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# Method validation and monitoring of thiamethoxam insecticide residues in fresh and processed button mushroom using LC-MS/MS

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**Abstract**
A quick, effective analytical technique was developed and validated to determine thiamethoxam residues in fresh and processed (mushroom in brine and dry mushroom powder) button mushroom by adopting modified QuEChERS method in LC-MS/MS. Various parameters such as linearity, limit of detection, limit of quantification, precision, and accuracy were considered to validate the modified method. The linearity of the calibration curve was established and shown good relationship with the $R^2$ value 0.998 whereas LOD and LOQ was 0.003 and 0.01 µg/g respectively. Mean recoveries of thiamethoxam were ranged 89.30 to 108.3, 95.96 to 116.37 and 97.17 to 114.26% at spiking level from 0.01 to 0.1 µg/mL whereas RSD values ranged from 4.04 to 8.31, 5.67 to 8.70 and 3.38 to 9.97 for fresh button mushroom, button mushroom in brine and dry button mushroom powder, respectively. The validated method was used to monitor the mushroom samples. The results revealed that, there were no residues of thiamethoxam insecticide in any samples.

**Keywords:** LC-MS/MS, fresh button mushroom, button mushroom in brine, dry button mushroom powder, QuEChERS, thiamethoxam, monitoring

## Introduction
Mushrooms are recognised as a good dietary food in addition to an important cradle of bioactive compounds of therapeutic value (Cheung *et al*., 2010) [3]. They are regarded as good supplement to cereals, due to their abundance in proteins, minerals, fibre and essential amino acids. Their consumption is increasing recently in the market as both fresh and processed products. China stands first in total production of mushroom in the world (Singh *et al*. 2021) [15]. In case of India, mushroom production is about 0.28 MT per annum (Indiastat, 2021-22) [6]. In India, button mushroom accounts for about 90 percent of total mushroom production in India (Mehta *et al*., 2011) [11]. Mushroom is prone to a number of insect pests that are known to cause significant crop losses (Nongthombam *et al*., 2021) [12]. The major pests attacking mushroom are sciarids (*Lycoriella* sp.), Phorids (*Megaselia* sp.), springtails (*Lepidocyrtus* sp.) and mites (*Tyrophagus* sp.) (Rijal *et al*., 2021) [13]. To prevent yield loss, pesticides are frequently utilized mainly throughout the growing process of mushroom during cultivation. Use of pesticides in uncontrolled manner results in causing pesticide residues on farm products (Chen *et al*., 2014) [4].
Thiamethoxam is a pro-insecticide belonging to second generation thionicotinyl group which was synthesized during 1991 (Maienfisch *et al*, 2001) [9]. Thiamethoxam acts by binding to nicotine acetyl choline receptors affecting nervous system of insects. (Taillebois *et al*., 2014) [17] It is a broad spectrum insecticide which is effective against insect families such as Hemiptera, Diptera, Lepidoptera, Coleoptera and Thysanoptera. It was widely used in button mushroom cultivation for the control of sciarid as these pests cause enormous damage to mushroom fruiting bodies (Chen *et al*., 2023) [5]. Apart from benefits in controlling pests, it has negative effects on pollinators, aquatic invertebrates and mammals (Thompson *et al*., 2016). However, multiple analytical techniques have been described for locating and measuring thiamethoxam residues in different matrices, including fruits, vegetables, pulses, and cereals, but a few studies were present in button mushroom. Thiamethoxam residues were analysed using liquid chromatography equipped with a diode array detector (HPLC-DAD) in tomato (Malhat *et al*., 2014) [10], UHPLC coupled with mass spectrometer in different mushrooms (Lu *et al*., 2018) [18], LC-MS/MS in banana stem and fruit (Suganthi *et al*., 2018) [16], liquid

chromatography equipped with a diode array detector (HPLC-DAD) in potato (Abd-Alrahman *et al*., 2014) [1]. However, little studies have examined thiamethoxam residues in button mushroom, but there are no studies prevalent to processed button mushroom (Mushroom in brine and dry mushroom powder), as per our knowledge. Hence, this research was conducted to validate a method for analysis of thiamethoxam residues in fresh and processed button mushroom (Mushroom in brine and dry mushroom powder) using LC-MS/MS.

## Material and Methods

### Chemicals and Reagents

Certified Reference Materials (CRM) were purchased from Sigma Aldrich, Bangalore, India. Acetonitrile (C2H3N) of LC-MS grade (LiChrosolv) was procured from Germany. The Formic acid (≥ 99.00% purity) was bought from Fisher chemicals. Anhydrous sodium chloride (NaCl) and magnesium sulphate ($MgSO_4$) were purchased from Merck in Mumbai, India, and heated for four hours at 650°C in a muffle furnace before being kept in airtight desiccators until use. Graphitized Carbon Black (GCB) and Primary Secondary Amine (PSA) (particle size of 4) were purchased from Agilent Technologies (Palo Alto, CA, USA).

The thiamethoxam reference standard (99.7%) was bought from Dr. Ehrenstorfer in Augsburg, Germany. The MS-grade acetonitrile and formic acid were bought from Sigma Aldrich in Mumbai, India. Merck India Ltd. (Mumbai, India) supplied the magnesium sulphate and anhydrous sodium chloride, which were preheated at 650 °C for four hours before use. Primary secondary amine (PSA) and graphitized carbon black (GCB) were obtained from M/s. Agilent Technologies (Agilent Technologies India Pvt. Ltd., Chennai, India). Ultrapure water (18 Ω) was obtained from the Millipore Q purification system, (Merck Millipore, Burlington, USA).

Primary stock solution of thiamethoxam (99.7%) purity was prepared by dissolving in 10.1 mg of analyte in 25 ml Liquid Chromatography Mass Spectrometry grade acetonitrile to prepare 400 µg/ ml of primary stock solutions. The flask was labeled and stored in a deep freezer at a temperature of -20 ˚C. Working standard solutions of five different levels were prepared by adding LCMS grade acetonitrile for LC-MS/MS compound with the appropriate quantity of the required solution. The matrix-matched standard solutions at 0.01 to 0.1 µg/ml concentrations were prepared by adding the blank extracts of different matrices of fresh and processed button mushroom (Mushroom in brine and powder) separately.

### Sample pretreatment and cleanup sorbents Optimisation

Fresh (250 g) and processed (250 g) button mushroom samples of the same batch were collected from the markets. The collected mushroom samples were taken to the laboratory. The samples were homogenized separately in a blender and carried out residue analysis. The processed samples were analysed immediately and/or stored at -20 °C for further analysis.

The QuEChERS method (Anastassiades *et al*., 2003) [2] was refined and adopted for this analysis. A representative sample of 5 g had been taken in a 50 ml centrifuge tube, 20 ml of acetonitrile was added and the mixture was vortexed for one minute. Then, four grams of anhydrous $MgSO_4$ and one gram of NaCl were added, again thoroughly mixed by the vortexer and centrifuged for 10 minutes at 6000 rpm. Six ml of the supernatant aliquot from the centrifuged sample was transferred into a 15 ml centrifuge tube prefilled with 600 mg of anhydrous magnesium sulphate, 100 mg of PSA and 10 mg of GCB. After being vortexed for one minute, the mixture was centrifuged at 3000 rpm for 10 minutes. From the upper extract, 4 ml was taken, and transferred into a turbovap tube where it was concentrated to near dryness under a gentle stream of nitrogen in a turbovap evaporator at 35˚ C for 30 minutes. The final volume was reconstituted to about one ml using acetonitrile for LCMS/MS. The residue was then filtered through a 0.2 µm PTFE nylon filter and placed in 1.5-ml glass autosampler vial for LC-MS/MS.

### Conditions of LC-MS/MS

Method validation of insecticide residue analysis was conducted on the Water alliance e2695separation model (Waters corp., Milford, MA, USA) with an integrated solvent and sample management system, coupled with Mass Spectrometry Detector with electrospray ionisation (ESI+) interface in positive ion mode. Chromatographic separation for LC was done in an analytical column XTerra C 18 (Waters Corp., Milford, MA, USA) with a pore size of 5 µm and a dimension of 4.6 x 250 mm. To decrease the viscosity of the fluid inside the column, the temperature of the column oven was maintained at 30 °C. Mobile phases A and B were water and acetonitrile, respectively. Formic acid @ 0.1% was used as an ionising agent. A flow rate of 0.5 ml/min was used with the isocratic mobile phase of 30% A and 70% B. The analytical run time was 22 min. Masslynx® version 4.1 software was employed for analytical instrumental control, sample data collection and analysis.

The MS conditions were as follows: the capillary voltage was 3.90 KV, the cone voltage was 24 V, the cone gas was 80 L/hr, and the desolvation gas was 1100 L/hr; the source and desolvation temperatures were maintained at 120 ˚C and 500 ºC respectively. The flow of collision gas was 0.18 ml/min. Multiple reaction monitoring transitions were employed, combined with retention time matching, to confirm the analytes. The MS/MS parameters were standardised for tuning purposes by using the Intellistart® software.

### Method validation

The analytical method used to determine thiamethoxam residues in fresh and processed button mushroom was validated in the laboratory by evaluating the analytical method's performance in accordance with SANTE, 2021 [14] criteria. In this study, method validation was done by validating the parameters such as linearity, limit of detection (LOD), limit of quantification (LOQ), accuracy, precision and uncertainity. Linearity of detector response was assessed by injecting in a total of five calibration working standard solutions at concentrations of 0.01, 0.025, 0.05, 0.075, and 0.1 µg/mL in LC-MS/MS with three replicate injections per concentration. The LOD and LOQ of spiked fresh, and processed button mushroom were computed by injecting samples at a spiking concentration of 0.01 µg/mL, following six replications. In order to calculate, LOD and LOQ, the standard deviation, standard error and x variable were used (SANTE, 2021) [14].

Prior to being spiked with a standard solution, samples were homogenised. The samples were spiked with the analyte at concentrations of 0.01, 0.025, 0.05, 0.075, and 0.1 µg/mL before being extracted and cleaned. Six times each spiking

sample was used. Based on analyte value estimates performed during recovery analyses, the relative standard deviation (RSD%) was used to evaluate the procedure's accuracy. Computation of the relative standard deviation, matrix effect, recovery percentage, and residue concentration were carried out by employing data which was obtained from the chromatogram.

## Results and Discussion

### Linearity, LOD and LOQ

The linearity study was carried out by injecting five different concentrations of 0.01, 0.025, 0.05, 0.075, and 0.1 µg/mL of standard solutions with three replications. The instrument response was determined separately against five different concentrations of the standard mix. The linearity of the calibration curve was established and shown good relationship with the $R^2$ value 0.998 whereas LOD and LOQ was 0.003 and 0.01 µg/g respectively. Lu *et al.* (2018) [18] reported that LOD of thiamethoxam for fresh button mushroom was 0.04 µg/g and LOQ was 0.1 µg/g by adopting UHPLC-MS/MS which were comparable to our study. The standard chromatogram of thiamethoxam was shown in figure 1.

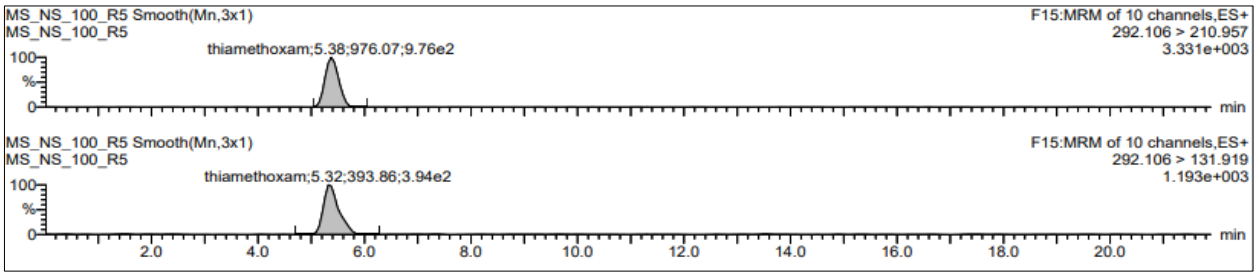


**Fig 1:** LC-MS/MS Standard chromatogram of Thiamethoxam (0.01 µg/g)

**Matrix effect:** In this study, matrix effect for thiamethoxam residues in fresh button mushroom, button mushroom in brine, dry button mushroom powder samples varied from -12.63 to 19.10, -16.46 to -2.64 and -19.82 to -1.63 respectively (Table 1). In fresh button mushroom, enhancement of ion response was observed, and in, processed button mushroom (mushroom in brine and powder) matrix suppression of ion response was observed for thiamethoxam.

### Precision and Accuracy

The mean recovery of five replicates of the pesticide fortified at five levels was used to illustrate the accuracy of the developed method. A quantitative analysis must be able to satisfy mean recovery values between 70 and 120% for each level of spiking, according to SANTE (2021) [14]. Mean recoveries of thiamethoxam was in the range of 89.30 to 108.3, 95.96 to 116.37 and 97.17 to 114.26 percent at spiking level from 0.01 to 0.1 µg/ml whereas RSD values were in the range of 4.04 to 8.31, 5.67 to 8.70 and 3.38 to 9.97 for fresh button mushroom, button mushroom in brine and dry button mushroom power respectively (Table 2).

**Table 1:** Linearity, LOD, LOQ and matrix effect of fresh and processed button mushroom (mushroom in brine and mushroom powder) of thiamethoxam residues

| Matrix | Calibration range (mg/L) | Regression equation | Correlation coefficient ($R^2$) | Matrix effect (%) | LOD | LOQ |
|---|---|---|---|---|---|---|
| Fresh button mushroom | 0.01-0.1 | 6482.x - 23.56 | 0.991 | -12.63 to 19.10 | 0.03 | 0.01 |
| Button mushroom in brine | 0.01-0.1 | 2902.x + 7.656 | 0.996 | -16.46 to -2.64 | 0.03 | 0.01 |
| Dry button mushroom powder | 0.01-0.1 | 3057.x - 12.80 | 0.990 | -19.82 to -1.63 | 0.03 | 0.01 |

**Table 2:** Recovery and Precision of thiamethoxam residues in fresh and processed button mushroom (Mushroom in brine and dry mushroom powder)

| Matrix | Spiking level | | | | |
|---|---|---|---|---|---|
| | 0.01 | 0.025 | 0.05 | 0.075 | 0.1 |
| | Recovery (%) Mean ± SD | Recovery (%) Mean ± SD | Recovery (%) Mean ± SD | Recovery (%) Mean ± SD | Recovery (%) Mean ± SD |
| Fresh button mushroom | 108.3 ± 8.31 | 103.64 ± 4.04 | 89.30 ± 7.66 | 99.47 ± 7.59 | 101.30 ± 4.50 |
| Button mushroom in brine | 116.37 ± 5.67 | 107.32 ± 6.56 | 102.85 ± 7.53 | 95.96 ±8.40 | 109.19 ± 8.70 |
| Dry button mushroom powder | 97.17 ± 4.88 | 110.16 ± 3.38 | 101.01 ± 9.97 | 100.50 ± 3.56 | 114.26 ± 4.81 |

### Application to real samples

In order to provide the demonstration of effectiveness and applicability of modified method, monitoring is carried out by collecting samples. A total of 25 Fresh button mushroom samples were collected randomly from various markets of Coimbatore and 10 processed button mushroom samples were collected from different markets located around the Coimbatore. The developed method was used to examine thiamethoxam residues. There was no detection of thiamethoxam residues in any of the samples analysed.

### Conclusion

A quick, effective, and reliable analytical method was standardized in the present study which can determine thiamethoxam residues in fresh and processed (Mushroom in brine and dry mushroom powder) button mushroom. The obtained recoveries and RSD values were in the acceptable range. The method parameters were validated by considering European Union regulations criteria and also suitable for monitoring in fresh and processed (Mushroom in brine and dry mushroom powder) button mushroom.

**Declaration**

The authors declare that they have no conflict of interests.

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