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Monitoring of Pesticides in the Cultivation of Nopal Vegetable (*Opuntia ficus-indica* (L.)) Mill, Morelos, México

Irene Iliana Ramírez-Bustos [1], Víctor López-Martínez [1], Porfirio Juarez-Lopez [1], Irán Alía-Tejacal [1], Dagoberto Guillén-Sánchez [1], Hugo Saldarriaga-Noreña [2,*] and Ismael León-Rivera [2]

[1] Posgrado en Ciencias Agropecuarias y Desarrollo Rural, Facultad de Ciencias Agropecuarias, Universidad Autónoma del Estado de Morelos, Av. Universidad 1001, 62209 Cuernavaca, México; irene.ramirezb@uaem.edu.mx (I.I.R.-B.); victor.lopez@uaem.mx (V.L.-M.); porfiriojlopez@yahoo.com (P.J.-L.); ijac96@yahoo.com.mx (I.A.-T.); dagoguillen@yahoo.com (D.G.-S.)

[2] Centro de Investigaciones Químicas, Instituto de Ciencias Básicas y Aplicadas, Universidad Autónoma del Estado de Morelos, Av. Universidad 1001, 62209 Cuernavaca, México; ismaelr@uaem.mx

* Correspondence: hsaldarriaga@uaem.mx; Tel.: +52-777-329-7997

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Abstract: The presence of pesticide residues in vegetable and fruit products, as a consequence of inappropriate application in some cases, constitutes a risk to the health of the exposed population. In Mexico, the official norm, NOM-003-STPS-1999, only allows the use of pesticides with the phytosanitary registry, the responsible state commission for the control of the process and use of pesticides and toxic substances, which recommends doses and permitted crops. Despite the above, it is still common to find pesticide residues in some vegetable products. In this study, the following were detected: Chlorpyriphos, Dimetomorph I, Malathion, Omethoate, Carbendazim, and Imidacloprid in Nopal. The study was carried out in two collection centers located in the state of Morelos. In total, sixty samples were taken, thirty for each collection center, for a period of 10 months. To determine the pesticide residues, the analytical methodology was used, according to the guide, SANTE/11945/2015; in a laboratory accredited by the Mexican Accreditation Entity A. C. in the norm, NMX-EC-17025-IMNC-2006. The procedure for extracting analytes was carried out using the method, QuEChERS. The highest concentration of the pesticides detected in the samples obtained from the non-Certified Supply Center were Chlorpyrifos 0.309 mg/kg (MRL 0.01), Dimetomorf I 0.029 mg/kg (MRL 0.01), Malathion 0.155 mg/kg (MRL 0.01), Omethoate 0.032 (MRL 0.01), Carbendazim 0.090 mg/kg (MRL 0.01), and Imidacloprid 0.058 mg/kg (MRL 0.01). Thirty percent of the samples analyzed showed pesticide residues; the most frequent were Carbendazim. The results for the estimated daily intake (EDI) oscillated between 6.5×10^{-5} and 1.3×10^{-4} mg/kg body weight for the vegetable, Nopal. In principle, it could be concluded that the consumption of Nopal with pesticide residues does not represent any toxicological risk for human health, however, the risk cannot be ruled out due to the intake of other vegetables and fruits that are cultivated in the Mexican Republic, which probably present pesticide residues, which together would raise potential risks to human health.

Keywords: pesticide residues; QuEChERS; *Opuntia ficus-indica* (L.) Mill; risk assessment

1. Introduction

The use of pesticides generates residues represents a risk to the environment and human health [1]. To ensure the safety of agricultural products and their derivatives, maximum residue limits (MRLs) have been established worldwide [2,3].

In México, the official norm, NOM-003-STPS-1999, only allows the use of pesticides with phytosanitary registry, buckskin, the responsible state commission for the control of the process and use of pesticides and toxic substances, which recommends doses and permitted crops [4]. Inappropriate use may involve health risks, environmental pollution, and interfere with international trade [5]. If a vegetable exporter is not careful enough to comply with the differing standards in different countries, such mistakes could lead to the detection of pesticides on vegetables for which maximum allowable levels had not been established, resulting in violations [6]. Thus, it is important to analyze and monitor pesticides in foods; for accurate assessment of exposure, levels, and health risks, accurate analytical results are needed [7]. Recently the Nopal acquired importance in the international market, mainly in the United States of North America, where the consumption increased by 128.0%. This behavior is attributed to the increase of the Latin population and acceptance of Mexican food in this country [8]. According to the national monitoring carried out each year by the National Health Service Food Safety and Quality (SENASICA, for its acronym in Spanish), reported in 2007, the presence of residues of Methyl parathion, Methamidophos, and Omethoate, in Nopal vegetable (*Opuntia ficus-indica* (L.) Mill) were noted [9].

Pesticides are extensively used to protect foods against pests and diseases. For this reason, a training program to obtain accreditation of production units, in the application of Good Use and Management of Agrochemicals (BUMA, for its acronym in Spanish), was implemented through the State Committee of Plant Health of the State of Morelos in 2016, [10].

To avoid exposure of the population to pesticide residues due to the ingestion of contaminated foods, the implementation of adequate analytical methodologies is required. Currently, the laboratories that perform the analysis of pesticides must comply with a series of guidelines that ensure the quality of their results, according to ISO/IEC-17025:2005 [11]. For the determination of pesticides in these very complex matrices, the application of multi-waste methods is needed. These methods require rigorous analytical validation. Aware of this need, the General Directorate for Health and Consumers (SANTE) established the guidelines for the validation of an analytical method and the quality control procedures that must be carried out for the analysis of pesticide residues in agricultural products and their derivatives [1].

The acceptance criteria for each of the validation parameters includes the repeatability of the method as a percentage of the coefficient of variation (RSD < 20%) and recovery percentages between 70–120% [1]. In this regard, the guidance document published by the Organization for Economic Cooperation and Development (OECD) states that any analytical method used to analyze pesticide residues in complex matrices, such as extracts obtained from plant products, requires the development of method analytics that demonstrate that they work well for the stated objective [12]. The analytical techniques that have shown the best results for this type of compounds are gas chromatography and liquid chromatography, both coupled to mass spectrometry, given their high sensitivity and selectivity. Prior to chromatographic analysis, exhaustive purification of the extracts is required, specifically in complex matrices, with the intention of eliminating the effects of the matrix caused by the co-extraction of other compounds, which can interfere in the actual response of the compounds of interest. One of the most used extraction techniques for the determination of pesticides in vegetable products is liquid-liquid extraction (LLE), followed by a "clean-up" with extraction in solid phase (SPE) or gel permeation chromatography (GPC). Recently, a general extraction procedure has been implemented, called "QuEChERS" (Quick, Easy, Cheap, Effective, Rugged, Safe) due to its simplicity, requiring few stages of sample processing, and efficiency of the removal of impurities in complex samples [13,14].

In the present study, the methodology for the determination of pesticide residues in the Nopal vegetable was developed in two collection centers, one of which receives certified production units and

the other of non-certified production units. The pesticides analyzed were Carbendazim, Chlorpyrifos, Dimetomorf I, Imidacloprid, Malathion, Omethoate, and Atrazine as an internal standard. For the extraction and cleaning of the extracts, the "QuEChERS" method was adapted. The calibration was carried out with the multi-standard addition of the different pesticides on the Nopal vegetable, at six concentration levels. Likewise, recovery percentages were evaluated by enriching the Nopal vegetable with known quantities of pesticides.

2. Materials and Methods

2.1. Standards

The analyzed pesticides were Carbendazim 99.2%, Clorpyrifos 99.1%, Dimetomorf I 99.5%, Imidacloprid 99.1%, Malathion 97.8%, Omethoate 99.1%, and Atrazine as the internal standard 99.2% (AccuStandar, Inc., New Haven, CT, USA). The used solvents were obtained from TEDIA High Purity Solvents: Toluene, acetonitrile, methanol, acetic acid, formic acid, and water (pesticide grade, Carson city, CA, USA).

2.2. Collection of Samples

The monitoring was carried out in two Gathering Centers located in the state of Morelos, México. The first of them, "San José", located in Tlayacapan (18.971059° E, −98.98835° N), which is supplied by producers accredited in Good Use and Management of Agrochemicals; the other site, "La Espina Verde SPR de RL", in Tlalnepantla (18.971059° E, −98.98835° N), which is supplied by non-certified producers. The methodology established by the Codex Alimentarius for the determination of pesticide residues (CAC/GL 33-1999) suggests the minimum size of laboratory samples with a minimum amount of 1.0 kg for legumes and vegetables [15]. In the laboratory, the samples were treated with standardized procedures based on the guidance document on validation procedures and quality control of analytical methods for the determination of pesticide residues in food and feed [1]. From each Gathering Center, 1.5 kg (10 pieces) of vegetable cactus (*Opuntia ficus-indica* (L.) Mill) were randomly selected. These were placed inside sterile polyethylene bags, refrigerated, and transported to the laboratory to be analyzed.

Samples were processed and analyzed at the National Reference Center for Pesticides and Pollutants (CNRPC, for its acronym in Spanish), which belongs to the National Service of Health, Safety, and Agrifood Quality (SENASICA, for its acronym in Spanish), accredited laboratory in the standard NMX-EC-17025-IMNC-2006 /ISO/IEC17025:2005, which establishes the requirements that the testing and calibration laboratories must follow.

2.3. Sample Preparation and Purification

The original Nopal matrix was prepared as follows: 1.5 kg of Nopal were homogenized in a blender. The extraction procedure of the pesticides from the samples used was the "QuEChERS" method. After the homogenization of the sample in a blender, 10 g were weighed in a 50 mL Polytetrafluoroethylene (PTFE) centrifuge tube, 10 mL of acetonitrile containing atrazine (Internal Standard 0.000133 mg/kg), 1 g of Sodium Citrate ($Na_3C_6H_5O_7$), 1 g of Sodium Chloride (NaCl), and 4 g of Magnesium Sulfate ($MgSO_4$) were added to each tube, then each tube was vigorously stirred for 2 min, and later taken to a bath of ultrasound for 5 min. Finally, the stirred samples were centrifuged at 3500 rpm for 2 min.

From the previous solution, an aliquot of 3 mL in a plastic tube was taken, which contained 900 mg of Magnesium Sulfate ($MgSO_4$), 150 mg of PSA (primary and secondary amine), and 150 mg of resin C18 and 80 g of activated carbon; it was stirred for 1 min in a vortex. It was centrifuged at 3500 rpm for 2 min. The supernatant was filtered through a nylon membrane (0.2 µm), the filtrate was divided into two equal aliquots of 1.5 mL, and one was used for analysis by gas chromatography and the other for liquid chromatography.

2.4. Preparation of the Calibration Plots

Initially, there were individual solutions of each analyte at a concentration of 2000 µg/mL. From these a standard solution of 20 µg/mL was prepared, and from this last one a series of dilutions were made until a stock solution of 20 ng/mL was obtained. The calibration samples were prepared by appropriate dilution of the stock solution in blank matrix to avoid matrix effects. The concentrations of the calibration curves ranged between 0.00011, 0.00032, 0.00107, 0.00218, 0.00641, and 0.01202 mg/kg.

The ratios of the concentration of each compound on the concentration of the internal standard were used versus the relationships of the areas of each standard over the area of the internal standard, to perform linear regression graphs, for which the regression equations were obtained for each composition.

2.5. Analysis by Gas Chromatography

The detection and quantification of Malathion, Chlorpyrifos, and Dimetomorph was performed with a multiple reaction monitoring (MRM) method on the Agilent 7890A/7000C GC triple quadrupole mass spectrometer system (GC/QqQ, Agilent, Santa Clara, CA, USA), with electron ionization (EI). The separation was achieved on a 15 m × 250 µm × 0.25 µm film thickness GC column (Agilent, Santa Clara, CA, USA). The oven temperature program was established as follows: Initially, 80 °C for 1 min, with increments of 15 °C/min until 180 °C (there it stayed for 2 min). Subsequently, increases were made for 5 °C/min until it reached 330 °C (per 5 min). The MSD transfer line was at 250 °C and ion source was set at 320 °C. The QqQ collision gas was nitrogen (99.9999%, INFRA, México City, México) at 1.5 mL/min and carrier gas was Helium (99.9999%, INFRA, México City, México) at 2.25 mL/min. EI energy was 70 eV, and quadrupole temperatures were set at 150 °C. Product ion and collision energy experiments were performed to determine the optimum two product ions, collision energies, and ratios between quantifier and qualifier ions. The National Institute of Standards and Technology (NIST) 2011 mass spectral database (NIST, Gaithersburg, MD, USA) was used when it contained library spectra for the analytes. Multiple reaction monitoring (MRM) transitions, collision energy for each transition, and average retention times (RT) are presented in Table 1.

Table 1. Summary of the selected parameters for each compound.

Analyte	Rt (min)	First Transition (*m*/*z*)	Collision Energy (V)	Second Transition (*m*/*z*)	Collision Energy (V)	Quantifier Ion
Atrazine	7.770	200.0→122.1	10	200.0→94.0	20	122.1
Malathion	9.772	126.9→99	24	157.8→47	12	99.0
Chlorpyrifos	9.776	313.8→257.8	15	313.8→285.8	5	257.8
Dimetomorph I	18.279	300.9→165	30	302.9→164.9	20	165.0

2.6. Analysis by Liquid Chromatography

The analysis of Omethoate, Carbendazim, and Imidacloprid was done in a Waters UPLC-MS/MS (XEVO TQ-MS Mass Spectrometer, Waters Corporation, Milford, MA, USA). The pesticides were analyzed in electrospray ionization in positive mode (ESI), nitrogen was used as desolvation gas at flow of 100 L/h (500 °C), while argon was used as collision gas at a flow of 0.15 mL/min. A chromatographic column C18 (Acquity, UPLC BEH C18 1.7 µm, 2.1 × 100 mm, Waters Corporation, Milford, MA, USA) was used for the separation of the compounds, the column was kept at 60 °C, and the injection volume was 10 µL. Two eluents were used: 0.1% formic acid in water-methanol (98:2) (A) and 0.1% formic acid in methanol (B). The flow rate was 0.35mL/min. A linear gradient was used to elute the compounds: 0–2.30 min, 20% A:80% B, 2.30–2.80 min, 100% B, 2.80–4.50 min, 20% A:80% B. Collision cell energy and fragmentation voltage were optimized in the dynamic Multiple Reaction Monitoring mode (MRM) for each pesticide (Table 2).

Table 2. Optimized ultra performance liquid chromatography tandem mass spectrometer (UPLC-MS/MS) parameters for the selected pesticides.

Analyte	RT (min)	First Transition (*m*/*z*)	Collision Energy (V)	Second Transition (*m*/*z*)	Collision Energy (V)	Quantifier Ion
Omethoate	1.760	214→155	14	214→183	12	183
Carbendazim	2.200	343→151	18	192→160	18	160
Imidacloprid	3.080	256.1→175	18	256.1→209	14	209

2.7. Evaluation of Recovery Percentages

The recovery was determined using 3 replicates at one concentration level (0.003 mg/kg) for all compounds in the homogenized Nopal vegetable. Then, each sample was processed as explained in Section 2.5. With the concentration values observed after the extraction and the added concentration, recovery percentages were calculated for each compound. The results of the three replicates were used to calculate the accuracy of the method, expressed as relative standard deviation (% RSD).

2.8. Toxicological Risk Assessment

The human health risk was evaluated based on the concentration of pesticides residues in Nopal, according to the methodology established by the Food and Agriculture Organization [16]. Estimated daily intake (EDI) was found by multiplying the residual pesticide concentration (mg/kg) by the food consumption rate (kg/day) and dividing by a body weight of 60 kg for the adult population. The average daily Nopal vegetable intakes for adults (LP) considered was 0.025 kg/person/day, according to the agrifood atlas for México [17].

$$EDI = \frac{LP \times HR}{bw} \quad (1)$$

where *LP* is the highest large portion reported (97.5th percentile of eaters), in average kg intake per day (kg/day); *HR* is the highest residue in composite sample of edible portion found in the supervised trials used for estimating the maximum residue level, in mg/kg; and *bw* is the body weight.

3. Results and Discussion

3.1. Optimization of the Analytical Conditions

The identification and quantification of the pesticides was based on the criterion specified in the decision of the European Commission [1]. The retention time of the extracted analyte must correspond to that of the calibration standard with a tolerance of ±0.1 min. The relative intensity of the ions must comply with a permitted tolerance of ±30% (using two product ions).

The limit of detection (LOD) was calculated from the calibration graph obtained for each compound according to Miller and Miller (2004), using the concentration that provides a signal that is equal to the signal corresponding to the blank ($Y_B = S_{y/x}$), plus three times the standard deviation of the blank ($S_B = a$) [18]:

$$\text{LOD} = Y_B + 3 \times S_B \quad (2)$$

$Y_B = S_{y/x}$: Random error in the direction of "y"; and
$S_B = a$: Intercept.

Table 3 shows the analysis mode, retention time (RT), Limit of Detection (LOD), recovery percent, and precision as the relative standard deviation (RSD) for the analyzed pesticide; Chlorpyriphos, Dimethomorph I, and Malathion were detected by gas chromatography, while Omethoate, Carbendazim, and Imidacloprid were determined by liquid chromatography, with both techniques coupled to mass spectrometry.

Table 3. Summary of the optimized parameters for the determination of pesticides residues in Nopal.

Analyte	Analysis Mode	LOD mg/kg	% Recovery (n = 3)	% RSD (n = 3)
Omethoate	UPLC	0.000016	85.4	7.45
Carbendazim	UPLC	0.000005	86.4	7.35
Imidacloprid	UPLC	0.000001	87.3	6.42
Malathion	GC	0.000006	88.3	6.82
Chlorpyrifos	GC	0.000004	84.8	9.94
Dimetomorph I	GC	0.000019	86.6	7.05

LOD: Limit of detection; % RSD: Relative standard deviation.

The percentages of recovery ranged between 84.8% and 88.3% for Chlorpyrifos and Malathion, respectively. On the other hand, the LOD were between 0.000001 and 0.00002 mg/kg for Imidacloprid and Dimetoforf I, respectively, with RSD <20%, which is congruent with the established values by the United States Department of Agriculture (USDA) [19]. All LOD were below the Maximum Residue Limits (MRLs) established by Codex Alimentarius for vegetable products [15]. Meanwhile, all the correlation coefficients for the analyzed compounds were greater than 0.991.

3.2. Concentrations of Pesticide Residues in Nopal Collected in Morelos

Of the 60 samples of Nopal vegetable (*Opuntia ficus-indica* (L.)) analyzed, residues of pesticides were found in 18 of them (30%), which correspond to the Gathering Center Espina Verde SPR of RL, collection Center no Certificate in Good Use and Management of Agrochemicals.

The pesticide that was presented in a greater number of samples was Carbendazim (18), followed by Chlorpyrifos and Imidacloprid (15), Malathion (4), Dimetomorph I (2), and Omethoate (1), respectively (Figure 1).

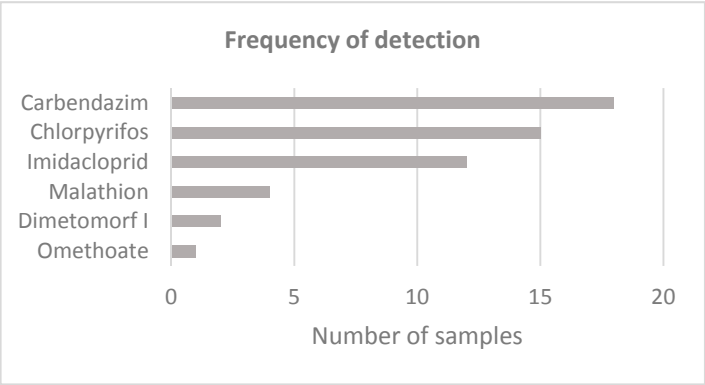

Figure 1. Frequency of detection of pesticides in Nopal.

The highest concentration of the pesticides detected in the samples obtained from the non-Certified Supply Center were Chlorpyrifos 0.309 mg/kg, Dimetomorf I 0.029 mg/kg, Malathion 0.155 mg/kg, Omethoate 0.032, Carbendazim 0.090 mg/kg, and Imidacloprid 0.058 mg/kg (Table 4). It should be mentioned that for Nopal there are no values for MLRs; however, the Mexican regulations established for these situations assume the value of 0.01 mg/kg [10]. Considering this criterion, we calculated the average concentration for each compound in all samples analyzed and we observed that all pesticides were above of the reference MRLs established (Table 4).

Table 4. Residual concentrations of pesticides observed in the Gathering Center Supplied by non-certified producers.

Sample	Pesticides Detected by Gas Chromatography (mg/kg)			Pesticides Detected by Liquid Chromatography (mg/kg)		
	Chlorpyrifos	Dimetomorf I	Malathion	Omethoate	Carbendazim	Imidacloprid
1	0.00242	0.02884	0.08624	0.032	0.03275	0.02725
2	0.30869	0.01393	0.08148	ND	0.03739	0.02732
3	0.00263	ND	0.09068	ND	0.03710	0.02722
4	0.00422	ND	0.15509	ND	0.03305	0.02720
5	0.00317	ND	ND	ND	0.03760	0.03106
6	0.00201	ND	ND	ND	0.03463	0.02742
7	0.00203	ND	ND	ND	0.05290	0.02711
8	0.00329	ND	ND	ND	0.03290	0.03921
9	0.21922	ND	ND	ND	0.03285	0.05814
10	0.00347	ND	ND	ND	0.04527	0.02744
11	0.20986	ND	ND	ND	0.09034	0.02731
12	0.03057	ND	ND	ND	0.03284	0.03751
13	0.02781	ND	ND	ND	0.03291	ND
14	0.05944	ND	ND	ND	0.03285	ND
15	0.04056	ND	ND	ND	0.08740	ND
16	ND	ND	ND	ND	0.05087	ND
17	ND	ND	ND	ND	0.03296	ND
18	ND	ND	ND	ND	0.04901	ND
Average	0.06129	0.02139	0.10337	0.03200	0.04365	0.03202

Chlorpyrifos (MRL 0.01), Dimetomorph I (MRL 0.01), Malathion (MRL 0.01), Omethoate (MRL 0.01), Imidacloprid (MRL 0.01), ND: Not detected.

The obtained results reveal the presence of pesticide residues in the Nopal vegetable in the samples taken at the non-certified Gathering Center, which indicates that the control measures that were implemented in the Good Use and Management of Agrochemicals did not detect pesticide residues. The World Health Organization considers Carbendazim, Chlorpyrifos, and Dimetomorph I as not very toxic, and Imidacloprid and Malathion as moderately toxic, meanwhile Omethoate is considered to be highly toxic and an endocrine disruptor [20].

Despite the large market that the Nopal has, there are few studies conducted in Mexico that report the concentration of pesticide residues in vegetable products. When comparing the average concentration obtained for each compound in the present study with other studies, we observed, for example, that Aldana-Madrid et al. (2008), in fresh Nopal obtained from trading companies of Hermosillo, Sonora, Mexico, reported average concentrations for Malathion of 0.014 mg/kg and Chlorpyrifos of 0.017 mg/kg, which reveals that in the Morelos state, the concentration was almost an order of magnitude larger [21]. Meanwhile, Angeles-Nuñez et al. (2014) reported average concentrations for Omethote (0.030 mg/kg), Clropyrifos (0.015 mg/kg), and Malathion (0.014 mg/kg) in Nopal from crops located in the state of Mexico during 2008 [22]. This suggests that farmers still have poor practices in the use of agrochemicals. According to the list of agricultural pesticides (SENASICA, 2012), Omethoate, Chlorpyrifos, and Malathion are not authorized for this crop, indicating the lack of compliance with national regulations [23]. This same situation is presented, considering other markets as the United States of America, which restricts the use of Chlorpyrifos Ethyl and Methyl Parathion and Bifenthrin does not allow the use of Omethoate, while only allowing Carbaryl, Diuron, Glyphosate, and Metaldehyde, with residue limits 12.0, 0.05, 0.5, and 0.07 mg/kg, respectively (US-EPA, 2011) [24]. These results indicate that if farmers do not take the measures established in the regulations, they will not be able to export their products to the national and international markets due to the proven negative impact of some of these compounds on human health.

3.3. Dietary Risk Human Health Assessment

The EDI values calculated for each one of the pesticides oscillated between 6.5×10^{-5} and 1.3×10^{-4} mg/kg body weight. The results obtained for the EDI were compared with the acceptable daily intake (ADI) values established by the WHO [25]. These comparisons reveal that the toxicological risk to human health from Nopal consumption is between 100–1000 times lower than the ADI (Table 5). In principle, it could be concluded that the consumption of Nopal with pesticide residues does not represent any toxicological risk for human health; however, the risk cannot be ruled out due to the intake of other vegetables and fruits that are cultivated in the Mexican Republic, which probably present pesticide residues, together raising potential risks to human health.

Table 5. Comparison of the results of estimated daily intake (EDI) with the admitted daily intake (ADI) for pesticide residues detected in Nopal in the state of Morelos.

	EDI mg/kg Body Weight	**ADI [25] mg/kg Body Weight**
Chlorpyrifos	1.3×10^{-4}	0.1
Dimetomorf I	1.2×10^{-5}	0.2
Malathion	6.5×10^{-5}	0.3
Omethoate	1.4×10^{-5}	IND
Carbendazim	3.8×10^{-5}	0.03
Imidacloprid	2.4×10^{-5}	0.06

IND: inadmissible; [25], WHO-2012.

4. Conclusions

The analytical method developed allowed the determination of the amount of residues of six pesticides in the Nopal vegetable. The clean-up stage using the QuEChERS method allowed good efficiencies in the recovery of the compounds to be obtained. The percentages of recovery of all pesticides analyzed were lower than 86.3% with coefficients of variation below 10%, indicating good repeatability of the optimized methodology. It can also be mentioned that all concentrations of pesticides detected were above the MRLs suggested by the Mexican regulations. Only pesticide residues were observed in the Collection Center that was not qualified for the Good Use and Management of Agrochemicals.

The presence of multiple residues in some of the samples analyzed is the consequence of the application of different types of pesticides to protect crops from the different pests and diseases that attack them. The results obtained indicate that farmers are not taking into account the precautions regarding the proper use of pesticides, which can cause health problems for both farmers and consumers of the products.

The frequency of application of pesticides in vegetable products can be twice a month or once a week, depending on the type of crop. This problem is magnified when farmers have little knowledge of pesticide management.

Currently, one of the priorities is the development of strategies to reduce the use of pesticides, the development of training programs that allow farmers to better use these substances, as well as the search for chemical or biological alternatives for pest control. In this sense, it is important to support the regulatory entities, which are the ones that establish the guidelines for the management and distribution of pesticides. Consumers of agricultural products should be aware of the practical measures that should be taken into account to reduce contamination by pesticides in fresh agricultural products, especially fruits and vegetables that can be consumed raw.

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