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Carbaryl and Chlorpyrifos pesticides quantification in honey by liquid chromatography-tandem mass spectrometry (LC-MS/MS)

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Abstract

Honey is a complex food matrix that may be exposed to pesticide residue through agricultural activities surrounding apiaries and foraging areas. Reliable analytical methods are therefore required for the detection and quantification of pesticide residues in honey. The present study evaluated the applicability of liquid chromatography-tandem mass spectrometry (LC-MS/MS) for the targeted quantification of two insecticides, carbaryl and chlorpyrifos in honey. A honey sample without any pesticide residues was initially analyzed to establish the background response matrix and both analytes were found to be below the quantification limit. The honey matrix was subsequently spiked with carbaryl and chlorpyrifos at four concentration levels of 10, 100, 1000 and 10,000 $\mu\text{g kg}^{-1}$. Carbaryl was quantified at 7.84, 93.48, 977.62, and 9144.71 $\mu\text{g kg}^{-1}$ at respective spiked levels, corresponding to apparent recoveries of 78.40, 93.48, 97.76, and 91.45% respectively. Chlorpyrifos was quantified at 6.55, 91.91, 824.51, 8759.82 $\mu\text{g kg}^{-1}$ corresponding to apparent recoveries of 65.50, 91.91, 82.45, and 87.60%, respectively. The mean apparent recovery across the investigated levels was 90.27% for carbaryl and 81.87% for chlorpyrifos. Carbaryl showed a consistent retention time of 10.6 min, while chlorpyrifos eluted at 17.4 min. The results demonstrated satisfactory quantitative agreement between nominal and measured concentrations over a broad concentration range. The study demonstrates the applicability of LC-MS/MS for targeted determination of carbaryl and chlorpyrifos in honey and provides a basis for further validation and routine residue monitoring.

Keywords: Honey, Carbaryl, Chlorpyrifos, LC-MS/MS, pesticide residues, residue analysis

1. Introduction

Honey has a characteristic sweet flavor with medicinal and therapeutic values has been consumed through ancient times across the world. The nectar collected through bees from flowers is regarded as honey (Codex, 2019; Wakgari & Yigezu, 2021a) ^[4, 15]. The environmental impact for extensive farming has resulted in contamination of honey with pesticides. It may occur through either direct application of pesticide near beehive for treating diseases or the consumption of nectar, guttation fluid and pollen from pesticide contamination. The consumption of such pesticide-contaminated honey has been linked to causing neurological disorders in human beings and it has resulted in immunological disorders and carcinogenic stripping the therapeutic and medicinal of honey (Wakgari & Yigezu, 2021b) ^[16].

Pesticide exposure is an important consideration in apiculture because honeybees frequently forage in agricultural landscapes where insecticides, fungicides and herbicides are applied. Pesticides may reach bees through direct contact, contaminated nectar and pollen, water sources or environmental drift (Al-Waili *et al.*, 2012; Drugs, 2020; Mukherjee, 2009; Verma & Bhardwaj, 2015) ^[2, 6, 10, 14]. Residues can subsequently be transported into the hive and potentially become associated with bee products. Consequently, the analytical monitoring of pesticides in honey is relevant both to food safety and to understanding chemical exposure within agricultural ecosystems. Carbaryl and Chlorpyrifos are two insecticides of particular analytical interest. Carbaryl is a carbamate insecticide, whereas Chlorpyrifos belongs to the organophosphorus class. Both compounds are associated with inhibition of acetylcholinesterase and have been widely investigated in the context of pesticide exposure and residue monitoring.

The consumer safety aspect has led to establishing the maximum residue level (MRL) by the governing bodies to ensure permissible safe levels and to prevent the commercialization beyond the established limits (Codex, 2019) [4]. However, the determination of pesticides in honey presents several analytical challenges. Honey contains a high concentration of sugars together with a range of proteins, amino acids, organic acids, phenolic compounds, minerals and other minor constituents. These components may be co-extracted during sample preparation and may interfere with chromatographic separation or affect ionization in electrospray-based mass spectrometry. Consequently, analytical procedures intended for pesticide determination in honey require adequate selectivity and sensitivity.

Liquid chromatography coupled with tandem mass spectrometry is particularly suitable for targeted pesticide analysis because chromatographic separation can be combined with compound-specific mass transitions (Tette *et al.*, 2016) [12]. The use of multiple reaction monitoring (MRM) allows sensitive detection of target compounds in complex matrices and has become an established approach for pesticide residue analysis. The present study was therefore to investigate the quantitative response of carbaryl and chlorpyrifos in honey following controlled fortification at four concentration levels. An unfortified honey sample was first analyzed to establish the background response of the matrix. The fortified samples were subsequently analyzed by LC-MS/MS, and the measured concentrations were compared with the corresponding nominal concentrations to determine apparent recovery across a broad analytical range (da Silva *et al.*, 2016) [12].

2. Materials and Methods

2.1 Honey Sample

The multifloral honey sample was collected directly from a local beekeeper at Nettigere, Karnataka, India. The sample was collected immediately after harvesting, stored in sterile amber glass bottles, transported under ambient conditions, and maintained at 25 °C until analysis.

2.2 Chemicals

Analytical-grade carbaryl, chlorpyrifos acetonitrile (ACN), formic acid, ammonium formate, and methanol were obtained from Sigma-Aldrich. Ultrapure water was used throughout the study.

2.3 Study Design

The analytical experiment was designed as a controlled spiked study using honey as the sample matrix. The honey (HM1) was initially analyzed for the presence of carbaryl and chlorpyrifos and both were found to be in below quantifiable limits. Following the confirmation of the absence of quantifiable background residues, separate honey portions were spiked with carbaryl and chlorpyrifos at concentrations of 0.01, 0.1, 1, and 10 ppm each respectively. The resulting analytical samples were designated HM2, HM3, HM4, and HM5 for carbaryl, HM6, HM7, HM8, and HM9 for chlorpyrifos. The quantitative response for these four-level concentrations in honey were evaluated.

2.4 Liquid Chromatography-Tandem Mass Spectrometry Analysis

A Ultra High Performance Liquid Chromatography (UHPLC) system by Shimadzu Corporation, Kyoto, Japan

was used for the Liquid chromatography part of the system. This was comprised of two Shimadzu UHPLC: Nexera UHPLC system Column: Shim-pack (C18-120, 4.6 x 150 mm, 3 µm particle size). The column temperature was maintained at 40 °C and flow rate was 0.7 mL min⁻¹ and injection volume being 10 µL (Tette *et al.*, 2016) [12]. This was coupled with Mass Spectroscopy System (Make: Shimadzu Corporation, Kyoto, Japan, Model: LCMS 8040, Triple Quadrupole). The system has ion spray voltage: + 4.5 kV/-3.5 kV and dwell time of 5 msec with pause time being 1 msec comprising block temperature of 400° C, detector voltage of 1.3kv, nebulizer gas flow rate of 1.5 litre min⁻¹. This coupled system resulted in Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) system (FSSAI, 2016) [7].

2.5 Sample Preparation for LC-MS/MS Analysis

A 2.5g sample was taken into a 50 mL centrifuge tube. To which 10 milliliters of ACN was added. Tubes were then capped and shaken by vortex for 5 min. To each tube, an Agilent Bond Elut QuEChERS EN extraction salt packet, containing 6 g anhydrous MgSO₄, 1.5g sodium acetate was added directly (Anastassiades *et al.*, 2003) [3]. Tubes were sealed tightly and shaken vigorously for 5 min by vortex to ensure that the solvent interacted well with the entire sample and crystalline agglomerates were broken up sufficiently. Sample tubes were centrifuged at 4000 rpm for 5 min. 5 mL aliquot of the upper ACN layer was transferred into a centrifuge tube and add Bond Elut powder; the 2 mL tube contained 25 mg of PSA and 150 mg of anhydrous MgSO₄. The tubes were capped tightly and vortexed for 1 min. The tubes were centrifuged at 4000 rpm for 5 min. Then the supernatant was filtered through 0.22 µ filter and transferred into an autosampler vial. The samples were capped and vortexed thoroughly, to prepare for LC/MS/MS analysis (FSSAI, 2016; Gawel *et al.*, 2019) [7, 8, 1].

2.6 Calculation of apparent recovery

The apparent recovery at each spiked level was calculated by comparing the LC-MS/MS measured concentration with the spiked concentration into honey matrix, which is given by the Equation 1.

$$\text{Apparent recovery} = \frac{\text{Measured concentration}}{\text{Spiked concentration}} \times 100 \text{ (Percentage)} \dots (\text{Eq.1})$$

3. Results and Discussion

The initial analysis of the honey (HM1) showed below quantifiable results for both carbaryl and chlorpyrifos. Carbaryl was associated with a retention time of 10.6 min, while chlorpyrifos was associated with a retention time of 17.4 min. No quantifiable concentration was reported for either analyte (Figure 1 & 2). This demonstrates that the concentrations subsequently measured in HM2-HM9 were associated with deliberate addition of the target pesticides rather than with quantifiable background residues in the honey matrix. The use of residue free matrix is an important component of pesticide residue analysis as it provides a basis for evaluating analytical response following spiking (Venu *et al.*, 2019) [13].

Carbaryl was quantified at all four investigated spiked levels (Table 1). For the spiked concentration of 10 µg kg⁻¹, the LCMS/MS was able to quantify 7.84 µg kg⁻¹, giving an apparent recovery of 78.40%. The chromatographic retention time was 10.6 min (Almeida *et al.*, 2020) [1]. At

spiked level of $100 \mu\text{g kg}^{-1}$, the measured concentration increased to $93.48 \mu\text{g kg}^{-1}$, having an apparent recovery of 93.48%. The $1000 \mu\text{g kg}^{-1}$ of spiking resulted in carbaryl

concentration of $977.62 \mu\text{g kg}^{-1}$, with recovery of 97.76% and for the spiking of $10000 \mu\text{g kg}^{-1}$, the LC-MS/M yielded 91.45% of recovery (Figure 1).

Table 1: Quantification of pesticide at different spiked levels in honey using LC-MS/MS

Sample	Spiked Concentration ($\mu\text{g kg}^{-1}$)	Retention Time (min)	Measured Concentration ($\mu\text{g kg}^{-1}$)	Apparent Recovery (%)
HM1	0	10.6	-	-
HM2	10	10.6	7.84	78.40
HM3	100	10.6	93.48	93.48
HM4	1000	10.6	977.62	97.76
HM5	10000	10.6	9144.71	91.45
HM1	0	17.4	-	-
HM6	10	17.4	6.55	65.50
HM7	100	17.4	91.91	91.91
HM8	1000	17.4	824.51	82.45
HM9	10000	17.4	8759.82	87.60

Similarly Chlorpyrifos was quantified at all four spiked levels (Table 1). For $10 \mu\text{g kg}^{-1}$, the LC-MS/MS was able to quantify $6.55 \mu\text{g kg}^{-1}$, resulting in an apparent recovery of 65.50%. However, the increase in the level of spiking concentration yielded higher recovery (Almeida *et al.*, 2020; Hrynko *et al.*, 2018) [1, 9]. As it was observed for $100 \mu\text{g kg}^{-1}$ of spiking recovery was $91.91 \mu\text{g kg}^{-1}$. The higher spiking levels of 1000 and $10000 \mu\text{g kg}^{-1}$ concentrations yielded recovery of 824.51 and $8759.82 \mu\text{g kg}^{-1}$ respectively with apparent recovery of 82.45 and 87.60% (Figure 2).

The spiking studies demonstrated unique quantitative responses for the two pesticides. Carbaryl exhibited an apparent recovery above 90% at three of the four spiked levels, whereas chlorpyrifos exceeded 90% at only $100 \mu\text{g kg}^{-1}$ concentration level. Nevertheless, the two compounds showed broadly similar behavior at the higher concentration levels, with apparent recoveries generally falling between approximately 82 and 98%. The difference at the lowest spiked concentration level may be associated with the

greater relative influence of analytical variability at lower analyte concentrations (Gawel *et al.*, 2019) [8]. The mean apparent recovery obtained for carbaryl was 90.27%, compared with 81.87% for chlorpyrifos. These results were within the commonly accepted 70-120% recovery interval range at all four spike levels with exception of $10 \mu\text{g kg}^{-1}$ concentration for chlorpyrifos (Pihlström *et al.*, 2021 n.d.) [11]. The results demonstrate the feasibility of measuring pesticide concentrations in a high-sugar food matrix using LC-MS/MS (Hrynko *et al.*, 2018) [9]. The honey sample matrix generated measurable and concentration dependent analytical responses. This design provides a direct comparison between the native matrix and pesticide-spiked matrix and minimizes the possibility of attributing the measured residues to naturally occurring contamination. This difference between nominal and measured concentration can therefore be used to evaluate the apparent recovery of the analytical procedure (Hrynko *et al.*, 2018; Pihlström *et al.*, 2021 n.d.) [9, 11]

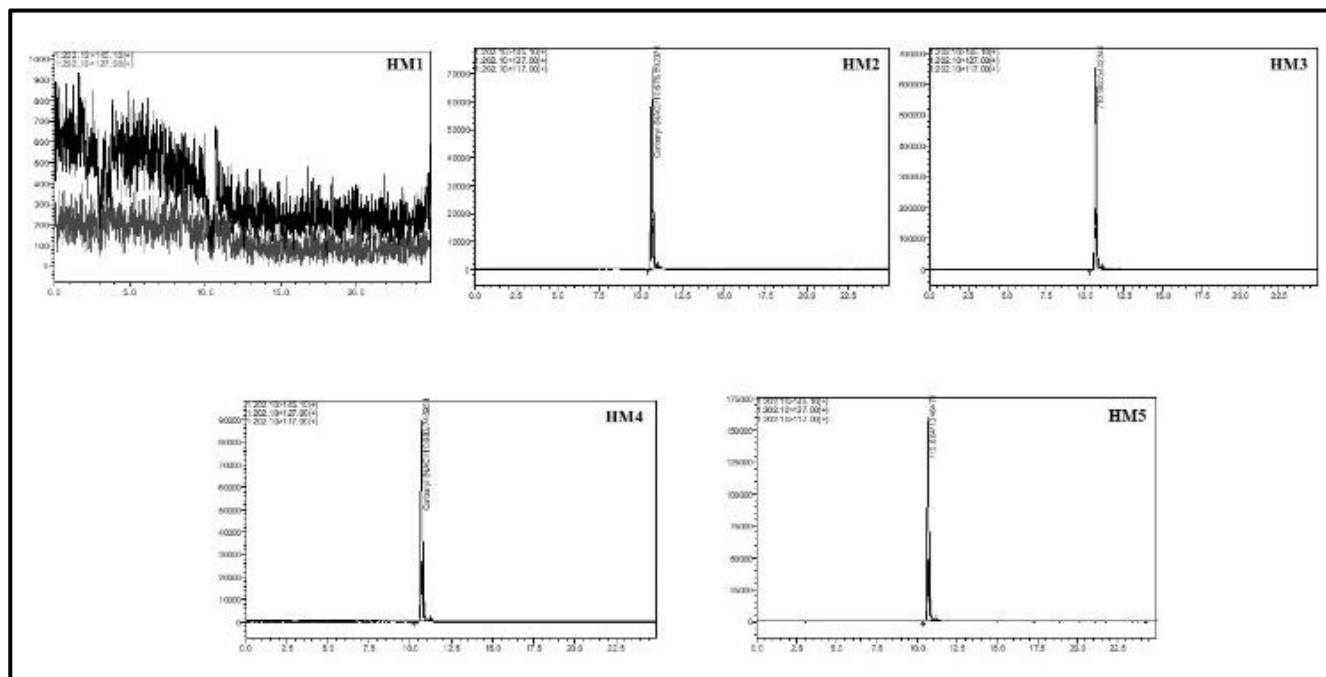


Fig 1: LC-MS/MS Spectra for different concentration levels of Carbaryl in Honey Matrix

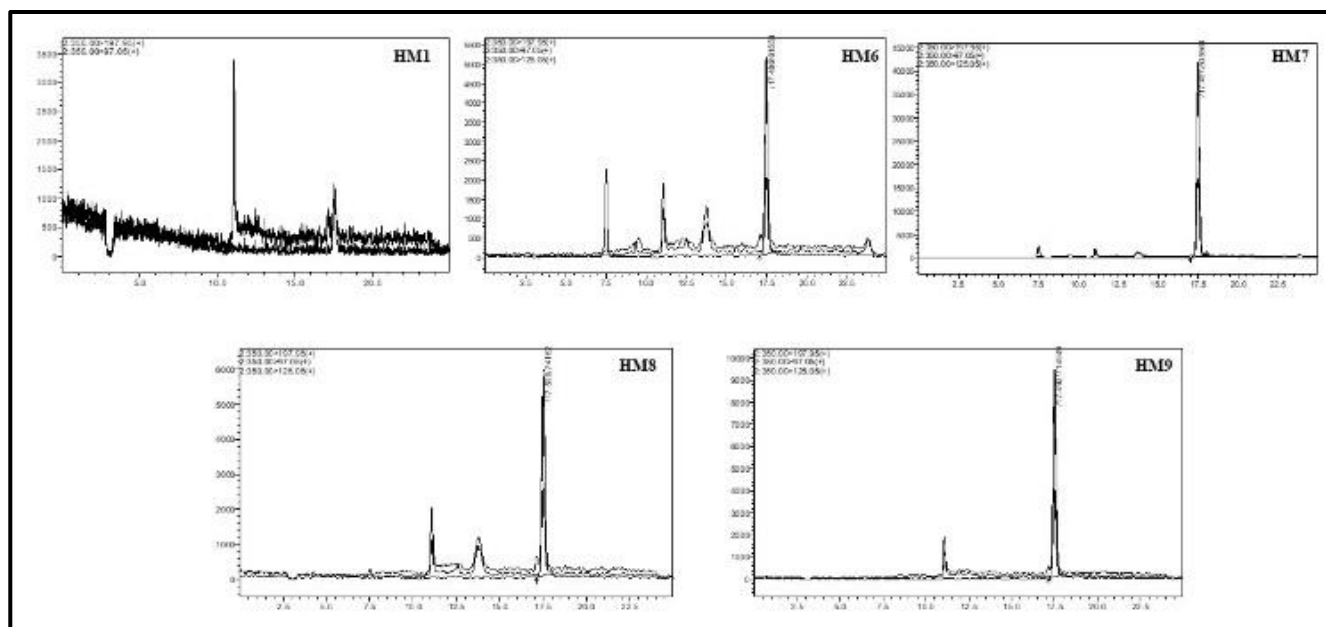


Fig 2: LC-MS/MS Spectra for different concentration levels of Chlorpyrifos in Honey Matrix

4. Conclusion

The present study demonstrated the applicability of LC-MS/MS for the targeted quantification of carbaryl and chlorpyrifos in a honey matrix following controlled spiking. The honey sample showed below quantifiable limit for both target pesticides, conforming the absence of quantifiable background residues in the matrix used for analysis. Following spiking, both compounds were successfully quantified over a range of 10-10000 $\mu\text{g kg}^{-1}$. Carbaryl showed measured concentrations of 7.84, 93.48, 977.62 and 9144.71 $\mu\text{g kg}^{-1}$ corresponding to apparent recoveries of 78.40, 93.48, 97.76 and 91.45%, respectively. Chlorpyrifos produced measured concentrations of 6.55, 91.91, 824.51, and 8759.82 $\mu\text{g kg}^{-1}$, corresponding to apparent recoveries of 65.50, 91.91, 82.45, 87.60%, respectively. The mean apparent recovery was 90.27% for carbaryl and 81.87% for chlorpyrifos. The consistent retention times of 10.6 min for carbaryl and 17.4 min for chlorpyrifos further supported chromatographic identification of the target compounds. These findings demonstrate that LC-MS/MS can provide quantitative measurement of pesticides in honey across broad concentration range.

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