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Novel Method Development for Extraction and Analysis of Pesticide Residues in Human Serum Samples

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Abstract: In recent years, exposure to pesticides has gained widespread attention due to their adverse health effects. Long-term exposure to pesticides has shown hazardous effects on vital functions of the human nervous and reproductive systems. Therefore, it is crucial to determine the extent of pesticide exposure in humans. Primarily, it is quite challenging to determine trace levels of pesticide residues in biological matrices. Hence, a quick, multi-residue extraction procedure was experimented for pesticide residue analysis in human serum. Herein, the original QuEChERS extraction method was modified for achieving the best possible recoveries. A total of 15 representative pesticides from each class were selected and fortified into the human serum samples. The extraction was performed by employing acidified solution containing acetonitrile and ethyl acetate followed by vortex and centrifugation. The obtained aqueous layer was collected and vapourised to dryness and d-SPE clean-up was conducted utilising PSA. The extracted sample was injected into the GC-MS/MS system under MRM mode. The method development parameters such as linearity, % RSD, accuracy, LOD, LOQ and % ME were assessed. The results obtained for the serum matrix were found to be within the criteria mentioned in European Union SANTE/12682/2019 guidelines for method validation. The developed solitary method is quick, simple and highly efficient for routine pesticide residue analysis. Hence, a wide spectrum of pesticides can be analysed utilising the proposed method for human serum.

Key words: Pesticide residues, modified QuEChERS, biological matrix, GC-MS/MS, MRM mode.

Introduction

Since the widespread consumption of pesticides in recent years, the global usage of pesticides has reached up to 3 billion kg (Iqbal et al., 2020) in particular, carbamate and pyrethroid pesticides are the most common insecticides used worldwide. They may cause chronic poisoning in farmers and acute poisoning in homicidal or suicidal cases. The determination of trace levels of these pesticides in human blood and urine is very challenging. This study focuses on a simultaneous quantitation method that was developed and validated for multi-class nine pesticides belonging to organophosphate, carbamate and pyrethroid classes in human blood and urine. Target pesticides were extracted from blood and urine using a modified QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe. The development of pesticides aimed to safeguard crops against harm caused by pests, insects, rodents, etc., however, they have been shown to persist in the environment (Chu et al., 2020; Fang et al., 2017). Organochlorine (OC), organophosphorus (OP), synthetic pyrethroids (SPs) and carbamates are the major classes of pesticides used in the modern agricultural routine. Amongst the populations of developing countries, exposure to pesticide residues remains a huge concern

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(Arrebola et al., 2012). Many countries have banned the usage of OC pesticides as they are environmentally sustainable and can get into human tissues because of their highly persistent nature. OC exposure has shown to interfere with human sex hormones activity and may affect reproductive functioning in both male and female (Freire et al., 2014). Pesticides enter the human body through the oral route by ingestion of food and water, inhalation in the form of aerosol or dust particles and skin exposure by dermal contact or absorption (Li et al., 2020). The government as well as consumers are concerned about the levels of pesticide contamination. Hence, pesticide residue monitoring, as well as risk assessment, are very important aspects for determining the quality of agricultural produces in various countries to safeguard human health (Chu et al., 2020).

Establishing contaminants of pesticides in the biological matrices is more strenuous as a result of the complexity and diverse properties of the matrix (Zhang et al., 2018). There is an urgent need for more reliable and cost-effective methodologies. In this study, we developed and validated a screening method for analysis of over 450 pesticides in precipitation using gas chromatography with tandem mass spectrometry (GC-MS/MS. A human matrix such as serum can be suitably utilised for the study of its chronic exposure to pesticide residues. In response, different methods were established for pesticide residue detection at trace levels from environmental as well as human samples (Fang et al., 2017). The methods routinely used for the evaluation of pesticide residues primarily depend on the employment of gas or liquid chromatography together with tandem mass spectrometry. Utilising tandem mass analysers in an MRM mode will enhance the capability of pesticide residue detection with high sensitivity and specificity for each target analytes (Sivaperumal et al., 2015). Most of these methods are typically developed, applicable to only one pesticide class of a limited number (Zhang et al., 2018). There is an urgent need for more reliable and cost-effective methodologies. In this study, we developed and validated a screening method for analysis of over 450 pesticides in precipitation using gas chromatography with tandem mass spectrometry (GC-MS/MS). The target analytes selected in this research included 15 multiclass pesticides, chosen on the basis of their high consumption and toxicity. The present research focussed on the development of a novel extraction method for the analysis of multiple pesticide residues in serum matrix by using GC-MS/MS spectroscopy.

Materials and Methods

Chemicals and Reagents

The analytical standards of individual pesticides were acquired from AccuStandard, USA (Table 1).

Table 1: Information of the selected multiclass pesticides for GC-MS/MS analysis

Sr. no.	*Name of compound*	*Molecular formula*	*CAS no.*	*Retention time (min)*
1	Acephate	$C_4H_{10}NO_3PS$	30560-19-1	5.991
2	Acetamiprid	$C_{10}H_{11}ClN_4$	135410-20-7	16.587
3	Buprofezin	$C_{16}H_{23}N_3OS$	69327-76-0	14.135
4	Butachlor	$C_{17}H_{26}ClNO_2$	23184-66-9	13.298
5	Chlorpyrifos	$C_9H_{11}Cl_3NO_3PS$	2921-88-2	11.41
6	Cypermethrin	$C_{22}H_{19}Cl_2NO_3$	52315-07-8	19.845
7	Dichlorovos	$C_4H_7Cl_2O_4P$	62-73-7	4.922
8	Ethion	$C_9H_{22}O_4P_2S_4$	563-12-2	14.939
9	Fipronil	$C_{12}H_{17}Cl_2F_6N_4OS$	120068-37-3	12.363
10	L-Cyhalothrin	$C_{23}H_{19}ClF_3NO_3$	68085-85-8	17.873
11	Monocrotophos	$C_7H_{14}NO_5P$	6923-22-4	7.711
12	Phorate	$C_7H_{17}O_2PS_3$	298-02-2	7.936
13	Profenofos	$C_{11}H_{15}BrClO_3PS$	41198-08-7	13.864
14	Quinalphos	$C_{12}H_{15}N_2O_3PS$	13593-03-8	12.7
15	Triazophos	$C_{12}H_{16}N_3O_3PS$	24017-47-8	15.275

Acetonitrile (MeCN) LC Optima grade was obtained from Fisher Scientific, USA. Ethyl acetate (EtAOc) and acetic acid (ACS grade) were bought from Fluka, Germany. PSA was obtained from Agilent Technologies, USA, while magnesium sulphate (anhydrous, ≥99.5%, ReagentPlus) from Sigma-Aldrich and acetone (AR grade) was purchased from B & J, USA.

Instrumentation

Remi centrifuge, analytical balance (AUX 220, Shimadzu), micropipettes (Eppendorf) and concentration evaporator (Zymark Tarbovab LV) were utilised in this study.

Standard Preparation

The stock solution (1000 mg/L) of 15 individual pesticides was prepared either in acetone or acetonitrile depending upon the nature of solubility. From the stock standard solution, the intermediate standard solutions (100 mg/L) were prepared and refrigerated at 2-4 ºC.

Sample Treatment

The institutional ethical clearance was obtained for the study. The sample treatment is based upon the modified QuEChERS extraction method. For the method validation purposes, blood samples were collected from healthy volunteers, serum was separated and stored at –20 ºC. In the optimised extraction method, 1 mL volume of serum was taken in 15 mL centrifuge tube, covered by 3 mL acidified solution of MeCN and EtAOc (3:1, $^{V}/_{V}$) along with 350 mg of $MgSO_4$ (2% acetic acid was added into the solution of MeCN and EtAOc for acidification). The tube was gently shaken for a minute and kept for centrifugation at 5500 rpm (10 min). The resultant supernatant was collected and completely evaporated. The reconstitution of the sample tube was carried out with 1 mL of EtAOc and PSA (50 mg) was added for the clean-up. The tube was gently shaken for a minute and kept for centrifugation at 5500 rpm (15 min). The clear supernatant obtained was filtered and 1 µL of the extracted sample was introduced into the GC-MS/MS system through MRM mode.

Chromatography

The analytical tool utilised for GC-MS/MS analysis was executed using Shimadzu (TQ8040) operated through MRM mode. The developed GC-MS/MS optimisation parameters for the present work are described in Table 2. About 1 µL extracted sample was introduced into the system for analysis. Acquisition and processing of data were accomplished utilising GC-MS solution software of version 4.45.

Table 2: Optimised GC-MS/MS parameters under MRM mode

GC-MS/MS Parameters	
Injection temperature	270 °C
Ion source temperature	230 °C
Interface temperature	250 °C
Solvent cut time	3.00 min
Injection mode	Splitless
Sampling time	1.00 min
Flow control mode	Linear velocity
Pressure	14.2 psi
Total flow	49.5 mL/min
Column	SH-Rtx®-5Sil
Column specification	30m × 0.25mm × 0.25 µm
Carrier gas	Helium
Column flow	1.50mL/min
Collision gas	Argon
Column Oven temperature	70 °C
Oven temperature program	70 °C (1min), 190 °C at 25 °C /min (3 min), 220 °C at 8 °C/min and 300 °C at 10 °C /min (1 min)

Method Validation

The validation of the developed method was performed as per European Union guidelines mentioned in the document (SANTE/12682/2019). The method validation parameters such as LOD, linearity, LOQ, matrix effect (ME), % RSD and accuracy were estimated. The linearity ranging from 10 to 500 ng/mL was estimated by plotting a calibration curve. The accuracy was determined by spiking the serum sample to establish recovery at four different spiking levels (10, 100, 250 and 500 ng/mL). The precision expressed as % RSD was carried out by injecting seven replicates for 10 and 100 ng/mL spiked concentrations. The ME was also addressed as it can impact the quality of quantitative results by signal enhancement/suppression. The ME was evaluated by comparing the values obtained from neat standard calibration and matrix-matched standard calibration against the spiked standard concentration at the same level. The % ME was calculated according to the formula:

$$\% \text{ ME} = ([\text{Obtained standard concentration}/\text{Spiked concentration}]-1) \times 100$$

The values obtained were divided into three categories: low ME (± 20%), moderate ME ± (20-50%) and high ME (± 50% or more). The serum sample extraction method along with validation parameters are illustrated in Figure 1.

Results and Discussion

Modified QuEChERS

Originally, the QuEChERS method development was associated with the determination of pesticide residue in commodities namely vegetables and fruits, which majorly contain more than 90% water. This dispersive-solid phase extraction (d-SPE) method employed a combination of $MgSO_4$ and PSA both at extraction and clean-up step for eliminating water, matrix, pigments and other components. Limited research has been carried out experimenting with the applicability for the utilisation of the QuEChERS method in biological matrices. Hence, a modified version of QuEChERS was experimented with to derive the best possible results by employing a minimum amount of solvents and reagents.

MRM Mode Optimisation

Optimisation of the GC-MS/MS instrumental parameters was performed for the best possible separation of all the analytes under study. Initially, identification and quantitation were performed under full scan mode of all the analytes. Figure 2 represents the total ion chromatogram (TIC) of the distinct peaks obtained for the 15 pesticides in a scan mode. Discrete peaks for each analyte were achieved though accompanied by background interferences and poor sensitivity. In order to address the matrix interferences, the MRM

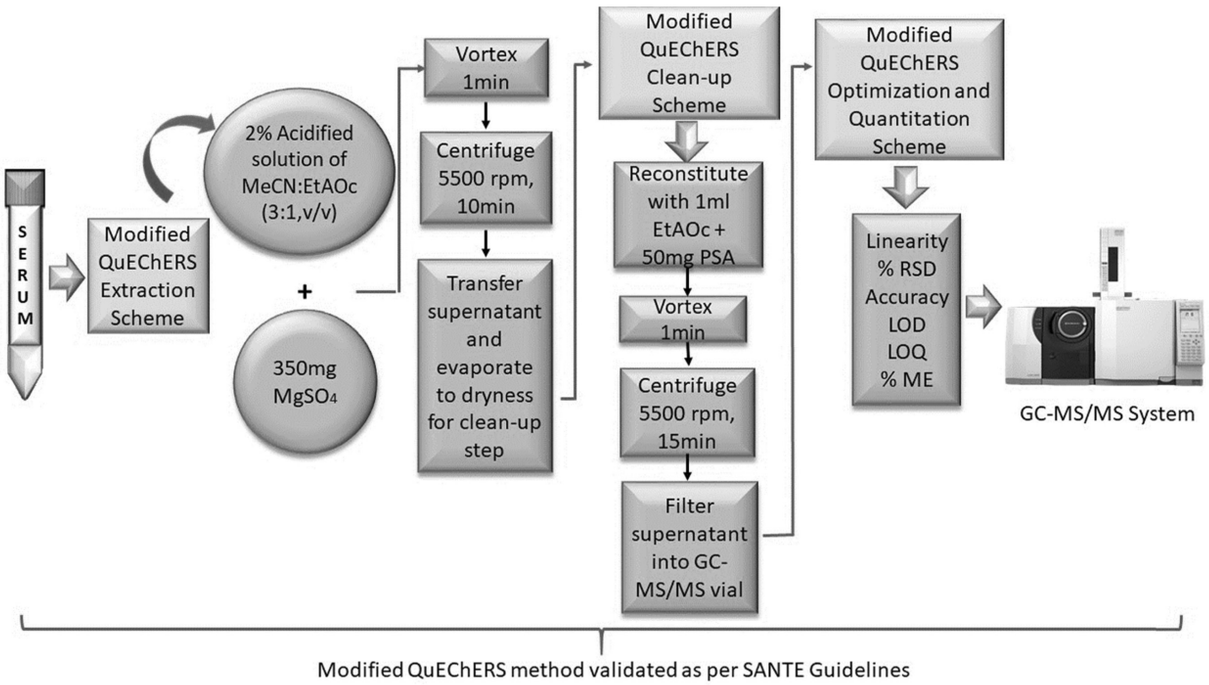

Figure 1: Schematic diagram of sample extraction and validation.

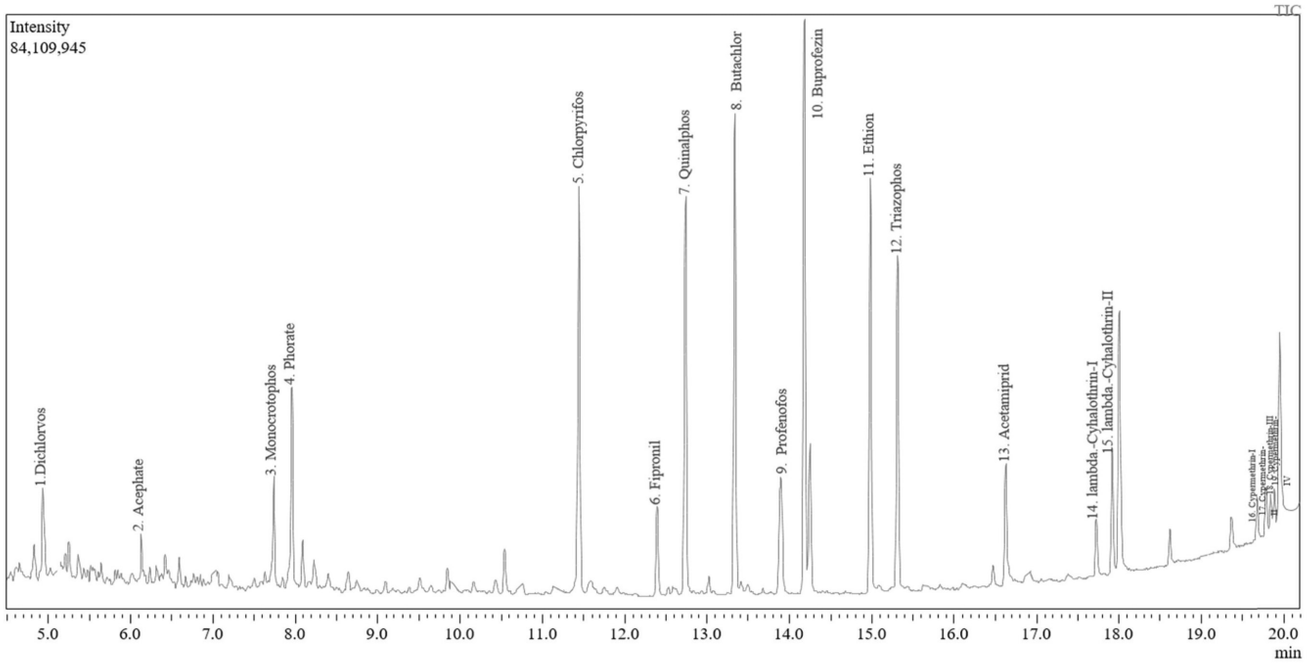

Figure 2: TIC of 15 target pesticides under full scan mode.

optimisation was performed as it helps in excluding background interference while analysing complicated matrices. MRM mode employs triple quadrupole scrutinisation wherein mass spectra selective to the target analyte is chosen. Parent ion, daughter ions and optimal collision energies were established by varying collision energy between 3 to 45V at 3V intervals for each target analyte. The first quadrupole selectively fragments the parent ion into corresponding daughter ions in the collision cell. The selected daughter ions pass through the second quadrupole and this transition is referred to as ion transition. Based upon the m/z ratio, the daughter ion providing the highest intensity was identified. This daughter ion undergoes further fragmentation in the third quadrupole wherein the highest intensity ion is designated as quantifier ion and the other two ions are designated as qualifier ion providing qualitative as well quantitative analysis, characteristic to the target analyte.

Validation Results

Table 3 summarises all the method validation results. The linearity was plotted in the different range from 10 to 500 ng/mL demonstrating good linearity for all the target analyte with correlation coefficients (R^2) more than 0.99. The recovery values obtained at 10, 100, 250 and 500 ng/mL spiking levels ranged between 66-129, 68-100, 69-95 and 79-106%, respectively. Figure 3 shows the MRM chromatogram of the blank serum sample, the standard and a spiked sample of an analyte ethion at 10 ng/mL level. The LODs and LOQs values ranged from 0.9-5.4 ng/mL and 5.0-18.1 ng/mL, respectively. The repeatability expressed as % RSD for the studied pesticides was found to be ≤20. The % RSD values ranged between 3.8-12.7 and 1.2-11.4 at 10 ng/mL and 100 ng/mL spiking levels showing the prospective potentiality of the proposed method for quantitative analysis. The % ME observed indicated enhancement or suppression of values due to the matrix effect. For matrix matched calibration, the matrix effect was found to be low except for analytes namely acephate, dichlorvos and phorate showing moderate signal suppression. The % ME was found to be ranging between –45 to 20, –15 to 14, –51 to 13 and –19 to 11 at 10, 100, 250 and 500 ng/mL levels, respectively. For neat calibration, the matrix effect was found to be high presenting matrix enhancement. The % ME was found to be ranging between -10 to 631, -40 to 295, -22 to 206 and 31 to 535 at 10, 100, 250 and 500 ng/Ml levels, respectively. Figure 4 shows the comparison between matrix-matched and neat calibration in the form of a clustered column chart. The outcome of % ME showed that matrix matched calibration had a low matrix effect compared to neat calibration.

Studies related to the application of the QuEChERS extraction method in biological samples are very

Table 3: Method validation results of fifteen multiclass pesticides using GC-MS/MS under MRM mode

Sr. no.	*Name of Compound*	*Linearity (r2)*	*LOQs (ng/mL)*	*Recovery (%) (ng/mL)*				*% RSD (n=7) (ng/mL)*	
				10	*100*	*250*	*500*	*10*	*100*
1	Acephate	0.9998	9.3	103	102	78	79	4.5	3.7
2	Acetamiprid	0.9951	7.7	117	104	95	90	7.2	3.3
3	Buprofezin	0.9950	14.7	111	97	89	103	12.7	11.4
4	Butachlor	0.9998	9.3	120	91	81	99	7.7	1.2
5	Chlorpyrifos	0.9999	5.8	117	96	87	97	4.9	0.9
6	Cypermethrin	0.9982	9.9	110	92	85	100	6.9	7.3
7	Dichlorovos	0.9996	5.0	78	73	69	90	3.8	1.7
8	Ethion	0.9997	7.8	111	95	82	99	6.9	1.7
9	Fipronil	0.9995	8.1	123	101	87	101	6.5	1.9
10	L-Cyhalothrin	0.9999	7.6	115	100	84	98	8.2	2.9
11	Monocrotophos	0.9997	5.5	115	105	91	92	4.7	2.8
12	Phorate	0.9996	5.9	71	83	75	90	7.2	1.6
13	Profenofos	0.9998	18.1	105	101	89	99	8.6	3.5
14	Quinalphos	0.9999	7.1	126	97	87	99	5.6	2.3
15	Triazophos	0.9996	8.0	115	108	91	95	6.9	3.7

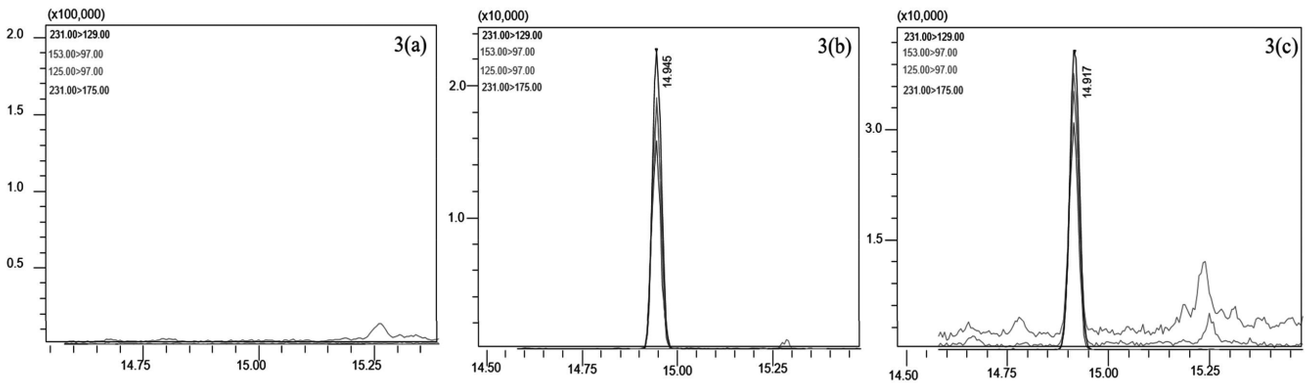

Figure 3: MRM chromatogram of Ethion in (a) blank sample, (b) neat standard and (c) spiked sample at 10 ng/mL.

limited. Chang et al. (2016)'s study on the effects of OP pesticides on human health have attracted attention. However, methods focusing on the determination of various OP pesticides in human blood serum are still scarce, and the exposure level of various OP pesticides in humans remains largely unknown. We have developed a method for the quantification of 20 OP pesticides in human blood serum simultaneously. The determination was performed using solid-phase extraction with a small blood serum sample volume (200 μL) established a pesticide residue SPE method from human serum for the evaluation of 20 organophosphorus pesticides with

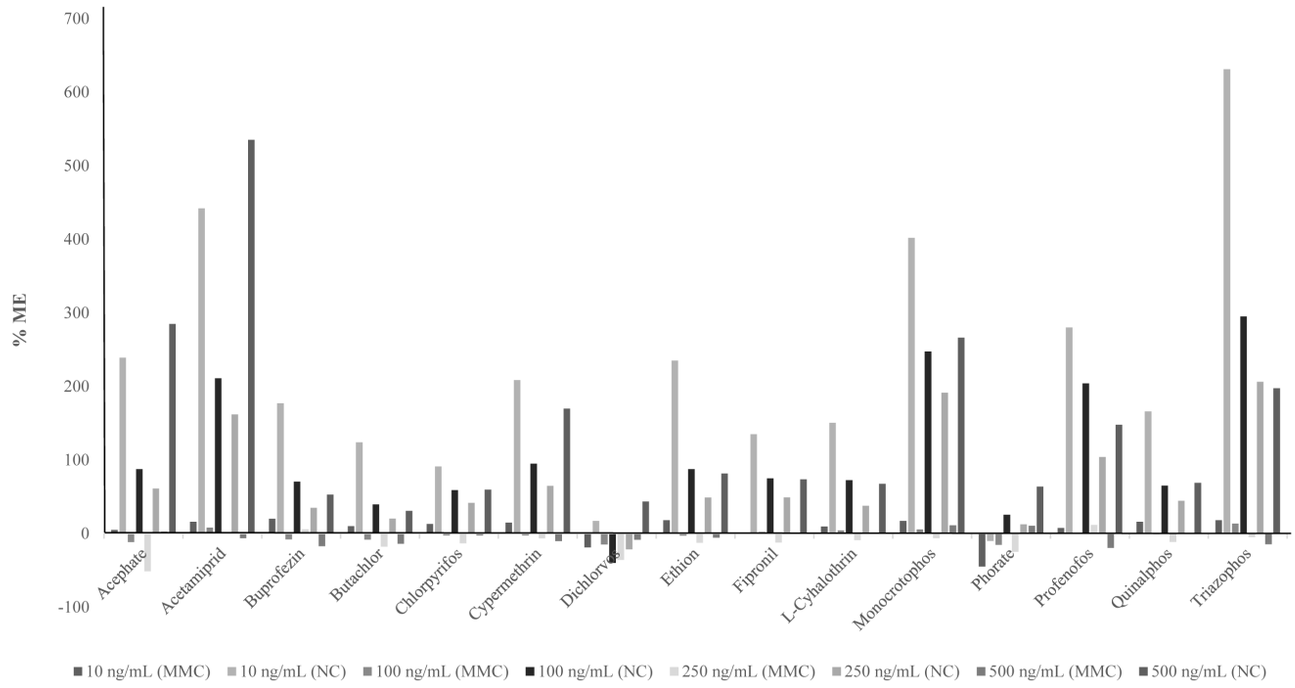

Figure 4: Clustered column chart representing a comparison between matrix matched calibration (MMC) and neat standard calibration (NC).

the help of GC-MS/MS. The results indicated the LOD values (0.01-2.70 ng/mL) and LOQ values (0.05-9.00 ng/mL) for the targeted 20 pesticides. Kusano et al. (2019) proposed a modified QuEChERS method along with clean-up (d-SPE) for 14 pesticides in whole blood using GC–MS/MS. The outcome of the study showed that the recovery ranged between 25-118% for all 14 pesticides. LOD values (0.09-1.4 ng/mL) whereas LOQ values (1.0-5.0 ng/mL) for the targeted pesticides were obtained. Srivastava et al. (2017) optimised a mini QuEChERS method for the evaluation of 31 different classes of pesticides in human plasma via GC–MS/MS. The mean recovery for all the targeted analytes ranged from 74 to 109 %. The LOD and LOQ ranged from 0.04 to 4.10 ng/mL and from 0.12 to 13.53 ng/mL respectively. For the whole blood sample, Valente et al. (2015) developed a SPE procedure for 10 organophosphorus pesticides using GC-MS. The results displayed that LOD and LOQ ranged from 2.46-5.19 ng/mL and 7.46-13.63 ng/mL respectively. Yu et al. (2017) developed the QuEChERS method in a blood sample for the detection of nine pesticides residues using GC-MS spectroscopy. The research outcome showed that LODs and LOQs for the seven pesticides ranging between 11-163 and 36-538 ng/mL, respectively. All the previous studies conducted are comparable to our present work which utilises modern technological advancement.

Conclusion

The method validation parameters showed results satisfying the guidelines set by European Union (SANTE/12682/2019) for pesticide residue analysis. The proposed methodical approach using modified QuEChERS is simple, less time-consuming and efficiently executable, making the method appropriate for routine analysis. The traditional system of employing GC or GC in combination with MS for pesticide residue analysis fails to bring out well defined identification resulting from the matrix effect. The utilisation of MRM mode ordinarily removes the background matrix interference from the sample providing unequivocal identification and evaluation. Considering the achieved method validation outcomes, the developed method can be successfully utilised for multiclass pesticide residue determination in serum matrix.

Conflict of Interests

All the authors state that while submitting the article they have no competing interest.

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References

Arrebola, J.P., Cuellar, M., Claure, E., Quevedo, M., Antelo, S.R., Mutch, E., Ramirez, E., Fernandez, M.F., Olea, N. and L.A. Mercado (2012). Concentrations of organochlorine pesticides and polychlorinated biphenyls in human serum and adipose tissue from Bolivia. *Environmental Research*, **112:** 40-47. https://doi.org/10.1016/j.envres.2011.10.006

Chang, C., Luo, J., Chen, M., Wu, K., Dong, T., He, X., Zhou, K., Wang, L., Chen, D., Zhou, Z., Wang, X. and Y. Xia (2016). Determination of twenty organophosphorus pesticides in blood serum by gas chromatography-tandem mass spectrometry. *Analytical Methods*, **8(22):** 4487-4496. https://doi.org/10.1039/c6ay00825a

Chu, Y., Tong, Z., Dong, X., Sun, M., Gao, T., Duan, J. and M. Wang (2020). Simultaneous determination of 98 pesticide residues in strawberries using UPLC-MS/MS and GC-MS/MS. *Microchemical Journal*, **156(May):** 104975. https://doi.org/10.1016/j.microc.2020.104975

Fang, J., Wu, Q., Zhao, Y., Zhao, H., Xu, S. and Z. Cai (2017). Comparison of different mass spectrometric approaches coupled to gas chromatography for the analysis of organochlorine pesticides in serum samples. *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences*, **1040:** 180-185. https://doi.org/10.1016/j.jchromb.2016.12.001

Freire, C., Koifman, R.J., Sarcinelli, P.N., Rosa, A.C.S., Clapauch, R. and S. Koifman (2014). Association between serum levels of organochlorine pesticides and sex hormones in adults living in a heavily contaminated area in Brazil. *International Journal of Hygiene and Environmental Health*, **217(2-3):** 370-378. https://doi.org/10.1016/j.ijheh.2013.07.012

Iqbal, S., Iqbal, M.M., Javed, M., Bahadur, A., Yasien, S., Najam-ud-din, Hurr, A., Ahmad, N., Raheel, M. and G. Liu (2020). Modified QuEChERS extraction method followed by simultaneous quantitation of nine multi-class pesticides in human blood and urine by using GC-MS. *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences*, **1152:** 122227. https://doi.org/10.1016/j.jchromb.2020.122227

Kusano, M., Sakamoto, Y., Natori, Y., Miyagawa, H., Tsuchihashi, H., Ishii, A. and K. Zaitsu (2019). Development of "Quick-DB forensic": A total workflow from QuEChERS-dSPE method to GC–MS/MS quantification of forensically relevant drugs and pesticides in whole blood. *Forensic Science International*, **300:** 125-135. https://doi.org/10.1016/j.forsciint.2019.03.048

Li, A.J., Banjabi, A.A., Takazawa, M., Kumosani, T.A., Yousef, J.M. and K. Kannan (2020). Serum concentrations of pesticides including organophosphates, pyrethroids and neonicotinoids in a population with osteoarthritis in Saudi Arabia. *Science of the Total Environment*, **737:** 139706. https://doi.org/10.1016/j.scitotenv.2020.139706

Sivaperumal, P., Anand, P. and L. Riddhi (2015). Rapid determination of pesticide residues in fruits and vegetables, using ultra-high-performance liquid chromatography/time-of-flight mass spectrometry. *Food Chemistry*, **168:** 356-365. https://doi.org/10.1016/j.foodchem.2014.07.072

Srivastava, A., Rai, S., Kumar Sonker, A., Karsauliya, K., Pandey, C.P. and S.P. Singh (2017). Simultaneous determination of multiclass pesticide residues in human plasma using a mini QuEChERS method. *Analytical and Bioanalytical Chemistry*, **409(15):** 3757-3765. https://doi.org/10.1007/s00216-017-0317-7

Valente, N.I.P., Tarelho, S., Castro, A.L., Silvestre, A. and H.M. Teixeira (2015). Analysis of organophosphorus pesticides in whole blood by GC-MS-μECD with forensic purposes. *Journal of Forensic and Legal Medicine*, **33**: 28-34. https://doi.org/10.1016/j.jflm.2015.03.006

Yu, T., Wang, T., Huang, Z., Huang, N., Zhang, H., Luo, Z., Li, H., Ding, S. and W. Feng (2017). Determination of multiple pesticides in human blood using modified QuEChERS method with Fe_3O_4 magnetic nanoparticles and GC–MS. *Chromatographia*, **80(1):** 165-170. https://doi.org/10.1007/s10337-016-3206-x

Zhang, H., Watts, S., Philix, M.C., Snyder, S.A. and C.N. Ong (2018). Occurrence and distribution of pesticides in precipitation as revealed by targeted screening through GC-MS/MS. *Chemosphere*, **211:** 210-217. https://doi.org/10.1016/j.chemosphere.2018.07.151