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Research Article

Method validation and monitoring of emamectin benzoate in mature banana fruit with peel and pulp through Liquid chromatography-Mass spectrometry/ Mass spectrometry (LC-MS/MS)

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Abstract
Emamectin benzoate has been frequently used in the banana ecosystem to combat the damage of pseudostem weevil. Therefore, the present study was conducted to validate the method, to assess harvest time residues and monitor emamectin benzoate residues in mature banana peel and pulp samples through LC-MS/MS. The validated method was used to determine emamectin benzoate residue in market banana samples. The study used Waters Alliance LC and Acquity TQD with an electrospray ionization interface in the positive ion mode. An isocratic flow of 0.1% formic acid (HCOOH) in water and 0.1% HCOOH in acetonitrile (CH3CN) was utilised for separation. CH_3CN was utilised to extract emamectin benzoate residue from the samples, and a dispersive solid-phase extraction technique was used for subsequent cleanup. Linearity tests were performed with standard solutions containing 0.01 to 0.1 g mL^{-1}, with three replicates for each concentration. For mature banana peel & pulp and mature banana pulp matrices, satisfactory recoveries of 79.85 to 95.09% and 89.20 to 100.94%, respectively and high precision relative standard deviations of 0.56 to 2.34% and 2.33 to 6.88%, respectively were obtained. For mature banana (peel and pulp, pulp alone) fruits, the lower detection and quantification limits were (0.003, 0.008), and (0.002, 0.007). The validated approach was utilised to analyse mature banana fruit samples obtained from emamectin benzoate treated fields and banana samples purchased from the local market. Results showed satisfactory validation of parameters like linearity, the limit of detection and quantification, and recovery for determining emamectin benzoate residues in banana fruit.

Keywords: Emamectin benzoate, LC-MS/MS, Mature banana, Recovery, Residues

INTRODUCTION

Banana is a very popular fruit on the global market, ranking second only to rice, wheat, and maize in terms of importance as a food crop. The term 'banana' refers to the fruit of an evergreen monocotyledonous, perennial, gigantic herb that is entirely subtropical and belongs to the genus Musa of the Musaceae family (Singh *et al*., 2016). It is grown in over 130 countries, mostly in the tropical and subtropical zones, with its origin in South-East Asia. Because of its inexpensive cost and great nutritional value, bananas are a very popular fruit among consumers. It also has high export potential. Baby food is made using banana fruit powder. Banana consumption lowers the risk of heart disease and is recommended for people with high blood pressure, arthritis, ulcers, gastroenteritis, and kidney problems (Suganthi *et al*, 2018). Banana pulp (BP), which is the edible part of the fruit, has an abundant amount of nutrients. Studies conducted on Banana pulp have elucidated different aspects ranging from its use as an ingredient for food enrichment to extraction and isolation of many health-beneficial components, such as different types of starch, cellulose and bioactive compound (Khoozani *et al*., 2019). Phenolics are significant secondary metabolites found in high concentrations in banana, compared to other fruits. Phenolic chemicals have been related to a variety of health advantages, including the prevention of heart disease, cancer, diabetes, and obesity (Boots *et al*., 2008).

The main constraint to the banana production system is pest and disease stresses. It is especially susceptible to banana stem weevil, *Odoiporuslongi collis* (Oliver), banana corm weevil, *Cosmopolites sordidus* (Germar), aphid, and a few other important insect pests. Banana stem weevil is a monophagous pest of banana, whereboth larvae and adults cause severe crop damage, hampering production and productivity. Weevil is a threat to commercial banana cultivars such as nendran, red banana, poovan, and dwarf Cavendish, with a yield loss from 10% and scaling upto90% (Chowdhury, 2015; Padmanaban, 2018).

For decades, banana crop was treated with pseudostem injections of monocrotophos, phorate, carbofuran, quinalphos, cypermethrin, dimethoate and triazophos (Kannan *et al*., 2021). Insecticides were injected into the stems of plants at monthly intervals from the fifth to seventh month of crop age (Reddy *et al*., 2020). However, in India, pesticides previously used in bananas, such as monocrotophos and triazophos, were restricted/banned(Central insecticide board and registration committee, 2022).Emamectin benzoate, a green insecticide compound effective against pseudostem borer, is now used by farmers in major banana-growing districts of Tamil Nadu in India.Emamectin benzoate is a macrocyclic lactone insecticide derived from the avermectin series of natural products (Dybas et al., 1989).As a foliar insecticide, it acts by stimulating the release of γ-aminobutyric acid to inhibit neurotransmitters, causing insect paralysis and subsequent death, and protects crops from coleopteran (Grosman and Upton, 2006) and lepidopteran pests (Jansson *et al*., 1996). Owing to the low-application dosage and broad-spectrum activity, emamectin benzoate has gained significant popularity in agricultural production. Trunk injection of emamectin benzoate was effective against emerald ash borer and Pinewood nematode in forest ecosystems (Ouyang *et al*., 2023).

Zhou *et al*. (2016) and Deng *et al*. (2020) developed an ultra-high-performance liquid chromatography-tandem mass spectrometry (UPLC/MS/MS) method to study the residue and dissipation of benzoate in tea and rice. Liu*et al*. (2012) and Wang *et al*. (2012) used LC-MS to determine the residues of emamectin benzoate in food and water. Carbendazim residues were detected in market banana samples from Tamil Nadu, India (Paranthaman*et al*., 2012). Chlorpyriphos residues were detected in Canary Island banana peels and pulp (Hernandez-Borges *et al*., 2009). In light of this, emamectin benzoate was chosen for the current studies based on the preliminary survey conducted in banana-growing regions across seven agro-climatic zones in Tamil Nadu, India. The present study aimed to develop simplified extraction and cleanup technique for assessing pesticide residues in mature banana with pulp, peel and pulp, to determine harvest time residues in mature banana fruit samples in emamectin-treated plants and to monitor insecticide residues in market samples of mature banana fruit.

MATERIALS AND METHODS

Chemicals

The reference standard of emamectin benzoate was purchased from Dr. Ehrenstorfer (Augsburg, Germany). Formic acid and acetonitrile of MS grade were purchased from Sigma Aldrich (Mumbai. India). Magnesium sulphate and anhydrous sodium chloride (heated at 650°C for 4 hours and kept in a dessicator) were procured from Merck India Ltd. (Mumbai, India). Graphitized carbon black (GCB) and primary secondary amine (PSA) were purchased from M/s. Agilent Technologies (Agilent Technologies India Pvt Ltd, Chennai, India). Ultra-pure water was collected using a Merck Millipore system. The sample extracts were filtered using nylon filters (0.2 µm) from PALL Life Sciences (PALL Life Sciences, Banglore, India). Stock solution (400 µg mL^{-1}) of emamectin benzoate was prepared by dissolving the technical-grade substance in acetonitrile. This was labeled and kept at -20°C in a freezer. The intermediate stock solution and working standard solution were made from the stock solution.

Apparatus and chromatographic conditions

The liquid chromatographic (LC) analysis was performed using the Waters Alliance 2695 Separation system, which consists of an autosampler, quaternary pump, and membrane degasser. Using an ESI interface, an acquity TQD was linked for the mass spectrometry. A 5 m (4.8 x 250 mm) X Terra analytical C_{18} column was used for the analyte separation. The column's temperature was set at 30°C. A mobile phase containing 0.1% HCOOH in water (A) and 0.1% HCOOH in CH_3CN (B) was used for separation, following an isocratic flow of 30% A + 70% B and the flow rate was 0.5 mL min^{-1}. An injection volume of 10 µL and a 5 minutes run time were used to elude the analyte.

Optimization of MS instrument conditions

The source block and desolvation temperatures were set at 150 and 500°C, respectively, and the desolvation (drying gas) and cone gas flows were set at 1100 and 80 L h^{-1} and 0.20 ml min^{-1}, respectively. The collision gas was argon, and the desolvation and cone gases were nitrogen. Tune Master (Mass Lynx software) was used to optimize the lens voltages and the flow rate of the 20 µL min^{-1} syringe pump infusion. The initial tunings were performed by infusing a working standard solution. Chromatograms were taken while the Electrospray ionization (ESI) interface was in positive and full scan modes. The analyte was identified by configuring the chromatographic run in MRM (Multiple reaction monitoring) mode.

Extraction and cleanup of mature banana fruit with pulp, peel and pulp

With a few minor changes, the QuEChERS approach (Anastassiades *et al*., 2003) was employed for sample extraction and cleanup. A representative mature banana peel and pulp sample of 10g was accurately weighed in a 50 mL centrifuge tube and 10mL of acetonitrile was added. Using a silent crusher (Germany-made Heidolph brand), the samples were homogenised at 15,000 rpm for five minutes. The centrifuge tube was vortex-shaken for one minute after adding 4 g of anhydrous magnesium sulphate and 1 g of sodium chloride and then centrifuged for ten minutes at 6000 rpm. After centrifugation, 6ml of the supernatant solution was transferred into a 15 ml centrifuge tube containing 10 mg of GCB, 100 mg of PSA, and 600 mg of anhydrous magnesium sulphate. Finally, 4 mL of the extract was transferred into test tube and evaporated at 40 °C in a Turbovap (Caliper life sciences, USA) concentrator using nitrogen gas until it was nearly dry. The final volume was reconstituted to 1 ml with acetonitrile, filtered through a 0.2 µm filter membrane, and then put into a 1.5 mL glass auto sampler vial for LC/MS-MS analysis.

For the cleanup of mature banana with pulp samples, 6 mL of the supernatant extract was added with 100 mg of Primary secondary amine (PSA) and 600 mg of anhydrous magnesium sulphate. Then, 4 mL of supernatant was transferred and concentrated using nitrogen gas in a glass tube and then reconstituted with acetonitrile to make 1 mL. This extract was transferred into a 1.5 mL glass autosampler vial for LC/MS-MS analysis after filtering via a 0.2 µm filter membrane.

Method validation

The analytical method used to determine pesticide residues in mature banana fruit was validated in the laboratory by characterising the method's performance in accordance with Directorate- General for Health and food safety (SANTE, 2021)requirements. The technique validation findings are used to evaluate the quality, dependability, and consistency of the analytical results acquired from the analysis. The present study verified the analytical method by calculating and assessing performance characteristics such as linearity, limit of detection (LOD), limit of quantification (LOQ), matrix effect, recovery, precision, and uncertainty. All studies were performed on identical blank mature banana fruit samples free of residues.

The linearity of the detector response for the test analytes was evaluated by injecting five calibration working standard solutions in LC-MS/MS at concentrations of 0.01, 0.025, 0.05, 0.075, and 0.1 g mL^{-1} with three replicate injections per concentration.

The LOD and LOQ were calculated by injecting samples of mature banana fruits with pulp, peel and pulp at the lowest spiking concentration of 0.01 g mL^{-1} with seven replications. The standard deviation, standard error, and regression were used to calculate the LOD and LOQ (SANTE, 2021). By examining the solvent and matrix-match requirements of the test analytes, the matrix effect (ME) was calculated using the formula:

$$x = \frac{(\text{Peak area of matrix standard} - \text{Peak area of solvent standard})}{\text{Peak area of solvent standard}} * 100 \quad \text{Eq. 1}$$

Before extraction and cleaning, untreated ripened banana samples were homogenised and spiked at concentrations of 0.01, 0.025, 0.05, 0.075, and 0.1 g mL^{-1}. For each spiking concentration, seven replications were performed. Blank analyses were performed alongside the spiked samples to evaluate for matrix interference. The relative standard deviation (RSD%) was used to assess the procedure's accuracy based on the results of the recovery analyses.Calculations for the recovery percentage, matrix effect, relative standard deviation, and residue concentration were made using the information from the LC-MS/MS chromatogram. The residue quantification procedure was carried out using the following equation.

$$\text{Residues (mg g}^{-1}) = \frac{s}{td} \times \frac{Wstd}{Ws} \times Vs \quad \text{Eq.2}$$

As - Sample peak area
Astd - Standard peak area
Wstd - Weight of standard (µg mL^{-1})
Ws - Weight of the sample (g)
Vs - Volume of the final extract in mL

Field experiment

The field experiment was conducted in Agriculture Research Station, Bhavanisagar, India(11.485324°; 77.137722°). Banana plants cv Nendran were planted in an area of 1500 m^2. Emamectin benzoate at 16 g L^{-1} (As per the farmers' practice) was injected at the recommended dose of 4 mL/tree using banana stem injector at 5, 6 and 7th months post-planting. Common agricultural and fertilization practices were followed. Representative samples of mature banana fruits, weighing approximately 2 kg were collected from the experimental plots at the harvest stage. The samples were then taken to the laboratory and stored at 4 °C until analysis.

Monitoring of market samples

For this study, the mature banana pulp (25 nos.) sold for fruit purposes were obtained from several marketplaces in Tamil Nadu, India. The samples (1–2 kg each) were packed in polythene bags, labelled, and delivered to the Pesticide toxicological laboratory (Tamil Nadu Agricultural University, Coimbatore) for analysis. The samples were homogenised and kept at 40°C in glass containers. The samples were analysed for emamectin benzoate residue estimation using the above-validated method (Anastassiades *et al*., 2003).

RESULTS AND DISCUSSION

The LC-MS/MS instrument was optimised to determine the optimal instrument settings for target analyte identification. Tuning was accomplished using the mass spectrometer's MRM mode and direct infusion of individual analyte standard solution of emamectin benzoate. Better fragmentation was achieved with collision energies ranging from 2 to 80V. The typical ions detected using ESI positive mode for emamectin benzoate were [M+H]+ parent ions.

Good linearity of the calibration curve for emamectin benzoate was established and suitable linear regression coefficient (r^2) values were obtained (0.961 to 0.994). The method's specificity was determined by analysing blank samples. The absence of background peaks at retention durations of the measured analytes above a signal-to-noise ratio of 3 demonstrated the absence of interferences in the selective ion monitoring approach. In reagent blank and blank samples, the response was 30% (SANTE, 2021). The validated method's excellent sensitivity was used to quantify trace amounts of pesticide residues in mature banana fruit matrices.Prior to the present study, emamectin benzoate in mature bananas with pulp, peel and pulp samples had not been validated with LC-MS/MS system. Paranthaman *et al.* (2012) utilised 100 mL of CH3CN in 50g of material to extract residues from banana. In the present study, only 10 g of the sample and 10 mL of CH_3CN were used for extraction. Fu *et al*. (2016) validated pyraclostrobin residue determination in banana peel and pulp using 10g of sample in 10 ml of acetonitrile in high-performance liquid chromatography.

In the present investigation, the LOD and LOQ of mature banana fruit with peel and pulp were 0.003 and 0.008 µg g^{-1}, respectively, while mature bananas with peel were 0.002 and 0.007 µg g^{-1}. Suganthi *et al*. (2018) reported lower limits of detection and quantification of thiamethoxam in mature banana fruit with pulp of 0.002 and 0.008 µg g^{-1}, consistent with the present findings.The recovery of mature banana with peel and pulp ranged from 79.85 to 95.09%, while the recovery of pulp alone ranged from 89.20 to 100.94% in the present study (Table 2). Satisfactory recoveries of thiamethoxam ranging between 90.94 – 109.22% were obtained for the banana mature fruit pulp matrix by Suganthi *et al*. (2018) and these findings are concurrent with the present study. As per SANTE (2021) (70 – 120%), satisfactory recoveries were obtained for the mature banana fruit with pulp, peel, and pulp matrices. Jyot *et al*. (2014) also reported an acceptable recovery of emamectin benzoate in okra (84.20 to 94.56%) using HPLC at the fortification level 0.05 – 1 µg g^{-1}. Similarly, good recoveries of emamectin benzoate in immature grapes (99.77 – 102.44 %) and mature grapes (92.19 – 103.07 %) were reported with acetonitrile as an extraction solvent (Reddy *et al*., 2021). Wang *et al*. (2021) validated emamectin benzoate in tender and older cowpea with satisfactory recoveries ranging from 83.55 – 103.69 %.

The sample matrix can significantly impact pesticide analysis and the results' quality (Smeraglia *et al*., 2002; Santilio *et al*., 2014). Matrix interferences can suppress or enhance analytical signals, resulting in low or high analyte recoveries (Zhang *et al*., 2011). The present study showed matrix effect for emamectin benzoate residues at different spiking levels was below ± 20 for all the matrices tested (Table 1). In mature banana matrices, ion enhancement and ion response suppression were observed for emamectin benzoate.The mature banana fruit (peel and pulp, pulp) from emamectin benzoate stem injected plant contained no residues at harvest stage. It is safe to consider for pseudostem

Table 1. Linearity, LOD, LOQ and matrix effect of mature banana fruit with pulp, peel and pulp on emamectin benzoate

Matrix	Calibration range (mg L^{-1})	Regression equation	Correlation coefficient (R^2)	Matrix effect (%)	LOD	LOQ
Mature banana fruit with peel and pulp	0.01-0.1	y = 1448.94x + (-5318.06)	0.994	-18.69 – (-13.78)	0.003	0.008
Mature banana fruit with pulp	0.01-0.1	y = 1105.94x + (-1425.65)	0.961	-11.78 – 11.19	0.002	0.007

LOD – Limit of detection; LOQ – Limit of quantification

Table 2. Recovery of emamectin benzoate from banana mature fruit with peel and pulp

Spiking level (ug/g)	Recovery (%)									
	R1	R2	R3	R4	R5	R6	R7	Mean	SD	RSD
0.01	94.47	93.87	96.63	95.81	93.80	94.12	96.92	95.09	1.34	1.40
0.025	86.32	86.17	86.87	85.97	86.10	85.12	84.16	85.82	0.90	1.04
0.05	86.78	86.77	87.51	87.91	87.67	87.11	86.72	87.21	0.49	0.56
0.075	76.84	82.36	81.73	78.72	80.45	79.49	79.38	79.85	1.87	2.34
0.1	86.66	86.26	89.08	90.70	80.18	88.23	87.58	87.81	1.66	1.89

SD - Standard deviation; RSD – Relative standard deviation

Table 3. Recovery of emamectin benzoate from banana mature fruit with pulp

Spiking level (ug/g)	Recovery (%)									
	R1	R2	R3	R4	R5	R6	R7	Mean	SD	RSD
0.01	97.91	91.00	93.27	92.54	93.46	96.05	86.86	93.01	3.55	3.82
0.025	83.65	87.02	89.26	89.90	89.90	90.74	93.91	89.20	3.19	3.58
0.05	99.83	99.89	99.51	94.48	97.60	97.90	101.65	98.70	2.30	2.33
0.075	94.27	98.02	112.43	97.90	93.54	106.57	103.85	100.94	6.94	6.88
0.1	101.81	100.51	103.28	97.93	92.98	99.28	90.65	98.06	4.65	4.74

SD - Standard deviation; RSD – Relative standard deviation

injection of emamectin benzoate upto 8 months for management of pseudostem borer.Suganthi *et al*. (2018) also reported stem injection of thiamethoxam in banana plants at 6th and 7th months, showing no residues in the mature banana pulp matrix at harvest stage. Malhat *et al.* (2013) reported that residues of emamectin benzoate reached below MRL in tomato fruits within 10 days after spraying. Zhou *et al*. (2016) observed rapid dissipation of emmectin benzoate in tea with a half-life ($t_{1/2}$) of 1 – 1.3 days.

In the present study, the analysis of commercially available mature banana pulp (25 in number) samples obtained from different retail marketplaces in Tamil Nadu indicated the efficiency of the standardised approach in assessing trace quantities of emamectin benzoate. The emamectin benzoate residues have not been found in any mature banana fruit samples.There is no MRL (Maximum residue limit) value for emamectin benzoate in banana fruit fixed by Codex Alimentarius Commission (Masson-Matthee, 2007).

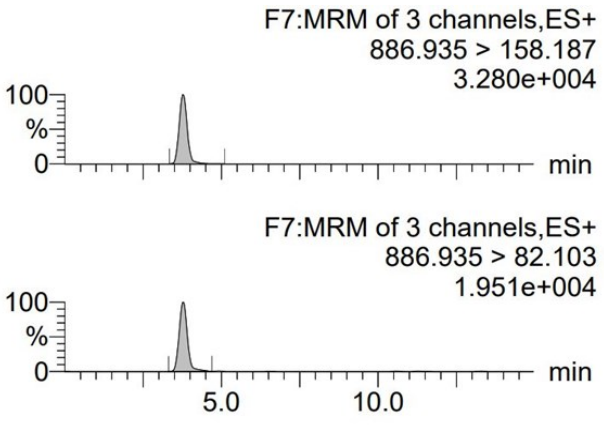
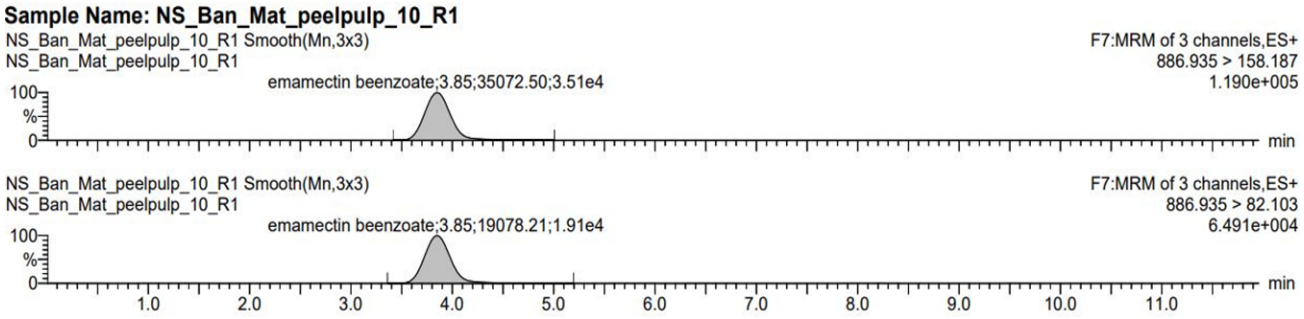


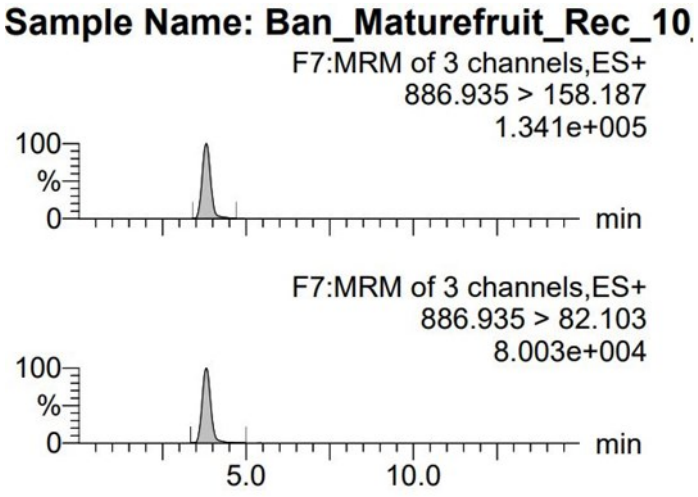
Fig. 1. *LC-MS/MS Standard chromatogram of emamectin benzoate (0.01µg g^{-1})*

Conclusion

In conclusion, this study describes an LC-MS/MS-based rapid, easy, and sensitive analytical approach for



A. Mature banana fruit with peel and pulp



B. Mature banana fruit with pulp alone

Fig. 2. *LC-MS/MS recovery chromatogram of emamectin benzoate in mature banana fruit with pulp, peel and pulp (0.01µg g^{-1})*

measuring emamectin benzoate in mature banana fruit. The optimized dispersive solid-phase extraction procedure is also inexpensive for determining emamectin benzoate residues in banana fruit. The emamectin benzoate mature banana fruit matrix produced good LOQ and LOD values. Satisfactory validation parameters, such as linearity, recovery, precision and LOQ were established for emamectin benzoate in mature banana peel and pulp matrices. No study has been reported till now about determining emamectin benzoate residues in banana fruit matrices. The present study is a novel one that deals with banana fruit matrices from a pesticide residue perspective. Thus, LC-MS/MS-based study will be helpful to various stakeholders, including scientific, regulatory, research and farming.

Conflict of interest

The authors declare that they have no conflict of interest.

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