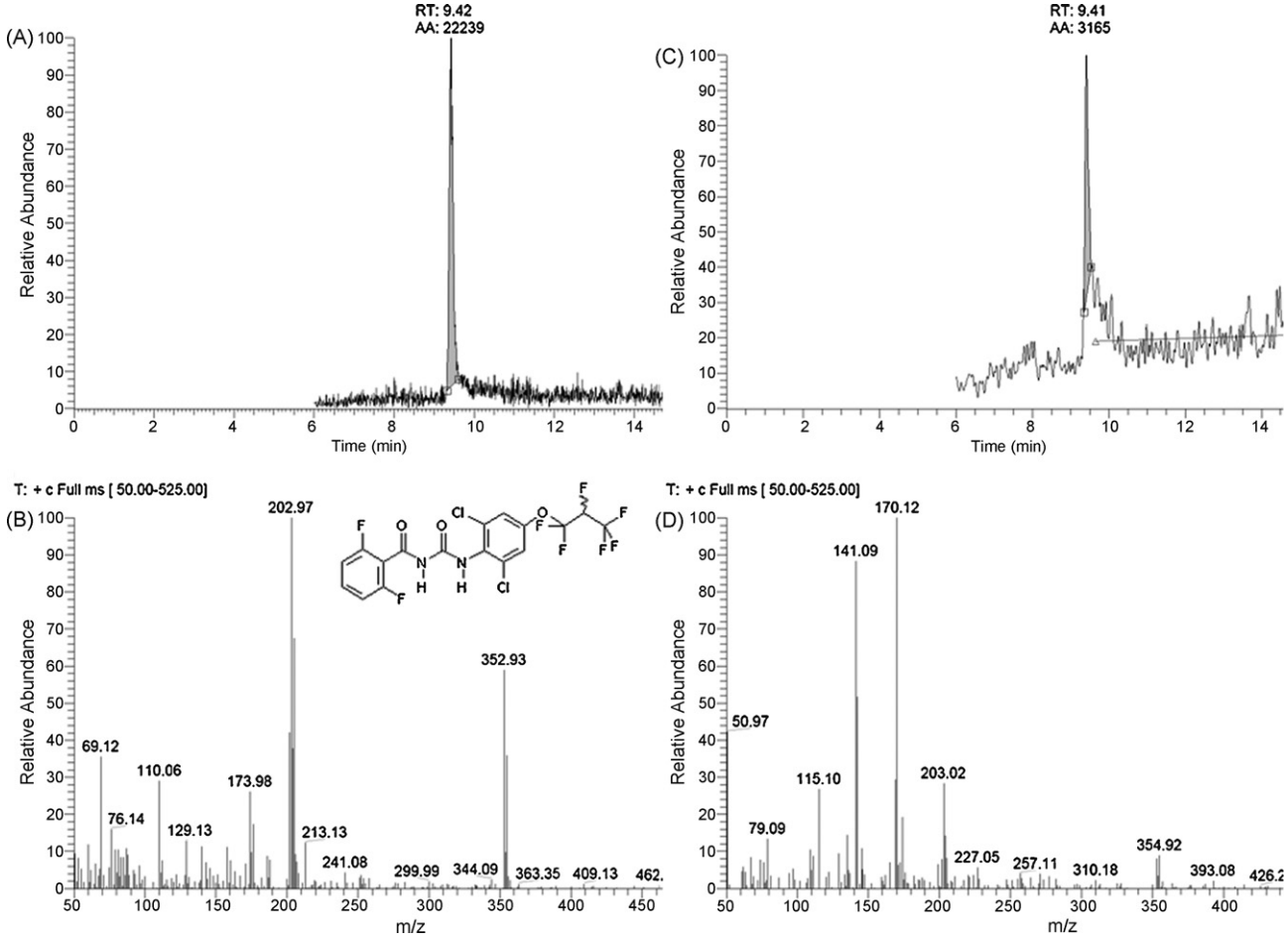

Fig. 1. Representative GC–MS chromatograms. (A) Chromatogram of lufenuron at conc. 500 ng/mL showing retention time (9.42 min) and peak area (of *m/z* 203). (B) Corresponding mass spectra (EI) showing base peak (*m/z* 203) and other major fragments (*m/z* 352, *m/z* 110, *m/z* 173). The inset shows structure of the lufenuron. (C) Chromatogram of wheat flour sample spiked with 150 ng/g lufenuron. (D) Corresponding mass spectra of lufenuron extracted from wheat flour matrix. Some extra mass fragments from matrix also appeared along with standard spectra of lufenuron.

Table 1
Linearity parameters of lufenuron analysis on GC–MS

Parameter	Value	Error
Concentration range (ng/mL)	50–1000	
Slope	45.51	0.25
Y Intercept	−260.89	145.00
Correlation coefficient	0.998	
S.D.	251.34	
Data points (*N*)	11	

Table 2
Accuracy and precision of lufenuron analysis

	Addition of lufenuron		
	150 ng/g	300 ng/g	450 ng/g
Set1	150.6	308.5	437.4
Set2	144.4	294.0	447.8
Set3	147.1	287.1	432.2
Mean	147.4	296.6	439.1
S.D.	3.1	10.9	7.9
Accuracy (% recovery)[a]	98.2	98.9	97.6
Precision (% R.S.D.)	2.1	3.7	1.8

[a] Accuracy of the method was calculated as % accuracy = (measured value/true value) × 100. Total average recovery = 98.23 (% R.S.D. = 2.52).

analysis, several parameters like PTVI-LV injector program and oven temperature program and interface temperature were optimized. The retention time (RT) and the typical mass fragments of lufenuron were applied as the criteria for its identification. The base peak of lufenuron (i.e. *m/z* 203) was selected for the quantification of peak area. Retention time was 9.40 ± 0.05 min under set gas chromatographic conditions and no interfering peak was found at the same RT. Base peak of lufenuron was found to be *m/z* 203 at the optimized MS conditions with the major fragments, *m/z* 353, *m/z* 174, *m/z* 110, *m/z* 158 and *m/z* 69 (Fig. 1B and D).

3.3. Linearity, accuracy, precision, LOQ and LOD studies

The 11 point calibration curve showed excellent linearity of the Peak Area with increasing lufenuron concentration ranging from 50 to 1000 ng/mL. The results of least square regression analysis are shown in Table 1. This external calibration showed a close positive correlation between two variables as indicated by coefficient of determination (r^2) value, which was greater than 0.998. The calibration curves were used to determine the % recovery of spiked lufenuron at different concentrations from the wheat flour prepared at the time of experiment and the amount was calculated by multiplication with the dilution factor (= 2). The analytical results for the determination of lufenuron at the three spiking levels are presented in Table 2. Excellent average recovery (98.23%) with good precision (% R.S.D. = 2.52) was obtained with the proposed method. The mean recoveries for the triplicate determinations were within 1.5% difference, and the mean relative standard deviations (R.S.Ds) ranged from 1.81 to 3.69% for the three spike levels.

The method provided sufficient specificity since there were no interfering peaks at the retention time of lufenuron. The sensitivity of the method was reflected by the values of LOD and LOQ. LOD was considered to be ca. 5 ng/mL on the basis of signal to noise ratio (S/N ∼3). LOQ of the method was 50 ng/mL (the lowest validation point on the calibration curve).

Table 4
One-way ANOVA on mortality of 7th, 10th and 15 days samples

Mortality data	Mean	Variance	Sample size (*N*)
Mortality on 7th day (%)	74.5	41.4	10
Mortality on 10th day (%)	92.3	30.4	10
Mortality on 15th day (%)	100	0	10

At the 0.05 level, the means on the 7th and 10th day are significantly different. $F = 71.6$ and $p = 1.61 \times 10^{-11}$.

3.4. Effect of residual lufenuron on larval survival:

The LC_{50} of lufenuron for the 2-day-old *T. castaneum* larvae was found to be 60 ng/g (data not shown). The results of the effect of residual lufenuron on the larval survival are shown in Table 3. In order to establish the fact that whether time influence the mortality rate significantly, one-way ANOVA was carried out on the larval mortality data (Table 4). The results indicated that the mean values of the percent mortality observed on 7th, 10th and 15th day were significantly different. In each case,

Table 3
Effect of residual activity of lufenuron on the survival of *Tribolium castaneum* larvae

Interval (in days)	Mortality on 7th day (%)	Mortality on 10th day (%)	Mortality on 15th day (%)
0	66.7 ± 12.6	90.0 ± 8.7	100.0 ± 0.0
10	81.7 ± 7.6	96.7 ± 5.8	100.0 ± 0.0
20	66.7 ± 17.6	78.3 ± 17.6	100.0 ± 0.0
30	78.3 ± 7.6	93.3 ± 6.6	100.0 ± 0.0
40	76.7 ± 12.6	90.0 ± 10.0	100.0 ± 0.0
50	76.7 ± 10.4	96.7 ± 5.8	100.0 ± 0.0
60	85.0 ± 10.0	93.3 ± 5.8	100.0 ± 0.0
70	73.3 ± 10.4	96.7 ± 2.9	100.0 ± 0.0
80	73.3 ± 10.4	93.3 ± 7.7	100.0 ± 0.0
90	66.7 ± 10.4	95.0 ± 5.0	100.0 ± 0.0
Average mortality	74.5 ± 6.4	92.3 ± 5.5	100.00 ± 0.0

Each data point is mean ± S.D. of three independent experiments. The mortality in the control was less than 5%.

100% mortality was observed on 15th day, when larvae were released at 10 days interval for total period of 90 days in contrast to control which showed less than 5% mortality in some cases. The results clearly indicated the potential of lufenuron for the treatment of wheat for controlling *T. castaneum* infestation. Most of the workers have so far studied the effect of BPUs and traditional pesticide residues in wheat grains for insect infestation but not in treated and milled wheat, which is important because the milling process results in high temperature which might possibly degrades the pesticide in some cases. Our finding indicates that lufenuron is not degraded in milling process, which is further confirmed by GC–MS analysis.

3.5. Residual life of lufenuron in wheat flour

Pesticide residue study is one of the important steps among different aspects of the pesticide chemistry, which gives insight into toxic doses, persistence and amount of toxicant in products as well as provides baseline data to assess the risk factors for consumer safety. As previously described $LC_{50} \times 5$ (300 ng/g) was the dose of lufenuron selected for treatment of wheat. However, it was found that the residual concentration of applied lufenuron in wheat flour was neither uniform nor in descending order. This may be because, the whole wheat (2 kg) was treated and milled, and there was a possibility of uneven dispersal of the EC formulation of lufenuron. The results of residual analysis of lufenuron in tested samples are shown in Table 5. However, the residual concentration of lufenuron was sufficient to produce 100% mortality of *T. castaneum* till 15th day exposure, indicating the residual amount, although not uniformly present in the sample, was sufficient to bring about 100% mortality up to 3 months. This indicates that the low concentration of lufenuron, within the range of MRL values of lufenuron set for vegetables (appox. 500 ng/g) is sufficient to control *T. castaneum* larvae in wheat flour.

There have been few reports concerning the dissipation of BPU insecticides in the agricultural products and the fate of these insecticides in vegetables [16–18]. Pesticide residue diminishes with time, although the rate of loss may differ from compound to compound and may vary according to environmental conditions. From the present study it can be concluded that there is no significant loss of lufenuron in the wheat flour when stored at room temperature (20–30 °C) for 3 months, as it is the maximum period for which wheat flour is usually stored under Indian conditions. Further studies on the residue analysis for longer periods are essential in order to calculate the half-life of lufenuron in wheat flour.

Similar kind of studies on other BPUs, *viz.* chlorofluazuron, flufenoxuron, hexaflumuron, triflumuron and teflubenzuron indicates that these compounds at the concentration of 1 ppm are superior to 10 ppm malathion in the protection of treated grains against *S. oryzae* for 12–18 months of post-treatment [19]. Viability of wheat grains treated with the Insect Growth regulators (IGRs) was not affected, as indicated by the germination rate being 90%, favors the use of these compounds for seed grain treatment. [5]. In the class of IGRs, comparatively the CSIs are better controlling agents than Juvenile Hormone Analogs (JHAs) against stored product insect pest populations [20]. BPU compounds show low toxicity to mammals, and they are widely used in agricultural practice. There is further scope to set the MRL values for these compounds in food grains.

In conclusion, the present study provides the methodology for the residual analysis of lufenuron in wheat flour as well as the biological efficacy opens up a new vista for its possible use in protection of the stored food commodities.

Table 5
Residual analysis of lufenuron in treated wheat flour by gas chromatography–mass spectrometry at various post-treatment intervals

Interval (in days)	Residue of lufenuron (ng/g)[a]	Recovery of lufenuron residue (%)
0	285.7 ± 7.5	95
15	255.3 ± 6.5	85
30	315.3 ± 8.1	105
60	230.0 ± 11.0	69
90	207.7 ± 6.1	77

[a] Each data point is mean ± S.D. of triplicate injections. Recovery of residue was calculated by considering 300 ng/g added lufenuron in wheat (true value).

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References

[1] S.R. Dean, R.W. Meola, H. Sittertz-Bhatkar, R. Schenker, J. Med. Entomol. 35 (1998) 720.
[2] S.R. Dean, R.W. Meola, S.M. Meola, H. Sittertz-Bilatkar, R. Schenker, J. Med. Entomol. 36 (1999) 486.
[3] D.G. Stansfield, Canine Pract. 22 (1997) 34.
[4] Y. Ben-Ziony, B. Arzi, J. Am. Vet. Med. Assoc. 217 (2000) 1510.
[5] K.A.M.S.H. Mondal, S. Parween, Int. Pest Manage. Rev. 5 (2000) 255.
[6] M. Martinez-Galera, T. López-López, M.D. Gil-Garcia, J.L. Martinez-Vidala, P.P. Vazquez, J. Chromatogr. A 918 (2001) 79.
[7] A.I. Valenzuela, Y. Picó, G. Font, Rapid Commun. Mass Spectrom. 14 (2000) 572.
[8] A. Sannino, M. Bandini, Rapid Commun. Mass Spectrom. 19 (2005) 2729.
[9] C. Bicchi, C. Balbo, A. Damato, O. Panero, Chromatographia 43 (1996) 439.
[10] A. Balinova, J. Chromatogr. A 823 (1998) 11.
[11] N.G. Tsiropoulos, P.G. Aplada-Sarlis, G.E. Miliadis, J. AOAC Int. 82 (1999) 213.
[12] R.T. Arbogast, in: J.R. Gorham (Ed.), Ecology and Management of Food Industry Pests, FDA Bulletin 4, Association of Official Analytical Chemists, Arlington, VA, 1991, p. 131.
[13] S.G. Salokhe, J.K. Pal, S.N. Mukherjee, Invert. Reprod. Dev. 43 (2003) 141.
[14] V.P. Shah, AAPS J. 9 (2007) E43.

[15] M. Danaher, L.C. Howells, S.R.H. Crooks, V.C. Flajs, M. O'Keeffe, J. Chromatogr. B 845 (2007) 1.
[16] P.G. Aplada-Sarlis, G.E. Miliadis, N.G. Tsiropoulos, J. Agric. Food Chem. 47 (1999) 2926.
[17] N.G. Tsiropoulos, P.G. Aplada-Sarlis, G.E. Miliadis, J. Agric. Food Chem. 47 (1999) 4583.
[18] T. López-López, J.L. Martinez-Vidal, M.D. Gil-Garcia, M. Martiïnez-Galera, J.A. Rodriïguez-Lallena, Pest Manage. Sci. 60 (2004) 183.
[19] A.A. Eisa, I.M.A. Ammar, Phytoparasitica 20 (1992) 7.
[20] K.J. Kramer, H.E. McGregor, Environ. Entomol. 8 (1979) 274.