

## LABORATORY VALIDATION OF AN LC-MS/MS METHOD FOR SIMULTANEOUS DETERMINATION OF CARBAMATE RESIDUES IN VEGETABLES

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### ABSTRACT

**Background.** The extensive use of carbamate pesticides in agriculture has increased serious public concern regarding the environment and food safety. Carbamate pesticides have been reported to cause deleterious effects on the immune system and fertility issues in both humans and animals. A liquid chromatography/tandem mass spectrometry (LC-MS/MS) method for the simultaneous determination of carbamate residues in vegetables has been developed and validated with quantification levels respecting the action levels established for the maximum residue limits (MRL) according to the European Union, World Health Organization (WHO), and Vietnam.

**Materials and methods.** In the proposed method, the pretreatment protocol was shortened to reduce time, and chemical usage was also minimized to decrease cost. The validation was performed according to the Commission Implementing Regulation (EU) 2021/808, which includes linearity, limits of detection (LOD), limits of quantification (LOQ), recovery, and repeatability.

**Results.** Based on the LC-MS/MS experiments, the matrix-matched calibration method showed good performance in the concentration from 5 to 200 µg/kg for vegetable samples, with a correlation coefficient of  $R^2$  value of  $> 0.996$ . The limits of quantification for all carbamate pesticides were 5 µg/kg. Recoveries of all carbamate pesticides were from 91 to 109% and repeatabilities (RSD) were  $< 10\%$ .

**Conclusion.** The validation data indicate the developed LC/MS/MS method is suitable for the determination of carbamate pesticides in vegetables.

**Keywords:** carbamate residue, LC-MS/MS, method validation, vegetable

### INTRODUCTION

Carbamates are insecticide chemicals used to control pests in crops, home gardens, and in a domestic environment. It is used on ornamentals, lawns, fruit and vegetables, and around public buildings. It is also used in the control of insects on domestic animals. Carbamate residues that may remain on the foods destined

for human consumption after application could be a major food safety risk and may have adverse impacts on the environment such as water, soil, and air, which cause an imbalance in the ecosystem (Ecobichon, 2001). Extensive research has focused on investigating carbamate toxicity. All publications have shown

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evidence that carbamate pesticides have deleterious effects on the immune system (Cha et al., 2000; Dhoub et al., 2016). It was reported that pesticides exhibit a toxic effect characterized by the inhibition of the enzyme cholinesterase (AChE) in the hydrolysis of the neurotransmitter acetylcholine, which is responsible for the transmission of nervous impulses (Okazaki et al., 2000). Many nations and organizations, including the European Union, World Health Organization, and Vietnam have established standards/regulations for the maximum residue limits (MRL) in vegetables and fruits due to their potential health risks to the human body (Ministry of Health of Vietnam, 2016). The determination of pesticide residues in agricultural products is of high priority to ensure food safety and protect customers against possible health risks. Therefore, to ensure the compliance of marketed food commodities with the law on food safety, the development of a sensitive, rapid, reliable, and simple method for monitoring trace levels of pesticides in foods is required.

Several analytic approaches used to determine carbamate residues in agricultural products and water included gas chromatography with derivatization (Lanqas et al., 1996; Zhang and Lee, 2006; Liu et al., 2005) and high-performance liquid chromatography (HPLC) with various detectors such as a fluorescence detector (Vassilakis et al., 1998; Caballo-López and Luque De Castro, 2003), a UV/Vis detector (Gou et al., 2000; Bernal et al., 1997; Ouertani et al., 2016; Kongphonprom et al., 2016; Ruengprapavuta et al., 2020), LC-MS (Liu et al., 2005), and LC-MS/MS (Dearmond et al., 2015; Wu et al., 2013). However, carbamate pesticides are easily decomposed due to thermal instability during direct GC analysis (Wu et al., 2013). In another method, LC/UV, HPLC with fluorescence detection by post-column derivatization is widely applied for the analysis of carbamate pesticides (Caballo-López and Luque De Castro, 2003; Vassilakis et al., 1998). The disadvantages of both methods are that they are time-consuming and have low sensitivity. Compared with LC-MS, LC-MS/MS has both high selectivity and sensitivity for target analytes, including pesticides residues in fruit and vegetables (Dearmond et al., 2015; Wu et al., 2013; Stachniuk and Emilia, 2016; Ramadan et al., 2016). Among them, LC-MS/MS-multiple reaction

monitoring (MRM) mode is currently considered the most suitable technique to detect multiple classes of target compounds due to its highly selective and sensitive detection of analytes. This indicates that the LC-MRM-MS/MS technique provides clear identification and reliable confirmation (Stachniuk and Emilia, 2016). It was reported that the presence of matrix components could affect the ionization of the target analytes when atmospheric pressure chemical ionization (APCI) or electrospray ionization (ESI) was used (Dearmond et al., 2015; Wu et al., 2013; Stachniuk and Fornal, 2016; Ramadan et al., 2016). It was also considered that very tiny matrices from biological samples can damage the pump head and the column of the HPLC instrument. To reduce matrix effects, more time or greater cost are required because complex treatments of samples (e.g., organic solvent, solid-phase extraction, combining QueEChERS (quick, easy, cheap, effective, rugged, and safe) procedure or using graphitized carbon black (GCB) to remove pigments) are needed prior to analysis of analytes. Taking these factors into account, this work aimed to develop and validate a robust and sensitive LC-MS/MS method for simple, fast, and reliable determination of the carbamate residues in vegetables for routine application purposes. Six carbamate residues (methomyl, aldicarb, carbaryl, fenobucarb, carbosulfan, and methiocarb) are usually required to be controlled in many countries through regulatory surveys, including Vietnam. A vegetable, pak choy, was selected for this study.

## MATERIALS AND METHODS

### Chemicals and reagents

Methomyl, aldicarb, carbaryl, fenobucarb, carbosulfan, and methiocarb were purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany). The minimum purity of all standards was 98.0%. Distilled water, acetonitrile (ACN), methanol (MeOH), formic acid (FA), acetic acid ( $\text{CH}_3\text{COOH}$ ), magnesium sulfate ( $\text{MgSO}_4$ ), sodium acetate ( $\text{CH}_3\text{COONa}$ ), primary secondary amine (PSA), and graphite carbon black (GCB) sorbents were purchased from Merck (Darmstadt, Germany). All other reagents were analytical reagent grade and were without further purification.

### Standard solutions

Each individual stock solution of 1000 µg/mL was prepared in ACN and then stored in darkness at –20°C. Intermediate standard-mix solutions at concentrations of 1 and 10 µg/mL were prepared with a combination of six individual stock solutions. The mixed standard solutions were stored at –20°C and diluted daily prior to experiments by further diluting them with ACN to give appropriate working solutions.

### Sample preparation and clean-up

Pak choy vegetables were purchased from a local market in Vietnam and were minced using a mechanical grinder. Blank samples were chosen from the samples in which there were no carbamate pesticides. An AOAC method was applied and modified to extract carbamate pesticides from vegetables (AOAC, 2010). For sample preparation, 5 ±0.1 g of minced raw vegetable were transferred into a 50 mL conical tube. The extraction of carbamate pesticides was obtained by the addition of 15 mL of 1% CH<sub>3</sub>COOH in ACN to the samples. A portion of 3.0 g of anhydrous MgSO<sub>4</sub> and 0.75 g of CH<sub>3</sub>COONa were gradually added and the tubes of samples were tightly capped. They were then carefully vortexed for 1 min, shaken by a mechanical shaker for 30 min, and then centrifuged at 4500 rpm for 5 min. One mL of supernatant was collected and transferred into a new tube containing 150 mg of anhydrous MgSO<sub>4</sub>, 50 mg of PSA, and 50 mg of GCB. The tube was vortexed for 30 sec. and centrifuged for 10 min. The resultant solution was filtered using a 0.22 µm membrane filter (Minisart RC 15, Sartorius, Germany), and an aliquot (10 µL) of the filtrate was injected into LC-MS/MS.

### Method validation

#### Linearity

Although quantification methods using isotopically labeled internal standards (IS) must compensate for the effects of matrix contaminants, the use of isotopically labeled techniques can be limited due to the cost-effectiveness and availability of isotopically labeled compounds. Therefore, an absolute standard curve method using plank vegetable (pak choy) was employed for pesticide assay to avoid a matrix effect from contaminants in the sample. The blank vegetables were used to

prepare mix-matched calibration solutions by spiking them with the intermediate standard-mix solutions of 1 and 10 µg/mL to give the final concentrations of 5, 10, 20, 50, 100, and 200 µg/kg in samples. Carbamate pesticides spiked in blank samples were extracted with 1% CH<sub>3</sub>COOH in ACN, as mentioned above. In the clean-up step, blank vegetable samples were spiked with carbamates, similarly to the above-mentioned sample preparation.

#### Limits of detection and limits of quantification

Limits of detection (LODs) and limits of quantification (LOQs) were determined by analyzing blank samples spiked at 5 µg/kg, for which a signal to noise ratios was > 3 (S/N > 3) and > 10 (S/N >10), respectively.

#### Recovery and repeatability

Accuracy and precision were expressed as the percentage of recovery and repeatability, respectively. The recovery and repeatability were evaluated by spiking blank vegetables with mixed standard solutions at three final concentration levels of 10, 15, and 20 µg/kg in ten replicates at each level.

#### LC-MS/MS analysis

A system for successive determination of six pesticides in the vegetables was performed on an ExionLC analytical HPLC system, using an API Sciex Triple Quadrupole 3200 mass spectrometer equipped with electrospray ionization (USA), and data were collected in a positive ionization mode. Chromatographic separation was performed on a Phenomenex C<sub>18</sub> column (3 µm particle size, 2 × 50 mm) maintained at 40°C. For optimizing chromatographic separations of the target analyte, elution of 6–95% MeOH containing 0.1% FA at a flow rate of 0.4 mL/min was conducted as follows (time in minutes per percent solvent B): 0.0/6, 0.5/6, 0.6/55, 2.0/75, 4/100, 4.4/100, 5.0/5, and 7.0/5 (total run time = 7 min). The MS/MS parameters were as follows: capillary voltage, 5.500 V; curtain gas, 25 psi; collision gas, 5 psi; nebulizer gas (gas 1) and heater gas (gas 2), 50 psi each; and dry temperature, 450°C. The MRM mode was used for quantification of the target analytes. Precursor ions (*m/z*), product ions (*m/z*), corresponding declustering potentials (DPs), and collision energies (CEs) were shown in Table 1.

**Table 1.** Retention time and MS parameters of six carbamate pesticides

Analytes	Retention time, min	Ion	Productions, <i>m/z</i>	Quantitative ions, <i>m/z</i>	DP, V	CE, eV
Fenobucarb	2.76	positive	208.1>95.1	208.1>95.1	31	21
			208.1>152.1			13
Methiocarb	2.87	positive	226.0>121.1	226.0>121.1	50	23
			226.0>169.3			15
Carbaryl	2.48	positive	202.1>145.1	202.1>145.1	35	17
			202.1>127.2			31
Methomyl	1.94	positive	163.0>88.1	163.0>88.1	21	17
			163.0>106.2			15
Aldicarb	2.25	positive	208.1>89.1	208.1>89.1	16	15
			208.1>115.9			23
Carbosulfan	4.53	positive	381.1>118.2	381.1>118.2	71	27
			381.1>75.9			23

## RESULTS AND DISCUSSION

### LC-MS/MS analysis

The LC-MS/MS method was developed to confirm data for the determination of carbamate residues in vegetables. The precursor ions, product ions, and collision energy were chosen and optimized by the injection of a mixed standard solution of 1 µg/mL directly into mass spectrometry. The precursor ions were the highest *m/z* and most intense ion beam, which were in a positive ionization mode. The product ions and collision energies were automatically optimized using the analyst system software (Analyst). The LC parameters were also optimized to obtain peaks of Gaussian shape, and the high signal to noise (S/N) at a concentration of 5 µg/kg spiked at pak choy samples with values above 10. To optimize the LC separation of target analytes, several trials were required to select a suitable mobile phase composition and linear elution gradient for developing a sensitive and selective method. This was due to the complexity of the chemical compounds in biological samples and the affinities of the components toward various solvents. Ahn et al. (2020) used a mobile phase consisting of ammonium acetate as ion-pair reagents in water and ACN to improve peak shape and facilitate resolving closely eluted compounds (Ahn et al., 2021).

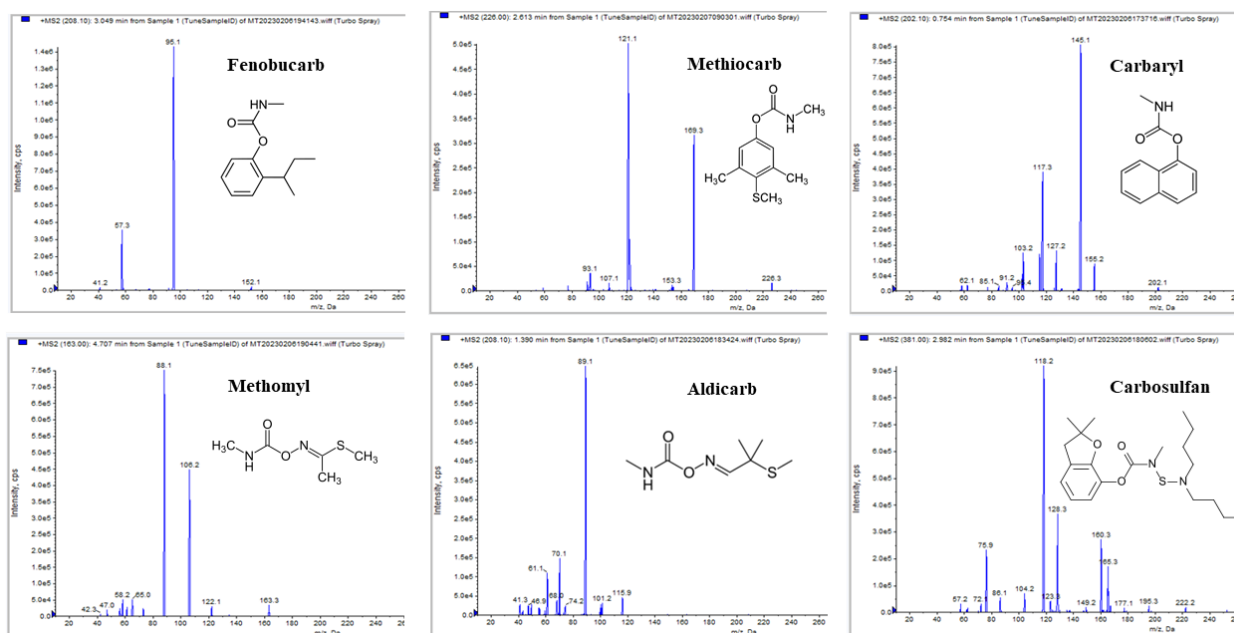
Another study by Tran et al. (2015) used a mobile phase composed of 0.1% (v/v) acetic acid in water (eluent A) and MeOH (eluent B) (Tran et al., 2015). However, the result showed the peak of methomyl was not symmetric due to strong interaction with the residual silanol group on the packing surface of the LC column. It was considered that this may be due to the low proportion of water in the mobile phase; inorganic acid or salt easily precipitates from the mobile phase, resulting in very tiny precipitated particles that can damage the pump head of the HPLC instrument (Yan et al., 2010). It was demonstrated that chromatographic resolution did not change significantly, and the MS signal for most pesticide residues decreased by a factor of 5–10 when ACN/water was compared to that of MeOH/water (Liu et al., 2005, Jansson et al., 2004). This may be due to the fact that ACN is a weaker proton donor than MeOH (Liu et al., 2005). In addition, MeOH is less toxic and inexpensive compared with ACN. MeOH was used as an organic solvent for the mobile phase of this study. Liu et al. (2005) reported that the signal of MH<sup>+</sup> ions and the signal to noise (S/N) of analytes increased when FA was added at a lower concentration of 0.2% (v/v) in the mobile phase (Liu et al., 2005). Thus, in this study, formic acid (FA) was added at 0.1% in the mobile phase to improve ionization in positive-ion mode.

Collisionally-induced dissociation mass were observed in product ion mode, and all target analytes were ionized in positive ion mode (Table 1). As shown in Table 1 and Figure 1, peaks of pure fenobucarb, methiocarb, carbaryl, methomyl, aldicarb, and carbosulfan spiked at 5 µg/kg in vegetable samples were observed at the retention time of 2.76, 2.87, 2.48, 1.94, 2.25, and 4.53, respectively. The product ions with the stronger signals (fenobucarb,  $m/z$  95.1; methiocarb,  $m/z$  121.1; carbaryl,  $m/z$  145.1; methomyl,  $m/z$  88.1; aldicarb,  $m/z$  89.1; and carbosulfan,  $m/z$  118.2) were selected as the ions for quantification; the others were used for confirmation (Fig. 1–2). These results were in agreement with the retention time and product ions reported by other studies (Ahn et al., 2021; Tran et al., 2015; Ma et al., 2020). As shown in Figure 2, our results also showed good symmetric peaks, indicating that the MS/MS could give high selectivity and sensitivity for the detection of pesticides in vegetable samples.

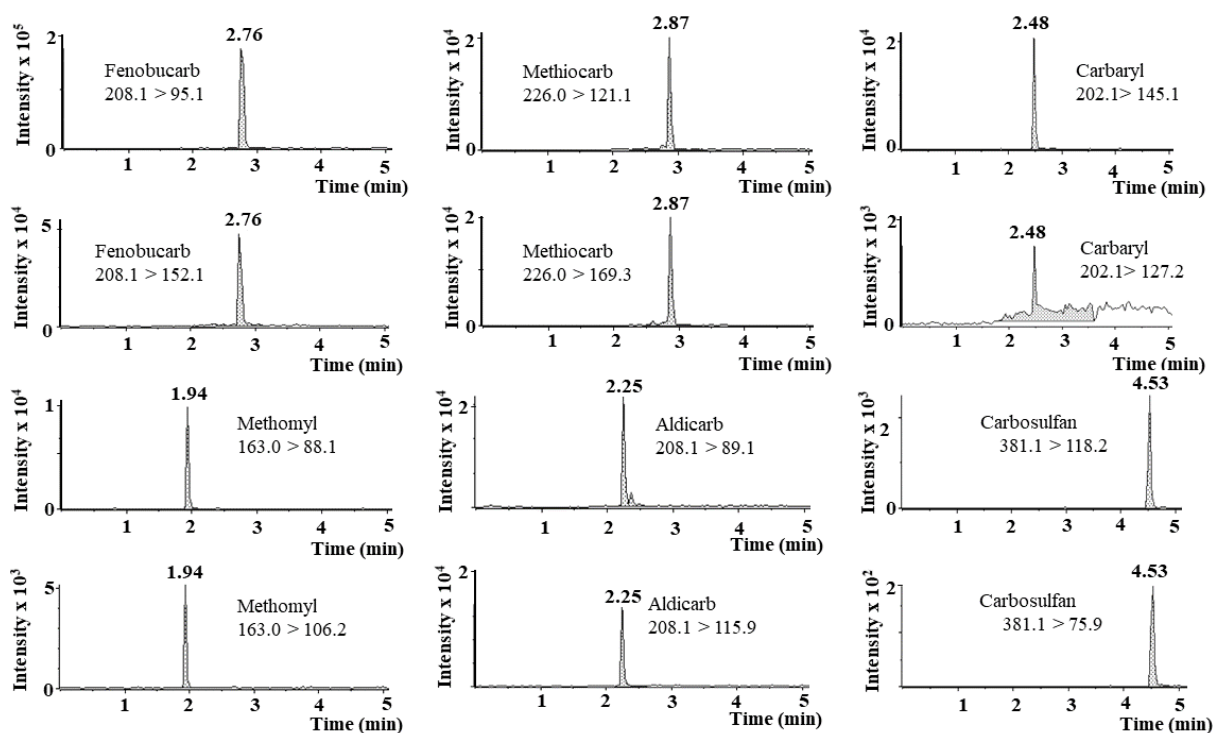
### Sample extraction and cleaning-up

The extraction procedure of carbamate pesticides from vegetables plays an essential part in analytical

methods. To extract pesticides from samples, some researchers have applied one of two versions of the public QuEChERS method, including the original version by Anastassiades et al. (2005) and the AOAC version based on QuEChERS method (AOAC, 2010). The original QuEChERS method uses a single-step buffered ACN extraction and salting out liquid-liquid partitioning from the water in the sample with  $MgSO_4$ , while the AOAC QuEChERS version uses 0.1%  $CH_3COOH$  in ACN extracts, together with a mixture of  $MgSO_4$  and  $CH_3COONa$  prior to the clean-up step. Tran et al. (2015) used a modified QuEChERS method, using lead acetate in the extraction step to absorb polyphenols, caffeine, and pigments in green tea (Tran et al., 2015). The study showed that the use of lead acetate was important to obtain a better cleaning and a higher recovery because green tea is rich in polyphenols and caffeine. A study by Ma et al. (2019) showed that using multi-flug filtration cleanup (m-PFC) with different tip-filtration could compensate for matrix-matched calibration (Ma et al., 2020). However, low recoveries of some pesticides were still observed using the modified QuEChERS or m-PFC with different



**Fig. 1.** Chemical structures and product ion mass spectra of carbamate pesticides with collision-induced dissociation



**Fig. 2.** Exactions chromatogram of six carbamate pesticides spiked in the vegetable sample at a final concentration of 5 µg/kg

tip-filtration methods. In another study, Ahn et al (2020) used a mixture of methanol and dichloromethane (1:99, v/v) for residue reconstitution from dried extraction under N<sub>2</sub> gas, applied an ENVI-Carb/LC-NH<sub>2</sub> solid-phase extraction (SPE) cartridge for cleaning of the target analytes, and then reconstituted it with ammonium formate solution/methanol after the eluent was evaporated under N<sub>2</sub> gas. As a result, proper recoveries of three target pesticide residues were obtained. However, this technique was time-consuming due to complex steps and requiring many chemicals. In the present study, we used PSA sorbent plus 150 g of anhydrous MgSO<sub>4</sub> and 50 mg of graphite carbon black (GCB) for the sample clean-up step. It was proved that PSA has good absorbance and that it can clean up the interfering components in the matrix of fruits and vegetables (Liu et al., 2006; Zhou et al., 2018). GCB has a strong affinity toward planar molecules and thus effectively removes pigments (e.g., chlorophyll, carotenoids) as well as sterols that are commonly present in

foods (Anastassiades et al., 2005). In contrast, it was also reported that GCB might retain structurally planar pesticides, leading to reduced recoveries (Anastassiades et al., 2005). After testing recoveries of the target pesticides following (1) PSA and (2) PSA + GCB, PSA + GCB was selected due to its proper recovery (data not shown). It was very important to consider that tiny matrices such as chlorophyll and/or carotenoids from plant samples can damage the pump head and the column of the HPLC instrument. Thus, the combination of PSA sorbent and GCB would be suitable for the sample clean-up step. However, for vegetable samples with low anthocyanin, chlorophyll, or carotenoid content such as cabbage or radish, it may not be necessary to use GCB because this can reduce the recovery of target analytes. It is also recommended to add Z-Sep<sup>+</sup>, consisting of both C18 and zirconia bonded to the same silica particles, or a mixture of C18 + PSA for cleaning extracts of highly fatty vegetable commodities (Moreno-González et al., 2014).

### Method validation

The validation was performed according to the Commission Implementing Regulation (EU) 2021/808 (European Commission, 2021). The LC-MS/MS conditions and the sample clean-up procedure for analysis of the target carbamate pesticides in the vegetable samples were optimized. For selectivity, every target carbamate pesticide had two signals from product ions (Table 1 and Fig. 1), and ion ratios of the target compounds spiked in the samples (as shown in Fig. 2) were also used for confirmation. The limits of detection (LOD) (S/N ratio of 3) and limit of quantification (LOQ) (S/N ratio of 10) were estimated by fortifying the pak choy samples with each analyte at a level of 5 µg/kg. As shown in Table 2, all carbamate pesticides could be quantified at the level of 5 µg/kg. These LODs and LOQs of all the carbamate pesticides were lower than the MRLs established by the EU and by many other countries. Thus, this value is acceptable compared to the default maximum residue level (MRL = 10 µg/kg). LODs and LOQs in this study are consistent with other results reported in the analysis of pesticide residues in vegetable samples (Ahn et

al., 2021; Tran et al., 2015; Ma et al., 2020). A linear range of 5 to 200 µg/kg was used in the investigation. The response of matched linearity was evaluated by the determination coefficient ( $R^2$ ) that was equal to or higher than 0.996. These results indicated that the linearity was good for all analytes in the range of tested concentration, as proved by the above coefficient correlation for all calibration curves. The recovery and repeatability were performed by spiking with pure analytes in blank samples at three concentration levels of 10, 15, and 20 µg/kg in ten replicates at each level. The recoveries and repeatabilities (relative standard deviations, RSD%) obtained in the present study were presented in Table 3. The mean recoveries of all pesticides in the present study were between 97 and 109%, and the relative standard deviations (RSD%) were lower than 10%. The repeatabilities and recoveries in this study were highly satisfactory, compared to those of the previous reports using LC-MS/MS method (Ahn et al., 2021; Tran et al., 2015; Ma et al., 2020). It proved that this method had accuracy appropriate for the quantification of pesticide multi-residues in vegetables.

**Table 2.** Signal to noise (S/N) of each target analyte by LC-MS/MS-MRM at a final concentration of 5 µg/kg in vegetable samples

Number (n = 10)	Fenobucarb	Methiocarb	Carbaryl	Methomyl	Aldicarb	Carbosulfan
1	588.3	144.5	748.1	271.2	298.8	132.5
2	465.8	152.5	721.3	253.7	318.8	130.2
3	486.7	140.5	692.2	211.5	378.6	76.5
4	383.2	150.5	777.5	258.3	377.5	147.0
5	291.9	155.5	687.5	208.2	322.2	150.5
6	590.5	175.5	832.6	283.5	367.1	135.5
7	582.6	150.2	722.3	242.5	308.5	78.5
8	500.9	127	767.2	252.8	340.5	150.5
9	526.1	146.5	683.5	240.2	294.1	142.5
10	554.5	135.5	734.4	228.5	281.8	132.5
<i>m/z</i>	208.1>95.1	226>121.1	202.1>145.1	163>88.1	208.1>89.1	381.1>118.2
	208.1>152.1	226>169.3	202.1>127.2	163>106.2	208.1>115.9	381.1>75.9

**Table 3.** Linearity, recovery (R), and repeatability (RSD) at 3 concentration levels (n = 10) of six carbamate pesticides

Analytes	Linear range µg/kg	Correlation coefficient R <sup>2</sup>	Spiked, µg/kg	Found, µg/kg	Recovery R, %	Repeatability RSD, %
Fenobucarb	5–200	0.999	10	9.79 ±0.23	97.92	8.92
			15	16.36 ±0.31	109.13	2.18
			20	20.08 ±0.27	100.40	1.66
Methiocarb	5–200	0.999	10	10.11 ±0.30	101.14	8.92
			15	16.36 ±0.49	109.07	2.47
			20	20.38 ±1.10	101.90	4.23
Carbaryl	5–200	0.997	10	9.55 ±0.58	95.48	5.09
			15	16.40 ±0.31	109.33	2.74
			20	20.09 ±0.73	100.45	5.13
Methomyl	5–200	0.996	10	9.76 ±0.33	97.59	4.28
			15	16.17 ±0.52	107.70	4.05
			20	19.25 ±0.67	97.80	4.28
Aldicarb	5–200	0.998	10	9.12 ±0.39	91.23	3.83
			15	15.36 ±0.38	102.40	5.38
			20	19.54 ±0.51	97.70	3.12
Carbosulfan	5–200	0.997	10	10.02 ±0.60	100.20	9.75
			15	16.26 ±0.95	108.53	6.80
			20	20.15 ±0.67	100.75	4.50

## CONCLUSION

In the present study, a simple, fast, and sensitive analytical method involving LC-MS/MS was developed and validated for the simultaneous determination of carbamate pesticides in vegetables. To ensure the removal of matrix interferences from vegetable samples, the combination of PSA sorbent and GCB was applied in the sample preparation and clean-up. On the basis of the results obtained for sample preparation and clean-up and method validation including linearity, detection capability, recovery, and repeatability, it was concluded that the described method is suitable for the multi-residue analysis of carbamate pesticides in vegetables.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

## ETHICAL APPROVAL

This article does not contain any studies with human participants or animals performed by any of the authors.

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