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Simultaneous determination of multiclass pesticide residues in human plasma using a mini QuEChERS method

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Abstract Blood is one of the most assessable matrices for the determination of pesticide residue exposure in humans. Effective sample preparation/cleanup of biological samples is very important in the development of a sensitive, reproducible, and robust method. In the present study, a simple, cost-effective, and rapid gas chromatography–tandem mass spectrometry method has been developed and validated for simultaneous analysis of 31 multiclass (organophosphates, organochlorines, and synthetic pyrethroids) pesticide residues in human plasma by means of a mini QuEChERS (quick, easy, cheap, effective, rugged, and safe) method. We have adopted a modified version of the QuEChERS method, which is primarily used for pesticide residue analysis in food commodities. The QuEChERS method was optimized by use of different extraction solvents and different amounts and combinations of salts and sorbents (primary–secondary amines and C_{18}) for the dispersive solid-phase extraction step. The results show that a combination of ethyl acetate with 2% acetic acid, magnesium sulfate (0.4 g), and solid-phase extraction for sample cleanup with primary–secondary amines (50 mg) per 1-mL volume of plasma is the most suitable for generating acceptable results with high recoveries for all multiclass pesticides from human plasma. The mean recovery ranged from 74% to 109% for all the analytes. The limit of quantification and limit of detection of the method ranged from 0.12 to 13.53 ng mL^{-1} and from 0.04 to 4.10 ng mL^{-1} respectively. The intraday precision and the interday precision of the method were 6% or less and 11% or less respectively. This method would be useful for the analysis of a wide range of pesticides of interest in a small volume of clinical and/or forensic samples to support biomonitoring and toxicological applications.

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The CSIR–Indian Institute of Toxicology Research communication number for the manuscript is 3445.

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Introduction

Pesticides are some of the most important agrochemicals used in agriculture for minimizing farm and postharvest losses of crops and ensuring effective crop production. Injudicious and indiscriminate use of pesticides and lack of use of safety appliances during manufacturing, storage, transportation, and agricultural application increases the threat of exposure of the human population to pesticides. Moreover, imprudent use of pesticides during agricultural applications may result in the occurrence of their residues in agriculture commodities and may lead to dietary exposure of human populations [1–4]. Therefore, it is important to determine the pesticide

concentrations in samples of human origin. A relevant way to investigate pesticide exposure is by biological monitoring studies. Biological monitoring studies require biomarker exposure assessment (normally the pesticide or its metabolite) in human biological matrices such as blood, plasma, serum, urine, or tissues. Although most human tissues are difficult to access, plasma is one of the biological materials that can be obtained from humans for these types of studies [5–7].

An efficient, simple, sensitive, reproducible, accurate, and cost-effective analytical method is required for the quantitative determination of pesticides in samples of biological origin for the generation of reliable data for use in biological

monitoring and toxicokinetic studies [8–10]. It is also important to emphasize that pesticides have diverse physical and chemical properties. There is a need for pesticide residue analysis to determine multiple groups of compounds simultaneously rather than one analyte or a single class at a time. Multiclass, multiresidue methods are a modern analytical approach addressing this need [11–13]. Ideally, a multiclass, multiresidue method should be simple, cost-effective, and easy to perform, require the minimum amount of solvents and sample, and be able to cover a wide range of analytes. Different methods have been reported to determine pesticide residues in plasma using C_{18}-based solid-phase

Table 1 List of pesticides, retention time (*RT*), time window, and multiple reaction monitoring (*MRM*) transitions for identification and quantification and their collision energy (*CE*)

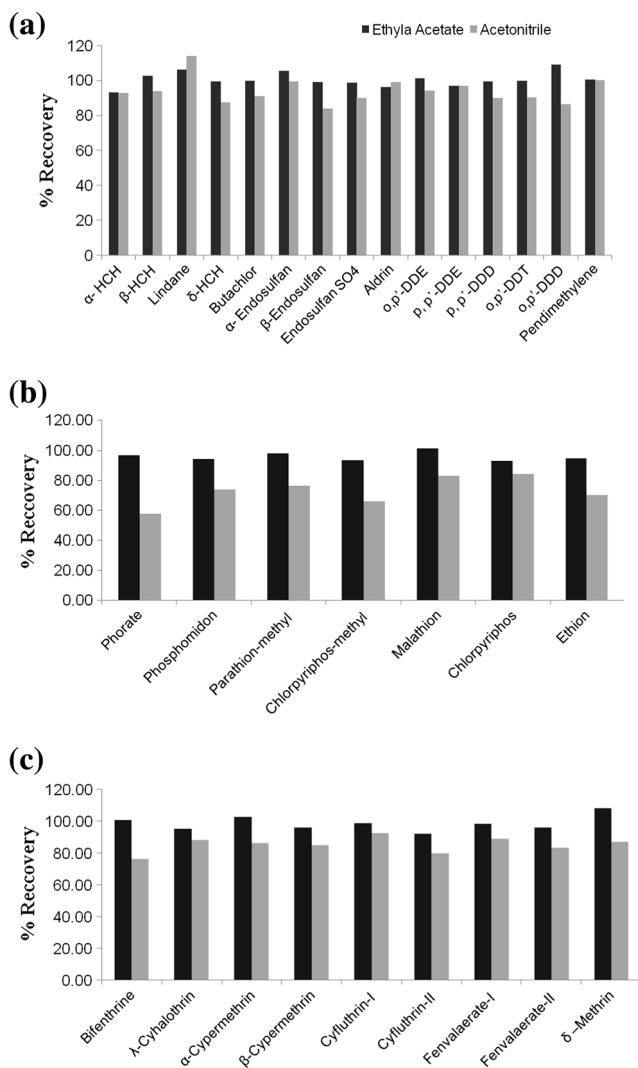| Pesticide	RT (min)	Time window (min)	MRM transitions at 70 eV and CE[a]	
			Identification	Quantification
α-Hexachlorocyclohexane	19.34	3	181 > 145 (20)	219 > 181 (10)
Phorate	19.23	4	260 > 75 (10)	260 > 121(10)
β-Hexachlorocyclohexane	20.51	3	181 > 145 (20)	219 > 181 (10)
Lindane	20.91	3	219 > 181 (10)	181 > 145 (20)
δ-Hexachlorocyclohexane	22.31	3	219 > 181 (10)	181 > 145 (20)
Phosphomidon	24.34	3	264 > 127 (12)	264 > 147 (12)
Parathion-methyl	26.48	4	263 > 109 (109)	263 > 125 (125)
Chlorpyriphos-methyl	26.56	4	286 > 125 (15)	286 > 147 (20)
Malathion	28.50	3	173 > 125 (10), 173 > 158 (12)	173 > 127 (12)
Aldrin	30.34	4	263 > 191 (15)	263 > 193 (15)
Chlorpyriphos	30.71	4	314 > 197 (15), 314 > 258 (15)	314 > 197 (15), 314 > 258 (15)
Pendimethylene	30.90	4	281 > 252 (12)	281 > 252(12)
o,p′-DDD	31.64	3	235 > 165 (20)	235 > 165 (20)
α-Endosulfan	32.27	4	241 > 147 (15), 241 > 206 (10)	241 > 195(15)
Butachlor	32.60	4	176 > 147 (15)	176 > 160(10)
o,p′-DDE	33.71	3	318 > 176 (15)	318 > 246(20)
p,p′-DDE	34.05	3	235 > 165 (20)	237 > 199 (20)
β-Endosulfan	34.66	4	241 > 170 (20), 241 > 206 (10)	241 > 174(20)
p,p′-DDD	36.31	3	235 > 165 (20)	237 > 199 (20)
Ethion	36.83	4	231 > 97 (15)	231 > 153(15)
Endosulfan sulfate	40.22	4	272 > 237 (15)	272 > 237(15)
o,p′-DDT	40.74	4	235 > 165 (20)	237 > 199 (20)
Bifenthrin	44.49	4	181 > 165 (20)	181 > 166 (10)
λ-Cyhalothrin	48.14	3	449 > 181 (12), 449 > 197 (14)	181 > 152 (20), 197 > 141 (10)
Cyfluthrin I	49.15	3	226 > 206 (15)	226 > 163 (10)
Cyfluthrin II	49.31	3	226 > 206 (15)	226 > 163 (10)
α-Cypermethrin	49.57	4	181 > 163 (15)	181 > 152 (10)
β-Cypermethrin	50.24	3	181 > 163 (15)	181 > 152 (10)
Fenvalerate I	50.57	3	419 > 125 (15)	419 > 167 (15)
Fenvalerate II	51.29	3	419 > 125 (15)	419 > 167 (15)
δ-Methrin	51.94	3	253 > 93 (20), 253 > 181 (15)	253 > 147 (15), 253 > 174 (10)

[a] The CE is given in *parentheses*

Fig. 1 Comparison of extraction efficiency of acetonitrile and ethyl acetate for extraction of pesticide residues form human plasma at a spiking level of 10 ng mL^{-1} ($n = 3$). Percent recoveries of **a** organochlorines and herbicides, **b** organophosphates, and **c** synthetic pyrethroids

the accuracy and reproducibility of results. Hence, in the present study special attention is given to a sample preparation technique for simultaneous analysis of multiple pesticide residues in small volumes of human plasma. All the key experimental parameters, such as pH, type of extraction solvent, and amount of salts and adsorbing reagents, were optimized one at a time.

Materials and methods

Reagents and solutions

All solvents and chemicals used in this study were of analytical grade. Acetonitrile, ethyl acetate, *n*-hexane, acetone, and acetic acid were procured from Sisco Research Laboratories (Mumbai, India). Sodium chloride (NaCl) and magnesium sulfate (MgSO$_4$) were procured from Sigma-Aldrich (Bangalore India). Primary–secondary amine (PSA; 40 μm) and C$_{18}$ solid phases were procured from Agilent Technologies (Santa Clara, CA, USA). Pesticide standards, organochlorines (α-hexachlorocyclohexane, β-hexachlorocyclohexane, lindane, δ-hexachlorocyclohexane, *p,p*′-DDD, *p,p*′-DDE, *o,p*′-DDD, o,*p*′-DDE, *o,p*′-DDT, α-endosulfan, β-endosulfan, endosulfan sulfate, aldrin), organophosphates (phorate, phosphomidon, parathion-methyl, malathion, chlorpyrifos methyl, chlorpyriphos, ethion), synthetic pyrethroids (bifenthrin, γ-cyhalothrin, cyfluthrin I, cyfluthrin II, α-cypermethrin, β-cypermethrin, fenvalerate I, fenvalerate II, δ-methrin), and herbicides (butachlor, pendimethylene) were procured from Supelco (Bellefonte, PA, USA). Control human plasma samples for method development were obtained from Era's Lucknow Medical College and Hospital, Lucknow, India.

Extraction and cleanup of pesticide residues

One milliliter of plasma sample was added to a 15-mL conical bottom polypropylene centrifuge tube and mixed with 3 mL of 2% acidified ethyl acetate and 0.4 g of MgSO$_4$ [11]. Ethyl acetate was acidified by addition of concentrated acetic acid. The tube was shaken on a Rotospin rotary mixer from Tarsons Products (Kolkata, India) for 5 min at 50 rpm and then centrifuged at 6000 rpm for 10 min. An 3-mL aliquot of the organic layer was separated and evaporated to dryness with use of a Turbovap nitrogen flow evaporator from Caliper Life Sciences (Mountain View, CA, USA). The residue was reconstituted in 1 mL of ethyl acetate and mixed with 50 mg of PSA for sample cleanup. This mixture was shaken on a Rotospin rotary mixer for 5 min at 50 rpm, followed by centrifugation at 8000 rpm for 10 min. The

extraction (SPE), Florisil-based SPE, mixed-phase SPE, and liquid–liquid extraction as sample preparation techniques. These traditional methods are time-consuming, laborious, complex, and expensive, and are unable to extract analytes with a wide polarity range [14–19].

Therefore, in this study a gas chromatography and tandem mass spectrometry (GC–MS/MS)-based multiclass, multiresidue analytical method has been developed and validated with use of a mini QuEChERS (quick, easy, cheap, effective, rugged, and safe) method as a sample preparation technique for simultaneous quantitation of multiclass, multiple pesticide residues in human plasma. Blood plasma is a complex matrix containing lipids, carbohydrates, and proteins among its many constituents that may cause significant matrix interferences and decrease
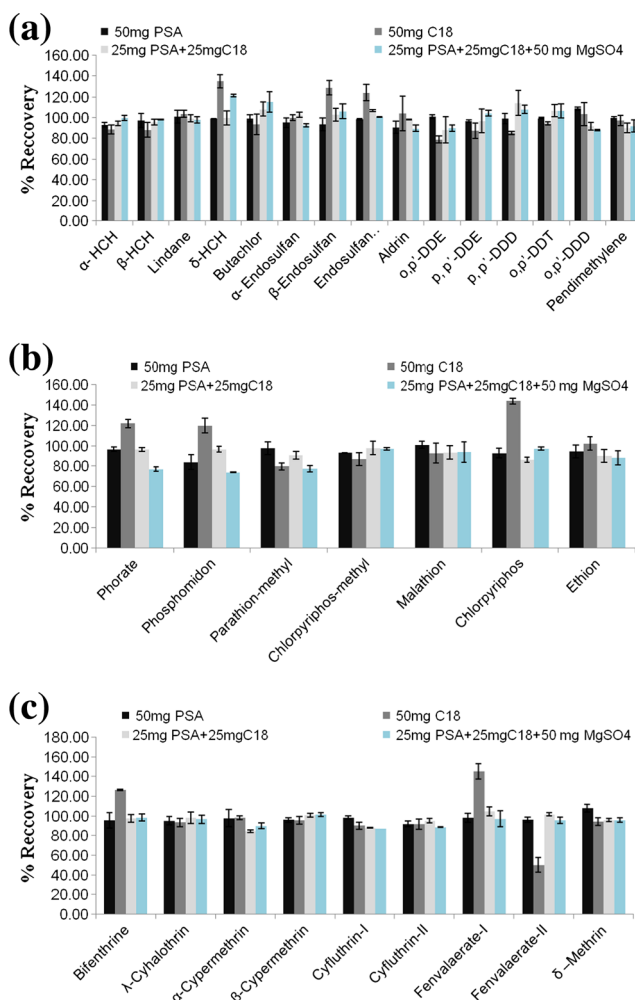
Fig. 2 Comparative evaluation of primary–secondary amine (*PSA*), C$_{18}$, MgSO$_4$, and their combinations in various amounts for cleanup of human plasma samples at a spiking level of 10 ng mL^{-1} (*n* = 3): **a** organochlorines and herbicides, **b** organophosphates, and **c** synthetic pyrethroids

concentration of organic solvent added to blank plasma samples for matrix-matched calibration standards was 10%.

GC–MS/MS analytical conditions

GC–MS/MS analyses were performed with a Trace Ultra gas chromatograph connected to a triple-quadrupole Quantum XLS mass analyzer (Thermo Scientific, Gainesville, FL, USA). Analytes were separated with use of an MS-5 capillary column (30 m × 0.25-mm inner diameter, 0.25-μm film thickness) with a stationary phase of 5% phenyl and 95% methyl polysiloxane. The sample injection was performed with a programmable temperature vaporization inlet in splitless mode at 60°C, which increased to 90°C at the rate of 14.5°C s^{-1} in the evaporation phase. The temperature was further increased to 260°C at a rate of 10°C s^{-1} in the transfer phase, and in the cleaning phase, the temperature was increased to 280°C at a rate of 14.5°C s^{-1}. Helium gas (purity 99.999%) was used as the carrier gas at a flow rate of 1.2 mL min^{-1}. The oven temperature program was as follows: the initial temperature was kept at 55°C for 3 min, and subsequently increased to 150°C at a rate of 10°C min^{-1} and further increased to 230°C at a rate of 3°C min^{-1} and held for 5 min. Finally, the temperature was increased to 300°C at a rate of 10°C min^{-1} and held for 10 min. The total run time was 61 min. The ion source temperature and the transfer line temperature were set at 220 and 290°C respectively. All samples were analyzed in selected reaction monitoring mode. Retention times, the collision energy, the time widow, and the selected reaction monitoring transitions used for identification and quantification are given in Table 1.

Method validation parameters

The analytical method was validated for parameters including selectivity, accuracy, precision, linearity, limit of detection (LOD), and limit of quantification [22–24]. A matrix-matched calibration curve for each analyte was plotted to demonstrate the linearity of method in the concentration range from 2 to 500 ng mL^{-1}. Each concentration was run in triplicate and the mean of responses were used to plot of the calibration curve. The LOD was calculated as per the method described elsewhere [24]. Seven independent spiked samples at a spiking level of 5 ng mL^{-1} were run for LOD calculation. The LOD calculation was done on the basis of multiplication of the standard deviations obtained for the measured concentration of spiked samples (*n* = 7) by the Student's *t* value, 3.14 (*t* value at 99% confidence level, six degrees of freedom) [24]. The limit of quantification was calculated by multiplication of the standard deviation by 10 [24].

resultant supernatant (0.9 mL) was separated and evaporated to dryness. The residue was reconstituted in 100 μL of ethyl acetate, and 2 μL was injected into the GC–MS/MS system for further analysis.

Matrix-matched standard preparation

A matrix-matched standard was prepared by the spiking of human plasma with a neat standard of pesticides [20, 21]. A neat stock solution of individual pesticides was prepared in *n*-hexane at 1000 μg mL^{-1} concentration and diluted to 10 μg mL^{-1} as an intermediate standard. A working matrix-matched standard of a pesticide mixture was then prepared at 2, 5, 10, 15, 25, 50, 100, and 500 ng mL^{-1}. The neat standard of pesticides was added in such a manner that the volume of the neat standard and the matrix was constant for each concentration of matrix-matched standard prepared. The
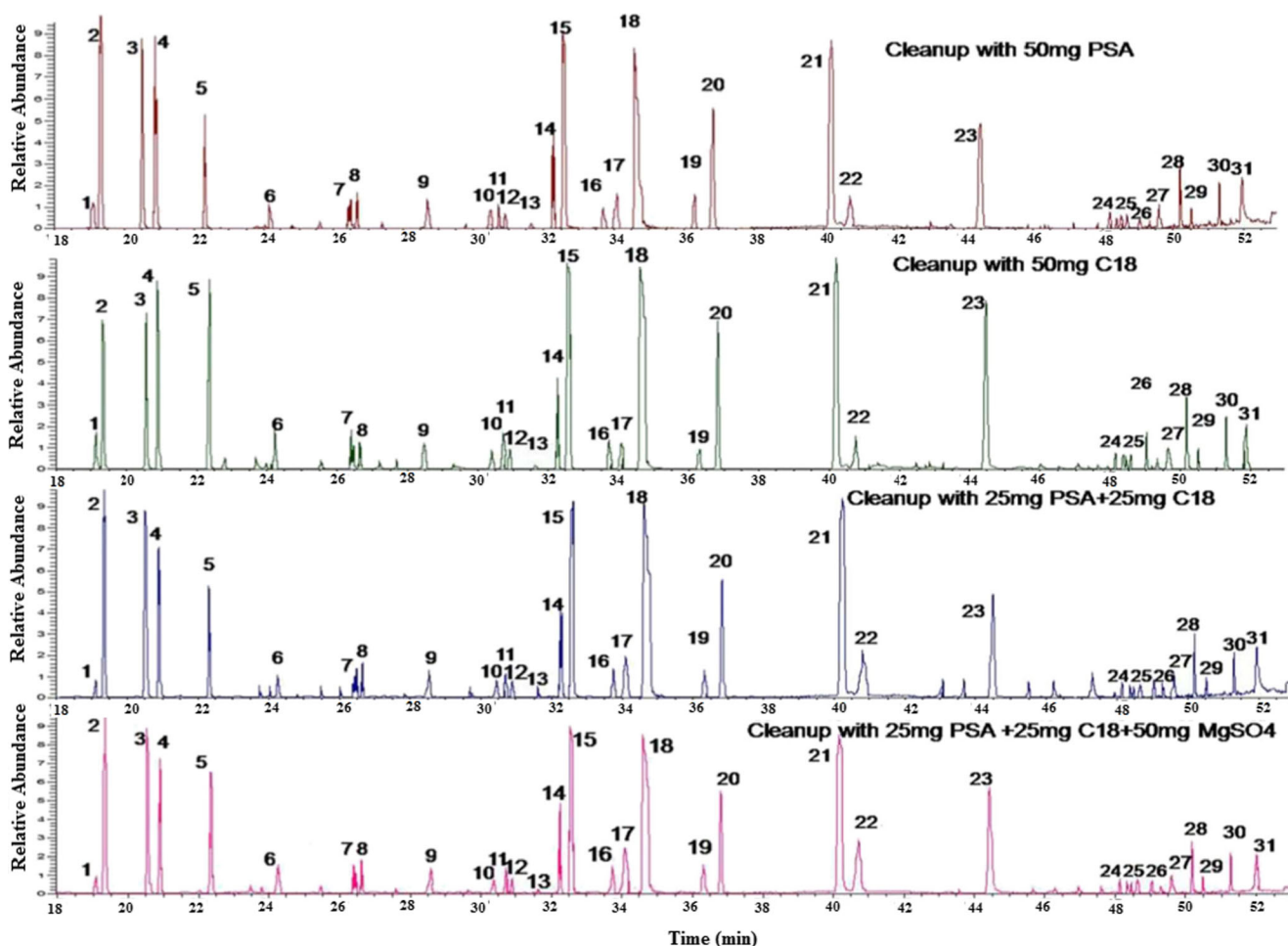
Fig. 3 Gas chromatography–tandem mass spectrometry separation profiles of pesticides by cleanup with PSA, C₁₈, PSA and C₁₈, and PSA, C₁₈, and MgSO₄ at a spiking level of 10 ng mL⁻¹ in human plasma. *1* α-hexachlorocyclohexane, *2* phorate, *3* β-hexachlorocyclohexane, *4* lindane, *5* δ-hexachlorocyclohexane, *6* phosphomidon, *7* parathion-methyl, *8* chlorpyriphos-methyl, *9* malathion, *10* aldrin, *11* chlorpyriphos, *12* pendimethylene, *13* o,p′-DDD, *14* α-endosulfan, *15* butachlor, *16* o,p′-DDE, *17* p,p′-DDE, *18* β-endosulfan, *19* p,p′-DDD, *20* ethion, *21* endosulfan sulfate, *22* o,p′-DDT, *23* bifenthrin, *24* λ-cyhalothrin, *25* cyfluthrin I, *26* cyfluthrin II, *27* α-cypermethrin, *28* β-cypermethrin, *29* fenvalerate I, *30* fenvalerate II, *31* δ-methrin

The interday precision and the intraday precision of the method were evaluated at concentration of 10 ng mL⁻¹. For the intraday precision, six replicates were analyzed on 1 day, whereas for the interday precision, six replicates were analyzed on five consecutive days. The results were expressed as the percent relative standard deviation. Recovery studies were preformed at spiking levels of 5, 10, 50, and 100 ng mL⁻¹. The percent accuracy of the method was measured at each level of spiking by the following formula: mean observed concentration/nominal concentration × 100.

Results and discussion

In the proposed method, pesticide extraction using ethyl acetate and partitioning with magnesium sulfate followed by dispersive SPE cleanup has been shown to extract the multiclass multiple pesticide residues simultaneously from a small volume of human plasma. Initially, we optimized the extraction solvent then its pH with a one variable at a time approach. Later we optimized the extraction salt type (MgSO₄, NaCl), their combination, and then the weak ion exchanger adsorbing reagents (PSA, C₁₈) and their amounts. After optimization of the extraction and cleanup parameters, the method was tested and validated.

Selection of extraction solvents and the pH

Acetonitrile and ethyl acetate were selected for optimization of the extraction solvent. The extraction efficiency of both solvents was determined by recovery estimates at a spiking level of 10 ng mL⁻¹. As shown in Fig. 1, overall the recoveries of all the analytes were better in ethyl

Table 2 Method validation parameters of pesticide residue estimation in human plasma

Pesticide	R^2	LOD (ng mL^{-1})	LOQ (ng mL^{-1})	Recovery at different spiking levels (%)				RSD of recovery estimation at different spiking levels (%)	Precision of method at a spiking level of 10 ng mL^{-1} ($n=6$), RSD (%)	
				5 ng mL^{-1} ($n=7$)	10 ng mL^{-1} ($n=7$)	50 ng mL^{-1} ($n=7$)	100 ng mL^{-1} ($n=7$)		Interday	Intraday
α-Hexachlorocyclohexane	0.964	1.26	4.15	80	90	93	103	7	1	4
Phorate	0.956	0.80	2.65	94	91	97	98	4	2	3
β-Hexachlorocyclohexane	0.972	0.04	0.13	93	92	102	102	6	3	6
Lindane	0.965	2.45	8.08	98	101	106	90	8	3	8
δ-Hexachlorocyclohexane	0.974	0.72	2.38	88	102	99	103	2	2	4
Phosphomidon	0.970	3.64	12.00	90	97	94	105	6	5	11
Parathion-methyl	0.990	0.97	3.21	90	101	98	98	2	4	3
Chlorpyriphos-methyl	0.963	0.92	3.06	81	97	93	105	6	4	8
Malathion	0.953	0.40	1.30	86	104	101	106	2	1	3
Aldrin	0.951	0.89	2.93	74	85	96	104	10	2	3
Chlorpyriphos	0.974	2.35	7.75	102	84	93	81	7	4	8
Pendimethylene	0.985	0.56	1.85	95	97	100	103	3	1	2
o,p'-DDD	0.986	0.54	1.77	105	102	99	98	2	2	6
α-Endosulfan	0.975	3.38	11.16	83	104	105	89	9	2	5
Butachlor	0.992	0.54	1.78	87	90	99	100	6	5	7
o,p'-DDE	0.977	0.87	2.87	87	94	101	102	4	6	8
p,p'-DDE	0.960	0.80	2.63	77	95	97	107	6	3	3
β-Endosulfan	0.970	1.91	6.32	94	96	99	91	4	3	7
p,p'-DDD	0.966	0.59	1.94	91	85	99	85	9	2	4
Ethion	0.950	1.78	5.86	94	100	95	98	3	4	6
Endosulfan sulfate	0.982	1.70	5.63	90	94	97	95	2	6	7
o,p'-DDT	0.976	4.10	13.53	94	97	100	91	5	4	11
Bifenthrin	0.959	0.36	1.88	97	102	101	94	4	2	6
λ-Cyhalothrin	0.974	2.70	8.91	86	101	95	102	4	4	8
Cyfluthrin I	0.992	2.70	8.90	91	92	99	94	4	2	6
Cyfluthrin II	0.961	3.05	10.05	96	106	92	100	7	2	4
α-Cypermethrin	0.976	1.50	4.95	88	97	103	87	8	2	2
β-Cypermethrin	0.976	1.72	5.66	102	99	96	89	5	2	5
Fenvalerate I	0.960	0.97	3.22	107	94	98	97	2	6	4
Fenvalerate II	0.984	1.98	6.52	99	101	96	100	3	3	7
δ-Methrin	0.988	1.33	4.38	102	97	108	99	6	1	4

RSD relative standard deviation

acetate than in acetonitrile. For example, organophosphates showed recoveries ranging from 57% to 84% in acetonitrile, whereas in ethyl acetate the recoveries ranged from 90% to 105%. The rest of the pesticides gave good recoveries (above 70%) in both solvent systems. On the basis of these outcomes, ethyl acetate was selected as the extraction solvent. Further, acetonitrile does not show better compatibility with GC–MS/MS as it has a high vaporization pressure, which significantly increases the internal pressure of the system. In programmed temperature vaporization injection mode, the injected solvent must have a low boiling point since it is desired to trap the solvent in the liner at acceptably high temperatures to eliminate the most of solvent by means of venting out. A solvent with a high boiling point may result in loss of highly volatile analytes. Therefore, the solvent should have a sufficiently low boiling point. On the basis of all requirements, ethyl acetate was found to be a better choice as the extraction solvent. Acidification of the extraction solvent using acetic acid at 0.1%, 0.5%, 1.5%, and 2% was also tested and optimized to ensure good and reproducible recovery for all pesticides. Ethyl acetate acidification with 2% acetic acid gave the best response and peak shape for all the analytes (data not shown) and hence was selected for subsequent experiments [25–27].

Selection of extraction salt

Selection of the salt type, amount, and combination of salts was done on the basis of the overall comparison of analyte responses at a spiking level of 10 ng mL^{-1}. Salt type, the combination of salts, and the concentration play an important role in proper phase separation and the salting-out effect, which significantly enhance the extraction efficiency of the method. By adjustment of the salt concentration, the polarity range of the method can be controlled. The polarity range of the method influences the clarity of the extract by controlling the amount of matrix components of different polarities in the extract [11]. We tried three different approaches: MgSO$_4$ (0.4 g) alone, NaCl (0.1 g) alone, and a combination of MgSO$_4$ (0.4 g) and NaCl (0.1 g). The overall percent recoveries and peak resolution of all analytes were found to be better with MgSO$_4$ (data not shown), and therefore MgSO$_4$ alone was selected as the extraction salt [27].

Selection of sample cleaning reagents

PSA, C$_{18}$, and their combination in various amounts were tested and optimized for sample cleanup. The efficiency of sample cleanup was evaluated by peak response/shape (Fig. 3) and percent recovery estimation (Fig. 2). We selected four combinations for optimization of the cleanup

step: (1) 50 mg PSA, (2) 50 mg C$_{18}$, (3) 25 mg PSA and 25 mg C$_{18}$, and (4) 25 mg PSA, 25 mg C$_{18}$, and 50 mg MgSO$_4$. MgSO$_4$ was used to remove any residual moisture. Sample cleanup with C$_{18}$ gave comparatively poor precision for δ-hexachlorocyclohexane, α-endosulfan, endosulfan sulfate, phorate, phosphomidon, chlorpyriphos, bifenthrin, and fenvalerate. The recoveries for these pesticides were greater than 110%, accompanied by a large standard deviation (1.39–22.83%), which could be due to poor cleanup leading to a matrix effect (ion enhancement) and therefore greater variability. Sample cleanup with the combination of 25 mg PSA and 25 mg C$_{18}$ and with the combination of 25 mg PSA, 25 mg C$_{18}$, and 50 mg MgSO$_4$ also showed poor precision and recovery, with a larger standard deviation (0.87–20.82%) for δ-hexachlorocyclohexane, butachlor, β-endosulfan, p,p′-DDE, p,p′-DDD, o,p′-DDT, o,p′-DDT phosphomidon, and chlorpyriphos-methyl. Sample cleanup with 50 mg PSA gave overall better precision, better recovery (84–109%), smaller standard deviation (0.52–10.52%), and cleaner chromatograms. Therefore, we selected 50 mg PSA as the cleanup medium (Fig. 3).

Method validation experiment

Optimized conditions for each parameter were used in the validation experiments. The results of the method validation are given in Table 2. The selectivity of the method was tested by analysis of the reagent blank and pooled human blank plasma samples. No significance interferences were observed at the retention times of the analytes. The method's matrix-matched calibration curve included concentrations of 2, 5, 10, 15, 25, 50, 100, and 500 ng mL^{-1}. Recovery studies were performed at concentrations of 5, 10, 50 and 100 ng mL^{-1}. The recovery values for all the analytes ranged from 74% to 109%. The percent relative standard deviations for recovery at different spiking levels ranged from 2 to 10. The percent accuracy values of recovery were within acceptable limits (70–120%) as per SANCO guidelines [22]. The intraday precision and the interday precision of the method ranged from 1% to 6% and from 2% to 11% respectively.

Conclusion

In conclusion, we have developed and validated a GC–MS/MS method for simultaneous analysis of multiclass multiple pesticide residues in human plasma using a mini QuEChERS technique. In this method, we adopted a miniaturized version of the QuEChERS technique for sample preparation that is very useful for covering the wide range of pesticide residues. From the validation results, conclude that this method would

be very useful for pesticide analysis in small volumes of clinical and/or forensic samples, with acceptable precision and accuracy along with high throughput.

Compliance with ethical standards The control human plasma samples for method development were provided by Era's Lucknow Medical College and Hospital, Lucknow, India. The study was approved by the Human Ethics Committee of the Indian Institute of Toxicology Research and was performed in accordance with ethical standards.

Conflict of interest The authors declare that they have no conflict of interest.

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