Assessing Washing Methods for Reduction of Pesticide Residues in Green Leafy Vegetables

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ABSTRACT

The use and import of pesticides have been increasing each year to increase agricultural production. The most highly consumed pesticide in Nepal is fungicide followed by insecticide, herbicide and others. Exposure to these pesticides may result in serious health effects. The main route of exposure is the consumption of contaminated fruits and vegetables. Many studies have shown to reduce the pesticide residues level through various culinary and processing treatments. This experiment was carried out to compare the effectiveness of four washing methods to reduce pesticide residues in vegetables. These comprised of running water method, washing by using $NaHCO_3$, NaCl, and vinegar solution. The leafy vegetable samples, Bok choy (N=61) were purchased from local markets and analyzed for the contents of the two pesticides, 2,4-D and atrazine. Samples were extracted using Quick, Easy, Cheap, Effective, Rugged, and Safe (QuEChERS) method and analyzed by liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) with stable isotopically-labelled internal standards. The quality of the method was examined in terms of accuracy, precision, linearity, limit of detection (LOD) and limit of quantification (LOQ). Washing vegetables under running tap water significantly decreased ($p<0.05$) 2,4-D residues by 50%. However, atrazine residues were not reduced significantly from washing under running water but washing with vinegar had a significant effect. This study suggests that washing vegetables before consumption is important to reduce intake of pesticides and associated health risks.

Keywords: 2,4-D, Atrazine, LC-MS/MS, Pesticide residues, Washing methods

1. Introduction

Pesticides are the potent agrochemicals used to protect the agricultural production from the damage by insects, weeds and disease carriers (Akoto et al., 2015). Use of pesticides helps to improve production yield and quality of agricultural products. However, pesticides may cause acute and delayed health effects in humans (Akoto et al., 2015). Nepal's economy is mainly based on agriculture with over 65% of the population is engaged in agriculture and contributing 28% of country's GDP (AICC, 2018). Different cereals such as rice, wheat, maize, millet, legumes, oilseeds and vegetables are cultivated. Due to higher rate of commercial agricultural practice and availability of fertile land, pesticide use is higher in the Terai region than in other regions (PPD, 2015). Consumption of pesticide has been increasing by about 10-20% per year (PQPMC, 2019). The import and application of pesticides in Nepal have grown considerably which shows increasing dependency of farmers on chemical pesticides for farming. It has been reported that the average use of pesticides is approximately 396 g of active ingredient per ha (PPD, 2015), and maximum pesticide has been used in vegetable farming.

In present time, research in weed management in Nepal is primarily focused on herbicides. Studies from 2018 show that herbicide use was almost 150, 000 active ingredient (ai) metric tons in Nepal an annual increase of about 6500 kg ai/year (PMRD, 2018). A total of 436 herbicides representing 30 active ingredients and chemical families have been registered in Nepal. 2,4-D is a selective systemic herbicide (Tayeb et al., 2011). It kills plants by increasing cell division in vascular tissue with an increase in cell wall plasticity, biosynthesis of proteins, and production of ethylene in plant tissues which ultimately leads to uncontrolled cell division. In vitro studies have confirmed that 2,4-D promotes the proliferation of androgen-sensitive cells which is a known estrogen receptor ligand (Kim et al., 2005). This establishes 2,4-D as an endocrine disruptor. Atrazine is a herbicide used to kill weeds. It falls in the triazine class which is synthetic systemic herbicide. It is widely used in agricultural practices specifically for broadleaf weeds (Hanson, 2020). The acute poisoning of atrazine at low doses causes diarrhea, vomiting, abdominal pain, eye and skin irritation and at high doses, breathing problems, muscle spasms, hypothermia, convulsion and death (Kennepohl et al., 2010). The most significant effect of atrazine in humans is the disruption of endocrine system and oxidative stress response (Jin et al., 2014).

The main route of human exposure to the pesticides is the consumption of vegetables and fruits (Wanwimolruk et al., 2016). Among all other crops, vegetables accumulate the highest levels of pesticides (Bhandari et al., 2019). This may increase the potential of pesticide toxicity to consumers if the vegetables are consumed freshly and daily (Wanwimolruk et al., 2016). The present study aimed to determine the contents of selected pesticides, 2,4-D and atrazine using Quick, Easy, Cheap, Effective, Rugged, and Safe (QuEChERS) method and liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) analysis. Bok choy was selected for this study as they are cheap, locally available and mostly consumed leafy vegetables in Asia (Wanwimolruk et al., 2016). In some dishes, these vegetables are consumed fresh, without cooking.

Most of the vegetables are first treated with different culinary and treatment practices before consuming, for example; boiling, washing, frying, peeling, refrigeration and baking. These processes are found to have the effects on the contents of pesticide residues (Keikothaile et al., 2010). Many studies have showed that household methods might be useful to wash off the pesticide residues in fruits and vegetables (Ryad & Mahmoud, 2016). This study evaluated the washing methods for the reduction of pesticide contaminants in vegetables. These included washing under running tap water, sodium bicarbonate ($NaHCO_3$), sodium chloride (NaCl) and vinegar. Comparisons between the contents of pesticides between washed and control unwashed samples were done to evaluate the effectiveness of washing methods.

2. Materials and methods

2.1 Sample collection

In this study, 61 vegetable samples were purchased from local markets. Bok choy (Brassica campestris subsp. Chinensis), one of the most consumed vegetables in Asia was selected for this study. All the samples were cut into small pieces, labeled, kept at -20ºC overnight, and then dried using freeze dryer (Labconco, USA) for 48-72 hourrs depending on the water content in the vegetables. The freeze dried samples were blended into powder-like form using blender (Waring, USA). The samples were stored in refrigerator at 4ºC until extraction. Wet weight and dry weight of the samples were recorded using 5-digit balance XS105DU (Mettler Toledo, USA).

Bok choy samples, which were detected with comparatively higher contents of pesticides, were subjected to washing experiments. This experiment was carried out to compare the effectiveness of four washing methods to decrease pesticide residues. These comprised of running water method, washing by using $NaHCO_3$, NaCl, and vinegar solution. These methods are commonly used to wash fruits and vegetables in Asian households (Vemuri et al., 2014; Wanwimolruk et al., 2015). Also these are very affordable and non-toxic chemicals. The procedures for the washing of vegetables were modified from the experiment done by Wanwimolruk et al. In the present study, both chopped and unchopped bok choy samples were used. The root section of the vegetable covered with soil is removed in both sample groups. For chopped sample group, bok choy samples were cut into 2-3 inches' pieces before the washing procedure (Table 1). For running water method, approximately 200 g vegetables were washed under running tap water for 2 min together with gentle rubbing with hand while running water, and the water was discarded. For washing with

$NaHCO_3$, half teaspoon (2.4 g) of $NaHCO_3$ was mixed with 5 L of water and the vegetables were soaked in the $NaHCO_3$ solution for 10 min. Then, vegetable samples were taken out of $NaHCO_3$ solution and immediately rinsed with 5 L tap water. For washing with vinegar, half teaspoon (2.5 ml) of cooking vinegar was added in 5 L of water and vegetables were soaked in the solution for 10 min. Then, vegetable samples were removed from the washing solution and immediately washed with 5 L tap water. Washing with NaCl, 100 g NaCl was mixed with 5 L water and samples were soaked in the solution for 10 min. Then, vegetable samples were removed from the washing solution and immediately washed with 5 L tap water. After the washing step, excess water in the washed vegetables was removed using blotting paper. Then the samples were processed, extracted using QuEChERS method and analyzed with LC-MS/MS technique.

Table 1. The washing procedures of vegetable samples modified from Wanwimolruk et al. (Wanwimolruk et al., 2015).

Vegetable sample	Washing agent	Washing method
Bok choy (n=3)	Running tap water	Vegetables were washed under running tap water for 2 min together with gentle rubbing with hand. Samples were taken out and washed in 5 L tap water.
	$NaHCO_3$	Half teaspoon (2.4 g) of $NaHCO_3$ was mixed with 5 L of water and the vegetables were soaked in the solution for 10 min. Samples were taken out and washed in 5 L tap water.
	NaCl	100 g NaCl was mixed with 5 L water and samples were soaked in the solution for 10 min. Samples were taken out and washed in 5 L tap water.
	Vinegar	Half teaspoon (2.5 mL) of cooking vinegar was added in 5 L of water and vegetables were soaked in the solution for 10 min. Samples were taken out and washed in 5 L tap water.

2.2 Sample extraction procedure using QuEChERS method

Recently, the QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe) method is a widely used method for extraction of pesticide residues (Anastassiades et al., 2003). QuEChERS is a multiresidue and effective method which gives high recovery and can be analyzed in short time (Anastassiades et al., 2003). This method includes a solvent extraction process with the use of acetonitrile and dehydrating salts ($MgSO_4$ and NaCl). The last part of the process is clean-up which is carried out to remove the co-extractives. Extraction and cleaning up process for sample preparation in our study were modified from the multiresidue QuEChERS procedure for determination of pesticides by Anastassiades et al. (Collimore & Bent, 2020). Recoveries were obtained from this method was mostly >95% and repeatabilities achieved were <5% for a wide range of spiked pesticides. For sample preparation, 1.0 g of dried homogenized vegetable sample was weighed and transferred into 50 mL polypropylene (PP) centrifuge tube and ultrapure water 10 mL was added into the tube. Next, mixed isotopically-labeled internal standard (50 ppb) was added into all samples and the mixed standard solution was added to prepare spike samples. The mixture was vortex mixed for a minute and shaken immediately in the incubator shaker SI500 from Stuart (UK) for 15 min at 250 rpm. Extraction solvent, 10 mL acetonitrile containing 1% formic acid, was mixed and then shaken for 15 min at 250 rpm in the incubator shaker. After that, a mixture 4 g of $MgSO_4$ and 1.0 g of NaCl were added carefully and again vortex mixed and shaken for 15 min at 250 rpm in the incubator shaker. The mixture was centrifuged using centrifuge Sorvall ST 16R (Scientific ThermorFisher, Germany) at 4500 rpm for 15 min at 4ºC. After centrifugation, 4 mL of raw extract solution which was the supernatant aliquot of acetonitrile, was transferred into a tube containing 100 mg of chitosan. The tube was tightly sealed, which needed to be further shaken for 10 min at 250 rpm in the incubator shaker and centrifuged at 4500 rpm for 15 min at 4ºC. Extracted solution of 500 µL, and 500 µL of mobile phase consisting of 30:70 of 5 mM Ammonium acetate: MeOH was transferred into microtube and then vortex mixed. Finally, the extract mixture was filtered through 0.2 µm PVDF membrane into amber vial and analyzed by using LC-MS/MS.

2.3 LC-MS/MS analysis

Determination of 2,4-D and atrazine was carried out simultaneously in vegetable samples using LC-MS/MS 6490 Triple Quadrupole (Agilent Technologies, USA). Maximal signal was obtained by optimization of the instrument setting. Negative mode results generated for 2,4-D were obtained from electrospray ionization (ESI) source, operating with the capillary voltages of 3000 V and nebulizer pressure of 25 psi. Gas temperature was set at 200ºC with the gas flow of 19 L/min. The chromatographic separation was performed on a reverse phased column, Kinetex C18 (4.6×150 mm, 2.6 µm) (Phoenomenex, USA) with the operating temperature at 30°C. The mobile phase consisted of two eluents, 5.0 mM ammonium acetate (solvent A) and acetonitrile (solvent B) delivered at a flow rate of 0.4 mL/min with a gradient system (0-2 min, 70%B; 2-6 min, 70-90%B; 6-8 min, 90%B; 8-9 min, 90-70%B; and equilibrated at 70%B for 6 min). The injection volume of each sample was 5.0 µL. The quantitation of 2,4-D was performed using a stable isotope-labeled internal standard by LC-MS/MS technique with the multiple reaction monitoring (MRM) mode. The precursor-to-product ion transitions at m/z 219.0 to 160.8 was used to determine 2,4-D in vegetable samples. The target ion transition with the highest intensity was used for the quantitation while the second target ion transition was used for confirmation. The presence of 2,4-D in vegetable samples was confirmed by comparing the ratios of target ion peak area to second target ion in both standard solutions and samples.

2.4 Quality control

Table 2. Criteria for quality control of analytical method

Evaluation	Criteria
Linearity	R2 > 0.995
Accuracy	Recovery% from spiked samples
Precision	Relative standard deviation (RSD)
Detection of pesticide	>Limit of detection (LOD)

2.5 Statistical analysis

All data are presented in terms of mean ± standard deviation (S.D). The differences between the sample groups in the washing experiment were assessed by a one-way analysis of variance (ANOVA), followed by Sidak's multiple comparisons test. Probability values of less than 0.05 were considered as statistically significant. GraphPad Prism 8.0.1 (GraphPad Software Inc., CA, USA) was used for the statistical analysis.

3. Results and Discussion

3.1 Quality control

Table 3. Criteria for quality control of analytical method

Test	2,4-D	Atrazine	Chlorpyrifos
Concentration	0.25 - 250 ng/mL	0.05 - 50 ng/mL	0.25 - 250 ng/mL
R2	0.9990	0.9991	0.9972
Recovery	104%	98%	100%
RSD	7%	14%	7%
LOD	1	0.02	4
LOQ	2	0.04	10

3.2 Effect of washing methods on pesticide residues in vegetables

This study investigated the effectiveness of washing methods on the reduction of pesticide residues in bok choy samples. This experiment consisted of four different washing methods: running tap water, $NaHCO_3$, NaCl and vinegar. Meanwhile, the bok choy samples were sub-divided into chopped and unchopped sub-groups. These four methods were compared on the basis of their potential to remove two pesticides. Figure 1A shows the changes in concentration of 2,4-D after washing the chopped bok choy samples with different methods. The residues of 2,4-D were found to be decreased from all washing methods. However, the reduction from washing with $NaHCO_3$, NaCl and vinegar were not significant (Figure 1A). The content of 2,4-D in vegetables washed under running tap water was significantly decreased ($p<0.05$) compared to the unwashed samples. The mean 2,4-D contents in samples after washing with running tap water was found to be approximately 50% of those control unwashed samples. For atrazine, the results were different in the experiment (Figure 1B). Washing the chopped bok choy samples with running tap water, $NaHCO_3$ and NaCl methods did not significantly reduced the content of atrazine in the bok choy samples. However, washing with vinegar had the significant effect ($p<0.05$) in atrazine content in the samples as compared to the control unwashed samples (Figure 1B). Mean content of atrazine after washing with vinegar in samples was reduced by approximately 52%.

(A) 2,4-D (B) Atrazine

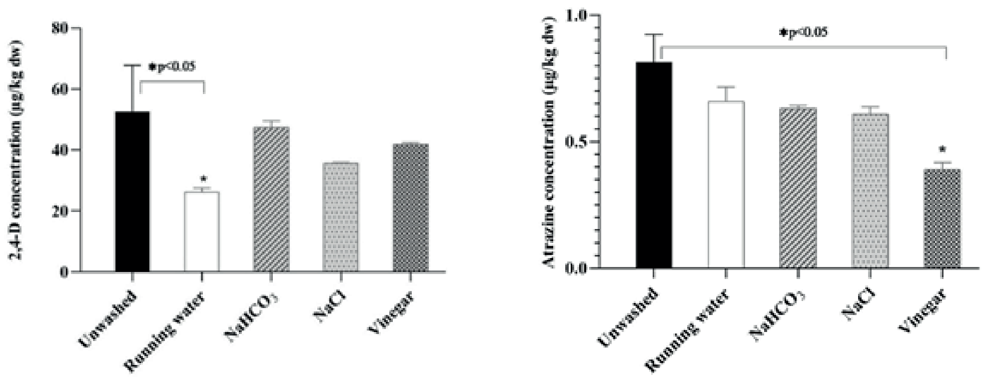

Figure 1. Effects of different washing methods on the reduction of pesticide residues in chopped bok choy samples: (A) 2,4-D and (B) Atrazine. The values are means and standard deviations.

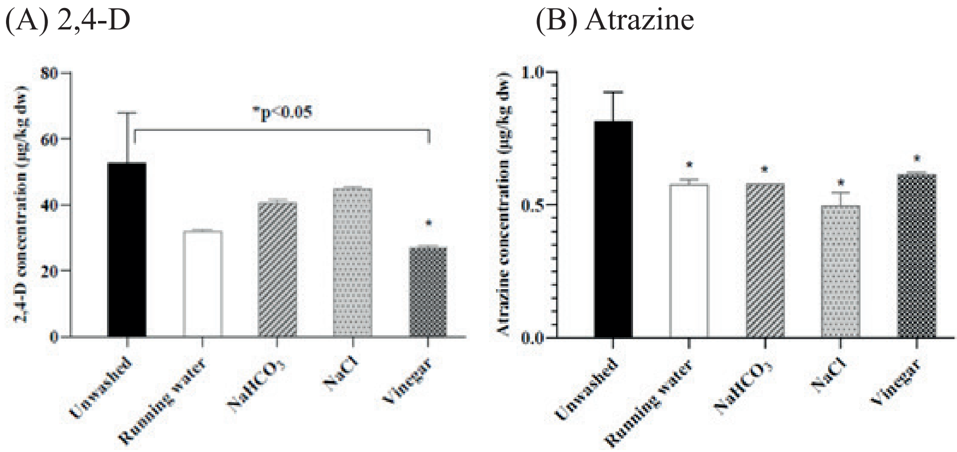

Figure 2. Effects of different washing methods on the reduction of pesticide residues in unchopped bok choy samples: (A) 2,4-D and (B) Atrazine. The values are means and standard deviations.

Figure 2A shows the changes in concentration of 2,4-D after washing the unchopped bok choy samples with washing methods. The residues of 2,4-D were found to be decreased from all washing methods. However, the reduction from washing with running water, $NaHCO_3$, and NaCl were not significant (Figure 2A). The content of 2,4-D in vegetables washed with vinegar was significantly decreased ($p<0.05$) compared to the unwashed samples. The mean 2,4-D contents in samples after washing with vinegar was reduced by approximately 49% of those control unwashed samples. For atrazine reduction, the results were different in the experiment (Figure 2B). Washing the unchopped bok choy samples with running tap water, $NaHCO_3$, NaCl, and vinegar methods significantly reduced the content of atrazine in the bok choy samples. Mean content of atrazine after washing with running water, $NaHCO_3$, NaCl, and vinegar in samples was reduced by approximately 30%, 30%, 39% and 25%, respectively.

3.3 Removal of pesticide residues from vegetables

Washing the vegetables is the simplest and affordable way to reduce the contamination of pesticides in vegetables. Keikothaile et al. have demonstrated that the washing fruits or vegetables could reduce pesticides which were loosely attached to the surface (Keikotlhaile et al., 2010). The present study has demonstrated that washing chopped leafy vegetables such as bok choy under running water and vinegar seemed to be the most effective ways to remove 2,4-D and atrazine, respectively, from the vegetables. The running water method involved washing the vegetables under running tap water for 10 min. Rubbing the vegetables with hands while washing is also helpful to remove the pesticide residues from the surface of vegetables. While, the vinegar washing method involved the soaking vegetables in diluted vinegar solution for 10 min. In this study, running tap water method was proven to be the most effective one to reduce the residues of 2,4-D. Approximately 50% of 2,4-D was reduced after washing under running tap water. The remaining 2,4-D residues after washing resulted from the contents of 2,4-D accumulated inside the vegetables which could not be washed as suggested by Yang et al. study (Yang et al., 2017). Results from Yang et al. showed that the effectiveness of washing is reduced with the increase in penetration depth and penetration amount. The results from our study suggested that the removal of 2,4-D by running tap water method was more effective than other washing methods used in this study. These included soaking with $NaHCO_3$, NaCl and vinegar. However, the results with atrazine appeared to be different. Soaking with diluted vinegar solution for 10 min was found to be the most effective method for the removal of atrazine in vegetables compared to the running tap water, $NaHCO_3$ and NaCl methods. The differences in the effectiveness of different methods for each pesticide may be related to the difference in their physiochemical properties such as water solubility, pKa and their solubility in acids and bases. Our results showed that washing chopped bok choy samples with running tap water was more effective to remove 2,4-D than atrazine. This may be explained by the fact that water solubility of 2,4-D (890 mg/L) is higher than that of atrazine (33 mg/L) (Gerberding, 2003; Gervais, 2008). In contrast, washing with diluted vinegar solution was more effective in removal of atrazine than 2,4-D. This could be due to the relative solubility in acetic acid (vinegar) solution and relative stability of these two compounds in acidic solution (Wanwimolruk et al., 2015).

In the experiment with unchopped samples, vinegar was more effective to remove 2,4-D residues. It might be due to the fact that 2,4-D is a systemic herbicide so running water, $NaHCO_3$, and NaCl could not remove the residues absorbed into the samples. Vinegar is an acidic solution so it could remove the residues on the surface of vegetables. On the other hand, all the four methods namely running tap water, $NaHCO_3$ and NaCl methods were able to remove atrazine methods significantly. This may be related to the presence of pesticides on the surface of vegetables which was successfully removed by washing. However, the differences of residues after washing methods were higher in chopped samples compared to unchopped samples. So, it can be said that vegetables should be chopped before washing to remove the pesticides effectively.

Chemicals used for washing procedures as well as pesticides have different physiochemical characteristics. Therefore, it is difficult to choose the most effective washing method for a particular pesticide. However, running tap water method is the most practical and economical way for washing vegetables before cooking or consumption. Moreover, water is a safe and comparatively better solvent to wash out pesticides by dissolving and discarding them away. Our results were consistent with other experiments in which they showed washing vegetables under running water could remove the pesticide residues. For instance, washing Chinese kale samples in water reduced 55% profenofos (Wanwimolruk et al., 2015). Rani et al. found that chlorpyrifos residues were reduced by 41 to 44% by washing tomatoes with water (Rani et al., 2013). The other report from Chavarri et al. demonstrated the reduction of chlorpyrifos residues by 24% after washing (Chavarri et al., 2005). A study showed the removal of carbyl residues by 33% after washing cucumbers under tap water for 1 min (Hassanzadeh et al., 2010).

4. Conclusion

We investigated the effectiveness of four washing methods to remove two systemic pesticides, 2,4-D and atrazine, from bok choy samples. The results in chopped bok choy samples demonstrated that washing under running tap water for 10 min effectively reduced 2,4-D residues whereas running tap water was not effective in removing atrazine. We determined that 50% 2,4-D was removed under running tap water and vinegar solution could decrease atrazine residues by 52%. Vinegar reduced 49% 2,4-D and all methods reduced 25-39% atrazine residues in unchopped samples. Thus, washing vegetables before consuming or cooking is a very crucial step for considerable reduction of pesticide residues. Chopping the vegetables before washing should be considered. Public education regarding washing methods of vegetables is strongly advisable as it might prevent health risks from pesticides.

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