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### MODIFIED QUECHERS EXTRACTION METHOD FOR THE EVALUATION OF CARBAMATE PESTICIDES IN FRUITS AND VEGETABLES GROWN IN TIRUPUR, SOUTH INDIA

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#### Abstract

An effective, simple and sensitive analytical method has been developed employing liquid chromatography coupled with tandem mass spectrometry and validated for estimation of 5 carbamate pesticides at trace levels in 3 fruits and 4 vegetables. Plackett-Burman design and central composite design was used to screen and optimize the significant factors in modified QuEChERS (quick, easy, cheap, effective, rugged and safe) extraction method. The method evaluation was done by matrix-matched calibration with linearity ranging from 3 to 500 µg/L with a correlation coefficient more than 0.991. The detection and quantification limit ranged from 1 µg/kg and 3 to 5 µg/kg, respectively. The mean recoveries were in the range of 78.98-109.64% with the relative standard deviation less than 12.62% for all pesticides. Further, the method developed was applied to analyze real samples cultivated in the plain areas of Tiruppur, Tamil nadu.

Key words: Carbamate, Pesticides, Fruits, Vegetables, Experimental design and QuEChERS

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## Introduction

Pesticides have become an indispensable part of modern farming and they play an important role in increasing agricultural productivity. Farmers have are using them to control weeds and insects, and significant increases in agricultural products have been reported. The world population growth in the 20<sup>th</sup> century could not have been possible without a parallel increase in food production<sup>(1)</sup>. About one-third of agricultural products are produced depending on the application of pesticides. There would be a 78% loss of fruit production, a 54% loss of vegetable production, and a 32% loss of cereal production if pesticides were not used<sup>(2)</sup>. It has been reported that approximately two million tons of pesticides are consumed annually throughout the world. Despite their popularity and widespread use, pesticides have raised serious concerns about health risks, as well as residues on food and in drinking water for the general population. Pesticide toxicity depends upon many factors such as use of pesticides, rate of application, spraying technique, climate conditions, humidity and properties of soil such as moisture, temperature and microbial activity<sup>(3)</sup>.

On the basis of chemical properties, pesticides are classified into organophosphate, carbamates, organochlorine, pyrethroid, Neonicotinoid. Amongst these, the carbamate class of pesticides is derivatives of carbamic acid and characterized by a carbamate ester bond as functional group. The effectiveness of Carbamate pesticides, coupled with their relatively cheap cost encourages farmers to use more of these pesticides when growing their field crops<sup>(4)</sup>. Yet, these pesticides pose severe risks to the farmer's health. These compounds act as reversible inhibitors of the enzyme, acetyl cholinesterase of the nervous system. Some carbamates are also suspected carcinogens. Long-term exposure of carbamates can produce delayed neurotoxicity, such as tingling, burning in the extremities and damage to the liver, kidney, immune system and bone marrow may occur<sup>(5)</sup>. Unlike other pesticides such as organophosphate, carbamate pesticides are not persistent in the environment however, their degradation is crucial due to their toxicity to living system. Carbamate pesticide degradation by microorganisms relies not only on the availability of microbes with suitable biodegradative enzymes, but also on the various ecological factors<sup>(6)</sup>.

Additionally, the extraction method also plays a major role in estimating the pesticide residues in the matrices. The use of QuEChERS (quick, easy, cheap, effective, rugged and safe) method has found to be an efficient pretreatment method as it is been developed to monitor the residual level in all kind of matrices. Moreover, the QuEChERS method provides an extensive analytical scope compared to the conventional extraction methods. However, the major drawback in the conventional QuEChERS method is large factor optimization and neglecting the interactions between factors, Lately, the use of multivariate analysis (screening and optimization designs) has been considered for the optimization of extraction method as it provides less experimental runs, interactions between factors and involves optimization of multiple factors simultaneously.

According to the studies, Carbofuran, which is a carbamate, has been reported to cause serious reproductive problems, while occupational exposure to Carbaryl has been reported to result in

nausea, vomiting, blurred vision, coma and difficulty in breathing<sup>(7)</sup>. At present, highly toxic carbamate class of insecticides is found to be predominant in the crop cultivations. In particular, Carbaryl, Carbofuran are majorly used in Indian agriculture to prevent crops from pests. Despite the application in crop cultivations, some of the carbamate pesticides (Aldicarb, Oxamyl, Methiocarb, etc.) have no prescribed MRLs for the cultivation of fruits and vegetables in India. Hence, it becomes essential to have continuous monitoring of pesticide residue to safeguard consumers from health effects.

Tirupur is a major knitwear centre in South India, with over 9000 small-scale units producing one-third of India's total apparel exports. Agriculture remains the most important sector of the Tirupur District economy; agriculture and related activities employ approximately 30% of the working population. The remaining 70% of the population is employed in the textile industry. As a result of the impact of dyeing units that discharge wastewater into the river, many problems arise in agricultural land. Vegetables and fruits are the most important nutrients of healthy diet but in some cases, they become toxic due to the presence of pesticides. Most of the studies revealed that the presence of pesticides in some vegetables is above the permissible limit. Excessive pesticides are used to increase yield; therefore, it is essential to evaluate pesticide residues in vegetables and fruits to ensure food quality and protect consumers from potential health risks.

The objectives of the present work are to develop a rapid liquid chromatography-tandem - mass spectrometry (LC-MS/MS) method for the simultaneous estimation of selected pesticides, develop an extraction method by modifying the QuEChERS extraction method with the aid of multivariate approach for the optimization of extraction method to achieve better recovery of analytes in less time, to perform stress degradation analysis and evaluate the pesticide residues in major fruits and vegetables cultivated in Tirupur, South India.

## Reagents and materials

The pesticide standard Aldicarb, Carbaryl, Carbofuran, Methiocarb, Oxamyl were purchased in form of gift from Global lab solutions Ltd. A standard stock solution of each pesticide was prepared using, methanol (1000µg/L) and stored at 8°C. Methanol and Acetonitrile of LC-MS grade were purchased from Thermo Fisher Scientific Pvt. Ltd. AR grade formic acid, anhydrous magnesium sulphate (MgSO<sub>4</sub>), sodium chloride (NaCl) was procured from SD Fine chemicals (Mumbai, India). Graphitized carbon black (GCB), primary secondary amine (PSA), C18 sorbents and syringe filters (0.45µm) were purchased from Sigma-Aldrich. Ultrapure water used for analysis was obtained from Milli-Q RO system (Millipore, Bangalore, India). The calibration standards were prepared by spiking the pesticide standards into the matrix-matched blank standards prepared from 4 vegetables and 3 fruits, known as matrix matched standards for quantification. The concentrations of the calibration curve standards were 3 to 500µg/L.

The statistical analysis for screening and optimization design was done by design expert software.

## 2.2. UFLC-MS/MS conditions

The quantification was performed using shimadzu 8030 LC system coupled with tandem mass spectrometry (Tokyo, Japan) equipped with LC-20AD pump, SIL-20AC autosampler, CBM-20 Alite controller, CTO-20AC column oven and SPD-M 20 PDA detector. The separation was achieved by YMC C18 Column (4.60mm×50mm, 5µm particle size) using the mobile phase (A) 0.05% formic acid and methanol (B) at the flow rate of 0.8mL/min. The following gradient programme was used: an initial composition of 80 % solvent B and 20% solvent A for 10 min. The separation and stabilization were achieved within 8.0min. The column temperature was set at 35°C. As shown in fig. 1 the compounds were eluted in the following order: Aldicarb (3.033min), Carbaryl(3.888min), Carbofuran (4.289min), Methiocarb (7.102min) Oxamyl (5.037min).

The quantification of the pesticides was carried out using a triple quadrupole mass spectrometry equipped with electrospray ionization (ESI) source, operating in a positive ionization mode. The control and acquisition of the instrument were carried using Lab solution data station. For nebulization and collision, nitrogen (99.95%) and argon gas (99.99%) were used. The following MS/MS conditions were optimized for the target compounds: The desolvation line (DL) temperature and heat block temperature were set at 3L/min and 15L/min, respectively. Multiple reaction monitoring (MRM) mode was operated for each compound. The molecular weight, precursor ions, product ions, collision energy (CE) of the analytes are stated in table 1.

Analyte	Molecular weight(g/mol)	Adduct Ion	Precursor ion(m/z)	Product ions(m/z)	ESI mode
Aminocarb	208.26	[M + H] <sup>+</sup>	209.16	89.00	positive
				116.05	
				41.70	
Carbaryl	201.22	[M + H] <sup>+</sup>	202.12	145.00	positive
				127.10	
				117.05	
Carbofuran	221.25	[M + H] <sup>+</sup>	222.21	165.05	positive
				123.05	
				55.15	
Methiocarb	225.31	[M + H] <sup>+</sup>	226.01	169.05	positive
				123.05	
				55.15	
Oxamyl	219.26	[M + H] <sup>+</sup>	220.10	146.10	positive
				203.05	
				145.05	

Table 1 Optimized mass parameters for the determined five carbamate pesticides using LC-MS/MS

### **Sample collection**

A total of 700 samples were collected randomly at appropriate seasons from different markets of four cities in Tiruppur district. The samples of fresh fruits included Amla, Banana and Sapota while fresh vegetables include Tomato, onion, Bhendi, Brinjal. The samples size was one kg (minimum) which was collected for both small and large size fresh products, which were immediately stored in ice-chest at 4°C and sent to laboratory for analysis.

### **Sample extraction and pre-treatment**

A portion of 10g of sub-sample was transferred to a 50mL centrifuge tube. Next, 10mL of ACN (acidified with formic acid 8%) was added and shaken thoroughly to ensure that the solvent contacted the whole sample. Subsequently, 5g, of MgSO<sub>4</sub> and 1.2g of NaCl were added, the tubes were shaken vigorously using vortex mixer for 1.2 min enhance the extraction process by phase partition. Anhydrous MgSO<sub>4</sub> is more effective in solvent partitioning and NaCl reduces the amount of polar interferences during the extraction efficiency of polar analytes. After centrifuging for 5 min at 5000 rpm, the aliquot (3mL) was transferred to a centrifuge tube containing 59mg PSA, 9mg GCB, 100mg C18 and 125mg MgSO<sub>4</sub>. The centrifuge tube was shaken for 1 min and centrifuged at 5000 rpm for 5 min. The supernatant collected was filtered through a nylon syringe filter and then transferred to auto sampler vials for analysis by LC-MS/MS.

The parameters PSA amount (59mg), formic acid percentage (8%) in extraction solvent, extraction time (1.2 min) and NaCl amount (1.2g) were predetermined in the multivariate optimization section by Plackett-Burman (P-B) design and central composite design (CCD).

### **2.5. Multivariate optimization**

Leek vegetable samples were used as matrix for optimization of extraction process due to their complexity. The optimization by multivariate approach was done using two-step process. Initially, the seven factors i.e., amount of PSA (mg), NaCl (g), GCB (mg), C18 (mg), anhydrous MgSO<sub>4</sub> (mg) along with formic acid (%) and extraction time (min) were studied using P-B design to screen the significant variables affecting the extraction efficiency of the pesticides. These factors were studied at two levels (low and high) to enhance the extraction efficiency of wide range of pesticides in multi-residue analysis. Later, the screened significant factors from P-B design were optimized through CCD. In order to get a suitable extraction method, chromatographic response (peak area) was used as a target to optimize the significant factors.

## **Results and discussion**

### **3.1 Experimental design**

#### **Screening design**

The seven factors i.e., amount of PSA (mg), NaCl (g), GCB (mg), C18 (mg), anhydrous MgSO<sub>4</sub> (mg) along with formic acid (%) and extraction time (min) were studied

using P-B design to screen the significant variables affecting the extraction efficiency of the pesticides (Table 5). These factors were studied at low and high level to enhance the extraction efficiency of wide range of pesticides in multi-residue analysis.

**Table 2. Experimental factor and levels of screening design**

Factors	Unit	Levels	
		Low (-)	High (+)
(A) PSA amount	Mg	0	100
(B) NaCl amount	G	0.5	2
(C) Formic acid	%	0	10
(D) Extraction time	Min	1	10
(E) GCB amount	Mg	0	150
(F) C18 amount	Mg	0	300
(G) Anhydrous MgSO <sub>4</sub> amount	Mg	100	300

## Optimization design

The significant factors {PSA amount (A), NaCl amount (B), formic acid percentage (C) and extraction time (D)} obtained from the P-B design were further studied and optimized using CCD. The selected factors were evaluated at different levels low (-), medium (0), high level (+) and at axial points -  $\alpha$  and +  $\alpha$ . A total of 30 experiments were performed considering the center points for estimating the experimental variance. ANOVA was used to access the relationship between accuracy and quality of the fitted model using coefficient of determination ( $R^2$ ).

From the multivariate response optimization it was found that PSA amount (59 mg), NaCl amount (1.2 g), formic acid percentage (8 %) and extraction time (1.2 min) with maximum recoveries of Aminocarb (101.80 %), Carbaryl (103.57 %) Carbofuran (102.80 %) Methiocarb (100.79 %) and Oxamyl (99.17 %) with a composite desirability of 0.9839. Based on the predicted values a new experimental run was performed where we obtained maximum recoveries of Aminocarb (104.87%), Carbaryl (105.74 %), Carbofuran (103.84 %), Methiocarb (98.32 %) and Oxamyl (97.26 %) with an experimental error of 3.62 %, 2.17 %, 2.60 %, 2.47 % and 1.85 % respectively, which are under 95 % confidence intervals, indicating the experimental fit.

## 3.2 Method performance

### 3.2.1 Calibration curve

The method responses were found to be linear over the concentration range of 3-500 $\mu$ g/L with correlation coefficient ( $r^2$ ) ranging from 0.9991 to 1.000 using diluent methanol and matrix-

matched calibration in blank extracts of the matrices, as shown in table s2 and the obtained results were in accordance to the guidelines.

### **3.2.2 Limit of detection (LOD) and quantification (LOQ)**

The detection limit were calculated three times the signal-to-noise ratio ( $S/N=3$ ) and the quantification limit were calculated at ten times at the above mentioned ratio ( $S/N=10$ ) in fruits and vegetable matrices. As summarized in table s2 LOD and LOQ ranged between  $1\mu\text{g}/\text{kg}$  and between  $3\text{--}5\mu\text{g}/\text{kg}$ , respectively.

Both the limits were much lower than the MRLs stated for the pesticide residue in the analyzed fruits and vegetables.

### **3.2.3 Recovery and precision**

For the recovery and repeatability determination, the samples (twelve vegetables and six fruits) were spiked in blank at three concentration levels (LOQ,  $50\times\text{LOQ}$  and  $100\times\text{LOQ}$ ) and analyzed at six replicates to evaluate the accuracy and precision, respectively. The recoveries of the method were found to be satisfactory in the range between 78.98 and 109.64% with relative standard deviations (RSDs) of less than 20%. The repeatability ( $n=6$ ) studies were found to be good with RSDs ranging from 0.84 to 12.62%. The satisfactory results contribute to the effective use of the designs in the optimization of modified QuEChERS technique with higher extraction efficiency.

### **3.3 Application of the method to real samples**

In order to access the potential application of the developed method and the feasibility of the pretreatment procedure, 700 samples of fresh fruits and vegetables were analyzed by the proposed method. An overview of the data obtained after the analysis of 700 samples is described in Table 4. In 273 (39.01%) number of samples pesticides were not detected and in 325(46.42%) samples pesticides residue were detected at or below the MRLs laid down by the European legislation. 102 samples (14.57%) contained pesticide residue above the maximum residual level in terms of commodity group. Among the 102 samples, Carbofuran (74.56%) and Carbaryl (61.96%) were highly found in the analyzed samples followed by Aldicarb (45.06%), Methiocarb (34.10%) and Oxamyl (20.48%). Brinjal and onion were mostly contaminated with pesticide as it is widely grown crops in this region. Farmers tend to use more pesticide to maintain its quality under the changed climate condition.

### **4. Conclusion**

A multi-residue analysis based on multivariate approach for modifies QuEChERS extraction method has been developed for the determination of five commonly used carbamate pesticides in fresh fruits and vegetables grown in tirupur, south India by LC-MS/MS. The significance of multivariate approach (equipped with a P-B design and CCD) proved to be influential in improving the extraction efficiency. The developed extraction method is found to be simple and robust with reduced analysis time, cost and suitability for pesticide monitoring in fruits and vegetables. Further, the applicability of the proposed method is demonstrated through analysis of fruits and vegetables grown in Tirupur, India. Majorly, the contamination level of carbamate pesticide residue was found below the MRLs of the EU regulatory body. However,

few commodities (Brinjal, onion, tomato and banana) were found to have pesticide residue higher than the MRLs. Hence, it is essential to control and reduce the level of pesticide use in these commodities by law enforcement. Nevertheless, residual monitoring is important to ensure minimal pesticide residue level in fruits and vegetables.

### **Conflict of Interest**

The authors declare that they have no known competing financial interests that could have appeared to influence the work reported in this paper.

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