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ORIGINAL ARTICLE

Liquid Chromatography–Mass Spectrometry/Mass Spectrometry Method Development for the Determination of Carbaryl Residue in Honey

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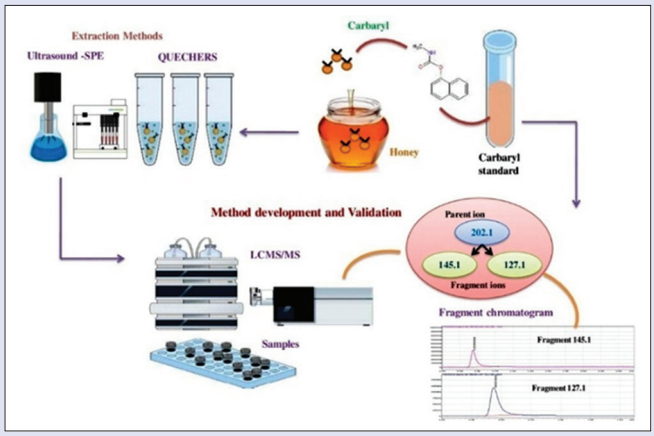
ABSTRACT

Background: Honey, the natural sweetener, obtained from *Apis mellifera* (honey bees) possesses many medicinal properties. Intensive use of carbamate insecticide in agricultural land not only contaminates the crop but also affects the honey and honey matrices. Hence, this study focused on the analysis of insecticide in honey. **Objective:** Liquid chromatography–mass spectrometry/mass spectrometry (LC-MS/MS) method development for the determination of Carbaryl insecticide in honey and Comparisons of different extraction techniques to determine the efficiency of extraction process. **Materials and Methods:** The LC-MS/MS method was developed by optimizing the multiple reaction monitoring (MRM) parameters. Further, the comparison study was done for the optimized extraction techniques such as quick, easy, cheap, effective, rugged, and safe (QuEChERS) method and ultrasound solid phase extraction (SPE) in the developed method. The validation was studied for the developed method as well as the extraction techniques to confirm the robustness of the developed method. **Results:** The validation study showed good accuracy for the developed method for the concentration from 2 to 9 ppb of the working solution. Limit of detection for the developed method was 0.08 and 0.05 ppb for the fragments 145.1 m/z and 127.1 m/z, respectively. Moreover, limit of quantification for the fragment 145.1 m/z was 0.24 ppb and for 127.1 m/z was 0.16 ppb. The average accuracies for the developed method of both the fragments (145.1 m/z and 127.1 m/z) were 98.51% and 98.15%, respectively. Recovery percentage for optimized QuEChERS ranged from 107% to 112% and for the ultrasound-SPE and from 107% to 118% of the honey samples which were spiked with three different concentrations of analyte. **Conclusion:** From the validation, it was confirmed that the developed method was robust and simple and provides better sensitivity and intensity and low consumption of chemicals. Thus, the developed method can be used for the routine analysis of carbaryl in honey.

Key words: Carbaryl, liquid chromatography–mass spectrometry/mass spectrometry, MRM, quick, easy, cheap, effective, rugged, and safe, ultrasound solid-phase extraction

SUMMARY

- The present study focused on liquid chromatography–mass spectrometry/mass spectrometry (MS) method development for detecting carbaryl insecticide residue in honey. Method development was done by optimizing MS parameters such as collision energy and dwell time. Further different extraction processes were compared in the developed method
- From the validation results of developed method, it can be concluded that the developed method is suitable for routine analysis and both the extraction methods can be employed for analyzing the carbaryl residue.



Abbreviations used: BHC: Beta-Hexachlorocyclohexane; DDT: Dichlorodiphenyltrichloroethane; DDVP: Dichlorvos or 2,2-dichlorovinyl dimethyl phosphate; DLLME: Dispersive liquid–liquid microextraction; QuEChERS: Quick, easy, cheap, effective, rugged, and safe; OCLLE: On column liquid–liquid extraction; SBSE: Stir-bar sorptive extraction; SFE: Supercritical fluid extraction; LCMS: Liquid chromatography–mass spectrometry; LCMS/MS: Liquid chromatography–mass spectrometry/mass spectrometry; PTFE filter: Polytetrafluoroethylene; LOD: Limit of detection; LOQ: Limit of quantification; MRM: Multiple reaction mode; SPE: Solid-phase extraction; RSD: Relative standard division; R^2: Correlation coefficient; Ng: Nanogram; Mg: Milligram; µg: Microgram; kg: Kilogram; ml/min: Milliliter/minute; ms: Millisecond; ppm: Parts per million; ppb: Parts per billion.

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INTRODUCTION

Honey is one of the most used products of the hive produced from the nectar of blossoms or from the secretion of living parts of plants by honey bees. It is mainly composed of monosaccharide and oligosaccharides, totaling 77%, with glucose and fructose having average contents of 30% and 38%, respectively.[1] It also consists of various chemical groups including amino acids, phenolic acids, and flavonoids in numerous honey sample.[2-4] The composition of honey

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may be affected by the climatic conditions, different types of flower, and regional conditions.[5] Hive products are produced in polluted environment, so it may be contaminated through air, water, and soil, which affects the raw sources of bees such as plant exudates, pollen, and nectar.[6] It is mainly contaminated by pesticides such as herbicides, insecticides, fungicides, acaricides, and types of veterinary drugs. Pesticides are mainly used for the protection of agricultural crops; however, the intensive utilization of pesticides (acaricides, fungicides, herbicides, and insecticides) not only contaminates the crops, soils, and water bodies but also affects the honey and honey matrices. Even if the concentration of pesticide residues in food matrices is low, still there are chances that it can lead to acute or chronic toxicity in humans.[7,8] Pesticides such as organonitrogen, organohalogen, organochlorine, organophosphorus, and carbamates are generally used in agricultural crops. Among all these pesticides, carbamates and organophosphorus are widely used in the agricultural field and have replaced the usage of organochlorine pesticides. The pesticides such as beta-Hexachlorocyclohexane and dichlorodiphenyltrichloroethane were banned in India around 1987–1988 due to their bioaccumulation in the environment.[9] Even though these pesticides were banned, they are still found in the environment.[9] These pesticides are still being transferred from soil to water and then it reaches the plants and animals. The bees which feed on these contaminated flowers transfer these pesticides into honey and thus to humans. Pesticide residues such as organophosphorus, dichlorvos or 2,2-dichlorovinyl dimethyl phosphate, and monocrotophos have been found to be present in honey samples from India during 1993–1995.[9] Carbaryl (1-naphthalenyl-N-methylcarbamate) is mostly used as an insecticide (carbamate family) against the pest on crops, fruits, and vegetables. Because it has few benefits over other pesticides such as less bioaccumulation potential and less mammalian toxicity. However, bioaccumulation in food matrices and water may lead to bioconcentration through food chain.[10]

The two major steps in analytical chemistry which plays a vital role in the detection of pesticide residues include (1) extraction methodology and (2) instrumentation method development. Based on the conventional extraction methods such as liquid–liquid extraction and solid-phase extraction (SPE), numerous advance extraction techniques have evolved to enhance the efficiency of extraction, methods such as dispersive liquid–liquid microextraction; liquid–liquid extraction–low-temperature purification; dispersive micro-SPE; quick, easy, cheap, effective, rugged, and safe (QuEChERS); solid-phase microextraction; on column liquid–liquid extraction; stir-bar sorptive extraction; and supercritical fluid extraction. Pesticides can be detected by instruments such as gas chromatography and liquid chromatography. Some other nonchromatographic techniques include optical sensor, immunosensor, and electrochemical sensor.[11] However, chromatographic techniques such as gas chromatography for volatile and semi-volatile analytes and liquid chromatography–mass spectrometry/mass spectrometry (LC-MS/MS) for thermally unstable, non-volatile, and high polarity analytes have shown superior performance based on its better sensitivity, due to its selective detectors and high separation power. Most of the studies have focused on the method development for group of pesticides, whereas only a few studies have reported the method development of individual pesticides. Based on the literature, the present study focuses on the method development for carbaryl molecule in honey under optimized MRM condition and comparison of extraction efficiency of different extraction techniques.

Objectives

- LC-MS/MS method development for carbaryl insecticides in honey
- Comparison of different extraction methods to determine the efficiency of carbaryl residue.

MATERIALS AND METHODS

Chemicals and standard solution

Pesticide standard (carbaryl) was procured from Sigma-Aldrich with ≥98% purity. Stock solution was prepared at the concentration 1 µl/l in methanol and stored at −20°C. The working solutions were prepared in different dilutions of stock solution. Solvents such as methanol, acetonitrile, and acetone (HPLC grade) were procured from Sigma-Aldrich. Reagent such as Milli-Q Water, ammonium formate (Merck), anhydrous magnesium salt (Agilent Technology), sodium acetate (Agilent Technology), C_{18} and primary–secondary amine (PSA) (Agilent technology), and formic acid (Sigma-Aldrich) were of analytical grade.

Instrument

Liquid chromatography–mass spectrometry/mass spectrometry

LC-MS/MS system (liquid chromatography coupled with triple quadrupole mass spectrometry) (Shimadzu LC-MS/MS 8040) with an electrospray ionization for both positive and negative ionization modes was employed by multiple reaction monitoring (MRM). LC consisted of an auto sampler, binary pump, and column oven. Chromatography was performed using C_{18} column with mobile phase consisting of Milli-Q Water (Phase A) and methanol (Phase B), both acidified with 0.01% formic acid and 10 mM ammonium formate at flow rate 0.5 ml/min. MS parameters such as collision energy (CE) (10–40 mV), dwell time (10–100 ms), nebulizing gas flow rate at 3.0 l/min, oven temperature (40°C), dissolvation line temperature (250°C), and heat block temperature (400°C) were fixed using software LabSolutions. The chromatography method was adapted from previously developed method.[12,13]

Solid-phase extraction technique

The cleanup process was performed by SPE technique (GX-271 Gilson, USA). Conditions such as volume of extraction solvent, washing solvent, and volume of sample were fixed using the software Gilson Trilution. The instrument consists of sample and collection holders, single probe coupled with VERITY® 4060 Single and VERITY® 4260 Dual Syringe Pump which can automate the extraction and liquid (solvents) handling process GX rinse pump where the flow rate can be set using software and GX injection module which permits the sample for SPE cleanup

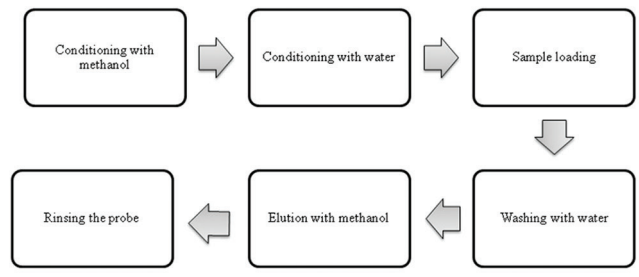


Figure 1: Solid-phase extraction cleanup process

and solvent bottle rack. Supelco VARIAN C_{18} cartridge was used for cleanup; here, column was conditioned with methanol and water to wet the bonded functional group which ensures the constant interaction. Then, washing with water was done to remove the interferences and elution was performed with methanol (carbaryl) which control both primary and secondary retention interactions between sorbent and analyte [Figure 1].

Method optimization

In this study, the method was developed by optimizing MS parameters to improve the sensitivity and quantitative accuracy of the fragments. Optimizing MS parameter such as CE and dwell time enhances the intensity and sensitivity (detector ion counting) of fragment ions. These parameters not only improve the sensitivity and accuracy but also prevent the interferences which significantly influence the quantitative analysis.[14] Optimization was studied by direct imbuing of standard solution (various concentration) into MS ensued by infusion via the column to determine their exact retention times (RTs), limit of detection (LOD), and limit of quantification (LOQ) of the targeted analyte (carbaryl insecticide) [Table 1].

Mobile phase preparation

The mobile phase used in the study was Phase A (Milli-Q Water) and Phase B (methanol). Both the mobile phases were acidified with 0.01% of formic acid and 10 mM ammonium acetate. To remove the air bubbles from the mobile phase, they were sonicated (20 min) followed by filtration through 0.45 µm polytetrafluoroethylene filters. The gradient elution program was used: 0.01 min, 50% B; 6 min 80% B; 10 min, 90%; and 10.5 min, 50% B.

Honey sample

Honey sample was purchased from the local market and stored at ambient temperature. Appropriate honey solutions were prepared by diluting with water and methanol in the ratio 1:1 depending on the extraction method. For the validation of developed method, honey samples were spiked with analyte (carbaryl) at three different concentrations (5 ppm, 10 ppm, and 20 ppm) and stored at 4°C until analysis.

Sample preparation

Extraction using quick, easy, cheap, effective, rugged, and safe method

The extraction of pesticide from honey was performed with a slight modification to that of procedure adopted by Tette *et al.*[15] and has been given in the following Flow Chart 1.

Ultrasound-assisted solid-phase extraction

Ultrasound-assisted SPE of pesticide from honey was conducted, and the detailed steps are given in the following Flow Charts 2 and 3.

Method validation

Stock solution was prepared by diluting 1 ml of standard solution in 100 ml of methanol and from the stock solution; working solution was prepared with different concentrations. The calibration was carried out by varying the concentration of working solution from 2 to 9 ppb, and the replication was done to avoid the random error. Fitting of the calibration curve and plotting of the residuals as a function of concentration were done. The linearity was obtained by means of regression line using the least square method. Calibration result shows the *y*-intercept, correlation coefficient (R^2), and slope of the regression line, and evaluation of linearity can be done by analyzing the deviation point from regression.[15] LOD means validation of trace concentration of analyte which can be confirmed by consecutive dilution of stock solution. LOQ can be examined with adequate accuracy by varying the concentration of standards.[16] Based on the calibration curve, both the LOD and LOQ can be determined. Accuracy was determined by injecting low concentration of working solution followed by "*n*" number of replications ($n = 10$) to determine the exact accuracy results for the developed method. Accuracy shows the compatibility between the test result and accepted reference value. Percentage recovery was also calculated for spiked samples to determine the efficiency of extraction method for the developed method.[16] It was determined by the given formula:

$$\%\ Recovery = \frac{Xr}{Xs} * 100$$

Xr = Recovered concentration.

Xs = Known (spiked) concentration.

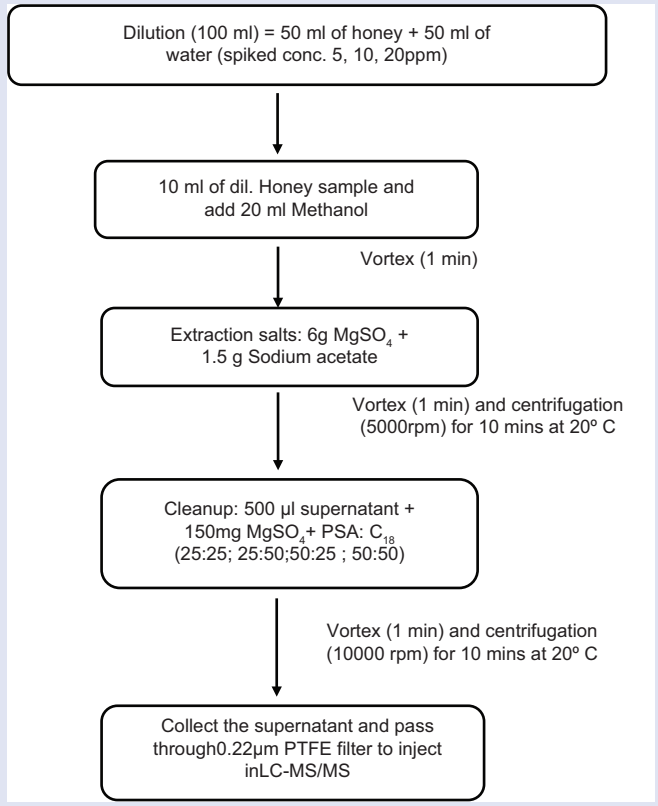
Table 1: Optimization of mass spectrometry parameters

Parameters	Low	High
CE (mV)	10	40
Dwell time (m s)	10	100

CE: Collision energy

RESULTS AND DISCUSSION

Carbaryl was analyzed by direct injection of individual standard solutions (1 µl/ml) at a flow rate of 0.5 ml/min with the mobile phase water/methanol (0.01 min, 50% B; 6 min 80% B; 10 min, 90%; and

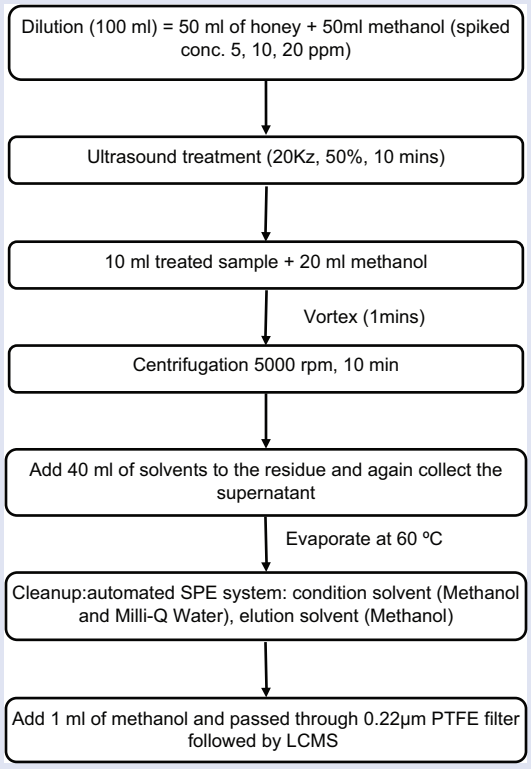


Flow Chart 1: Modified quick, easy, cheap, effective, rugged, and safe extraction method

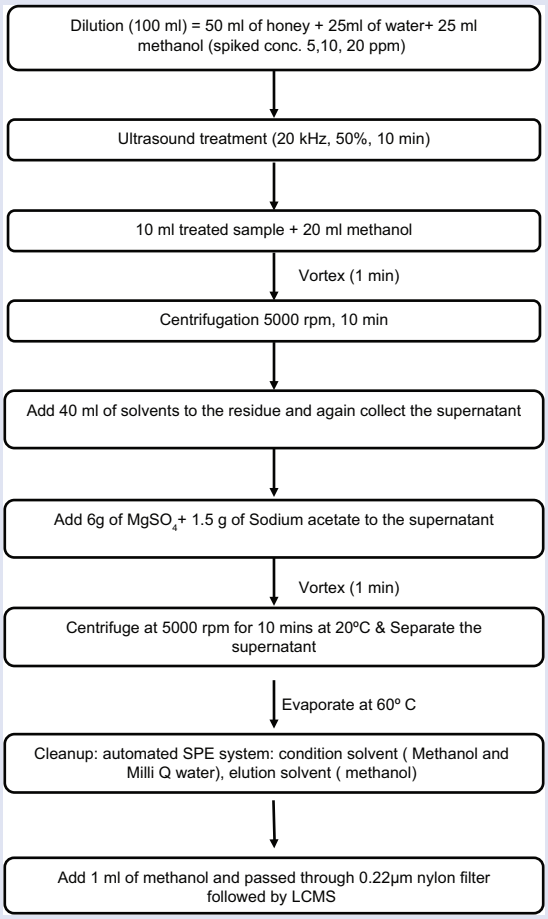
10.5 min, 50% B) with 0.01% formic acid and 10 mM ammonium formate. The molecular weight of most intense ion which is carbaryl precursor ion is 202(m/z) and the product ions of carbaryl were 145.1 (m/z), 127.1 (m/z). To increase the selectivity and intensity of fragments, LC-MS/MS method was developed by manual optimization of MS parameters such as CE and dwell time.

Optimization of MRM parameter

The intensity of peak and sensitivity of MRM-MS are influenced by tuning of transition-specific parameters, mainly CE which is employed during fragmentation.[17] Optimization of CE was done for both the fragment ions 145.1 m/z and 127.1 m/z by varying the collision energies from 10 to 40 mV at a fixed dwell time of 100 ms. This phenomenon takes place in collision cell where the analyte is collided with gas (N_2) molecules leading to fragmentation. The CE optimization results showed that the maximum peak intensity for the fragment 145.1 m/z emerged at low CE of 15 mV [Figure 2]. In case of the fragment ion 127.1 m/z, the maximum peak intensity [Figure 3] emerged at high CE of 34 mV [Table 2 and Figure 2]. Similarly, the optimization of dwell time was done for the fragment ions 145.1 m/z and 127.1 m/z by varying the time from 10 to 100 ms while keeping the CE fixed at the earlier optimized values of 15 mV and 34 mV, respectively. From the dwell time optimization study, it was observed that the maximum peak intensities for both the fragments 145.1 m/z and 127.1 m/z were at 80 ms with low RT of 0.437 min [Figure 3 and Table 2].



Flow Chart 2: Ultrasound solid-phase extraction method for Sample A



Flow Chart 3: Ultrasound solid-phase extraction method for the Sample B

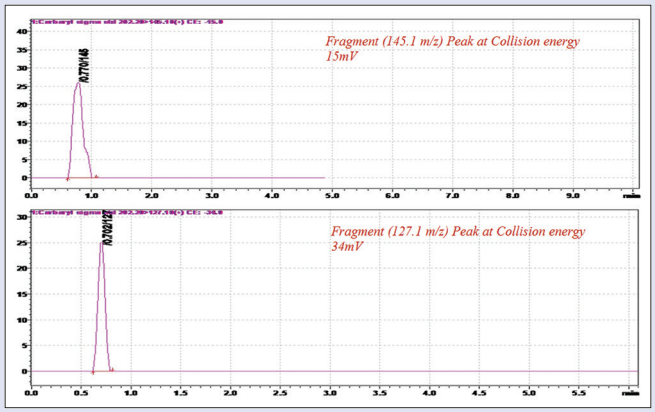


Figure 2: Fragment peaks at optimized collision energy

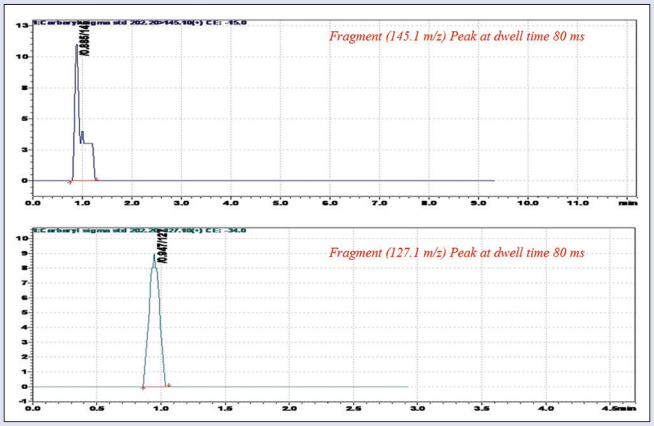


Figure 3: Fragment peaks at optimized dwell time

Hence, the CE was fixed at 15 mV for 145.1 m/z and 34 mV for 127.1 m/z. Moreover, the dwell time was fixed as 80 ms for the both fragments 145.1 m/z and 127.1 m/z [Table 3 and Figures 4, 5]. The developed method showed slight variation in CE and low dwell time when compared with previously developed method in water sample;[13] however, this variation can be expected due to change in the matrix type; in the present study, honey matrix was used, whereas in the Roudani *et al.*,[13] it was water sample. Further, the developed method showed better response when compared with the existing method of Madureira *et al.*[12] This may be due to the fact that the method developed by them was focused to analyze multiclass pesticides as compared to the present study which focused on carbaryl in honey. Hence, the optimized conditions were fixed for method validation studies.

Method validation

The linearity response of the developed method was examined with the calibration curve using eight points by varying the concentration of working solution from 2 to 9 ppb for the product ions. Linearity for the calibration curve was obtained with high R^2 in the range ≥0.99 for the fragment 145.1 m/z with relative standard division (RSD) % 6.76 [Figure 6a]. In case of fragment 127.1 m/z, R^2 was ≥0.98 with RSD % 7.98 for 127.1 m/z [Figure 6b]. LOD for fragment ion 145.1 m/z was 0.08 ppb, and for 127.1 m/z, it was 0.05 ppb, whereas LOQ for fragment ion 145.1 m/z was 0.24 ppb, and for fragment ion 127.1 m/z, it was 0.16 ppb. Average accuracies of both the fragments were 98.51% and 98.15% with the least standard deviation of 0.813 and 1.025 for the fragments 145.1 m/z and 127.1 m/z, respectively. From the calibration study, the developed method showed high R^2 which indicates the method efficiency. LOD and LOQ as well as accuracy profile for the developed method were better when compared with the previous developed method by Roudani *et al.*[13] for water sample. Hence, it can be concluded that the developed method stayed efficient for the routine analysis of carbaryl insecticide.

Table 2: Optimized response for the fragment ion

RT	Area	m/z	Area (%)	Absolute intensity	Relative intensity
0.437	2,189,613	202.20>145.10	100	85,926	100
0.437	572,373	202.20>127.10	100	24,052	100

RT: Retention time

Table 3: Optimized conditions

Precursor ion (m/z)	Product ion (m/z)	Dwell time	Q1 Prebias (V)	CE	Q3 Prebias (V)
202.2	145.1	80	−30	−15	−15
202.2	127.1	80	−30	−34	−34

CE: Collision energy

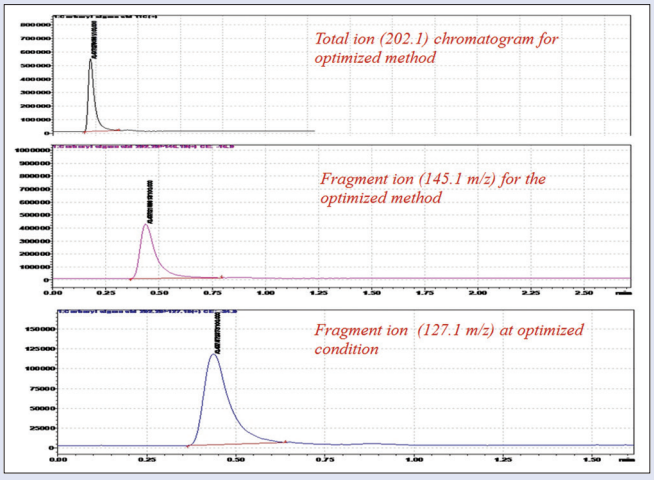


Figure 4: Total ion and fragment ions chromatogram for the optimized condition

Extraction efficiency

Quick, easy, cheap, effective, rugged, and safe

QuEChERS is an extraction method which is simple, rapid, robust, and efficient for the determination of pesticides with an acceptable recovery % of the analyte.[18-21] This technique consists of two steps which include (a) liquid–liquid partitioning by means of extraction solvent and salt and (b) cleanup process by dispersive SPE.[22] Cleanup sorbents (PSA and C_{18}) have been extensively used in QuEChERS extraction procedure for removal of sugars, fatty acids, and polar organic components.[23,24] Hence, in the present study, the concentrations of cleanup sorbents (PSA and C_{18}) were optimized to improve the purification process. The sample (honey) was diluted with water to reduce its viscosity, and methanol was chosen as an extraction solvent.[25] In addition to the extraction solvent, extraction salts such as magnesium sulfate and sodium acetate were added to enhance the extraction efficiency. The optimization study revealed that among the four different proportions, 50:25 ratio of PSA: C_{18} had better recovery %. Nectar consists of >60% of sugary compound; therefore, to remove them, higher concentration of PSA was required. However, in case of C_{18}, low concentration was sufficient enough to remove the lipid and other impurities as this may be due to the fact that nectar is low in lipid content. The modified method showed recovery % in the range 107%–114%, although in the case of Tette *et al.*[15] method, the recovery % was in the range 93%–99% for the carbaryl analyte. From this, it was clear that the modified extraction method developed with the combination of different chemicals makes extraction process simple and provides better recovery % even at low spiked concentration [Table 4].

Ultrasound solid-phase extraction

The studies have reported that ultrasound increases the extraction efficiency by reducing the time and volume of solvent. By its mechanical process, ultrasound improves the homogenization and cavitation effect which increases the extraction yield by increasing surface areas.[26,27] Fontana *et al.*[28] used ultrasound-assisted treatment to extract pesticide from honey followed by concentration before analyzing on GC-MS.

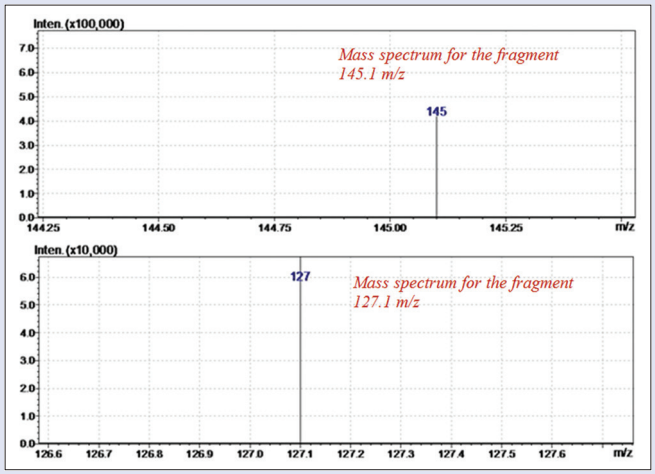


Figure 5: Mass spectrum for fragment ions at optimized condition

Table 4: Study on the recovery percentage of spiked sample

Samples	RT (min)*	Spiked concentration* + 0.01 ppb	Recovered concentration*	Recovery (%)*
QuEChERS extraction				
S1=PSA: C18 (25:25)	0.45	5.01	4.70	93.81
S2=PSA: C18 (25:50)	0.44	5.01	4.53	90.41
S3=PSA: C18 (50:25)	0.43	5.01	5.40	107.78
S4=PSA: C18 (50:50)	0.43	5.01	4.96	99.01
S5=PSA: C18 (25:25)	0.44	10.01	9.14	90.49
S6=PSA: C18 (25:50)	0.44	10.01	9.08	90.70
S7=PSA: C18 (50:25)	0.43	10.01	11.51	114.98
S8=PSA: C18 (50:50)	0.44	10.01	10.37	103.59
S9=PSA: C18 (25:25)	0.45	20.01	19.42	97.05
S10=PSA: C18 (25:50)	0.46	20.01	19.30	96.45
S11=PSA: C18 (50:25)	0.44	20.01	22.32	111.54
S12=PSA: C18 (50:50)	0.46	20.01	21.58	107.85
Ultrasound solid-phase extraction				
S13 (SPE)	0.45	5.01	5.55	110.77
S14 (SPE)	0.45	10.01	11.87	118.58
S15 (SPE)	0.43	20.01	21.63	108.09
S16 (SPE=methanol + water)	0.47	20.01	21.57	107.79

*Average. 0.01 ppm of carbaryl already present in sample. RT: Retention time; SPE: Solid-phase extraction; PSA: Primary-secondary amine

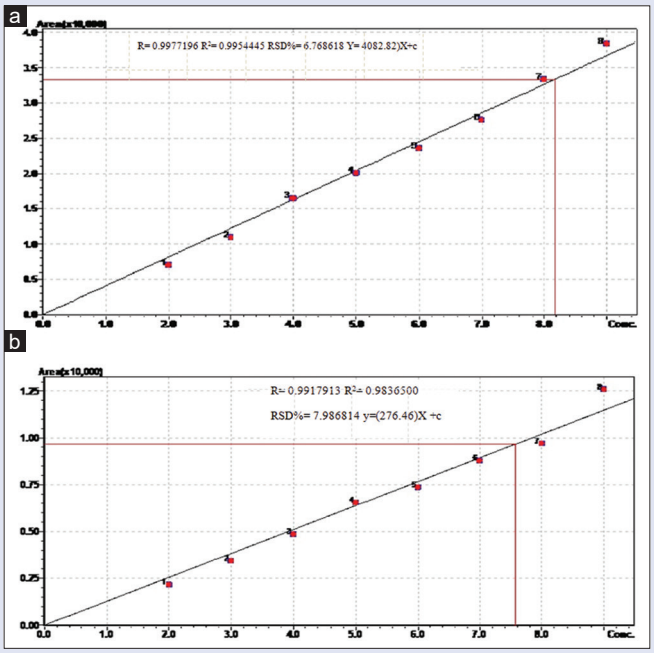


Figure 6: (a) Calibration curve for fragment 145.1 m/z. (b) Calibration curve for fragment 127.1 m/z

Rawson *et al.*[29] reported the improvement in extraction yield of oil in the ultrasound-assisted extraction of rice bran oil. All these advantages make ultrasound to play a vital role in different extraction methods.

In this extraction method, two ultrasound-SPE methods were employed to reduce the usage of chemicals and volume of extraction solvent. Most studies have used acetonitrile for the extraction of pesticides from honey since they deal with multiclass pesticides; however, in general, methanol is more preferable solvent in case of carbaryl compound.[12,13] Hence, Sample A consisted of honey:methanol in the ratio of 1:1 and Sample B consisted of honey:methanol:water in the ratio of 1:0.5:0.5. Both the samples were treated with ultrasound to increase the extraction efficiency by means of cavitation principle and for the better dissolution of honey sample.[30] Extraction process for the ultrasound-treated Sample A was done by SPE without any addition of extraction salt. Another extraction process for the ultrasound-treated Sample B was also done by SPE with the addition of extraction salts such as magnesium sulfate and sodium acetate. On comparing both the ultrasound-SPE methods, recovery % of the samples was similar ($P > 0.05$), but recovery % was slightly low when compared with QuEChERS method [Table 4]. The advantage of ultrasound-SPE over QuEChERS is low usage of chemicals when compared to QuEChERS which shows better recovery %. This showed that the extraction efficiency improved following ultrasound as treatment before SPE extraction. Previous studies used ultrasound as an aid for the extraction of pesticide metabolite as in Roudani *et al.*[13] The wattage of the ultrasound system was 200 W; however, in the present study, the wattage was 750 W; hence, more power was input hence improved extraction. From the extraction study, it was inferred that both the extraction techniques (ultrasound-SPE and QuEChERS) can be used for the routine analysis.

The main aim of this study was to specify the impact of sample preparation in developed method. For that, in the present study, the samples were spiked with three different concentrations of analyte (5, 10, and 20 ppm) in samples and optimized the condition of sorbents, dilution, and solvents. The results of recovery study of optimized condition clearly showed that both the extraction techniques were suitable for the analysis of carbaryl in the developed method. Both the extraction methods were examined for blank also, and it was confirmed that the presence of carbaryl was within the acceptable range (0.009–0.1 ppm).

CONCLUSION

The present study focused on LC-MS/MS method development for carbaryl insecticide by optimizing MRM parameters which is not only improves the sensitivity and intensity; it also prevents the interferences which significantly influence the quantitative analysis. Moreover, it also highlights the importance of the extraction methodology which can significantly affect the efficiency of the method in a complex matrix like honey. Furthermore, this study concluded that both ultrasound-SPE and QuEChERS extraction techniques yielded similar recovery %. Hence, both the extraction process can be applied for the analysis of carbaryl residue in honey. However, the advantage of ultrasound-SPE extraction over QuEChERS is it did not require salts for improvement of the recovery %.

Acknowledgements

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Conflicts of interest

There are no conflicts of interest.

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