KIMIKA Volume 33, Number 1, pp. 01-08 (2022)
 ISSN 2508-0911
https://doi.org/10.26534/kimika.v33i1.01-08

Method Development and Validation of Gas Chromatography Methods using Nitrogen Phosphorus Detector (GC-NPD) and Isotope Dilution Triple Quadrupole Mass Spectrometry (GC-IDMS/MS) for the Determination of Chlorpyrifos in Mango

Johanna Andrea C. Valdueza*[1,2], Jomar C. Sta Ana[2], Alleni T. Junsay[2], Aaron C. Dacuya[2], Benilda S. Ebarvia[1,2]

[1] *The Graduate School, University of Santo Tomas, España Boulevard, 1015 Manila Philippines*
[2] *Metrology in Chemistry, National Metrology Laboratory, Department of Science and Technology-Industrial Technology Development Institute (DOST-ITDI), Bicutan, Taguig City Philippines 1633*

*Author to whom correspondence should be addressed; email: juna0610valdueza@gmail.com/ johannaandrea.valdueza.gs@ust.edu.ph

ABSTRACT

Pesticides residue determination in fruits like chlorpyrifos is important due to its health effects on humans. Thus, there is a need for sensitive and accurate methods for better quantification. In this study, the performance of two gas chromatographic detection methods, nitrogen phosphorus detector (GC-NPD) and isotope dilution triple quadrupole mass spectrometer (GC-IDMS/MS), for the determination of chlorpyrifos in mango was evaluated to be used for the development of candidate reference material. Other than liquid-liquid extraction (LLE), Quick, Easy, Cheap, Efficient, Rugged and Safe (QuEChERS) sample extraction and dispersive solid phase extraction (dSPE) clean-up procedures were optimized to extract chlorpyrifos from the sample matrix. Comprehensive method validations were performed in GC-NPD and GC-IDMS/MS with HP-5 capillary column to establish linearity, the limit of detection (LOD), the limit of quantification (LOQ), accuracy, repeatability, and intermediate precision using freeze-dried mango samples. The LOD and LOQ were 2.35 and 3.47 μg kg^{-1} for GC-NPD, and 1.23 and 1.85 μg kg^{-1} for GC-IDMS/MS, respectively. Matrix-matched calibration curves showed excellent linearity of r^2=0.999 for both GC-NPD and GC-IDMS/MS protocols. Acceptable repeatability was obtained for three spike concentrations of 10, 30, and 60 μg kg^{-1} (n=10) and intermediate precision for 0.5-, 1- and 3-months in the same three-spike concentrations (n=3) expressed as relative standard deviation (RSD) ranging from 0.34-5.61 % for GC-NPD and 0.46-4.22 % for GC-IDMS/MS. Satisfactory mean recoveries (n=10) were achieved: 88.48-109.81% in GC-NPD and 97.61-108.40% in GC-IDMS/MS. GC-IDMS/MS and GC-NPD methods were fit for the purpose of quantifying chlorpyrifos in Philippine mango accurately with the use of isotope dilution technique and traceability to international standards by the application of gravimetric sample preparation.

Keywords: *chlorpyrifos; GC-NPD; GC-IDMS/MS; method validation; QuEChERS*

INTRODUCTION

Chlorpyrifos is a toxic chlorinated organophosphate insecticide and one of its applications is to sustain mango fruit production. The Philippine mango industry is the third largest fruit export of the country, after banana and pineapple, with 85% of the country's processed mango going to the export market (Briones et al, 2013). However, one major concern in mango fruit production is the persistent use of pesticides as means to protect from pests and diseases. Pesticide residues are monitored in mangoes due to their adverse effects on human health. Specifically, chlorpyrifos may be absorbed by all routes, including inhalation, ingestion, and dermal absorption in humans (Aswathi et al., 2019). Pesticide poisoning is a serious health problem because they are carcinogenic, neurotoxic, and may disrupt hormonal and enzymatic regulations when consumed by humans (Samsidar et al., 2018).

The setting of Maximum Residue Limits (MRLs) of pesticides in important crops is part of the government's regulatory process and accurate analytical results for a truthful assessment of exposure level and health risks are implemented (Cassou et al, 2017). The MRL of chlorpyrifos in mango in the Philippines is 50 μg kg^{-1} (PNS/BAFS, 2015). Highly accurate analytical methods and selective instrumental analyses are highly needed, especially in the case of pesticides, which deal with residual levels of 5 to 500 μg kg^{-1} (Grimalt et al., 2015). To ensure the reliability of the analytical results, method validation of pesticides analysis is essential (Yarita et al., 2014).

In this paper, the performance of the validation parameters for the determination of chlorpyrifos in mango was evaluated for two gas chromatographic methods, GC-NPD and GC-IDMS/MS, to be used for the development of candidate reference material. The use of reference material ensures the accuracy and reliability of analytical results, thereby ensuring MRLs of pesticides are not exceeded. In the development of reference material, the validated GC-NPD method was employed for the assessment of homogeneity and stability, and the validated GC-IDMS/MS was used for the assignment of the reference value. Furthermore, GC-IDMS/MS and GC-NPD results with associated measurement uncertainty were cross-checked.

METHODS

Chemicals and reagents. Pesticide standard of chlorpyrifos with 99.6±1.3% purity was obtained from the National Measurements Institute of Australia (NMIA). Isotopically enriched chlorpyrifos (Diethyl-D10) with 100 μg mL^{-1} concentration in nonane, atomic purity of 99%, and chemical purity of 97.2% from Cambridge Isotope Laboratories, Inc. was used for the IDMS/MS method. Chlorpyrifos standard from Sigma-Aldrich was used for the recovery analysis. Analytical HPLC grade ethyl acetate from RCILabscan was optimized as the solution and extracting solvent. A Type 1 water (de-ionized) was used throughout the analysis. Stock standard solutions with 100 mg kg^{-1} solution concentration were prepared gravimetrically and diluted in ethyl acetate were stored in the refrigerator (4 ˚C). Ultra-high purity helium and nitrogen; and high purity compressed air and hydrogen were from Linde Philippines Inc. QuEChERS Bond Elut AOAC and EN extraction packets were purchased from Agilent Technologies Inc. AOAC extraction packets contain 6.0 g magnesium sulfate and 1.5 g sodium acetate, while the EN extraction packets contain 4.0 g magnesium sulfate, 1.0 g sodium chloride, 1.0 g sodium citrate, and 0.5 g disodium citrate sesquihydrate.

***Optimization of the sample matrix*.** Ripe and unripe Carabao mangoes and unripe Indian mangoes were differentiated to select the applicable sample matrix in the study. Fresh mango fruits were purchased from a local market and analyses were done in the pulp or flesh part of the mangoes since the seed and peel are most likely separated from the fruit when consumed. Mango samples are freeze-dried for 96 hours at 10 ˚C in 0.220 mbar using a Martin Christ freeze-dryer and processed using Retsch knife mill and Retsch sieve shaker.

Optimization of the QuEChERS extraction method using GC-NPD. Two QuEChERS extraction methods based on AOAC and EN methods for pesticides in mango were compared to select the best method to use. Three (3) replicates for each type of QuEChERS method were analyzed. Each replicate in a 50-mL centrifuge tube contains 2 g of freeze-dried unripe Carabao mango sample, 1.0 g of 500 μg kg^{-1} spike standard solution, 8.0 g of de-ionized water, and placed in a vortex mixer for 5 mins. Extraction salts were added following AOAC Official Method 2007.01 (AOAC, 2007) and EN 15662:2018 (EN, 2018). The final extract was injected in GC-NPD and % recovery was evaluated.

Sample preparation with isotope using GC-IDMS/MS. In the extraction of samples with an isotope, the same sample preparation for GC-NPD was used. Sample blends were prepared by adding 0.8 g of 200 μg kg^{-1} chlorpyrifos calibration and isotope solutions to achieve a 1:1 area ratio of analyte/isotope, placed in a rotary mixer at room temperature for 1 hour and another 1 hour after addition of 8.0 g de-ionized water. Extraction salts were added following the EN 15662:2018 protocol as the optimized QuEChERS extraction method. The final extract was injected in GC-IDMS/MS.

GC-NPD conditions. Analyses were performed using Agilent Technologies 6890B GC-NPD equipped with HP-5 capillary column (30 meters long, 0.32 mm ID) coated with (5%-Phenyl) methylpolysiloxane *(*0.25 μm film thickness) and autosampler. Inlet liner used was a splitless single taper type with wool-to-trap non-volatile residue to prevent column contamination. General operating conditions were as follows; injector port temperature: 250 °C; carrier gas (helium): flow rate of 2 mL min^{-1}; combustion gases: hydrogen 3 mL min^{-1} and air 120 mL min^{-1} and make-up gas: N_2 20 mL min^{-1}; oven temperature program: initially at 70 °C, increased to 20 °C min^{-1} at 170 °C, held for 2 mins at a rate of 8 °C min^{-1} at 211 °C, then increased to 3.5 °C min^{-1} at 230 °C for 2 mins and held for 5 mins at 290 °C with a rate of 18 °C min^{-1}; and detector temperature: 320°C (S. Chinitis, personal communication, January 16, 2019). The OpenLab software controlled both the online and offline analyses.

GC-IDMS/MS conditions. GC-IDMS/MS analyses were performed using Agilent 6890B GC with Agilent 7010B Triple Quadrupole MS (QQQ) equipped with an autosampler. Analytes were separated in an Agilent GC capillary HP-5MS column (30 m x 0.25 mm ID x 0.25 μm). Ultra-high purity helium was used as the carrier gas at a flow rate of 2.25 mL min^{-1} and nitrogen as the collision gas at 1.5 mL min^{-1}. The oven temperature program and splitless single liner with wool were the same as in GC-NPD. The temperatures of the injection port and MS transfer line were both 280 °C. The electron impact ion source temperature was also set at 280 °C. A MassHunter workstation controlled both the online systems, quantitative and qualitative data analysis. Quantifications were analyzed using multiple reaction monitoring (MRM) mode. The MRM transitions and the optimum collision energies are listed in Table 1.

Table 1. Optimized MRM transitions and collision energies.

Analyte	RT (min)	Precursor Ions (m/z)	Product Ions (m/z)	Collision energy (eV)
Chlorpyrifos	11.896	196.7	168.9	15
		196.7	106.8	40
Chlorpyrifos-d10	11.958	197.7	169.9	15
		197.7	107.0	40

Method validation. The validation parameters performed were LOD, LOQ, working range by linearity in matrix, recovery, repeatability, and intermediate precision. LOD/LOQ measurements were done using unripe Indian mango since a higher concentration of chlorpyrifos was detected in unripe Carabao mango. On the other hand, for the evaluation of linearity, recovery,

repeatability, and intermediate precision, the blank matrix used was unripe Carabao mango. Working range by linearity was determined following the matrix-matching calibration to compensate with the matrix effects. Two (2) replicates of 9 concentration points of the sample with 6-80 µg kg^{-1} spike concentration of chlorpyrifos were used to evaluate linearity in the matrix by plotting in a calibration curve. Accuracy was obtained with recovery studies by spiking in 3 different concentration levels. On the other hand, precision was evaluated through repeatability and intermediate precision using incurred mango samples with an approximate sample concentration in 3 different concentration levels. All analyses were prepared gravimetrically.

RESULTS AND DISCUSSION

Method development. *Optimization of the sample matrix*. After freeze-drying, the pulp of unripe Carabao mango appeared as dried powdered while the ripe Carabao mango was moist, which could be due to its high sugar content. Since the ripe Carabao mangoes were not completely freeze-dried and cannot be processed, only the unripe Carabao and Indian mangoes were used for the recovery study. The average % recovery for unripe Carabao and Indian mangoes with 3 replicates each were 104.47 and 87.69%, respectively. Results on recovery analysis may be within the acceptable 80-110% recovery, however, the yellow pigment in the Indian mango final extracts even after dSPE clean-up was apparent. This could affect the accuracy of the response obtained and could decrease the lifetime of the GC column. In comparison, the unripe Carabao mango extract showed lesser pigments, thus it was used for method validation.

Optimization of the QuEChERS extraction method using GC-NPD. Two QuEChERS extraction (AOAC and EN) methods for pesticides in mango were compared to select which method can reduce the appearance of co-eluting peaks of chlorpyrifos in the chromatogram for better and easier quantification in the chromatographic technique. Sample extracted with the EN method has a distinctive peak for chlorpyrifos than the AOAC method as shown in Figure 1. The selection of a better method that is more suitable for the pesticide in mango was further analyzed with the quantitation of the % recovery. EN method had an average 93.05% recovery for chlorpyrifos while chlorpyrifos quantification by the AOAC method cannot be calculated because of co-eluting peaks. QuEChERS EN extraction method was selected for the whole study.

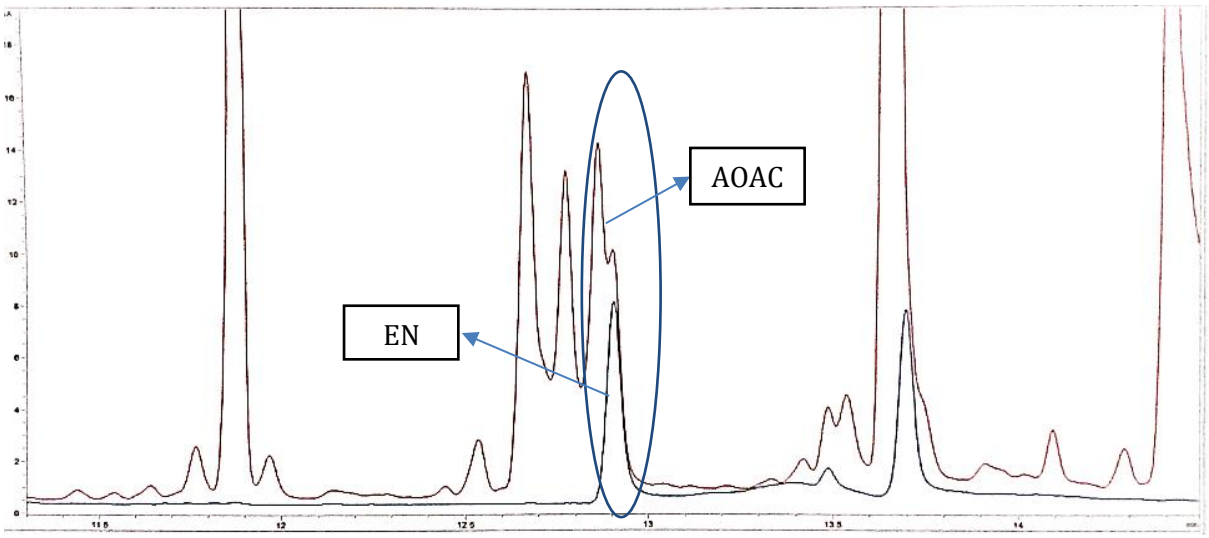

Figure 1. GC-NPD chromatogram using QuEChERS AOAC and EN methods

Optimization of the calibration method. External solvent calibration and matrix matching calibration were compared to select the calibration methods to be used in GC-NPD and GC-IDMS/MS. Both calibration methods showed a linear response with a correlation coefficient (r^2) of 0.999. The response of chlorpyrifos in matrix matching calibration is reduced in GC-NPD and

increased in GC-IDMS/MS as compared to using external solvent calibration making the use of matrix-matching calibration better to compensate with the matrix effects.

Optimization in IDMS/MS. Isotopically labeled analog of the chlorpyrifos was incorporated in the sample matrix as internal standards. Four replicates of calibration solutions (200 µg kg^{-1} solution concentration) were prepared using 1000 µg kg^{-1} intermediate chlorpyrifos standard solution. Two (2) grams of freeze-dried unripe Carabao mango, 0.8 g of calibration solution, 0.8 g of 200 µg kg^{-1} isotope solution were processed in duplicate for four calibration solutions prepared, to achieve a 1:1 area ratio of analyte/isotope in the sample blend. This ratio is directly related to the concentration of the analyte in the sample. Exact matching IDMS/MS can be employed where the sample blend is iteratively approximated until the isotope blend ratio meets the isotope ratio of the calibration solution (Vogl and Pritzkow, 2010). The sample concentration using IDMS was calculated using equation (1).

$$C_{chlorpyrifos} = \frac{m_{is-sol,spiked} \times m_{s-sol,std} \times AR_{sample} \times C_{s-sol}}{W_s \times AR_{std} \times m_{is-sol,std}} \quad (1)$$

Where:
$C_{chlorpyrifos}$ = concentration of chlorpyrifos in the sample (µg kg^{-1})
$m_{is\text{-}sol,spiked}$= mass of isotope solution spiked in the sample blend (g)
$m_{s\text{-}sol,std}$ = mass of standard solution spiked into the calibration blend (g)
AR_{sample} = area ratio of analyte/isotope in the sample blend
Cs-sol = concentration of the standard solution
W_s = mass of the sample (g)
ARstd = area ratio of analyte/isotope in the calibration blend
$m_{is\text{-}sol,std}$= mass of isotope solution spiked in the calibration blend (g)

Optimization of the GC-IDMS/MS parameters. MRM transitions of the analyte and isotope were optimized generated by mass-to-charge (m/z) ions. The parent ions were identified via MS1 scan and the product ions were identified via Product Ion scan. The collision energies were optimized from 5-30 electron volts via multiple reaction monitoring (MRM) scan. The GC-IDMS/MS chromatogram in Figure 2 showed retention times of chlorpyrifos-d10 and chlorpyrifos.

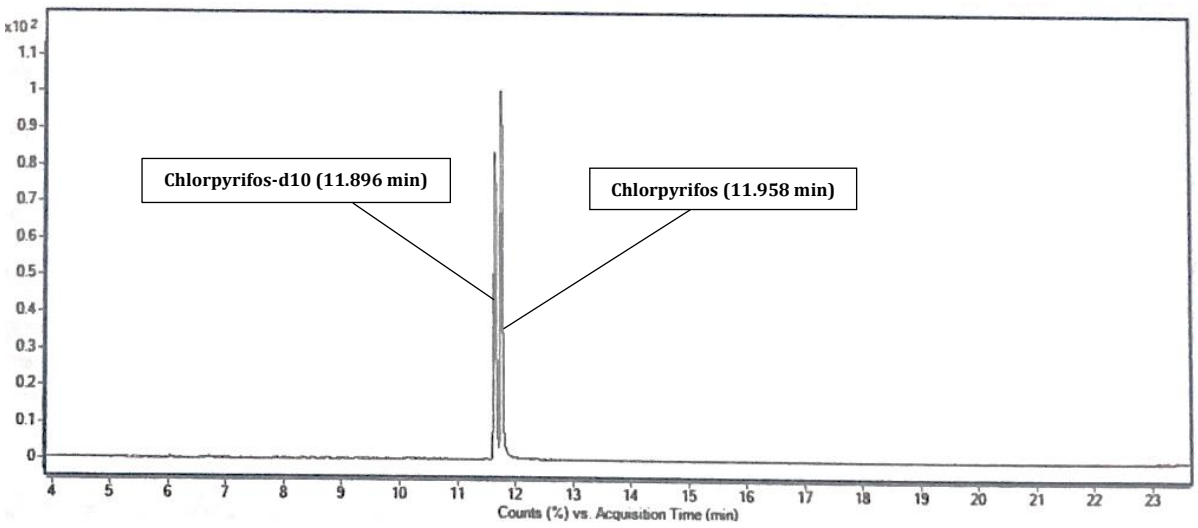

Figure 2. GC-IDMS/MS chromatogram of chlorpyrifos at and chlopyrifos-d10

Method validation. Method validation is the first part in the development of candidate reference material for chlorpyrifos in mango by the Philippine government through the Department of Science and Technology-Industrial Technology Development Institute (DOST-ITDI) as the

designated National Metrology Institute (NMI) of the Philippines. Since, DOST-ITDI has available GC-NPD, which is selective for nitrogen- and phosphorus-containing compounds such as chlorpyrifos and GC-IDMS/MS as a primary method for the reference value assignment used by NMIs in the world, both methods were utilized and validated.

Matrix-matching calibration was used to compensate errors associated with matrix effects in both GC methods (Pelit et al., 2012). The performance characteristics were conducted using a gravimetric approach following the Eurachem Guide "The Fitness for Purpose of Analytical Methods" and criteria based on AOAC "Official Methods of Analysis" (Ellison and Williams, 2019).

Each validation parameter was tabulated comparing the results in GC-NPD and GC-IDMS/MS.

Table 2. Summary of validation parameters results in GC-NPD and GC-IDMS/MS.

Parameter	*GC-NPD*	*GC-IDMS/MS*	*Criteria based on AOAC Official Methods*
LOD/LOQ			
instrumental LOD	2.28	1.19	
instrumental LOQ	3.37	1.80	
matrix LOD, µg kg^{-1}	2.35	1.23	
matrix LOQ, µg kg^{-1}	3.47	1.85	
Linearity			
sensitivity	1.3728	7114.6	
r^2	0.999	0.999	r^2 not be less than 0.995
Working range, µg kg^{-1}	5.38 – 73.33	4.27-59.50	
Recovery, %			
Low: 10 µg kg^{-1} (n=10)	98.90 - 105.49	102.37 – 107.89	80-110%
Mid: 30 µg kg^{-1} (n=10)	103.69 - 109.81	100.23 – 108.40	
High: 60 µg kg^{-1} (n=10)	88.48 - 93.98	97.61 – 104.96	
Repeatability (%RSD)			
Low: 10 µg kg^{-1} (n=10)	4.20	1.46	7.77 / 7.74
Mid: 30 µg kg^{-1} (n=10)	2.22	1.33	6.06 / 5.75
High: 60 µg kg^{-1} (n=10)	2.55	1.75	5.72 / 5.39
Intermediate Precision (%RSD)			
After 0.5 month			
Low: 10 µg kg^{-1} (n=3)	3.71	1.70	7.87/7.66
Mid: 30 µg kg^{-1} (n=3)	0.58	0.46	5.89/5.87
High: 60 µg kg^{-1} (n=3)	1.59	4.22	5.49/5.31
After 1 month			
Low: 10 µg kg^{-1} (n=3)	1.90	2.49	7.75/7.73
Mid: 30 µg kg^{-1} (n=3)	2.68	1.24	5.95/5.93
High: 60 µg kg^{-1} (n=3)	0.34	1.57	5.55/5.37
After 3 months			
Low: 10 µg kg^{-1} (n=3)	5.61	0.71	8.30/7.71
Mid: 30 µg kg^{-1} (n=3)	3.89	0.76	6.01/5.92
High: 60 µg kg^{-1} (n=3)	1.89	1.91	5.55/4.19

Through linear regression, the matrix-matching calibration curve injected in GC-NPD and GC-IDMS/MS were both linear and with acceptable correlation coefficients (r^2). For the recovery analysis, the % recovery values for both methods range from 88.48-108.40%, which are within the 80-110 acceptable % recoveries. This implies that the method efficiently extracts chlorpyrifos from the freeze-dried mango sample matrix in GCNPD and GC-IDMS/MS. Results on repeatability

in 3 different concentration levels showed %RSD lower than the acceptable RSD computed from Horwitz's equation. This means that the method is repeatable for the freeze-dried mango sample matrix in GCNPD and GC-IDMS/MS.

CONCLUSIONS

All the performance characteristics following the Eurachem Guide passed the acceptance criteria based on AOAC Official Methods of Analysis, therefore, detection using GC-NPD for assessment of homogeneity and stability and using GC-IDMS/MS for reference value assignment was found to be fit for its purpose to determine chlorpyrifos in mango following QuEChERS extraction and dSPE cleanup procedure.

REFERENCES

AOAC Official Method 2007.01. Pesticide residues in foods by acetonitrile extraction and partitioning with magnesium sulfate [Applicable for the following pesticides in grapes, lettuces, and oranges: atrazine, azoxystrobin, bifenthrin, carbaryl. 2007. Retrieved from https://nucleus.iaea.org/sites/fcris/Shared%20Documents/SOP/AOAC_2007_01.pdf [Date accessed: 1.11.22].

Aswathi A, Pandey A, Sukumaran RK. Rapid degradation of the organophosphate pesticide – chlorpyrifos by a novel strain of *Pseudomonas nitroreducens* AR-3. Bioresour Technol. 2019 Nov; 292:122025. https://doi.org/10.1016/j.biortech.2019.122025

Briones RM, Turingan PAS, Rakotoarisoa MA. FAO commodity and trade policy market structure and distribution of benefits from agricultural exports: The case of the Philippine mango. FAO commodity and trade policy research working paper number 42. FAO, UN; 2013. Retrieved from https://www.fao.org/3/ar709e/ar709e.pdf [Date accessed: 1.11.22].

Cassou E, Tran DN, Nguyen TH, Dinh TX, Nguyen CV, Cao BT, Jaffee S, Ru J. An overview of agricultural pollution in Vietnam: Summary report 2017. World Bank: Washington, DC; 2017. Retrieved from https://openknowledge.worldbank.org/handle/10986/29242 [Date accessed: 1.11.22].

Ellison SLR, Williams A, editors. Eurachem/CITAC Guide. Metrological traceability in chemical measurement. A guide to achieving comparable results in chemical measurement 2nd Ed. (2019). Retrieved from https://www.eurachem.org/images/stories/Guides/pdf/ECTRC_2019_EN_P1.pdf [Date accessed: 11.30.19].

EN 15662:2018. Foods of plant origin - Multimethod for the determination of pesticide residues using GC- and LC-based analysis following acetonitrile extraction/partitioning and clean-up by dispersive SPE - Modular QuEChERS-method. 2018. https://standards.iteh.ai/catalog/standards/cen/167a30bc-edf9-4cf8-b96b-cabd932f2f02/en-15662-2018

Grimalt S, Harbeck S, Shegunova P, Seghers J, Sejerøe-Olsen B, Emteborg H, Dabrio M. Development of a new cucumber reference material for pesticide residue analysis: Feasibility study for material processing, homogeneity and stability assessment. Anal Bioanal Chem. 2015 Jan; 407(11):3083–3091. https://doi.org/10.1007/s00216-015-8476-x

Pelit FO, Pelit L, Ertaş H, Ertaş FN. Development of a gas chromatographic method for the determination of Chlorpyrifos and its metabolite Chlorpyrifos-oxon in wine matrix. J Chromatogr B: Anal Technol Biomed Life Sci. 2012 Sept; 904:35–41. https://doi.org/10.1016/j.jchromb.2012.07.006

PNS/BAFS. Pesticide residues in mango: Maximum Residue Limits (MRLs). PNS/BAFS 160. Bureau of Agriculture and Fisheries Standards; Department of Agriculture (Philippines) 2015. Retrieved from http://www.bafs.da.gov.ph/bafs_admin/admin_page/pns_file/2021-02-24-PNSBAFS%20160-2015%20PesticideResidue%20MRLs_mango.pdf [Date accessed: 11.30.19].

Samsidar A, Siddiquee S, Shaarani SM. A review of extraction, analytical and advanced methods for determination of pesticides in environment and foodstuffs. Trends Food Sci Technol. 2018 Jan; 71:188–201. https://doi.org/10.1016/j.tifs.2017.11.011

Vogl J, Pritzkow W. Isotope Dilution Mass Spectrometry – A Primary Method of Measurement and Its Role for RM Certification. MAPAN – J Metrol Soc India. 2010 Nov; 25(3):135-164. https://doi.org/10.1007/s12647-010-0017-7

Yarita T, Otake T, Aoyagi Y, Kuroda Y, Numata M, Iwata H, et al. Development of soybean certified reference material for pesticide residue analysis. Talanta. 2014 Feb; 119:255–261. https://doi.org/10.1016/j.talanta.2013.11.011