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Simple determination of dichlorvos in cases of fatal intoxication by gas Chromatography-Mass spectrometry

Rajeev Jain [a,*], Bharti Jain [a,b], Vimukti Chauhan [a], Bhawna Deswal [a], Sukhminder Kaur [a], Shweta Sharma [b,*], Mohammad A. S. Abourehab [c]

[a] Central Forensic Science Laboratory, Plot #2, Sector 36-A, Dakshin Marg, Chandigarh 160036, India
[b] Institute of Forensic Science & Criminology, Panjab University, Chandigarh 160014, India
[c] Department of Pharmaceutics, College of Pharmacy, Umm Al-Qura University, Makkah 21955, Saudi Arabia

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ABSTRACT

Dichlorvos (DDVP) is an organophosphorous insecticide which is classified as "highly hazardous" Class 1B chemical by World Health Organization (WHO) and largely misused for the purpose of self-poisoning in developing countries. Forensic toxicology laboratories are routinely encountering cases of pesticide poisoning due to their fatal intoxication. Herein; a method is described based on vortex-assisted dispersive liquid–liquid microextraction (VA-DLLME) coupled with Gas Chromatography-Mass Spectrometry (GC–MS) for the determination of an organophosphorous insecticide; dichlorvos (DDVP) in human autopsy samples (blood, stomach content and liver). Under the optimum conditions, the method was found to be linear in the range of 0.5–10 μg mL^{-1} and 1.5–10 μg g^{-1} for blood and tissue samples, respectively. Limit of quantification was set at 0.55 μg mL^{-1} and 1.1 μg g^{-1} for blood and tissue samples, respectively. Intraday and inter-day precisions were less than 8 and 12 %, respectively. Good recoveries in the range of 86–95 % were obtained for the proposed procedure. The method has been satisfactorily applied for the determination of DDVP in autopsy samples from two different cases received in our laboratory. In comparison to previous methods; the proposed method is relatively short, high sample throughput, inexpensive and adheres to the principles of green analytical chemistry (GAC) for determination of DDVP in human autopsy samples. The method can be adopted in forensic toxicological laboratories for analysis of DDVP in autopsy samples. In addition, the green character of the proposed method was evaluated using ComplexGAPI procedure.

1. Introduction

Dichlorvos (O, O-dimethyl 2,2-dichlorovinyl phosphate; DDVP) is a commonly used insecticide worldwide belonging to the organophosphate family. In developing countries, DDVP is still a frequently used agricultural and household insecticide [1]. In addition to its intended function, DDVP has also occasionally been used as a poison for homicidal and suicidal intent. [2–5]. DDVP is available under many trade names, such as Vapona, Nuvan, Atgard, Phosvit, Sniper, etc. The World Health Organization (WHO) has classified it as "highly hazardous" chemicals in class 1B [1]. The irreversible inhibition of the enzyme acetylcholinesterase, which is responsible for the hydrolysis of the neurotransmitter acetylcholine, causes DDVP toxicity. As a result of the accumulation of acetylcholine in synaptic clefts, nerve function is disrupted, and symptoms of intoxication in the central and peripheral nervous systems are produced. Nausea, vomiting, diarrhoea, lacrimation, perspiration, salivation, and bradycardia are among the symptoms [1–6]. In cases of acute poisoning, death occurs due to inhibition of the respiratory centre in the brain stream, which results in respiratory paralysis and failure [3].

The analysis of DDVP from biological samples, especially human autopsy samples such as blood and tissue, is a tedious task owing to the complex nature of these matrices, and therefore limited reports are available in the literature for its analysis. From samples of porcine tissue, DDVP was extracted using solid phase extraction (SPE) and liquid–liquid extraction (LLE), and then evaluated using either gas chromatography (GC) or liquid-chromatography tandem mass spectrometry. (LC-MS/MS) [7,8]. Beside tissue samples, SPE based on specialized sorbents has also been applied for the extraction of DDVP and other organophosphates from vegetables and aqueous samples [9–11]. Additionally, DDVP was

* Corresponding authors.
E-mail addresses: rajeevjaincfsl@gmail.com (R. Jain), 25shweta@pu.ac.in (S. Sharma).

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extracted using LLE from matrices like environmental water, beverages, plasma, and human urine samples. [12–13]. However, these methods employ complex multistep protocols for extraction of pesticides, which are lengthy and require an additional pre-concentration step before final instrumental analysis. Furthermore, LLE in combination with gas chromatography-mass spectrometry (GC–MS) has been utilized for monitoring organophosphorous insecticide concentrations in plasma and serum samples of patients with acute poisoning [14–15]. In cases of fatal intoxication, however, plasma and serum are scarce; instead, whole blood and tissue materials are the most abundant samples. Since the nature of each biological sample is different, an exclusive analytical method has to be developed for the analysis of DDVP in human autopsy samples.

The implementation of microextraction techniques, which need the least amount of sample and extraction solvent (zero to microliters), can address the shortcomings of SPE and LLE that were stated above. Microextraction methods have also been shown to be quick, environmentally friendly, economical, and to give excellent enrichment factors, extraction efficiencies, and sample throughput [16–19]. Organophosphorous pesticides have been extracted using a variety of microextraction procedures, including solid-phase microextraction (SPME), single drop microextraction (SDME), and hollow fiber liquid phase microextraction (HF-LPME) [20–22]. However, these methods are limited to relatively simple matrices such as water samples. Additionally, SPME fibers require specific maintenance and preservation because they are delicate by nature, expensive, and fragile [23]. Similar to this, it is difficult to maintain the stability of tiny droplets of organic solvent during the extraction process with SDME [24]. Therefore, a thoroughly optimized and validated assay based on a simple and cost-effective microextraction technique is still needed for determination of DDVP in human tissue and blood samples which will be useful for toxicological studies involving its fatal intoxication.

Rezaee et al. established dispersive liquid–liquid microextraction (DLLME) in 2006. A scaled-down version of LLE; i.e. DLLME has grown immensely popular among analytical chemists [25]. An extractant, dispersion, and an aqueous sample make up the ternary components of the DLLME solvent extraction system. The traditional DLLME procedure entails quickly injecting a suitable extractant and dispersant mixture into a water sample that contains the target analytes. As a result, the extraction solvent is evenly distributed throughout the aqueous phase, creating an emulsion. The tremendously high contact area between the extractant and the aqueous sample at this time causes a rapid attainment of equilibrium and prompts the extraction of analytes [25].

DLLME is distinguished by its capacity to hyphenate with a variety of analytical equipment and its consumption of a tiny amount of extraction solvent (in the microliters range), ease of operation, low cost, and high enrichment factors. DLLME and its variants has been widely applied for determination of various analytes in diverse range of matrices including cypermethrin and its metabolites in rat tissue, clenbuterol in porcine tissue, polybrominated diphenyl ether in aquatic animal tissues, quinolones in swine muscles etc.[26–31]. However, to the best of our knowledge, there are only a small number of uses of DLLME from human tissue samples, including acetaminophen in autopsy samples, methadone and tramadol from postmortem vitreous humor, antidepressants in pericardial fluid, and parabens from breast tumor tissue. [32–35].

Organophosphorous pesticides have been used for pest management on a global scale for a long time. However, due to their easy and cheap availability, they have been extensively misused for the purpose of self-poisoning. Forensic toxicology laboratories routinely receive autopsy samples where such poisoning is suspected. As discussed above, the current analytical methods include LLE and SPE which are time consuming, laborious and consumes large volumes of toxic organic solvents. However, since the emergence of green analytical chemistry (GAC), various microextraction and miniaturized sample preparation methods have been developed and applied in forensic toxicology analysis [36].

Herein, an analytical method based on VA-DLLME-GC–MS is developed and validated for the quick, simple, affordable, and environmentally friendly determination of DDVP in complex matrices (human autopsy samples: blood, liver and stomach content). There are multiple factors which can affect the extraction efficiency of VADLLME such as: type and volume of extraction solvent, type and volume of disperser solvent, pH, ionic strength, speed and time of vortex agitation. In order to obtained best extraction efficiency for DDVP, all of the above parameters were studied and optimized. Finally, the developed method has been applied in two cases of fatal intoxication of DDVP to demonstrate the suitability of the proposed method.

2. Case history

Case 1. *A 21-year-old unmarried Indian man from Chandigarh City who was being treated for a chronic disease was admitted to a local hospital's emergency department for an unknown poisoning. The patient died during course of treatment. No external injuries or abnormalities were observed during postmortem examination. Lung, liver, spleen and kidney were found to be congested and around 100 mL of greenish liquid was present in the stomach. The approximate time interval between death and postmortem was 9 h at local hospital.*

Case 2. *In another case, a chronic alcoholic 50-year-old Indian male from Chandigarh was admitted to a local hospital after consuming an unknown poison. The patient was in gasping stage and his blood pressure and pulse were unrecordable. Smell of kerosene with increased oral secretion and eye miosis was observed. No external injuries were seen. However, the victim died during the treatment. During autopsy; congestion were observed in lungs, liver and kidneys. The stomach contained 250 mL of a bluish-green semi-solid material, and the mucosa was extremely congested.*

3. Materials and methods

3.1. Reagents and chemicals

Until otherwise stated, all chemicals and reagents used in this study were of analytical grade. Certified Reference Material of DDVP in powdered form was obtained from AccuStandard (New Haven, USA). Ethanol (EtOH), methanol (MeOH), acetonitrile (ACN), and acetone (ACE) were purchased from LobaChemie (Mumbai, India) and were used as disperser solvents. Whereas, chloroform (CF), dichloromethane (DCM), ethyl acetate (EA), hexane (HEX), cyclohexane (CHX) were used as extraction solvent and provided by Merck (Darmstadt, Germany). Ultrapure water was used throughout the work. Working standard solution of DDVP was prepared in MeOH at 100 μg $mL^{-1}$and stored at ~ 4 °C until analysis. This was used to fortify biological matrices for optimization and validation studies.

3.2. Biological samples

Postmortem blood and tissue samples of above described cases of DDVP fatal intoxication were collected by Department of Forensic Medicine and Toxicology at different Government Hospitals of Chandigarh (India). The collected samples along with authorization certificates were submitted by law enforcement agencies to our laboratory for toxicological examination. In order to prepare fortified samples for method development and validation purposes, similar matrices were used which were previously tested negative for DDVP and other drugs and poisons. The study has been approved by Institutional Ethical Committee of CFSL (Approval No. 1109) and was performed according to guidelines by ICMR (Indian Council of Medical Research) [37].

3.3. Systematic toxicological analysis (STA)

Exhibits from both cases were subjected for routine STA in our

laboratory for the detection of volatile poisons (EtOH and MeOH), abused drugs (opioids, heroin, cannabinoids, amphetaminesetc.), sedative and hypnotic drugs (barbiturates and benzodiazepines), antidepressants (amitriptyline, paroxatine etc.), antipsychotic drugs, gaseous poisons (cyanide and carbon monoxide), and inorganic poisons such as aluminum phosphide and heavy metals etc. According to the monograph, thin layer chromatography (TLC) was employed for drug and pesticide screening. Volatile poisons were analyzed by headspace-gas chromatography-flame ionization detector (HS-GC). TLC preliminary analysis revealed the presence of DDVP in both cases.

3.4. Vortex-assisted dispersive liquid–liquid microextraction (VADLLME)

Exactly 0.5 g of stomach content and tissue sample (i.e. liver) was homogenized in 5 mL of ACE using a tissue homogenizer (Remi Laboratory Instruments, Mumbai, India). The supernatant of this solution was separated by centrifugation at 5000 rpm for 5 min. One mL of this supernatant along with 100 μL of CF was instantly injected into 2 mL of ultrapure water with the help of a syringe. This solution was then vortex agitated at 2000 rpm for 2 min to promote the process of emulsification. The cloudy solution thus obtained was immediately centrifuged for 3 min at 5000 rpm. Approximately 80 μL of CF was sedimented at the bottom of centrifuge tube and 2 μL of it was injected into GC–MS system for analysis.

For blood samples, 0.5 mL of whole blood sample was placed in a 15 mL centrifuge tube followed by addition of 500 μL of MeOH or ACN for the purpose of deproteinization of blood protein. This was centrifuged for 5 min at 5000 rpm to get a clear supernatant. Two mL of this supernatant was subjected for DLLME as follows: a mixture of disperser solvent (1 mL ACE) and extraction solvent (100 μL of CF) was instantly injected into this supernatant. This was vortex agitated at 2000 rpm for 2 min to enhance the process of emulsification. Rests of the steps were similar to the protocol as described above. Schematic representation of VA-DLLME-GC–MS process has been shown in Fig. 1.

3.5. GC–MS analysis

GC–MS analysis of extracts obtained after DLLME was performed on Shimadzu Nexis GC – 2030 coupled with QP-2020 NX mass spectrometer having installed with SH-Txi-5Sil MS capillary column (30 m length × 0.25 mm internal diameter × 0.25 μm film thickness) with a stationary phase of 5 % phenyl and 95 % dimethylpolysiloxane. Exactly 2 μL of the extract under split mode (split value 10) was injected into GC–MS at an injection port temperature of 250 °C with the help of Shimadzu AOC 20i Plus Auto sampler. Vaporized analytes from the injection port were carried into the column with helium gas at a flow rate of 1 mL min^{-1}. Oven temperature was initially kept at 60 °C for 1 min, followed by an increment up to 250 °C at a rate of 15 °C/min, resulting into total runtime of 13.67 min. Electron Impact (EI) mode was used to ionize the analytes with electron energy of 70 eV. Temperatures of transfer line and ion source were kept at 200 °C, respectively with a solvent delay of 3 min. Mass spectra was obtained in the range of 50–500 amu in full scan mode. The peak of DDVP was observed at 7.53 min in total ion chromatogram (Fig. 2).

3.6. Method validation

Blood, stomach content and liver samples were fortified with DDVP at different concentrations. Tissue samples were spiked in the range of 1.5–10 μg g^{-1}, whereas blood samples were spiked in the range of 0.5–10 μg mL^{-1}. All the fortified samples were processed and analyzed according to the developed procedure. Limit of detection (LOD) and limit of quantification (LOQ) were used to express sensitivity of the method and were evaluated at the lowest concentration of DDVP with a signal to noise ratio 3:1 and 10:1, respectively. For each matrix, repeatability and reproducibility were studied at three different

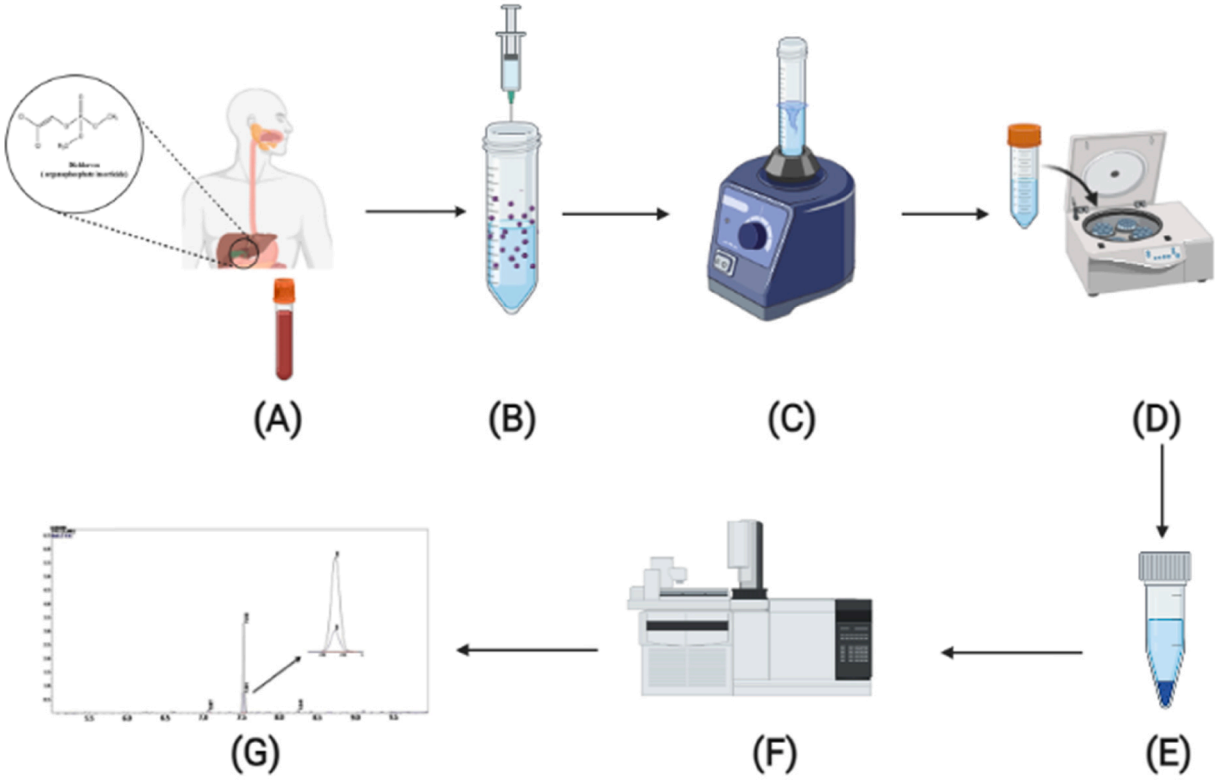

Fig. 1. Schematic representation of proposed protocol; (A) homogenization / deproteinization of tissue and blood samples, (B) VADLLME process using 1000 μL of ACE (homogenate) and 100 μL of CF, (C) vortex agitation at 2000 rpm for 2 min, (D) centrifugation at 5000 rpm for 3 min; (E) collection of sedimented phase for further analysis (F) GC–MS analysis (G) Total ion chromatogram (TIC) of DLLME extract.

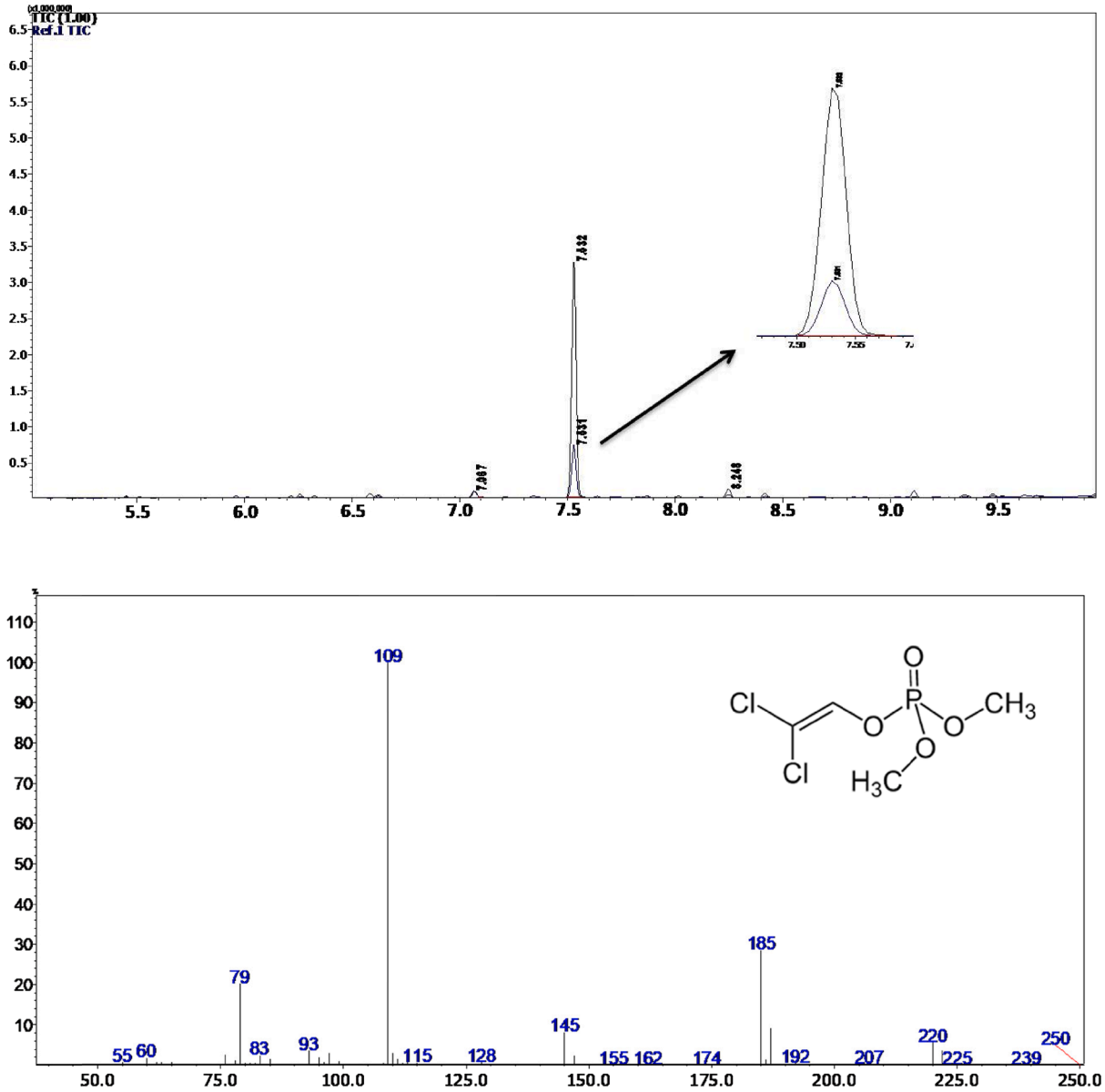

Fig. 2. Total ion chromatogram (TIC) of stomach content and standard DDVP obtained after VA-DLLME-GC–MS (above) and NIST mass spectra of DDVP (below).

concentrations in triplicates and were recorded as percent intra-day precision and inter-day precision, respectively (%RSD) following developed procedure.

4. Results and discussion

4.1. Selection of disperser solvent and optimization of its volume

The main goal of the disperser solvent in DLLME is to increase the surface area of contact between the extraction solvent and the aqueous sample. Utilizing disperser solvents that are concurrently miscible in the extraction solvent and aqueous phase allows for this. Herein, most commonly used four disperser solvents viz. EtOH, MeOH, ACN and ACE were evaluated. An experiment was conducted where 0.5 g of blank tissue sample was fortified with DDVP and homogenized in 5 mL of these disperser solvents (EtOH, MeOH, ACN and ACE). This sample was then centrifuged and supernatant was used as disperser solvents in DLLME process. Each disperser solvent (500 μL) along with DCM (200 μL, extraction solvent) was instantly injected into 5 mL of ultrapure water fortified with DDVP at concentration of 1 μg mL^{-1}. In order to intensify the emulsion formation the sample was vortex agitated for 1 min at 1500 rpm. This mixture was then centrifuged to get a clear sedimented phase for GC–MS analysis. It is evident from Fig. 3a that highest detector response for DDVP was obtained when ACE was used when compared to other disperser solvents. Therefore, ACE was selected as disperser solvent in all further experiments.

Additionally, an experiment was carried out using various volumes of ACE in the range of 200–1500 μL (200, 400, 600, 800, 1000, 1300, and 1500 μL) together with 200 μL of DCM in order to optimize the volume of ACE. The findings showed that as ACE volume increased from 200 μL to 1000 μL, the peak areas for DDVP also increased. Beyond this volume, they, however, usually start to decline. Additionally, when the volume of ACE increases, the amount of sedimented phase that was collected after centrifugation likewise decreases. Additionally, this makes it more difficult to collect sedimented phase for instrument analysis. As a result, the ACE volume was set at 1000 μL for all subsequent tests (Fig. 3b).

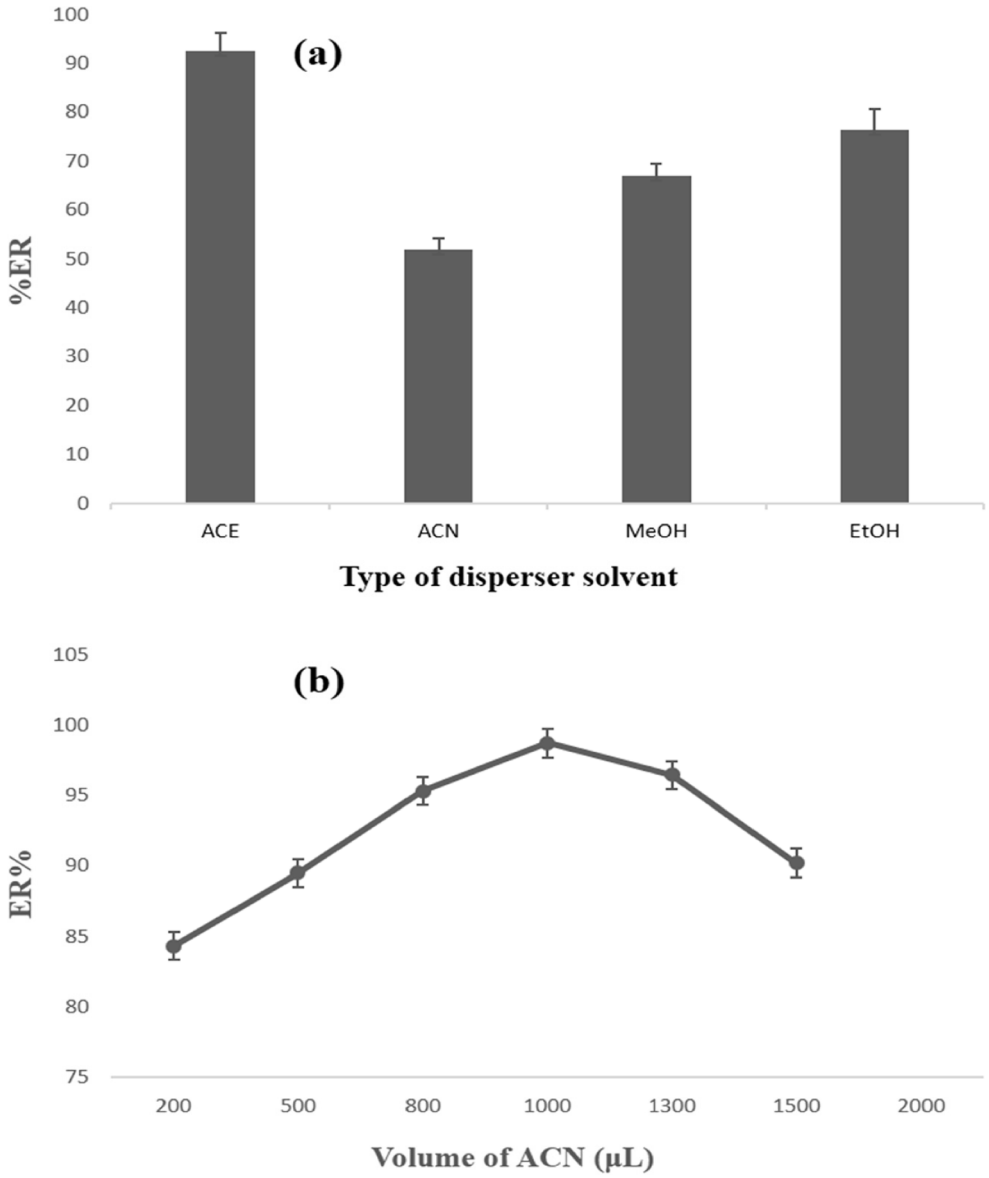

Fig. 3. Disperser solvent (a) selection of disperser solvent; and (b) study of volume of ACE (µL).

4.2. Selection of extraction solvent and optimization of its volume

In classical DLLME; extraction solvents with density greater than water are used for extraction; however, solvents with lower density than water have also been used due to their easy collection after centrifugation. Therefore, we compared a total of five extraction solvents, of which three (HEX, CH, and EA) had a lower density than water and two had a higher density (CF and DCM). An experiment was conducted by mixing 1000 µL of ACE obtained from above described process with each extraction solvent (200 µL). The sample was processes as described above and sedimented phase / supernatant phase was collected and analyzed by GC–MS. Results showed that CF as the extraction solvent yielded the maximum peak area with the fewest matrix interferences, followed by DCM and EA (Fig. 4a). Therefore, it was decided to use CF as extraction solvent for all further experiments.

After selecting CF as extraction solvents, a set of experiment was further conducted to optimize its volume which is crucial for improved extraction efficiency of DLLME. CF was mixed with 1000 µL of ACE in various volumes ranging from 100 to 500 µL, and DLLME was carried out as previously described. The resulting mixture was agitated on a vortex shaker for 1 min at 1500 rpm followed by centrifugation to get a clear sedimented phase for GC–MS analysis. Fig. 4b makes it clear that using CF at a volume of 100 µL resulted in the greatest response. Dilution causes the detector response for DDVP to decrease with increasing CF volume. Therefore, the volume of CF was set at 100 µL for all subsequent experiments.

4.3. Optimization of volume of water, vortex agitation time and speed

Herein, water is being used as a component of ternary solvent system and also facilitates the process of emulsification. Therefore volume of water was also optimized in the range of 2–5 mL. Maximum peak areas were obtained with volume of water was kept at 2 mL (Fig. 5a). It is also worth noting that as the volume of water increases, the amount of sedimented phase of CF decreases, resulting in lower peak areas. As a result, DLLME was performed with 2 mL of aqueous phase.

The DLLME technique's emulsification process can be promoted simply and affordably by vortex agitation. By increasing the interfacial area between these two phases, vortex agitation promotes the mass transfer of the analyte by facilitating the production of tiny droplets of extraction solvents that are dispersed in aqueous phase. This facilitates quick partition equilibrium within minutes [38,39]. Therefore, effect of

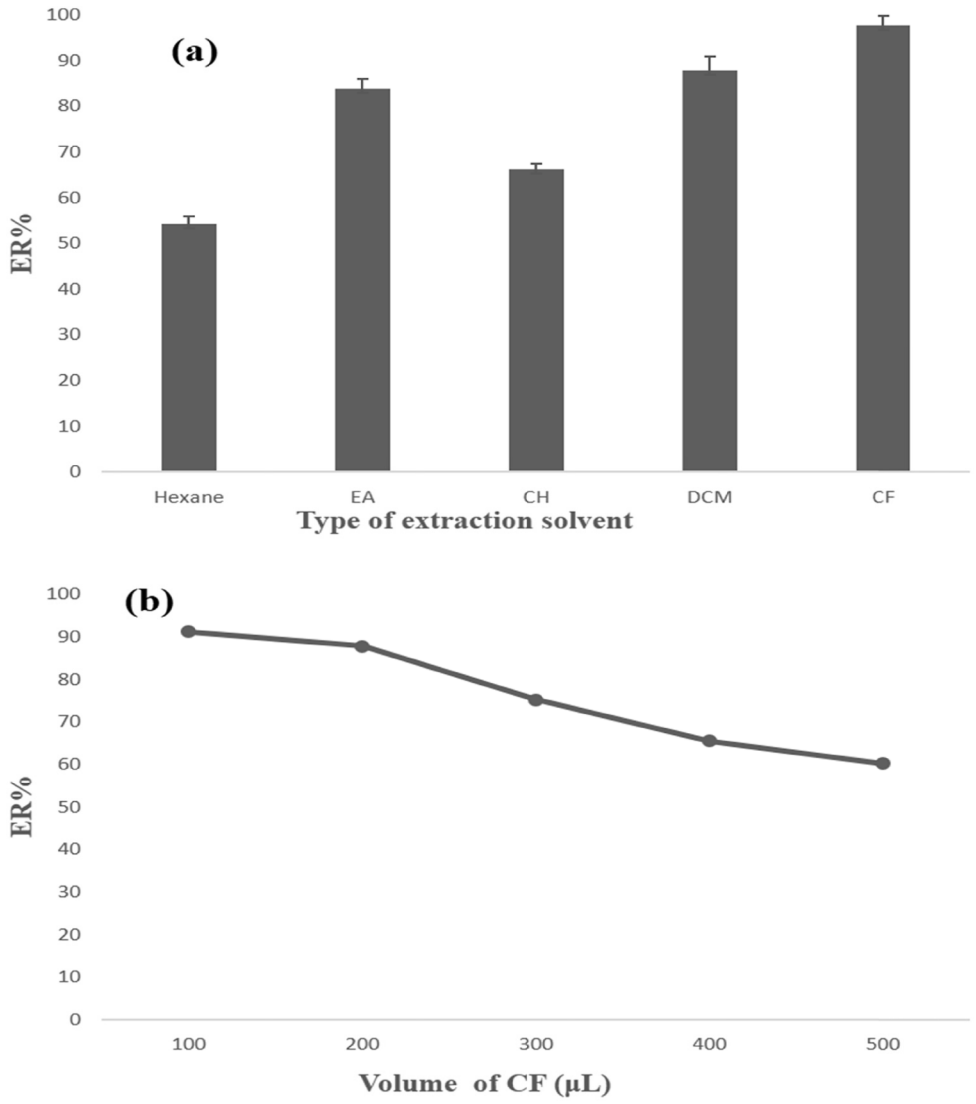

Fig. 4. Extraction solvent (a) selection of type of extraction solvent, (b) study of volume of CF (µL).

vortex agitation speed and time was investigated in the range of 1000–5000 rpm and 0.5–5 min, respectively, keeping the previously described parameters at their optimum values. Results indicated that response of DDVP increases as the vortex agitation speed increases up to 2000 rpm, however they stayed almost uniform above this speed. This indicates that sufficient equilibrium has been achieved at this point (Fig. 5b). Further, keeping the vortex agitation speed fixed at 2000 rpm; time of vortex agitation was also optimized. It was observed that extraction efficiency of DLLME increases from 0.5 to 2 min of vortex agitation time and stayed uniform beyond 2 min. Therefore, in further samples were vortexed for 2 min at 2000 rpm (Fig. 5c).

4.4. *Effect of pH and ionic strength*

The effect of pH on extraction efficiency of VA-DLLME was evaluated in the range of 4 – 8. An experiment was designed where pH of aqueous phase was adjusted by 1 M HCl and 1 M NaOH solution. All the parameters were kept at their optimum values as described earlier. The findings showed that the optimum amount of DDVP could be extracted when the aqueous phase's pH was kept at 5. (Fig. 5d).

Further, effect of salt addition was also evaluated by adding NaCl in the range of 0–10 % (*w/v*) in the aqueous phase. Addition of salt in the aqueous phase increases the extraction efficiency of DLLME by decreasing the solubility of the analyte. However, in this case, there was no significant improvement observed in the extraction of DDVP, and hence, no salt was added during the DLLME process for all further experiments (Fig. 5e).

4.5. *Performance of the proposed method*

The proposed method, VA-DLLME, has been thoroughly validated in human autopsy samples such as blood, stomach content, and liver fortified with known amounts of DDVP under optimized conditions for linearity, precision, recovery, and sensitivity. The autopsy samples were used in accordance with the ICMR guidelines [37]. External calibration graphs for DDVP in each matrix were prepared by plotting peak areas of DDVP on *y*-axis and corresponding concentration on *x*-axis. Satisfactory linearity in the range of 0.997–0.999 was observed (Table 1A). In order to keep the proposed method convenient and simple for the purpose of quantification of DDVP; no internal standard was added, as this would impose one more additional step in the protocol which could lead to higher errors in quantification. This choice was also supported by previous literature where external calibration methods were found more convenient than internal standard methods [40–42].

The LOD and LOQ were evaluated at signal to noise ratio of 3:1 and 10:1, respectively. LOD and LOQ for blood were found to be 0.17 and

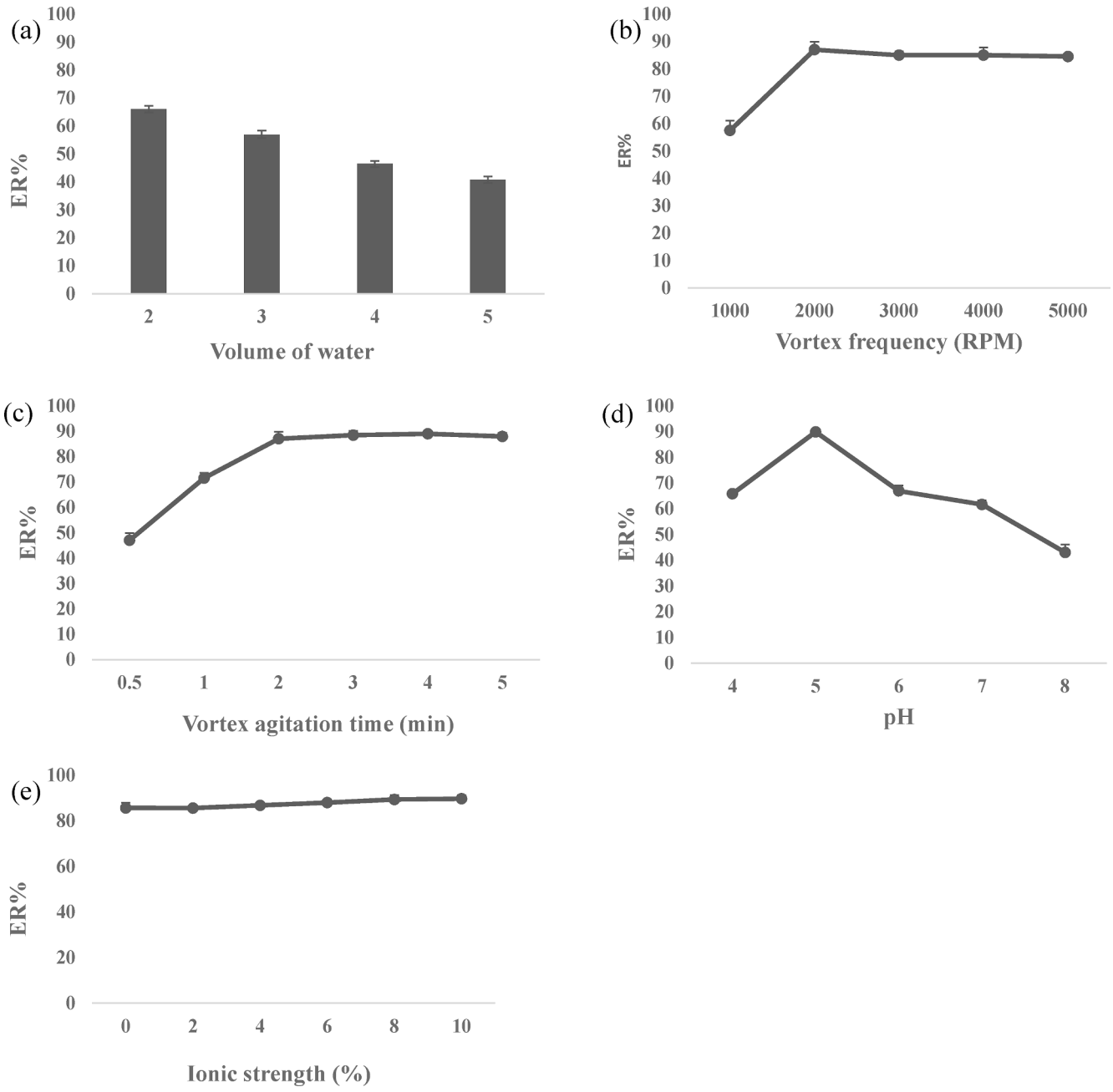

Fig. 5. Effect of (a) volume of water (b) vortex frequency (c) vortex agitation time (d) pH and (e) IS on DLLME.

Table 1A
Method validation parameters for VA-DLLME-GC–MS analysis of DDVP in autopsy samples ($n = 3$).

Matrix	Linear range	R^2	LOD	LOQ	Calibration curve	Intra-day			Inter-day		
						2	5	10	2	5	10
Blood[a]	0.5–10	0.999	0.17	0.5	y = (7823.6 ± 98.01)x + (26348 ± 451.9)	7.8	6.8	5.6	11.9	9.2	8.6
Sotmach content[b]	1–10	0.998	0.31	1.0	y = (4772.9 ± 80.6)x –(696.83 ± 530.5)	7.0	8.6	6.9	9.9	7.9	6.4
Tissue homogenate (Liver)[b]	1.5–10	0.997	0.33	1.1	y = (4397.8 ± 100.4)x + (8778.3 ± 481.52)	6.4	7.2	4.8	10.5	8.4	7.4

a = concentration expressed in μg mL^{-1}.
b = concentration expressed in μg/g.

0.55 μg mL⁻1. Similarly for stomach content and tissue homogenate (liver) samples the values of LOD and LOQ were 0.31 – 0.33 μg g^{-1} and 1 – 1.11 μg g^{-1}, respectively. Intra and inter-day precisions were evaluated at three different concentration levels of linearity range and were found to be less than 8 and 12 % ($n = 3$), respectively. Recovery studies were also performed at similar concentration levels as of precision. The relative recoveries which were obtained by comparing the analytical results of extract of blank sample spiked with DDVP with the same concentration of standard DDVP. Good relative recoveries in the range of 86–95 % were obtained by the proposed procedure (Table 1A).

Absolute recoveries were calculated as the ratio of concentration found to the true concentration of DDVP in blood and tissue samples (Table 1B). Stability of DDVP was accessed by analyzing the extract after 72 h of extraction. The peak areas of such samples were compared with those of freshly extracted samples by proposed procedure. Variation of $>$ 15 % in concentration accuracy was deemed to be unstable sample.

The proposed method has been compared with previously reported analytical methods for determination of DDVP in biological matrices and the comparison has been shown in Table 2. It can be observed from the comparison that, proposed method offered comparable sensitivity to all reported methods except at ref. [8], where LC-MS/MS was used for detection of DDVP, which is superior in sensitivity to GC–MS in this case.

4.6. Evaluation of green character of the method

The proposed method has been evaluated for its green character by using complementary green analytical procedure index (ComplexGAPI), which has been utilized as a comprehensive tool in many studies for this purpose [43–53]. This procedure enables a methodical assessment of the analytical protocol taking into account sampling preparation and analysis, reagents and solvents, apparatus, type of method (qualitative and/or quantitative); and pre-analytical processes. A pictogram made up of five pentagons which are produced by ComplexGAPI using a red, yellow, and green colour scale. The respective criteria of greenness are satisfied if the pentagon or hexagon is green. Fig. 6 displays the ComplexGAPI pictogram created for the suggested procedure.

The red color in the first pentagon corresponds to offline sampling since sample were collected from dead body of victims at local hospital and transported to the laboratory where the samples were preserved under refrigerated conditions until analysis. The red color in the second pentagon corresponds to the use of non-green solvents i.e. chloroform and acetone which were used as extraction and disperser solvent in this study. The red color in the central pentagon is due to the analytical method which required extraction.

4.7. Application to real forensic samples

Under its optimized and validated conditions, the proposed VA-DLLME-GC–MS method was successfully used to determine the presence of DDVP in autopsy samples collected from individuals who had died after consuming it, as stated in section 2 (case history). Routine STA of blood samples has detected EtOH at a concentration of 33.25 mg % in case no. 2. TLC was performed for screening of pesticides in tissue samples and DDVP was found positive in both cases. The amount of DDVP detected in blood, stomach contents and liver tissues of both cases is given in Table 3.Table 4 compares the amount of DDVP detected in postmortem specimens by proposed method with those reported in literature for similar fatal cases.

5. Conclusion

In the present study; VADLLME-GC–MS method has been developed and validated for the determination of DDVP in human autopsy samples. The novelty of the proposed method is consumption of least amount of organic solvent and sample, low-cost, simplicity, rapidity and high extraction efficiencies. The method involves homogenization of tissue samples in ACE and further use of this homogenate as disperser solvent in DLLME. The sample preparation and analysis time for a single sample is less than 30 min. The proposed method can be utilized by forensic toxicological laboratories for routine analysis of cases of organophosphorous poisoning.

Compliance with ethical standards

Table 1B
Recoveries of VA-DLLME-GC–MS procedure.

Matrix	Relative recovery (%)			Absolute recovery (%)		
	2	5	10	2	5	10
Blood[a]	88.3	90.6	95	89	99	100.2
Stomach content[b]	84.8	87.0	89.2	111.2	98.6	101.1
Tissue homogenate (Liver)[b]	86.1	89.5	93.4	101.8	104.2	99.2

a = concentration expressed in μg mL^{-1}.
b = concentration expressed in μg/g.

Table 2
Comparison of proposed method with previously reported method for DDVP in biological matrices.

Biological matrices	Extraction technique	Detection technique	Figures of merit	Reference
Blood, urine, stomach content, heart	LLE	HPLC-MS/MS	• Precision: 3.97 % • Accuracy: 93.9 % • LOD: 0.5 μg mL^{-1} • LOQ: 1 μg mL^{-1}	[2]
Blood, heart, kidney, lung	LLE	GC–MS	• Precision: 13.8 % • Accuracy: 100.2 % • Linearity: 1 – 10 μg mL^{-1} • LOD: 0.275 μg mL^{-1} • LOQ: 1 μg mL^{-1}	[3]
Blood, brain, heart, lung, liver, kidney, spleen, urine, stomach content	LLE	GC-FID	• Linearity: 1 – 80 μg mL^{-1}	[5]
Pork	SPE	GC	• Linearity: 0.2 – 1 μg mL^{-1} • Recovery: 100 – 120 % • Precision: 2.6 %	[7]
Animal tissues (pork, muscle and casing)	LLE	LC-MS/MS	• Precision: less than10.6 % • Recovery: 85–106 % • LOD: 0.07 μg kg^{-1} • LOQ: 5 μg kg^{-1} • Linearity: 25 – 500 μg/L	[8]
Blood, stomach content, liver	VA-DLLME	GC–MS	• LOD: 0.17 μg mL^{-1}& 0.33 μg g^{-1} • LOQ: 0.55 μg mL^{-1}& 1.1 μg g^{-1} • Recovery: 86 – 95 % • Precision: less than11.9 % • Linearity: 0.5–10 μg mL^{-1}& 1.5 – 10 μg g^{-1}	This study

Abbreviations: LLE: liquid–liquid extraction; HPLC-MS/MS: high performance liquid chromatography-tandem mass spectrometry; GC–MS: gas chromatography-mass spectrometry; GC-FID: gas chromatography-flame ionization detection; SPE: solid-phase extraction; LC-MS/MS: liquid chromatography-tandem mass spectrometry; VA-DLLME: vortex-assisted dispersive liquid–liquid microextraction.

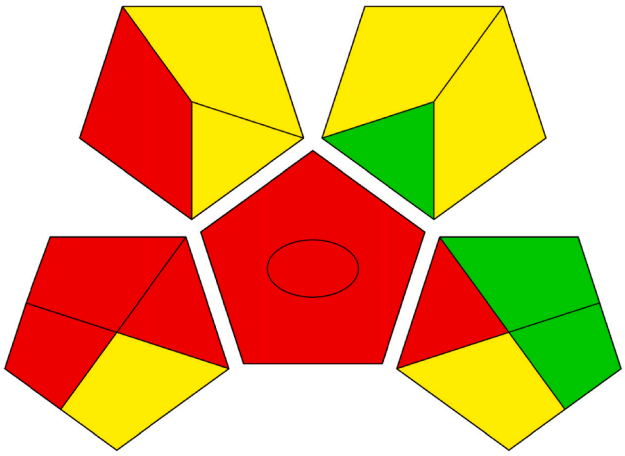

Fig. 6. ComplexGAPI pictogram for proposed procedure.

Table 3
Results of examination of autopsy samples two cases of DDVP fatal intoxication ($n = 3$).

Matrix	Case 1	%RSD	Case 2	%RSD
Blood	8.1 μg mL^{-1}	2.1	11.4 μg mL^{-1}	5.7
Stomach content*	82.2 g	3.4	69.08 g	4.8
Tissue homogenate (Liver)	22.35 μg g^{-1}	2.3	19.89 μg g^{-1}	1.9

Table 4
Comparison of concentration of DDVP found in postmortem specimens from present study with previously reported studies.

Sample	Present Study		Nara *et al*	Abe *et al*	Shimizu *et al*
	Case 1	Case 2	[2]	[3]	[5]
Blood (μg mL^{-1})	8.1	11.4	11.6	4.4	29
Stomach content (g)	82.2	3.4	7.35	38	–
Liver (μg g^{-1})	22.35	19.89	–	ND	20

*ND = Not detected.

The study was adhered to the guidelines of "National ethical guidelines for biomedical and health research involving human participants" issued by ICMR, India.

CRediT authorship contribution statement

Rajeev Jain: Conceptualization, Methodology, Investigation, Formal analysis, Validation, Visualization, Data curation, Project administration, Supervision, Writing – original draft. **Bharti Jain:** Investigation, Methodology, Formal analysis, Data curation, Validation, Software, Writing – review & editing. **Vimukti Chauhan:** Resources. **Bhawna Deswal:** Methodology, Validation. **Sukhminder Kaur:** Resources, Writing – review & editing. **Shweta Sharma:** Project administration, Data curation, Resources, Writing – review & editing, Investigation, Supervision. **Mohammad A. S. Abourehab:** Visualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The data that has been used is confidential.

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References

[1] H. U, Okoroiwu, I. A, Iwara, Dichlorvos toxicity: a public health perspective. *Interdisciplinary Toxicology*, *11* 2 2018 129–137.https://doi.org/10.2478/intox-2018-0009.
[2] A. Nara, C. Yamada, T. Kodama, K. Saka, T. Takagi, Fatal poisoning with both dichlorvos and phenthoate, J. Forensic Sci. 63 (6) (2018) 1928–1931, https://doi.org/10.1111/1556-4029.13781.
[3] E. Abe, C. Duverneuil, G. De La Grandmaison, J.C. Alvarez, A fatal dichlorvos poisoning: Concentrations in biological specimens, J. Forensic Sci. 53 (4) (2008) 997–1000, https://doi.org/10.1111/j.1556-4029.2008.00774.x.
[4] L. Kumar, S.S. Agarwal, K.H. Chavali, S.C. Mestri, Homicide by organophosphorus compound poisoning: a case report, Med. Sci. Law 49 (2) (2009) 136–138, https://doi.org/10.1258/rsmmsl.49.2.136.
[5] K. Shimizu, H. Shiono, T. Fukushima, M. Sasaki, H. Akutsu, M. Sakata, Tissue distribution of DDVP after fatal ingestion, Forensic Sci. Int. 83 (1) (1996) 61–66.
[6] B.K. Binukumar, K.D. Gill, Cellular and molecular mechanisms of dichlorvos neurotoxicity: Cholinergic, nonchlolinergic, cell signaling, gene expression and therapeutic aspects, Indian J. Exp. Biol. 48 (7) (2010) 697–709.
[7] Z. FengYun, G. Chuan, L. Qin, Y. CaiXia, N. Jun, "determination of dichlorvos in pork by solid-phase extraction-gas chromatography" J Food Safety, Quality 14 (2018) 3670–3674.
[8] G.M. Wang, H. Dai, Y.G. Li, X.L. Li, J.Z. Zhang, L. Zhang, Y.Y. Fu, Z.G. Li, Simultaneous determination of residues of trichlorfon and dichlorvos in animal tissues by LC-MS/MS, Food Additives and Contaminants - Part A Chem. Anal. Control, Exposure and Risk Assessment 27 (7) (2010) 983–988, https://doi.org/10.1080/19440041003671270.
[9] J. Xin, X. Qiao, Y. Ma, Z. Xu, Simultaneous separation and determination of eight organophosphorous pesticide residues in vegetables through molecularly imprinted solid-phase extraction coupled to gas chromatography, J. Sep. Sci. 35 (24) (2012) 3501–3508, https://doi.org/10.1002/jssc.201200754.
[10] Z. Xiong, L. Zhang, R. Zhang, Y. Zhang, J. Chen, W. Zhang, Solid-phase extraction based on magnetic core-shell silica nanoparticles coupled with gas chromatography-mass spectrometry for the determination of low concentration pesticides in aqueous samples, J. Sep. Sci. 35 (18) (2012) 2430–2437, https://doi.org/10.1002/jssc.201200260.
[11] Z. Xu, G. Fang, S. Wang, Molecularly imprinted solid phase extraction coupled to high-performance liquid chromatography for determination of trace dichlorvos residues in vegetables, Food Chem. 119 (2) (2010) 845–850, https://doi.org/10.1016/j.foodchem.2009.08.047.
[12] M. Katagi, H. Tsuchihashi, S. Hanada, H. Jinmori, K. Otsuki, Determination of dichlorvos, trichlorfon and their metabolites and degradation products in environmental water, drinks and human urine, Japanese J. Toxicol. Environ. Health 39 (1993) 459.
[13] L. K, Unni*, M. E. H, R. E. B, High-performance liquid chromatographic method using ultraviolet detection for measuring metrifonate and dichlorvos levels in human plasma. *Journal of Chromatography B: Biomedical Applications*, *573* 1992 99–103. https://doi.org/10.1016/0378-4347(92)80480-e.
[14] G. Yuan, R. Zhang, X. Chen, B. Wang, R. Guo, Brief Report A simple and economical method of gas chromatography-mass spectrometry to determine the presence of 6 pesticides in human plasma and its clinical application in patients with acute poisoning, Biosci. Trends 12 (2) (2018) 201–207, https://doi.org/10.5582/bst.2018.01043.
[15] P. S, Adole, S, Bora, V. A, Chaudhari, Clinical utility of validated gas chromatography–ion trap mass spectrometry in patients with anticholinesterase pesticides poisoning. *Analytical Biochemistry*, *621*(October 2020) 2021 https://doi.org/10.1016/j.ab.2021.114158.
[16] R, Jain, R, Singh, A, Kabir, "microextraction techniques in analytical toxicology: an overview" Microextraction Techniques in Analytical Toxicology 1-10 (2021).
[17] R. Jain, R. Singh, Microextraction techniques for analysis of cannabinoids, TrAC - Trends in Anal. Chem. 80 (2016) 156–166, https://doi.org/10.1016/j.trac.2016.03.012.
[18] R. Jain, R. Singh, Applications of dispersive liquid-liquid micro-extraction in forensic toxicology, TrAC - Trends in Anal. Chem. 75 (2016) 227–237, https://doi.org/10.1016/j.trac.2015.07.007.

[19] N.F. El Azab, A validated UHPLC-MS/MS method for simultaneous quantification of some repurposed COVID-19 drugs in rat plasma: Application to a pharmacokinetic study, Microchem. J. 178 (2022), 107321.
[20] S.M.S. Khademi, A. Salemi, M. Jochmann, S. Joksimoski, U. Telgheder, Development and comparison of direct immersion solid phase micro extraction Arrow-GC-MS for the determination of selected pesticides in water, Microchem. J. 164 (2021), https://doi.org/10.1016/j.microc.2021.106006.
[21] D.A. Lambropoulou, T.A. Albanis, Application of hollow fiber liquid phase microextraction for the determination of insecticides in water, J. Chromatogr. A 1072 (1) (2005) 55–61, https://doi.org/10.1016/j.chroma.2004.11.076.
[22] Q. Xiao, B. Hu, C. Yu, L. Xia, Z. Jiang, Optimization of a single-drop microextraction procedure for the determination of organophosphorus pesticides in water and fruit juice with gas chromatography-flame photometric detection, Talanta 69 (4) (2006) 848–855, https://doi.org/10.1016/j.talanta.2005.11.024.
[23] S. Ulrich, Solid-phase microextraction in biomedical analysis, J. Chromatogr. A 902 (1) (2000) 167–194, https://doi.org/10.1016/S0021-9673(00)00934-1.
[24] A, Jain, M, Gupta, KK, Verma "application of single drop microextraction in analytical toxicology" Microextraction Techniques in Analytical Toxicology 2021 131-145.
[25] M. Rezaee, Y. Assadi, M.R. Milani Hosseini, E. Aghaee, F. Ahmadi, S. Berijani, Determination of organic compounds in water using dispersive liquid-liquid microextraction, J. Chromatogr. A 1116 (1–2) (2006) 1–9, https://doi.org/10.1016/j.chroma.2006.03.007.
[26] RR, Jha, Thakur RS, Jain R "dispersive liquid-liquid microextraction and its variants" Microextraction Techniques in Analytical Toxicology 159-170 (2021).
[27] M.K.R. Mudiam, R. Jain, S.K. Maurya, H.A. Khan, S. Bandyopadhyay, R.C. Murthy, Low density solvent based dispersive liquid-liquid microextraction with gas chromatography-electron capture detection for the determination of cypermethrin in tissues and blood of cypermethrin treated rats, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 895–896 (2012) 65–70, https://doi.org/10.1016/j.jchromb.2012.03.015.
[28] M.K.R. Mudiam, R. Jain, A. Singh, H.A. Khan, D. Parmar, Development of ultrasound-assisted dispersive liquid-liquid microextraction-large volume injection-gas chromatography-tandem mass spectrometry method for determination of pyrethroid metabolites in brain of cypermethrin-treated rats, Forensic Toxicol. 32 (1) (2014) 19–29, https://doi.org/10.1007/s11419-013-0196-3.
[29] B. Liu, H. Yan, F. Qiao, Y. Geng, Determination of clenbuterol in porcine tissues using solid-phase extraction combined with ultrasound-assisted dispersive liquid-liquid microextraction and HPLC-UV detection, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 879 (1) (2011) 90–94, https://doi.org/10.1016/j.jchromb.2010.11.017.
[30] X. Liu, J. Hu, C. Huang, H. Wang, X. Wang, Determination of polybrominated diphenyl ethers in aquatic animal tissue using cleanup by freezing-dispersive liquid-liquid microextraction combined with GC-MS, J. Sep. Sci. 32 (23–24) (2009) 4213–4219, https://doi.org/10.1002/jssc.200900480.
[31] W.H. Tsai, H.Y. Chuang, H.H. Chen, J.J. Huang, H.C. Chen, S.H. Cheng, T. C. Huang, Application of dispersive liquid-liquid microextraction and dispersive micro-solid-phase extraction for the determination of quinolones in swine muscle by high-performance liquid chromatography with diode-array detection, Anal. Chim. Acta 656 (1–2) (2009) 56–62, https://doi.org/10.1016/j.aca.2009.10.008.
[32] R. Jain, A. Kumari, I. Khatri, Simple and rapid analysis of acetaminophen in human autopsy samples by vortex-assisted dispersive liquid–liquid microextraction-thin layer chromatography-image analysis, Sep. Sci. Plus 4 (2) (2021) 92–100, https://doi.org/10.1002/sscp.202000092.
[33] M. Akhgari, N.M. Sani, Z. Mousavi, Research Paper: Determination of methadone and tramadol in vitreous humor specimens using dispersive liquid liquid microextraction and ultrahighperformance liquid chromatography, Int. J. Med. Toxicol. Forensic Med. 11 (1) (2021) 1–9.
[34] P. Cabarcos-Fernández, M.J. Tabernero-Duque, I. Álvarez-Freire, A.M. Bermejo-Barrera, Determination of seven antidepressants in pericardial fluid by means of dispersive liquid-liquid microextraction and gas chromatography-mass spectrometry, J. Anal. Toxicol. 46 (2) (2022) 146–156, https://doi.org/10.1093/jat/bkab003.
[35] X. Shen, J. Liang, L. Zheng, H. Wang, Z. Wang, Q. Ji, Q. Chen, Q. Lv, Ultrasound-assisted dispersive liquid-liquid microextraction followed by gas chromatography–mass spectrometry for determination of parabens in human breast tumor and peripheral adipose tissue, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 1096 (2018) 48–55, https://doi.org/10.1016/j.jchromb.2018.08.004.
[36] R. Jain, R. Singh (Eds.), Microextraction Techniques in Analytical Toxicology, CRC Press, 2021.
[37] S. Behera, S. Das, A. Xavier, S. Selvarajan, N. Anandabaskar, Indian Council of Medical Research's National Ethical Guidelines for biomedical and health research involving human participants: the way forward from 2006 to 2017, Perspect. Clin. Res. 10 (3) (2019) 108, https://doi.org/10.4103/picr.picr_10_18.
[38] E. Yiantzi, E. Psillakis, K. Tyrovola, N. Kalogerakis, Vortex-assisted liquid-liquid microextraction of octylphenol, nonylphenol and bisphenol-A, Talanta 80 (5) (2010) 2057–2062, https://doi.org/10.1016/j.talanta.2009.11.005.
[39] Y. Zhang, H.K. Lee, Determination of ultraviolet filters in water samples by vortex-assisted dispersive liquid-liquid microextraction followed by gas chromatography-mass spectrometry, J. Chromatogr. A 1249 (2012) 25–31, https://doi.org/10.1016/j.chroma.2012.06.019.
[40] P. Haefelfinger, Limits of the internal standard technique in chromatography, J. Chromatogr. A 218 (1981) 73–81.
[41] O. Yoo, E.K.Y. Tang, M.N. Nguyen, S. Salman, A.J. Hua, B.S. von Ungern Sternberg, L.Y. Lim, HPLC-UV assay of tramadol and O-desmethyltramadol in human plasma containing other drugs potentially co-administered to participants in a paediatric population pharmacokinetic study, J. Chromatogr. B 1184 (2021), 122971.
[42] P. Franco, E. Porru, J. Fiori, A. Gioiello, B. Cerra, G. Roda, C. Caliceti, P. Simoni, A. Roda, Identification and quantification of oxo-bile acids in human faeces with liquid chromatography–mass spectrometry: a potent tool for human gut acidic sterolbiome studies, J. Chromatogr. A 1585 (2019) 70–81.
[43] J. Płotka-Wasylka, W. Wojnowski, Complementary green analytical procedure index (ComplexGAPI) and software, Green Chem. 23 (21) (2021) 8657–8665, https://doi.org/10.1039/d1gc02318g.
[44] J. Płotka-Wasylka, A new tool for the evaluation of the analytical procedure: green Analytical Procedure Index, Talanta 181 (2018) 204–209, https://doi.org/10.1016/j.talanta.2018.01.013.
[45] A. E, Allam, *Green Chemical Analysis and Sample Preparations* (R. N. El-Shaheny, Mahmoud H. El-Maghrabey V. Sivasankar (ed.); Issue June). Springer US. 2022 https://doi.org/10.1007/978-3-030-96534-1.
[46] M. Gamal, I.A. Naguib, Analytical Methods Comparative study of four greenness assessment tools for selection of greenest analytical method for assay of hyoscine N -butyl bromide †, Anal. Methods 369–380 (2021) https://doi.org/10.1039/d0ay02169e.
[47] K.P. Kannaiah, A. Sugumaran, H.K. Chanduluru, S. Rathinam, Environmental impact of greenness assessment tools in liquid chromatography – A review, Microchem. J. 170 (2021), 106685, https://doi.org/10.1016/j.microc.2021.106685.
[48] M. Sajid, P. Justyna, Green analytical chemistry metrics : A review, Talanta 238 (2021), https://doi.org/10.1016/j.talanta.2021.123046.
[49] B. Jain, R. Jain, A. Kabir, S. Sharma, Rapid determination of non-steroidal anti-inflammatory drugs in urine samples after in-matrix derivatization and fabric phase sorptive extraction-gas chromatography-mass spectrometry analysis, Molecules 27 (21) (2022) 7188.
[50] B. Jain, R. Jain, R.R. Jha, A. Ghosh, D. Basu, M.A.S. Abourehab, A. Bajaj, V. Chauhan, S. Kaur, S. Sharma, Cellulose paper sorptive extraction (CPSE): A simple and affordable microextraction method for analysis of basic drugs in blood as a proof of concept, J. Chromatogr. B (2022), 123551.
[51] B. Jain, R. Jain, R.R. Jha, A. Bajaj, S. Sharma, A green analytical approach based on smartphone digital image colorimetry for aspirin and salicylic acid analysis, Green Analytical Chem. 3 (2022), 100033.
[52] A.M. Hegazy, A.S. Batubara, M.A. Abdelgawad, M. El-Sherbiny, M.M. Ghoneim, A. M. Ahmed, M. Gamal, Recommended and verified stability indicating GC–MS procedures for green separation of quaternary mixture of naphazoline, ephedrine, methylparaben, and naphazoline impurity, Microchem. J. 183 (2022), 108058.
[53] A.H. Almalki, I.A. Naguib, F.S. Alshehri, B.S. Alghamdi, H.O. Alsaab, Y. S. Althobaiti, S. Alshehri, F.F. Abdallah, Application of Three Ecological Assessment Tools in Examining Chromatographic Methods for the Green Analysis of a Mixture of Dopamine, Serotonin, Glutamate and GABA: A Comparative Study, Molecules 26 (18) (2021) 5436.