Use of Enzyme-Linked ImmunoSorbent Assay technique to monitor pesticide residues in horticultural crops

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RECEIVED: 13 FEBRUARY 2020; REVISED: 9 DECEMBER 2020; ACCEPTED: 17 DECEMBER 2020

ABSTRACT

Organophosphates, carbamates (OP/Cs), and pyrethroids are among the most commonly used pesticides worldwide. These pesticides are highly toxic to insects but also other animals, including humans. The increasing public concern in recent years about possible health risks due to pesticide residues has deeply modified the strategies for crop protection. This study was conducted to assess ELISA as a rapid, economical, and safe analytical procedure as an alternative prior to chromatographic techniques for monitoring the residues of the target pesticides in horticultural crops. The study fulfilled its purpose. With the use of ELISA, more samples (higher than 50%) were quantified with OP/Cs, and pyrethroid residues compared to the chromatography techniques that mostly detected them. The developed ELISA exhibited accuracy (114% recovery with a 3% coefficient of variation for OP/Cs and 115% recovery with a 4.0% coefficient of variation for pyrethroids) and they are ideally suited as a fast, high-throughput, and low-cost (around 100 times lower) screening test for OP/C and pyrethroids residue prior to chromatographic analysis. Lineal relationships (slope 1.0089 with R^2 0.9983 for carbaryl [OP], and slope 1.1088 with R^2 0.9986 for cypermethrin) between the quantified values obtained by the chromatographic technics with the ELISA test values were observed.

Keyword: organophosphate, carbamate, pyrethroid, ELISA

RESUMEN

Organofosforados, carbamatos (OP/Cs) y piretroides se encuentran entre los plaguicidas más utilizados en todo el mundo. Estos plaguicidas son muy tóxicos para los insectos, pero también para otros animales, incluidos humanos. La creciente preocupación en los últimos años por los posibles riesgos para la salud debido a residuos de plaguicidas ha modificado profundamente las estrategias de protección de los cultivos. El estudio evalúa a ELISA como alternativa analítica rápida, económica y segura frente a técnicas cromatográficas para la monitorización de residuos de plaguicidas en cultivos hortícolas. El estudio cumplió su objetivo. Con el uso de ELISA, se cuantificaron más muestras (50% mayor) con residuos de OP/Cs, y piretroides en comparación con las técnicas cromatográficas que mayoritariamente los detectaron. ELISA desarrollado mostró precisión (114% de recuperación y 3% coeficiente de variación para los OP/Cs y 115% de recuperación con 4,0% coeficiente de variación por piretroides) y es ideal como prueba de control rápida, de alto rendimiento y bajo coste (100 veces menor) para los residuos de OP / Cs y piretroides frente al análisis cromatográfico.

Se observaron relaciones lineales (pendiente 1,0089; R2 0,9983 por carbarilo [OP], y pendiente 1,1088; R2 0,9986 por cipermetrín entre valores cuantificados por las técnicas cromatográficas con los valores de la prueba ELISA.

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Palabras clave: Organofosforado, carbamato, piretroides, ELISA

RESUM

Organofosforats, carbamats (OP / Cs) i piretroides es troben entre els plaguicides més utilitzats a tot el món. Aquests plaguicides són molt tòxics per als insectes, però també per a altres animals, inclosos humans. La creixent preocupació en els últims anys pels possibles riscos per a la salut a causa de residus de plaguicides ha modificat profundament les estratègies de protecció dels cultius. L'estudi avalua l'ELISA com a alternativa analítica ràpida, econòmica i segura davant tècniques cromatogràfiques per al monitoratge de residus de plaguicides en cultius hortícoles. L'estudi va complir el seu objectiu. Amb l'ús d'ELISA, es van quantificar més mostres (50% més gran) amb residus d'OP / Cs, i piretroides en comparació a les tècniques cromatogràfiques que majoritàriament els van detectar. L'ELISA desenvolupat va mostrar precisió (114% de recuperació i 3% coeficient de variació per als OP / Cs i 115% de recuperació amb 4,0% coeficient de variació per piretroides) i és ideal com a prova de control ràpida, d'alt rendiment i baix cost (100 vegades menor) per als residus d'OP / Cs i piretroides enfront de l'anàlisi cromatogràfica. Es van observar relacions lineals (pendent 1,0089; R2 0,9983 per carbaril [OP], i pendent 1,1088; R2 0,9986 per cipermetrín entre valors quantificats per les tècniques cromatogràfiques amb els valors de la prova ELISA.

Paraules clau: Organofosforat, carbamat, piretroides, ELISA

INTRODUCTION

Fruit and vegetables can be carriers of pesticide residues if they are treated with pesticides. To lower the residue good agricultural practices and respecting the ALARA principle are recommended. Pesticide applicators should take care of insecticides with action on the central nervous system (organophosphates, carbamates, pyrethroids, among others) [1,2]. Organophosphates, carbamates (OP/Cs), and pyrethroids are among the most commonly used pesticides worldwide due to their broad biological activity and low bioaccumulation potential [3]. However, these pesticides are toxic not only to insects but also to other animals, such as amphibians, birds, and mammals, including humans. Some of this pesticide affects the human nerve impulse transmission-inducing neurologic toxicity, the chronic neurodevelopmental disorder, possible dysfunction of the immune, reproductive, and endocrine system or cancer [3–5].

Exposure to OP/Cs and pyrethroids can occur through the ingestion of contaminated food or water, contact with skin, and inhalation. Symptoms of exposure include headache; dizziness; nausea; increased nasal, ocular, and bronchial secretions; vomiting, and others. The increasing public concern in recent years about possible health risks due to pesticide residues in food has deeply modified the strategy for crop protection, with emphasis on food quality and safety. The widespread concern for the health of society led to the strict regulation of maximum limits for pesticide residues in food commodities, potable and drinking water, soil, and general environmental media [3,5,6].

Cuba is not an exception to the use of OP, CPs, and pyrethroids, being these the families of insecticides more used in the control of plagues and diseases [7,8]. As a result, some journalists reported and the public expressed concerns about possible health risks due to pesticide residues, mainly in fresh crops (e.g. vegetables: tomato, sweet pepper, and cucumber). Since that, the government, together with the phytosanitary and human sanitary department, started to search for an analytical procedure to control and monitor pesticide residues [9,10].

Over the years, technicians and researchers have relied on several analytical methods, such as gas chromatography (GC) and liquid chromatography (LC) for the detection, separation, and quantification of pesticide residues in different matrices [3]. However, it is not always convenient to use such detection tools due to their high cost, expensive instruments, long analysis duration, complex sample pretreatment, and the requirement of skilled labor [11]. A group of authors (Dhull et al., 2014; Ge et al., 2014; Wang et al., 2014 and Xu et al., 2014) cited by Kumar [5], declare that fortunately, the need for simplified and portable detection techniques can be met through the use of biosensors, immunosensors, chemosensors, or electrochemical sensors.

The enzyme-linked immune sorbent assay (ELISA) has taken on new importance for pesticide analysis over the past decades [3]. ELISA is commonly used for the detection of pesticides, biological toxins, pathogen, and drug residues [11]. Sassolas et al., 2012 cited by Kumar[5], also mention that ELISA techniques offer remarkable advantages over chromatographic techniques. ELISA has been proved to be a low cost, sensitive tool suitable for high throughput analysis, which has been extensively designed to monitor food contamination [12], mainly in terms of fast response, specificity, low detection limits, and most attractively, cost-effectiveness [5,13]. ELISA allows us to easily automate the analysis of a massive number of samples and does not require time-consuming procedures and sophisticated equipment. Watanabe [14], refers to its applicability as an analytical method for a simple and quick inspection of pesticide residues in agricultural products before shipment, and Yan [15] emphasizes its benefits to protect ecosystems and prevent diseases. Moreover, the colorimetric detection of enzyme activity of immune reagents makes this assay highly sensitive [16].

The enzyme inhibition-based colorimetric detection of OP/Cs in pesticides has not been popularized until recently [17,18]. A colorimetric approach is ideal for consumers, as it is more visual and intuitive than chromatographic or spectroscopic methods. The color of sample solutions can be observed with the naked eye for qualitative determination or analyzed through digital images for quantification [19]. On the other hand,

the magnetic enzyme-linked immunosorbent assay has attracted interest [11]. Sullivan et al., 2007 cited by Kumar [5], show the feasibility of detecting chlorpyrifos using a commercial magnetic particle-based ELISA kit.

Taking into account that there is no previous history in Cuba of using the ELISA test to monitor and control pesticide residues, the results obtained in this study could serve as a basis for the integration of ELISA in pesticide residue monitoring procedures in Cuba. The study

intends to evaluate the suitability (rapid, low cost, easy and safe analytical procedure) of the ELISA technique (Abraxis ELISA kits) for the monitoring and control of residues of organophosphates, carbamates, and pyrethroids insecticides in vegetables locally grown in Cuba, prior to chromatographic techniques (gas chromatography or liquid chromatography).

MATERIAL AND METHODS

2.1 Sample collection

Thirty-four samples from three agricultural areas in Sancti Spíritus (Banao, Cabaiguán, and La Quinta) were collected during March 2019. The samples consisted of tomatoes (19), cucumber (5), and sweet pepper (10). Samples were blended and centrifuged at 10 000 rpm for 5 min. The subsample volume for the ELISA assay was taken from the clear upper layer, as well as a subsample for analysis with gas chromatography with Electron Capture Detector (GC-ECD) for the pyrethroids and ultra-performance liquid chromatography-tandem mass spectrometer (UPLC-MS/MS) for the OP/Cs. The ELISA tests were performed two days after collection in the Laboratories of the Centre for Energy and Industrial Process Studies of the University of Sancti Spíritus, Cuba. Subsamples to be analyzed with the chromatographic technique were kept frozen at -20 °C until analysis in the Laboratory of Crop Protection Chemistry of Ghent University.

2.2 Materials and instruments

The analytical chromatography grade OP/Cs and pyrethroid standards were purchased from Sigma-Aldrich (Belgium). Sigma–Aldrich also supplied sodium hydrogencitrate sesquihydrate ($C_6H_6Na_2O_7$ ½H_2O) 99 %, sodium chloride (NaCl) > 99 %, sodium citrate tribasic dihydrate ($C_6H_5Na_3O_7$ 2H_2O) > 99 %, and the highest analytical purity pesticides standards needed in the study. Magnesium sulfate anhydrous ($MgSO_4$) came from Merck (Belgium). HPLC grade acetonitrile (ACN) was supplied by VWR (BDH PROLABO, Belgium) and n-hexane > 99 % assay was obtained from Chem-Lab (Belgium).

2.3 ELISA

Abraxis Life Technologies™ provides field and lab-based ELISA testing kits for several pesticides tested in various matrices listed in the National Environmental Methods Index. Numerous articles prove the utility of Abraxis pesticide kits, especially for the analysis of glyphosate [20–23]. For OP/C, a colorimetric assay ELISA screen kit (Microtiter Plate: 96 Test) was purchased. The analysis of pyrethroid was performed using the Abraxis Pyrethroid Assay kit (paramagnetic particles attached with antibodies specific to pyrethroids) (100 Tests). The analysis was developed following the corresponding procedure explained in each test kit. Each procedure provides a table with the respective detection limits of some OP/C and pyrethroids. A Vortex-Genie 2 (VWR International; Edmonton) was used and a magnetic separator rack was supplied by Abraxis. The final reading was performed with a spectrophotometer (Rayleigh VIS723G, China) at 405 and 450 nm.

2.5 ELISA control

Four-point calibration curves (0.75, 2.5, 5.0 and 15 µg l^{-1}) were used. The average values (3 determinations) of each permethrin (pyrethroid) calibration sample in water (control) and the matrices were tested (tomato; cucumber and sweet pepper). A midrange standard (positive control: 3.0 µg l^{-1}) was developed to check the accuracy of the curve. The Abraxis Pyrethroid Assay has an estimated minimum detectable concentration, based on a 90% B/Bo of 0.75 µg l^{-1} (for permethrin), which was more than adequate according to the level expected in the fresh produce (10.0 µg l^{-1}). In the end, a statistical correspondence between the calibration curves of the studied matrices and the water control curve will be evaluated. The slopes of the calibration curves of the matrices must be between the confidence limits (upper and lower) of the slope of the control curve, for n-2 degrees of freedom and a probability of 95 %. The correspondence between the mean values, the coefficient of variation, and the recovery of the positive control in each calibration curve will be also evaluated.

The estimated minimum limit of detection based on a 20 % inhibition of the color developed for OP/C in 50% methanol is 0.3 µg l^{-1} for azinphos-methyl. For the positive control, in the food safety assessments, diazinon at 5.0 µg l^{-1} was used. When samples showed a percent inhibition lower than 20%, they were regarded as negative, and vice versa. With the relation between the absorbance values obtained for the negative control and the positive control, a linear calculation was performed to predict the OP/C values of the analyzed samples. As the OP/C ELISA test is a semi-quantitative method, the positive samples were analyzed by chromatography to quantify and confirm the pyrethroids and OP/Cs concentration concentrations obtained in the ELISA tests. The accuracy of ELISA results developed for OP/C will be evaluated through the correspondence (average concentration, recovery, standard deviation, coefficient of variation) between the value of positive ELISA control in the matrices concerning the concentration used (diazinon at 5.0 µg l^{-1}).

2.4 Comparative analysis

The QuEChERS method was used as a simple analytical extraction method for the detection of multiple pesticide residues in fruit, vegetables, and other matrices [24]. Besides, tomato, sweet pepper, and cucumber blank samples were spiked at 15 µg l^{-1} of permethrin and 5 µg l^{-1} diazinon (positive controls of the Abraxis kits) to confirm the kit performance. Extracts were analy-

zed by GC–ECD using Agilent Technologies 6890N, and a Waters ACQUITY UPLC-MS/MS. A detailed description of the analytical method and equipment conditions is described below.

A detailed description of the analytical method

Ten grams of vegetables of a homogenous made sample were weighed in standard centrifuge tubes (50 ml), and then 15 ml of acetonitrile (ACN) was added and shaken. The following salts were added to each sample to remove co-extracted contaminants: 1.5 g NaCl, 1.5 g C6H5Na3O7 2H2O, 0.750 g C6H6Na2O7 1/2H2O, and 6.0 g MgSO4. Samples were mixed and then separated shaking for 5 minutes by 300 rpm and centrifuged 5 min at 10 000 rpm. The solvent exchange is different for the LC-MS/MS and GC-ECD samples. For the LC-MS/MS samples 1 ml of the upper layer was sampled and added to a volumetric flask of 10 ml. 9 ml Milli-Q water was added to obtain a total volume of 10 ml. A subsample of +/- 1.5 ml was pipetted in an LC-MS/MS vial. For the GC-ECD samples, 5 ml of the upper layer was sampled to an evaporation bowl. The solvent (ACN) was evaporated in the Rotary evaporator and 5 ml of n-hexane was added to the bowls to recover the analyte. A subsample of +/- 1.5 ml was pipetted in a GC-ECD vial.

Ultra-performance liquid chromatography operating conditions

A Waters ACQUITY UPLC™, equipped with a quaternary pump and triple quadruple system with electrospray ionization (Waters Xevo® TQD) to perform sample analyses was used. The separation column, an Acquity UPLC BEH C18, 130Å (1.7 µm 2.1 mm 50 mm) was kept at 40°C. 10 µl per sample was automatically injected. The mobile phase components were (A) Milli-Q water with 0.1 % formic acid and (B) ACN with 0.1 % formic acid. A flow rate of 0.4 mL min-1 of 98 % mobile phase A for 0.25 min was used as a gradient set. From 0.25 min to 7 min, a linear gradient was used to 98 % mobile phase B, held for 1 min. Then a linear gradient was used to 98 % mobile phase A and held for 1 min. The capillary needle was maintained at +2 kV, curtain gas (N2) at 7 bars, and temperature 500 °C. The AIs were monitored and quantified using multiple reactions monitoring (MRM). Two different m/z transitions were selected for each analyte. The MS/MS-transitions, ionization mode, cone voltage, and collision energy are given in table 1.

Gas chromatography with electron capture detection

An Agilent Technologies 6890N gas chromatograph equipped with an Agilent Technologies 7683 Series autosampler injector, coupled to an electron capture detector (GC-ECD) was used. Separation was performed on a HP-5MS (5 % phenyl methyl siloxane) capillary column (30 m 0.25 mm 0.25 µm). As operating conditions, the column was initially set at a temperature of 60 °C and then the oven temperature was increased at a rate of 20 °C min-1 to 150 °C. Furthermore, it was increased at a rate of 15 °C min-1 to 250 °C, held for 2 min at 250 °C, followed by an increase at a rate of 30 °C min-1 to 270 °C and held constant for 10 min at 270 °C. Thereafter, it was increased at a rate of 30 °C min-1 to 280 °C and finally, it was held at 280 °C for 11 min. Injector and detector temperatures were maintained at 200 °C and 250 °C, respectively. Helium was used as a carrier gas at a flow rate of 1.1 mL min-1 and the injections were made in the split mode with a split ratio of 52.7:1.

Table 1 *MS/MS-transitions, ionization mode, cone voltage, and collision energy of the active ingredients tested*

Pesticide	Pesticide ion (m/z)	Fragment ion (m/z)	Ionization mode (/)	Cone voltage (eV)	Collision energy (eV)	Residence time (ms)
methomyl	163	88	ES+	20	10	0.017
	163	106	ES+	20	10	0.017
acephate	184.1	125.1	ES+	11	18	0.052
	184.1	143	ES+	11	8	0.052
pyrimethanil	200	82	ES+	45	24	0.015
	200	107	ES+	45	24	0.015
methiocarb	226	121	ES+	22	22	0.015
	226	169	ES+	22	10	0.015
pirimicarb	239.1	72	ES+	28	72	0.017
	239.1	182.1	ES+	28	15	0.017
fenpropimorph	304.2	57.2	ES+	50	30	0.015
	304.2	147.2	ES+	50	28	0.015
thiodicarb	355	87.9	ES+	20	16	0.015
	355	107.9	ES+	20	16	0.015
prochloraz	376	70.1	ES+	16	34	0.015
	376	307.1	ES+	16	16	0.015
difenoconazole	406	111.1	ES+	40	60	0.015
	406	251.1	ES+	40	25	0.015
carbaryl	202	117	ES+	22	28	0.08
	202	145	ES+	22	22	0.08
ametryn	228.1	68.1	ES+	32	36	0.013
	228.1	186.1	ES+	32	18	0.013
thiametoxam	292	132	ES+	22	22	0.038
	292	211.2	ES+	22	12	0.038
malathion	331	99	ES+	20	24	0.013
	331	127	ES+	20	12	0.013
dimethomorph	388.1	165	ES+	35	30	0.013
	388.1	300.9	ES+	35	20	0.013
dimethoate	230.1	125	ES+	18	20	0.012
	230.1	199	ES+	18	10	0.012
metalaxyl	280.1	192.1	ES+	20	17	0.012
	280.1	220.1	ES+	20	13	0.012
tebuconazole	308	70.1	ES+	40	22	0.015
	308	125	ES+	40	40	0.015
chlorpyrifos	349.9	97	ES+	30	32	0.037
	349.9	198	ES+	30	20	0.037
azoxystrobin	404	329	ES+	22	30	0.015
	404	372	ES+	22	15	0.015
carbendazim	192.1	132.1	ES+	27	28	0.08
	192.1	16.1	ES+	27	18	0.08
imidacloprid	256.1	175.1	ES+	34	20	0.038
	256.1	209.1	ES+	34	15	0.038
parathion	291.9	110	ES+	30	33	0.017
	291.9	236	ES+	30	14	0.017
diazinon	305	96	ES+	31	35	0.017
	305	169	ES+	31	22	0.017
propiconazole	342	69	ES+	40	22	0.017
	342	159	ES+	40	34	0.017
profenofos	372.9	127.9	ES+	36	40	0.017
	372.9	302.6	ES+	36	20	0.017
methamidophos	142	93.9	ES+	28	13	0.163
	142	124.9	ES+	28	13	0.163

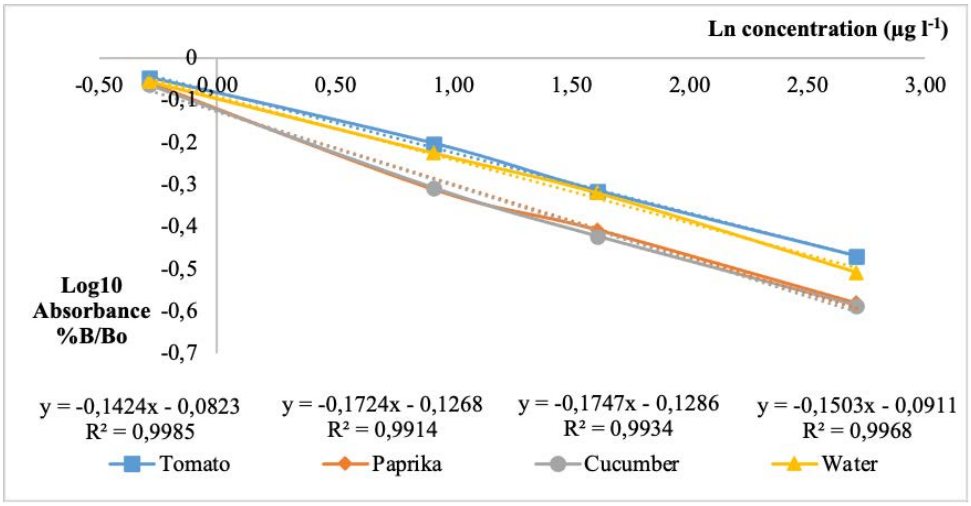

Figure 1 *Calibration curves in water and matrixes of the study performed during the ELISA kit pyrethroid test*

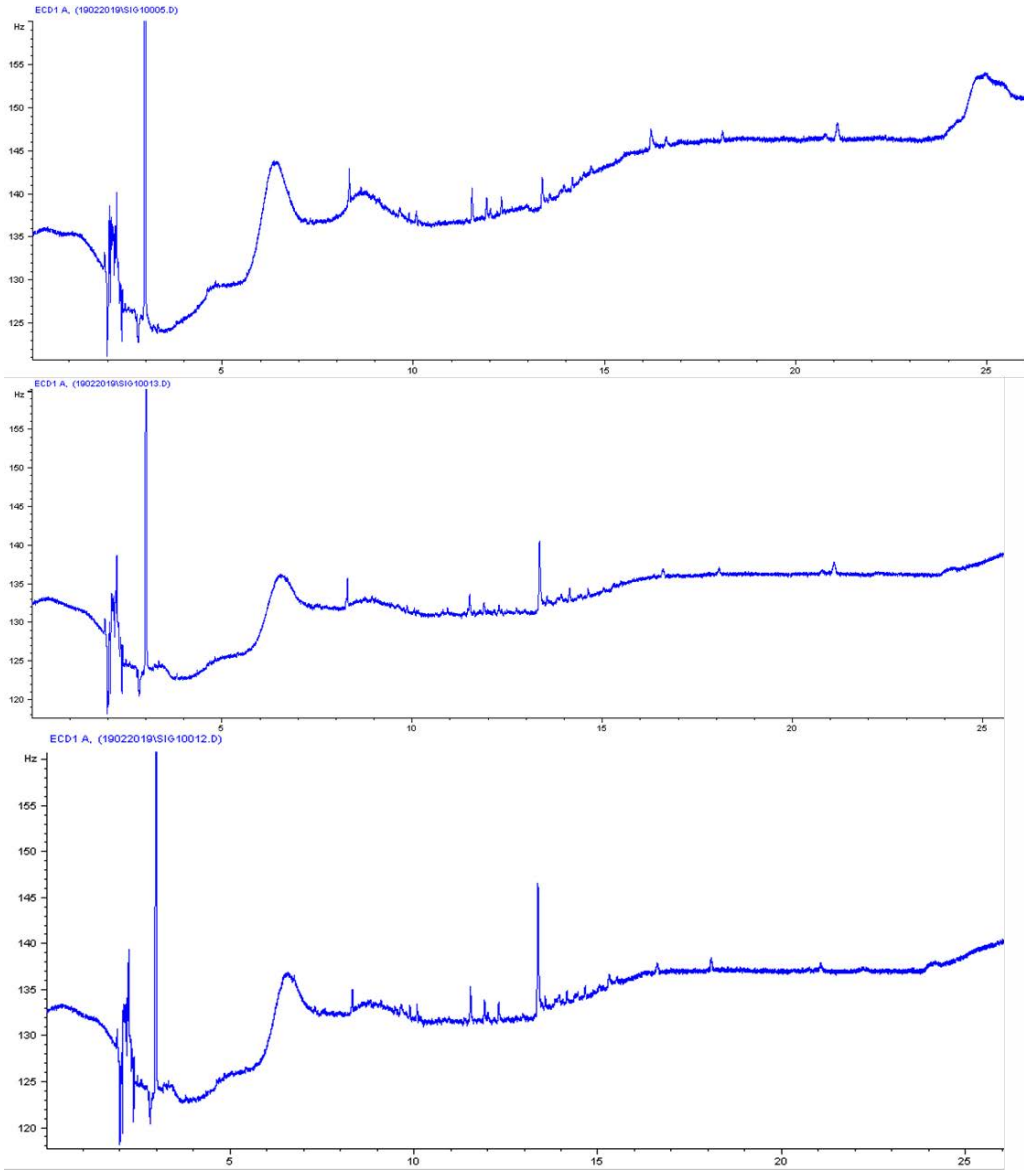

Figure 2 *GC-ECD Chromatograms from tomato, sweet pepper, and cucumber spiked at 15.0 ppb of permethrin (retention times of the isomers 20.8 and 21.1 minutes).*

RESULTS AND DISCUSSION

3.1 ELISA test for pyrethroids

Figure 1 shows the calibration curves for permethrin developed in water (control) and in the tested matrices (tomato; cucumber and sweet pepper) using the pyrethroid ELISA kit. As the kit method recommends, clean vegetable juices dilutions (1:1 in methanol) to be used as reference instead of clear water for the spectrophotometric measurements.

A statistical correspondence between the calibration curves from the matrixes and the control curve in water was found. The slope from the pepper, cucumber, and tomato calibration curves was between the confidence limits (upper: -0.12311 and lower: -0.17749) of the control slope curve, for n-2 degrees of freedom to the desired probability of 95 %. Good statistical correspondence was also confirmed when the average values obtained (3.46 μg l^{-1}) from the positive control evaluated (3.0 μg l^{-1}) in each calibration curve (n=4) showed a 4.0 % of the coefficient of variation and a 115 % of the recovery. Xu [23], also studied four ELISA kits brands to analyze organophosphates, carbamates, and pyrethroids. Their best results were obtained with the kits provided by Abraxis, with recovery percentage and coefficient of variation very similar to the values obtained in the present study. Figure 1 shows the percentages of inhibition of absorbance with respect to the negative control from 0.75 to 15.0 μg l^{-1} of permethrin in the evaluated matrixes. Based on those results, calibration curves from each matrix were used for the calculation of the corresponding concentration in the evaluated samples.

3.2 Comparison of results from ELISA with GC-ECD and UPLC–MS/MS

The accuracy of the ELISA results for pyrethroids could not be evaluated by GC-ECD. The GC-ECD equipment used has a limit of quantification (LOQ) (considered in this study as the low point in the calibration curve) for permethrin of 100.0 μg l^{-1}, around seven times higher than the spiked concentration evaluated (15.0 μg l^{-1}) in the ELISA test. Figure 2 shows the chromatograms from the evaluated vegetables in the ELISA test at the spiked concentration of 15.0 μg l^{-1}.

As it can be seen in figure 2, the chromatography can detect permethrin (20.8 and 21.1 min of retention times), but the relation signal area/noise value is not enough to quantify it; the low area's values obtained cannot be used to quantify it through the equation of the calibration curve. From the group of synthetic pyrethroids that are analyzed in the laboratory, permethrin is one of the least sensitive (ten times less than cypermethrin, bifenthrin, and others). In an Agilent report it is also mentioned that, from the group of pyrethroids analyzed by GC-ECD, permethrin is the active ingredient with the highest response factor (injected concentration/peak area) [25]. With the use of these ELISA values lower than the LOQ of the GC-ECD can be quantified. This test can help in the monitoring and control of pyrethroid residues in the vegetables studied.

Permethrin is widely used for hygienic control in sanitary in Cuba and together with other pyrethroids in phytosanitary control. Sometimes these pesticides are used incorrectly. They reach water bodies and/or remain as residue in certain crops [26–28]. Unlike in the EU, where the use of permethrin is not allowed, in Cuba permethrin and other pyrethroids can still be used. If Cuba is considering the export of some of these vegetables or fruits, to Europe, it should meet the EU Maximum residual levels (MRL) which fix permethrin at concentrations below the 50.0 μg l^{-1}. For this aim, ELISA can play an important role.

The accuracy of ELISA results developed for OP/Cs was evaluated by UPLC–MS/MS. Table 2 shows the results of the positive ELISA control analysis in the matrices evaluated by liquid chromatography. Percentages of inhibition were obtained in the ELISA tests, which could be verified by chromatography. An average concentration of 5.69 μg l^{-1} among the matrices evaluated, a 114 % recovery, and a 3 % coefficient of variation with respect to the concentration used may be obtained with the ELISA test. Similar results (recoveries of 97-116% with coefficients of variation of 4-10%), also from local vegetables (Chinese cabbage, cucumber, tomato, carrots), were reported in studies from China and India [17,18,23]. The authors also outlined in the studies, the benefits of ELISA tests in terms of cost-effectiveness, simple operation, rapid response, and lower limits of quantification and detection than chromatography techniques [17,18,23]. In this way, the accuracy of the ELISA test for OP/C could be proved. The inhibition percentages above were used as referents to decide to analyze these samples with LC-MS/MS.

3.3 Screened samples

As can be observed in table 3, with the use of ELISA a higher amount of samples with OP/Cs and pyrethroids residues were detected compared to the chromatography techniques. Luo et al. (2017) also mention that the immunoassay was capable to detect ethyl carbamates in a large number of samples. A small signal was obtained in the chromatograms of several samples at the retention time thiodicarb, methiocarb, acephate, dimethoate, oxamyl, and cypermethrin, but the ratio between signal/noise was quantitatively low to consider them as a real signal. In the brochure of the Abraxis kit for OP/Cs, it is indicated that the Limit of Detection Pattern Sensitivity for some pesticide residue like chlorpyrifos (methyl and ethyl), dichlorvos, diazinon, and others are between 0.4 to 0.6 μg l^{-1}. Pyrethroids showed the following sensibility: cypermethrin 4.75 μg l^{-1}, λ-cyhalothrin 9.2 μg l^{-1}, and bifenthrin 13.5 μg l^{-1}. Although these values can be detected, they are below the LOQ of the chromatographic technique used in this study. All detected values were below their MRL.

Figure 3 shows the relationship between quantified values obtained by chromatographic techniques and the ELISA values in table 3. As can be seen in Figure 3, linear relationships were found between those values. Carbaryl values show a slope of 1.0089 with an R^2 of 0.9983, and cypermethrin shows a slope of 1.1088 with an R^2 of 0.9986. Additionally, a satisfactory Pearson correlation r= 0.999 ($p<0.001$) was found. Other authors also obtained well-correlated results between ELISA

Table 2 *Average recovery values of the OP/C positive controls evaluated by LC-MS/MS*

Matrices	Positive control spiked (µg l-1)	ELISA inhibition (%) at 405 nm	LC values (µg l-1)	Mean	Recovery (%)	S.D	CoV
Cucumber		80 %	5.91				
Sweet pepper	5.00	78 %	5.62	5.69	114 %	0.0002	3 %
Tomato		74 %	5.54				

LC: liquid chromatography, S.D: standard deviation, CoV: coefficient of variation.

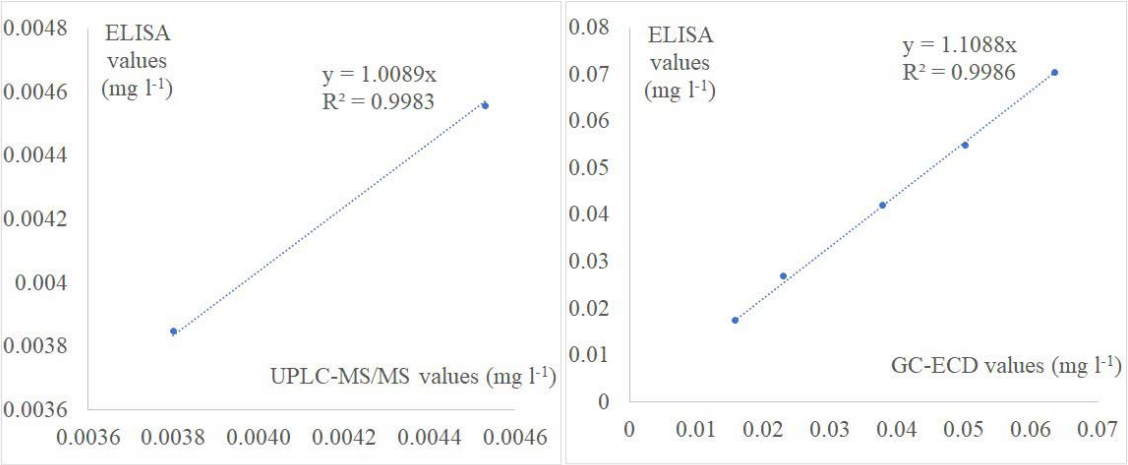

Figure 3 *Figure 2: Analysis of the linear relationship for the cross quantified values between ELISA test and their chromatographs results*

and chromatographic techniques in their sample analysis, suggesting good accuracy and reproducibility of the ELISA methods [12,29,30]

After this result, ELISA receives important attention, especially for residues of OP/Cs and pyrethroids, several of which are prohibited or of restricted use, mainly in the EU, where they received a default MRLs value. Thus, the developed ELISA exhibited good accuracy, is ideally suited as a fast, high-throughput, and low-cost screening test for OP/C and pyrethroids residues prior to chromatographic analysis to monitor and control the level of such residues.

3.4 General pesticide residue detected

Table 4 shows the rest of the pesticide residues detected by chromatography in the analyzed samples. As can be observed, residues of 13 different active ingredients (AIs) were detected. Fungicide was the most common group with nine AIs measured in the samples. Three of them were triazoles. Leyva Morales [31], also identify fungicides as the group with the highest frequency of use in northwestern Mexico; and Wahid [32], cite fungicides as the second pesticide group imported after herbicides in Suriname. Additionally, EFSA [33] reported in the 2015 annual report of pesticides in food, fungicides as the most frequent pesticides with concentrations equal to or greater than the LOQ found. Two insecticides (neonicotinoids) and two herbicides completed the list of the residues detected.

For seven of the samples analyzed, the AI residues belonged to the same mode of action (neonicotinoids and triazoles) were found. Farmers should be alerted to the hazard and risk of developing resistance to pests and diseases if AIs with the same mode of action are used on the same crop in one season. [1,2,34]. From 13 of the AIs detected in the collected samples, seven (fenpropimorph, chlorothalonil, thiamethoxam, carbendazim, propiconazole, ametryn, and alachlor) are forbidden for use in the EU. If the carbamate and organophosphates from Table 3 are also included, the number increases to 11 [35].

The present study also likes to alert the local authorities to the risk that is being incurred due to the absence of MRL values in the Cuban norm [36] for 14 of the 19 AIs detected. This hinders their control and monitoring. Among the AIs detected without Cuban values of MRLs, are those banned from use in the EU: acephate, ametryn, fenpropimorph, thiamethoxam, carbendazim, propiconazole, dimethoate, and alachlor. Alachlor had a value even higher than the EU MRL.

Although the presence of prohibited AI residues in the EU persists, with respect to previous studies in journals reviewed, the number of these has decreased. In samples of vegetables collected in the period 2016 - 2018, residues of endosulfan, methamidophos, parathion and parathion methyl, thiodicarb, permethrin, and lindane were additionally found. It is important to note that the current list of authorized pesticides in Cuba dates from 2016 to [37] and concerning the previous list [38] lindane, methamidophos, parathion, and methyl parathion are not authorized for use. Therefore, the presence of the residues mentioned in the samples collected between 2016 and 2018 meant a violation of the established laws and/or due to possible illegal activities. Positive is the

Table 3 *Organophosphate, carbamate, and pyrethroids residues detected in the screened samples by ELISA test and checked by chromatography*

Samples	Chromatographic				ELISA (mg l^{-1})	Chromatographic		ELISA (mg l^{-1})
	carbamate (Cs)	organophosphate (OP)				pyrethroid		
	carbaryl (mg l^{-1})	profenofos (mg l^{-1})	acephate (mg l^{-1})	dimethoate (mg l^{-1})		cypermethrin (mg l^{-1})	tau-fluvalinate (mg l^{-1})	
Sweet pepper	0.0038				0.0038		<LOQ	0.0019
Cucumber		<LOQ		<LOQ	0.0049			0.0026
Cucumber		<LOQ		<LOQ	0.0034			0.0042
Tomato					0.0025			
Tomato			<LOQ	<LOQ	0.0034	<LOQ		0.0089
Tomato				<LOQ	0.0052	<LOQ		0.0053
Tomato			<LOQ	<LOQ	0.0030			
Tomato			<LOQ	<LOQ	0.0035			
Tomato			<LOQ		0.0030			
Tomato				<LOQ	0.0027			
Tomato					0.0023			
Tomato				<LOQ	0.0025			
Tomato			<LOQ		0.0026			
Tomato				<LOQ	0.0043			
Sweet pepper		<LOQ			0.0027			
Sweet pepper	0.0045			<LOQ	0.0046		<LOQ	0.0102
Sweet pepper		<LOQ			0.0030			
Cucumber		<LOQ			0.0025			
Cucumber						<LOQ		0.0145
Cucumber				<LOQ	0.0024	0.0158		0.0174
Sweet pepper						0.0379		0.0422
Sweet pepper						0.0230		0.0270
Sweet pepper						0.0502		0.0550
Sweet pepper						0.0636		0.0705
EU/Cuban MRL (mg kg^{-1})								
Tomato								
Cucumber	0.01/5.0	10.00/10.0	0.01/-	0.01/-		0.50/0.2	0.10/-	
Sweet peppers	0.01/-	0.01/-	0.01/-	0.01/-		0.20/0.07	0.05/-	
	0.01/5.0	0.01/5.0	0.01/-	0.01/0.5		0.50/0.1	0.01/-	

Table 4 *Pesticide residues detected by GC-ECD and UPLC-MS/MS in the collected samples*

sample #	fenpropi-morph	imidaclo-prid	chlorotha-lonil	difenocon-azole	thiameth-oxam	carben-dazim	propicon-azole	azoxys-trobin	pyrimeth-anil	tebucon-azole	ametryn	alachlor	prochloraz
Sweet pepper	<LOQ	0.0190				0.0135		0.0022		0.0019			
Cucumber		0.0017			0.0099								
Cucumber		0.0019		<LOQ	0.0125								
tomato	<LOQ		0.018			<LOQ							
Tomato	<LOQ				<LOQ								
Tomato			0.036						0.0054				
Tomato	<LOQ		0.105										
Tomato	<LOQ	<LOQ											
Tomato	<LOQ		0.099										
Tomato	<LOQ								<LOQ				
Tomato	<LOQ		<LOQ										
Tomato	<LOQ												
Tomato	<LOQ		0.024			<LOQ		<LOQ					
Tomato			<LOQ										
Tomato	<LOQ	<LOQ											
Tomato	0.0025	<LOQ											
Tomato	<LOQ		0.015										
Sweet pepper		0.0208				0.0183		0.0027		0.0022			0.0012
Sweet pepper		0.0021		<LOQ	0.0103								
Sweet pepper		<LOQ			0.0034								
Sweet pepper	<LOQ	0.0022		0.0017	0.0102								
Cucumber		0.0025									<LOQ	0.018	
Cucumber	<LOQ												
Sweet pepper		0.0202		0.0054			0.0054						
Sweet pepper		0.0203		0.0036			0.0039						
Sweet pepper		0.0203		0.0054			0.0055						
Sweet pepper		0.0193		0.0061			0.0074						
EU/Cuban MRL (mg kg^{-1})													
Tomato	0.01/-	0.5/0.5	6.0/5.0	2.0/0.5	0.2/-	0.3/-	3.0/-	3.0/3.0	1.0/0.7	0.9/0.2	0.01/-	0.01/-	0.05/-
Cucumber	0.01/-	1.0/1.0	5.0/5.0	0.3/-	0.5/-	0.1/-	0.01/-	1.0/1.0	0.8/-	0.6/0.2	0.01/-	0.01/-	0.05/-
Sweet peppers	0.01/-	1.0/1.0	0.01/7.0	0.9/-	0.7/-	0.1/-	0.01/-	3.0/3.0	2.0/-	0.6/0.5	0.01/-	0.01/-	0.05/-

fact that the group of AIs (mainly OP/Cs) already banned in several countries, mainly in the EU and the United States, still existing in the current list of authorized pesticides in Cuba, are gradually decreasing from one list to another. That is part of a program for the reduction of synthetic pesticides with high toxicity, promoted by the government of Cuba in support of national and international environmental laws. It aims to guarantee food safety without compromising human health and environmental protection [27,39–42].

CONCLUSION

The study achieved its aim, with the use of Abraxis ELISA kits, it was possible to detect the presence of the residue of certain groups of compounds of interest like organophosphates, carbamates, and pyrethroids in the collected samples. ELISA proved to be a reliable low-cost analytical procedure for fast detection, control, and monitoring of the presence of pesticide residues in tomatoes, peppers, and cucumbers, before chromatographic techniques (gas or liquid chromatography). The ELISA kits tested showed the capacity for quantification at values below the detection limit of the chromatographic techniques used. However, further analysis to determine specific active ingredients and their quantity if it is needed can be continuing done by chromatographic techniques, where a general screen can be also obtained. More than half of the total residues detected in the collected samples indicated the use of synthetic pesticides which are nowadays banned in the EU. In the context of Cuban agriculture, ELISA can be well used as a tool to evaluate the use of pesticides, since carbamates, organophosphates, and pyrethroids are still used. Monitoring and control actions would mainly focus on guaranteeing that the crops to be exported will meet international residue limits, as well as on ensuring that the population does not ingest highly toxic pesticide residues.

FUNDING SOURCES

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

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