Indian Journal of Weed Science **49**(4): 329–334, 2017
DOI: 10.5958/0974-8164.2017.00087.9

Quantification of flufenacet residues in soil and wheat grain

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Received: 24 September 2017; Revised: 2 November 2017

ABSTRACT

The terminal residues of flufenacet were quantified in soil and wheat grains. Flufenacet was applied at 250 and 300 g/ha on 21 and 35 days after the sowing of wheat at the Research Farm of Punjab Agricultural University, Ludhiana. Matrix solid phase dispersion (MSPD) method was used for the extraction of flufenacet from soil and grain samples. The herbicide residues were quantified using High Performance Liquid Chromatography (HPLC) equipped with UV-Vis detector and were confirmed with gas chromatographic tandem mass spectrometry (GC-MS/MS). The average recoveries of flufenacet extracted from the matrix ranged from 80.9 to 93.0% and 88.0 to 96.2% when quantified using HPLC and GC-MS/MS, respectively with relative standard deviation less than 10%. Both HPLC and GC-MS/MS offer high reproducibility, however GC-MS/MS was more sensitive having limit of detection (LOD) and limit of quantification (LOQ) as 0.001 and 0.003 µg/g, respectively. Terminal residues of flufenacet in the soil and wheat grain samples were below the detectable limit. Thus, the use of flufenacet in wheat under sub-tropical humid conditions could be considered safe.

Key words: Flufenacet, GC-MS/MS, HPLC, MSPD, Residue, Soil, Wheat grain

India has world's largest cultivated area (30.23 million ha) under wheat and ranks third in production (93.50 million tonnes) after European Union and China, with a productivity of 3.09 t/ ha (DES 2017). Wheat is an important food grain crop in Punjab. It is grown on 3.51 million hectare, with an average annual production of 16.07 million tonnes and productivity of 4.58 t/ha (POP 2017).

Weeds are the major biotic constraint in wheat production, causing grain yield losses from 10 to 80% depending on the weed species and the weed density (Ladha *et al*. 2000, Timsina and Connor 2001). Among grassy weeds *P. minor* Retz. is of major concern in irrigated wheat under rice-wheat system in India (Singh *et al*. 1995, Chhokar *et al*. 2006). Sequential selection pressure exerted by the extensive use of herbicides (*viz*. isoproturon, clodinafop and sulfosulfuron) has led to the evolution of multiple herbicide resistant biotypes of *P. minor* (Chhokar and Sharma 2008). Continuous application of the same herbicide over a long period of time can result in resistance along with residue buildup in soil, which may harm the succeeding crops. So, there is a challenge to develop new herbicides with alternative modes of action which can be used in rotation with the existing herbicides. Flufenacet (4'-fluoro-*N*-isopropyl-2-[5-(trifluoromethyl)-1,3,4-thiadiazol-2 yloxy]acetanilide), is a new oxyacetanilide herbicide. It has been found effective for the control of a wide range of annual grasses in maize, wheat, rice, soybeans, cotton, sunflower, groundnut, tomato and potato (Deege *et al*. 1995, Forster *et al*. 1997, Michel 1998). It is applied pre or early post-emergence and acts as a seedling growth inhibitor by disrupting the biosynthesis of very-long-chain fatty acids in plants with inhibition of cell division and meristematic activity (Senseman 2007).

Flufenacet can cause endocrine disruption, methemoglobinemia and multi-organ effects in blood, kidney, spleen and heart (Christenson 1996), allergic skin reaction and eye irritation. It has been found to affect the fertility in aves and cause delays in the growth and development of aquatic organisms (USEPA 2011). It is a biodegradable compound, but undergoes slow degradation in the environment and in waste water treatment plants (Cheminova 2014). Hence, the residue estimation of this herbicide in soil and edible part of the crop is very essential to determine the duration of herbicide activity in soil and its effect on crop.

Determination of the herbicide residues is a challenging task as low maximum residue limits (MRLs) are imposed by regulatory agencies (Codex Alimentarius Commission 2005, European Union On-line 2005, Korea Food and Drug Administration 2005). Due to the low level of herbicides that may be found in soil/crop, the analytical methods used to

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monitor the herbicide levels in soil, water and food products should be able to identify and quantify the herbicide residues at very low levels (Saieva *et al*. 2004, Taylor *et al*. 2002). There are numerous reports on the efficacy of flufenacet against various weeds in cereal crops, but very few reports are available in literature on the methods of its analysis and its environmental fate in agro ecosystems. Rouchaud *et al*. (1999), Gupta *et al*. (2001) and Gupta and Gajbhiye (2002) performed residue analysis of flufenacet using gas-liquid chromatography (GLC), gas chromatography (GC) and chromatography–selected ion monitoring–mass spectroscopy (GC-MS-SIM). Since the persistence of herbicides is correlated not only with the climatic conditions but also with the management practices and soil physico-chemistry (Ampong-Nyarko and Datta 1991), the behavior of flufenacet in subtropical humid agroclimatic conditions of Punjab needs to be scrutinized. Keeping these points in view the present study was done in order to develop a novel, simple and sensitive MSPD (Matrix Solid Phase Dispersion) method for the efficient extraction of flufenacet and its determination using High Performance Liquid Chromatography (HPLC) followed by confirmation and quantification with GC-MS tandem mass spectrometery (GC-MS/MS).

MATERIALS AND METHODS

Chemicals

The analytical-grade flufenacet (99.1±5% purity) was supplied by Bayer India Ltd. Methanol, water HPLC grade and all other chemicals used in extraction were purchased from Finar Chemicals, Mumbai, India. Only redistilled solvents were used in the study.

Preparation of standards

A stock solution of flufenacet (1000 µg/ml) was prepared by dissolving 10 mg of analytical-grade herbicide in 10 ml of methanol HPLC grade and was stored at - 4 °C. Working standard solutions (0.001, 0.01, 0.1, 0.5, 1, 5, 10 µg/ml) were prepared by dilution with methanol HPLC grade.

Experimental field

A field experiment was conducted at Punjab Agricultural University, Ludhiana (30º 54‘ N latitude and 75º 48‘ E longitude, at a height of 247 meters above the mean sea level), Punjab, India during winter season of 2013-14 and 2014-15. The experimental soil was loamy sand having organic carbon (0.38%), pH (7.90) and EC (0.19 mmhos/ cm). Weekly weather data during the cropping season of 2013-14 and 2014-15 is given in **Figure 1**. Wheat variety (*HD-2967*) was seeded on 15 November 2013 and 2014 and the crop was raised following the recommended package of practices. Herbicides were applied using knapsack sprayer fitted with flat fan nozzle which was calibrated to deliver 375 L of spray solution/ha. Soil (0-20 cm) and grain samples for residue analysis were collected at harvest from the experimental plots, which were sprayed with flufenacet 250 and 300 g/ ha at 21 and 35 days after sowing (DAS) and also from unsprayed control plots. Four soil cores were taken randomly from each plot using the soil auger, excluding the outer 20 cm fringes of the plots. The soil from all cores within a plot was pooled, air dried under shade, powdered and sieved through a 2 mm sieve. The wheat grain samples collected at harvest were cleaned and crushed using mechanical blender.

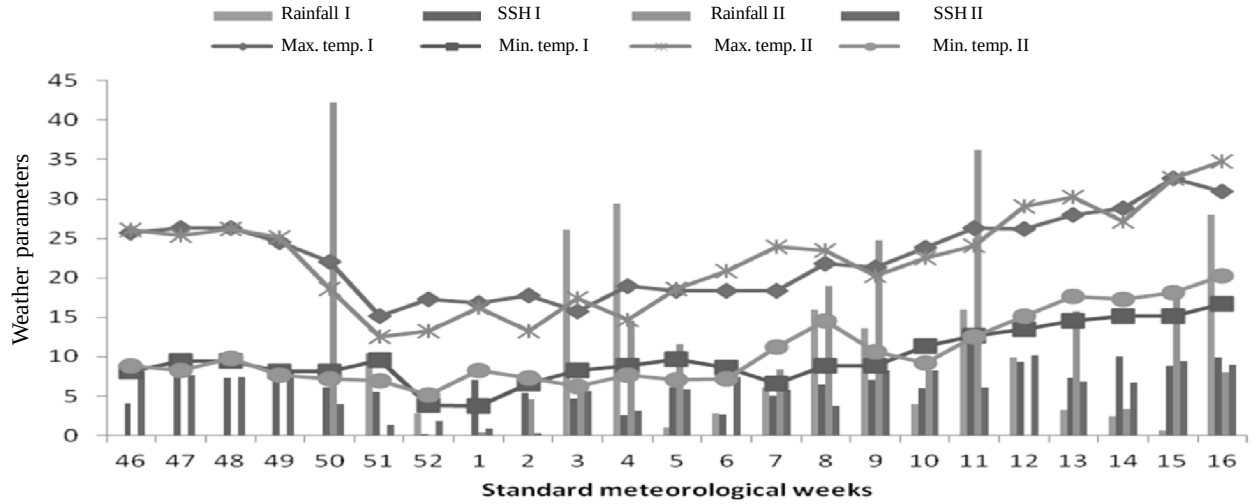


Figure 1. Weather data of 2013-14 (I), 2014-15 (II) during the cropping season at Ludhiana

Herbicide residue analysis

Extraction of flufenacet was done using matrix solid phase dispersion (MSPD). The soil/grain samples were blended with 5 g of florisil (60-200 mesh, HIMEDIA) activated at 200°C for 8 hours. A glass column (50 cm) was plugged with cotton on its lower end. To this column 2 g sodium sulphate, 1.0 g of charcoal and the blend were transferred in succession. The analyte was eluted with acetone:hexane (45 ml) (8:2) and the extract was collected and the solvent was evaporated to dryness using rotary vacuum evaporator at 40°C. The samples were reconstituted in 2 ml methanol and analyzed by HPLC and GC-MS/MS.

Instrument and operating conditions

Gas chromatography: Chromatographic analysis of flufenacet was carried out using GC-MS/MS (Agilent 7890 A series) at CCS Haryana Agricultural University, Hisar. Operating conditions included the use of a HP-5 column (30 m x 0.32 mm i.d. × 0.25 µm film thickness). Helium at the flow rate of 1 ml/ min was used as the carrier gas. Injections (2 µl) were made in the pulse split less mode. The samples were analyzed using the following oven temperature programme: an initial oven temperature of 70°C held for 2 minutes (min) with a ramp of 25°C per min up to 150°C, then a ramp of 15°C per min up to 200°C and finally a ramp of 8°C per min up to 280°C and held for 2 min. Detector: Mass 7000 GC-MS/MS; detector parameters were: source temperature, 230°C; emission current, 35 mA; energy, – 70 eV; repeller voltage, 11 V; ion body, 12 V; extractor, –7.2 V; ion focus, –7.4 V; quadrupole one (MS^1) temperature, 150°C; quadrupole two (MS^2) temperature, 150°C. The retention time under the present experimental condition was found to be 18.44 min.

High performance liquid chromatography: Water HPLC system with 20 ìl injection loop and UV/Vis detector was used. The separation of flufenacet was performed using reverse phase symmetry C18 (5.0 ìm, 4.6 mm × 250 mm column at 210 nm. The mobile phase consisted of methanol: water (80: 20) at the flow rate of 1 ml/min. Under these operating conditions, the retention time of flufenacet was found to be 4.08 min.

Standardization of method

Since the quantitative determination of flufenacet in a given soil or plant sample is directly dependent on the evaluation and interpretation of data, a reliable method is required which is reproducible and can be applicable to different samples. The method was fully validated according to the analytical method recommendations described in the SANCO guidelines in terms of linearity, precision (repeatability and reproducibility) and accuracy (SANCO 2013).

RESULTS AND DISCUSSION

Optimization of matrix solid phase dispersion (MSPD)

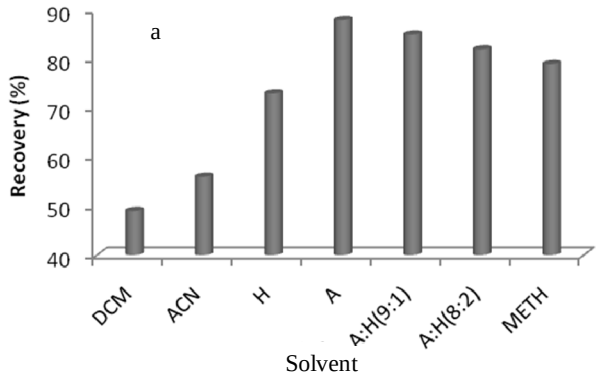
Preliminary experiments were carried out to select the optimum conditions for extraction of flufenacet using MSPD. In MSPD, type and volume of elution solvent is important for the efficient extraction of herbicide from sorbent. Several solvents such as acetone, hexane, acetone: hexane (9:1), acetone: hexane (8:2), acetonitrile, methanol and dichloromethane were evaluated as elution solvents for extraction of flufenacet from spiked soil and wheat grain samples (fortified at 0.01, 0.05, 0.5 and 0.1 µg/g). The percentage recoveries varied from 49 ± 4.01% to 88 ± 5.29% (**Figure 2a**). Use of acetone gave the highest recovery, but the co-elution of matrix co-extracts gave undesirable peaks close to retention time of target analyte in HPLC chromatograms and the results were not interpretable. Based on the results of percent recovery and HPLC chromatograms, acetone: hexane (8:2) was selected as the eluting solvent.

The optimization of elution volume was performed with acetone:hexane (8:2) as elution solvent. The results showed that the recovery of the target compound increased till an increase in volume of elution solvent up to 45 ml and thereafter, equilibrium was attained with further increase in volume (**Figure 2b**).

Method validation

Linearity: The linearity of the method was evaluated from the calibration curve using standard solutions over the concentrations ranging from 0.001 to 10 µg/ ml using HPLC and GC-MS/MS. The response was found to be linear in the range of 0.003 to 10 and 0.001-10 µg/ml in case of HPLC and GC-MS/MS, respectively, with the co-efficient of determination (R^2) > 0.99.

Limit of detection and quantification: The limit of detection (LOD) and limit of quantification (LOQ) were determined based on signal-to-noise ratio (S/N) of 3:1 and 10:1, respectively (Sahoo *et al*. 2013). The LOD and LOQ in this study were calculated as 0.003 and 0.01 µg/g, respectively, in case of HPLC and 0.001 and 0.003 µg/g, respectively, in case of GC-MS/MS.



DCM- Dichloromethane, ACN- Acetonitrile, H- Hexane, A-Acetone, METH-Methanol

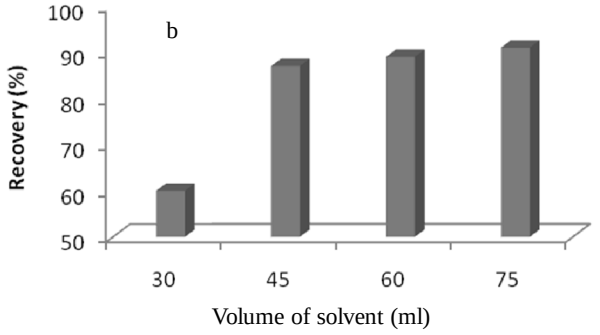


Figure 2 a and b. Effect of different elution solvent types and their volume on flufenacet recovery in MSPD

Accuracy and precision: The accuracy of the analytical method was estimated in terms of percent recoveries. The mean recoveries for the three replicates of spiked samples at different fortification levels (0.01, 0.05, 0.5 and 0.1 µg/g) ranged from 82 ± 4.3 to 96 ± 3.4% in soil, and 80 ± 2.7 to 93 ± 2.6% in wheat grains. The precision values expressed as relative standard deviation (RSD) were <10% irrespective of sample type and spiking levels indicating good reproducibility of the method **(Table 1)**.

Residues of flufenacet under field conditions

No quantifiable amount of residues of flufenacet was detected in the soil and grain samples at harvest by HPLC at both the application rates in 2013-14 and 2014-15 **(Table 3)**. The maximum residue limit (MRL) of flufenacet for wheat grain has been set as 0.05 µg/g by EFSA. Under the present experimental conditions, the residues in wheat grain at harvest were below the MRL set by EFSA (EFSA 2012). Therefore, it could be considered nontoxic to food and environment. Though the proposed method was carefully designed and no interference of matrix peak was observed in the samples and residues were well

Table 1. Average recovery of flufenacet from fortified soil and wheat grains

Matrix	Level of fortification (µg/g)	Recovery (%)	
		HPLC	GC-MS/MS
Soil	1	93.4±6.1	96.2±3.5
	0.5	91.8±5.5	94.7±4.6
	0.05	88.3±6.5	93.6±4.3
	0.01	82.6±4.3	92.8±2.1
Grain	1	92.5±1.7	93.1±3.4
	0.5	91.7±2.3	93.5±2.4
	0.05	85.3±7.2	90.9±2.0
	0.01	80.9±2.7	88.0±3.0

Each value is mean±SD of three replicates

Table 2. Programming parameters for MRM

Compound	Molecular mass	Precursor ion (m/z)	Collision energy	Monitoring ions (m/z) and relative abundance (in bracket)
Flufenacet	363	151 211	10 4	95 (50694.4), 136.1 (69036.4) 123.1 (17976.9)

Table 3. Flufenacet residues in soil and wheat grains at harvest

Flufenacet		Matrix	Residues (µg/g)	
Dose of application	Time of application		HPLC	GC-MS/MS
250 g/ha	21 DAS	Soil	<0.01	<0.003
250 g/ha	21 DAS	Grain	<0.01	<0.003
250 g/ha	35 DAS	Soil	<0.01	<0.003
250 g/ha	35 DAS	Grain	<0.01	<0.003
300 g/ha	21 DAS	Soil	<0.01	<0.003
300 g/ha	21 DAS	Grain	<0.01	<0.003
300 g/ha	35 DAS	Soil	<0.01	<0.003
300 g/ha	35 DAS	Grain	<0.01	<0.003

DAS-Days after sowing

below the MRL, still the confirmatory test plays essential part in quantification of residues in trace quantities. In the present study confirmatory method was developed using GC-MS/MS to identify and detect the flufenacet residues. Based on the LOD it was observed that GC-MS/MS was at least 3 fold more sensitive than HPLC/UV and could be used as alternative instrument for detection of flufenacet residues. The confirmation and quantification of flufenacet was achieved by developing a programming in SCAN, product ion and finally multiple reaction monitoring (MRM). Characteristic ions with relatively high intensity and strong anti-turbulence were selected as monitoring and quantitative ions (**Table 2** and **Figure 3**).

Analysis of soil and wheat grain samples using GC-MS/MS showed that residues were below 0.003 µg/g in 2013-14 and 2014-15 (**Table 3**). It revealed that none of the flufenacet application rates or timings could cause the herbicide to persist in detectable

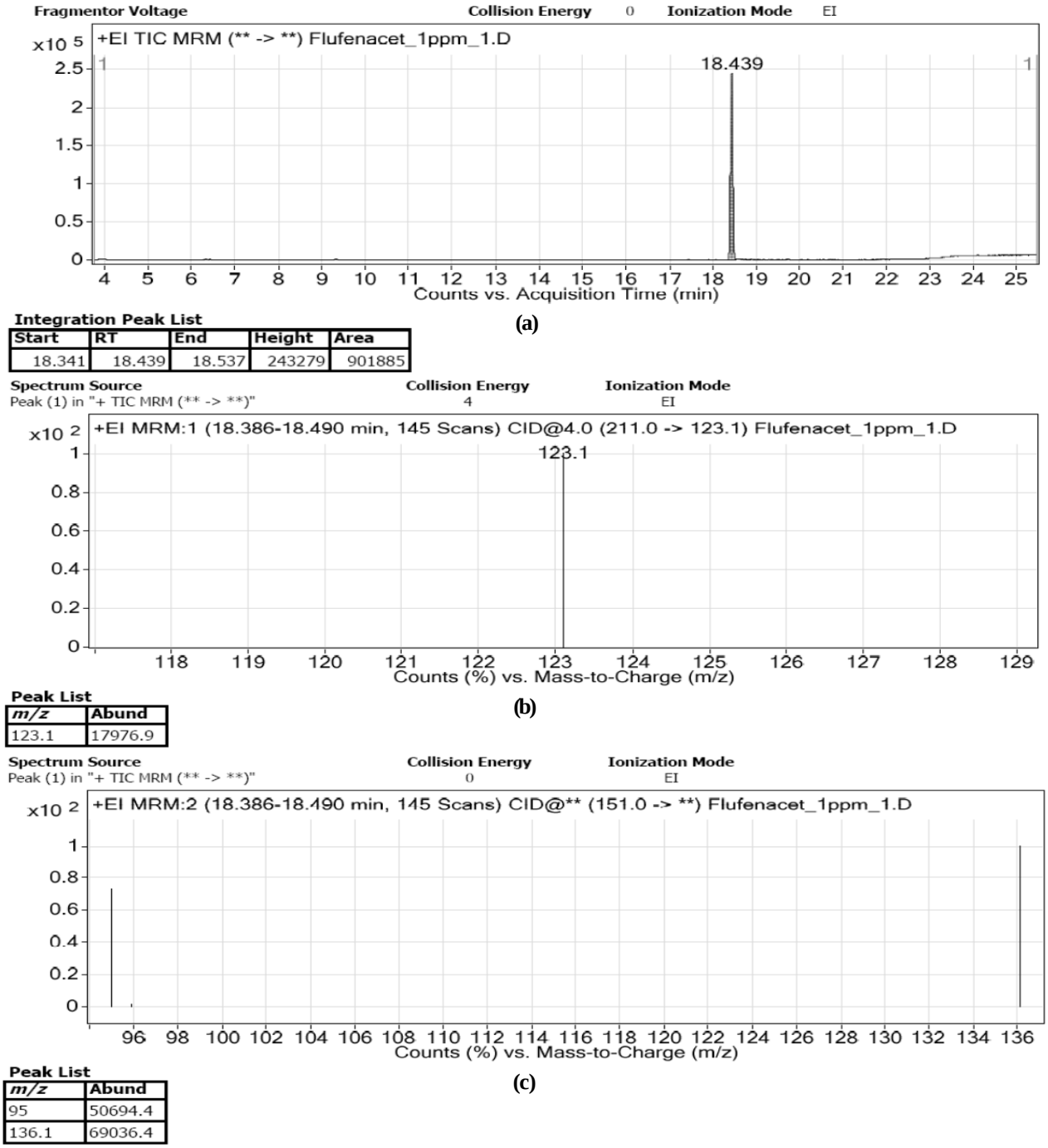


Figure 3. (a) User chromatogram of flufenacet; (b, c) Mass spectrums of flufenacet at different collision energies scanned in MRM

concentrations in the soil till harvest, thus confirming the rotational safety of flufenacet to succeeding crops, as well as the safety of wheat grains from the consumption point of view. This may be attributed to the dissipation of flufenacet, due to longer time duration between the herbicide application and harvest. These results are in conformity with the studies conducted by Gupta *et al*. (2001) and Rouchaud *et al.* (1999).

MSPD method has a good analytical performance in terms of accuracy, precision, selectivity, sensitivity and rapidity and could be used for the detection and quantification of flufenacet residues in soil and wheat grain. Both HPLC and GC-MS/MS offer high reproducibility, but GC-MS/MS was more sensitive for quantification of flufenacet residues. As the residue of flufenacet applied at 250

and 300 g/ha at 21 and 35 DAS were below 0.001 µg/g, flufenacet application at those doses and time could be considered as an option for controlling *P. minor* in wheat under subtropical conditions of Punjab.

ACKNOWLEDGEMENT

The authors are thankful to University Grants Commission, New Delhi, India for financial support to the student involved in this study.

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