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Effect of dissociation energy on ion formation and sensitivity of an analytical method for determination of chlorpyrifos in human blood, using gas chromatography–mass spectrometer (GC–MS in MS/MS)

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Abstract

A sensitive GC–MS method in MS/MS ion preparation was developed for quantitative estimation and qualitative determination of chlorpyrifos in human blood samples. This paper describes the combined effect of dissociation energy on ion formation of chlorpyrifos and sensitivity of this analytical method. Chlorpyrifos was extracted using methanol/hexane mixture from 0.2 ml human blood, deactivated with saturated acidic salt solution. The extract was then re-concentrated and analyzed by electron impact (EI) MS/MS gas chromatography–mass spectrometer. The MS/MS spectra of chlorpyrifos ion were recorded on different dissociation energy (30–100 V) to establish the structural confirmation and to demonstrate the effect of this energy on sensitivity, *S*/*N* ratio and detection limit for quantification of chlorpyrifos, which is first time reported. At different exciting amplitude (30–100 V), different behaviors of base peak, sensitivity, *S*/*N* ratio and detection limit of this method were observed for quantification of chlorpyrifos. The mass spectra recorded at dissociation energy <70 V, in between 70–80 V and >80 V correspond to the *m*/*z* 314 (100%), *m*/*z* 286 (100%) and *m*/*z* 258 (100%), respectively. The sensitivity, signal and detection limit of this method increased on 95 V at *m*/*z* 258. Therefore, *m*/*z* 258 was used for the quantification of chlorpyrifos. The detection limit for quantification was 0.1 ng/ml with *S*/*N*: 2 in human blood. The linear calibration curve with the correlation coefficient ($r > 0.99$) was obtained. The percentage recoveries from 95.33% to 107.67% were observed for chlorpyrifos from human blood. The blood samples were collected at different time intervals. The concentration of chlorpyrifos in poisoning case was 3300, 3000, 2200, 1000, 600, 300 ng/ml on day 1, 3, 6, 8, 10 and 12, respectively. On the 12th day of exposure of chlorpyrifos, 90.9% reduction in concentration was observed. On day 14th the chlorpyrifos was not detected in the blood of the same subject. Thus the present method is useful for detection of chlorpyrifos in critical care practices and also provides tremendous selectivity advantages due to matrix elimination in the parent ion isolation step in blood sample analysis for chlorpyrifos.

Keywords: GC–MS/MS; Chlorpyrifos; Dissociation energy; Human blood

1. Introduction

Chlorpyrifos [*O*,*O*-diethyl *O*-(3,5,6-trichloro-2-pyridinyl) phosphorothioate) is a widely used broad-spectrum organophosphorus insecticide [1]. Chlorpyrifos gets easily oxidized to its metabolites Oxon [*O*,*O*-diethyl *O*-(3,5,6-trichloro-2-pyridyl) phosphate] and TCP [3,5,6-trichloro-2-pyridinol] [2]. It was introduced in 1965 [3] and now a day's chlorpyrifos is one of the most commonly used pesticides throughout the word. Approximately 21–24 million pounds are used annually in the USA [4], and in India according to the Central Insecticide Board, New Delhi, approximately 1400 to 4500 tonnes are used over the same period. As the government of India has banned the chlorinated hydrocarbon insecticides like Aldrin, Chlordane, DDT, BHC and recommended chlorpyrifos as an alternate, the use of chlorpyrifos has increased. It is reported to be moderately toxic to humans [5]. Excessive exposure to chlorpyrifos can produce symptoms typical of acute organophosphate poisoning. Poisoning from chlorpyrifos may affect the central nervous system, the cardiovascular system, and the respiratory system [6], and also

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act as skin and eye irritant [7]. In India the cases of chlorpyrifos poisoning are increasing day-by-day [8]. A number of analytical methods such as gas chromatographic (GC) [9], negative-ion chemical ionization gas chromatography–mass spectrometry method [10], gas chromatography–electron capture detector (GC–ECD) [11], normal-phase liquid chromatography [12] and reversed-phase simple and rapid high-performance liquid chromatographic (HPLC) [13–15] was reported for the determination of the chlorpyrifos and their metabolites in biological samples. In addition to this, the gas chromatography–mass spectrometry (GC–MS) using electron impact mode [16] and the negative-ion chemical ionization gas chromatography–mass spectrometry (NCI-GC–MS) [17] methods were used for determination of chlorpyrifos and its metabolites in biological samples. They however, are not always useful for confirmation and quantification in emergency cases. None of the methods gives the combined picture of confirmation, ion formation, effect of dissociation energy on fragmentation schemes, sensitivity, detection limit and *S/N* ratio for the quantification of chlorpyrifos in blood samples. Therefore, our aim was to develop a more reliable and sensitive method for confirmation and quantification of chlorpyrifos and to demonstrate the effect of dissociation energy on ion formation and sensitivity of analytical method.

2. Experimental sections

2.1. Materials

Chlorpyrifos standard was obtained from Accu Standard Ltd., USA. HPLC grade methanol, toluene, hexane and ethyl acetate were purchased from Sigma–Aldrich, Mumbai, India. The analytical grade reagents were procured from Qualigens Fine Chemicals, Mumbai, India. A stock solution of 2000 ng/ml of chlorpyrifos standard was prepared in methanol. The concentration ranges of 0.1–1000 ng/ml were used for the development and standardization of this method.

2.2. Blood samples

Two to three millilitres of (control) blood samples (control subjects) were obtained from non-poisoning cases after taking informed consent from male and females (age group 18–55 years) through vein-puncture method. The control subjects were mainly the healthy attendants of the poisoning patients. The study blood samples were collected from the poisoning patients admitted to different hospitals of Ahmedabad (Gujarat) India. Blood samples were collected in heparinized vials on day 1, 3, 6, 8, 10, 12 and 14.

2.3. Extraction of chlorpyrifos from blood samples

The extraction of chlorpyrifos was carried out as per the reported method [17] with slight modifications. These modifications were in quantity of solvent used for extraction, quantity of blood samples, use of low speed rotorac for extraction and time required for mixing of analytes with vortex.

For hydrolysis, 175 μl acetic acid saturated salt solution (2.5 N acetic acid with sodium chloride solution) was added in 0.2 ml heparinized blood in 10 ml glass vial and the solution was mixed on a vortex mixture for 10 min. The analytes were extracted with 0.75 ml methanol and 4.5 ml hexane using low speed Rotorac shaker for 1 h. Hexane layer was separated by centrifugation (20 min at 1640 × *g*). The extract was dried under gentle stream of N_2 gas and the residue was reconstituted in 50 μl toluene.

2.4. GC–MS study

Extract of chlorpyrifos was analyzed on Varian CP-3800 gas chromatography equipped with Saturn 2000 (Varian Pvt. Ltd., USA) MS/MS ion trap mass detector. The data system contains all the software required for calibration, collection of GC–MS spectra and data processing for qualitative and quantitative analysis. Also having National Institute of Standards and Technology (NIST) library with more than one hundred and fifty thousand mass spectra for standard compounds [18].

Separation was achieved on high-resolution DB-5 MS capillary column (30 m × 0.25 mm i.d., 0.25-μm-film thickness (Varian Pvt. Ltd., USA). Ultra pure helium (99.999%) gas at a flow rate of 1 ml (10 psi head pressure) was used as a carrier gas for recording the mass spectra. The GC oven temperature was set at 80 °C for 5 min, programmed from 80 to 250 °C at 15 °C/min and hold for 20 min. The injection port temperature was 250 °C. The injector port was initially on (split ratio 10), then off and after that the split state was on for 1 min (split ratio 10). The total running time was 36.33 min and 1 μl analyte was injected for the analysis.

2.5. MS in scan mode

The MS first time was operated in the full-scan EI mode. The mass range was 50–500 amu (μ) with 1 s/scan with zero thresholds. The emission current of the ionization filament was set 10 mA generating electrons with 70 eV Energy. The transfer line, trap temperature and manifold were set at 270, 150 and 40 °C, respectively. The used electron multiplier voltage was 1500–1600 V for scanning the spectra.

2.6. MS/MS study

In order to develop a more sensitive method for determination of concentration of chlorpyrifos in blood samples, the MS method was first attempted in GC–MS with electron impact auto ionization in full scan mode. Interfering peaks from blood matrices, poor chromatograms due to peak tailing, peak broadening, and poor sensitivity complicated this method at ppb levels. Therefore, a more sensitive MS/MS method was developed for confirmation and to increase the sensitivity for low-level quantification of chlorpyrifos in blood samples.

Mass spectral analysis was performed in the electron impact (EI) mode with MS/MS ion preparation by using mass range 40–355 amu with background mass of 40 amu and scan rate equal to 0.50 s/scan. Axial modulation voltage and emission

current of the ionization filament set for MS/MS were 4 V and 30 mA, respectively. The parent ion storage was *m/z* 314 μ and used excitation storage level (storage RF) was 139 μ while the isolation window for the spectra was 3 *m/z*. The non-resonant MS/MS parameters used for selective fragmentation of parent ion chlorpyrifos analysis. The segment starting time for chlorpyrifos was 16.3 min and ending time was 16.5 min.

To demonstrate the effect of dissociation energy on relative abundance of molecular ions as well as fragment ions in MS/MS mode, the spectra of this compound was recorded on different dissociation energy (30–100 V), injecting similar concentration of analyte. Similarly, the MS/MS spectra of chlorpyrifos were recorded on different dissociation energy (30–100 V) on injecting same concentration to improve the signal, sensitivity and level of detection of this analytical method.

Calibration curves were constructed using area count and plotted against different concentrations range from 0.1 to 20 ng/ml. Similarly, the *S/N* ratios were also plotted against different concentrations. The percentage recovery of this method was determined using 0.1–20 ng/ml of chlorpyrifos.

3. Results and discussion

3.1. Qualitative MS/MS spectral analysis of chlorpyrifos

The MS spectrum of chlorpyrifos in scan mode is shown in Fig. 6(a) for comparison with MS/MS spectra, The full scan spectrum gives ion at *m/z* 352, 350, 314, 286, 258, 199 and 198. The presence of chlorpyrifos in blood samples was also confirmed by NIST library that contains more than one hundred and fifty thousand mass spectra of standard organic compounds [18].

The different behaviors of chlorpyrifos recorded on different dissociation energy are shown (Fig. 1). These results reveal three regions in the MS/MS spectra of chlorpyrifos in relation to percentage relative abundance of base peak and fragment ion peaks.

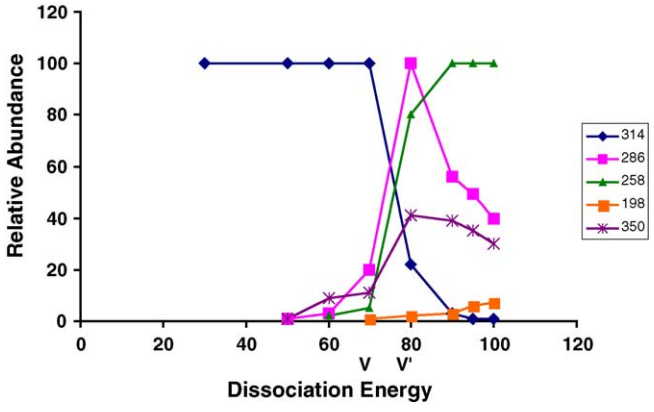

Fig. 1. Effect of the different dissociation energy (30–100 V) on ion formation of chlorpyrifos recorded using GC/MS–MS spectrometer in MS–MS mode. At low dissociation energy (<70 V), the base peak obtained at *m/z* 314. In between 70 and 80 V, the *m/z* at 286 behaved as a base peak. The peak at *m/z* 258 represents base peak on 95 V and used for quantification.

In region-1, dissociation energy lower than *V* (<70 V), a fragment ion at *m/z* 314 (100%) is obtained with no other fragment ion. This region in range of 50–70 V still shows the base peak at *m/z* 314 with certain fragment ions. Region-II, in the range between *V* and *V*′ (70–80 V) the *m/z* at 286 (100%) behaves as a base peak, however in region III, the condition is greater than >*V*′ (>80 V), the base peak at *m/z* 258 (100%) is obtained with several fragment ions.

The MS/MS spectra of chlorpyrifos in study samples are illustrated in Fig. 2(a)–(c) to understand the dissociation energy response on fragmentation behaviors of the compound. In Fig. 2a base peak is obtained at *m/z* 314 [$M^+ - HCl$] (100%) on used energy in between 30 and 70 V. The spectra recorded on 80 V has shown in Fig. 2(b) suggest that the *m/z* at 286 (100%) behaved as a base peak, besides this peak the ions at *m/z* 350 (4%), 352 (2%), 313 (4%), 314 (73%), 258 (81%), 204 (3%), 198 (2%) are also obtained. However, the spectra recorded on 95 V, the base peak at *m/z* 258 (100%) is obtained with the fragment ions at *m/z* at 350 (35%), 352 (36%), 314 (1%), 298 (5%), 294 (11%), 286 (49%), 206 (12%), 198 (2%).

This study suggests that the base peak changes with the change in dissociation energy condition, which may be due to the fact that the MS/MS parameters are obtained by using the Toolkit software. The Toolkit optimization procedure allows changing the energy on a scan-by-scan basis to optimize the analysis. The experiments conducted at source rf values corresponding to qz = 0.4 were calculated by the software. Therefore, only dissociation energy was changed during each acquisition. MS/MS non-resonance waveform in the Toolkit software was applied for significance of the acquisition.

From this study we conclude that the MS/MS operated at 95 V in region III is very useful for structural conformation of chlorpyrifos. In this study, the quantification and identification by the MS/MS method is achieved by the selection of three major ions related to chlorpyrifos 'three ion criteria' principle. For chloripyrifos *m/z* 314, 286 and 258 were isolated and assigned for confirmation while the *m/z* 258 was used for quantification. Similar type of study has been reported [19].

The ion formation of study sample is shown in Fig. 3. Protonated molecular ion peak corresponds at *m/z* 350 $(M+H)^+$ while the isotopic peak due to Cl^{37} was obtained at *m/z* 352. The *m/z* at 314 (scheme I, structure I) was due to the elimination of HCl from *m/z* 350, because three chlorine atoms are attached on 3, 5, 6 position of pyridinyl ring, which is deactivating group. Hence an unstable aryne like structure I was formed due to benzyne mechanism or cine substitutions. The *m/z* at 286 (II) and 258 (III) were obtained due to removal of ethylene molecule from *m/z* 314 to 286 (scheme I). The chloripyrifos possesses a sufficiently long chain to permit transfer of a β-hydrogen atom namely loss of ethylene with hydrogen rearrangement form through a three-member intermediates; similar fragmentation pattern noted previously with phenolate ion [20]. The three-member ring with phosphorous and oxygen may also be formed at *m/z* 258 (structure IV, scheme II) due to transfer of free radical hydrogen atom from methyl group to the 4 and 5 position of benzyne structure I (*m/z* 314). In scheme III, the

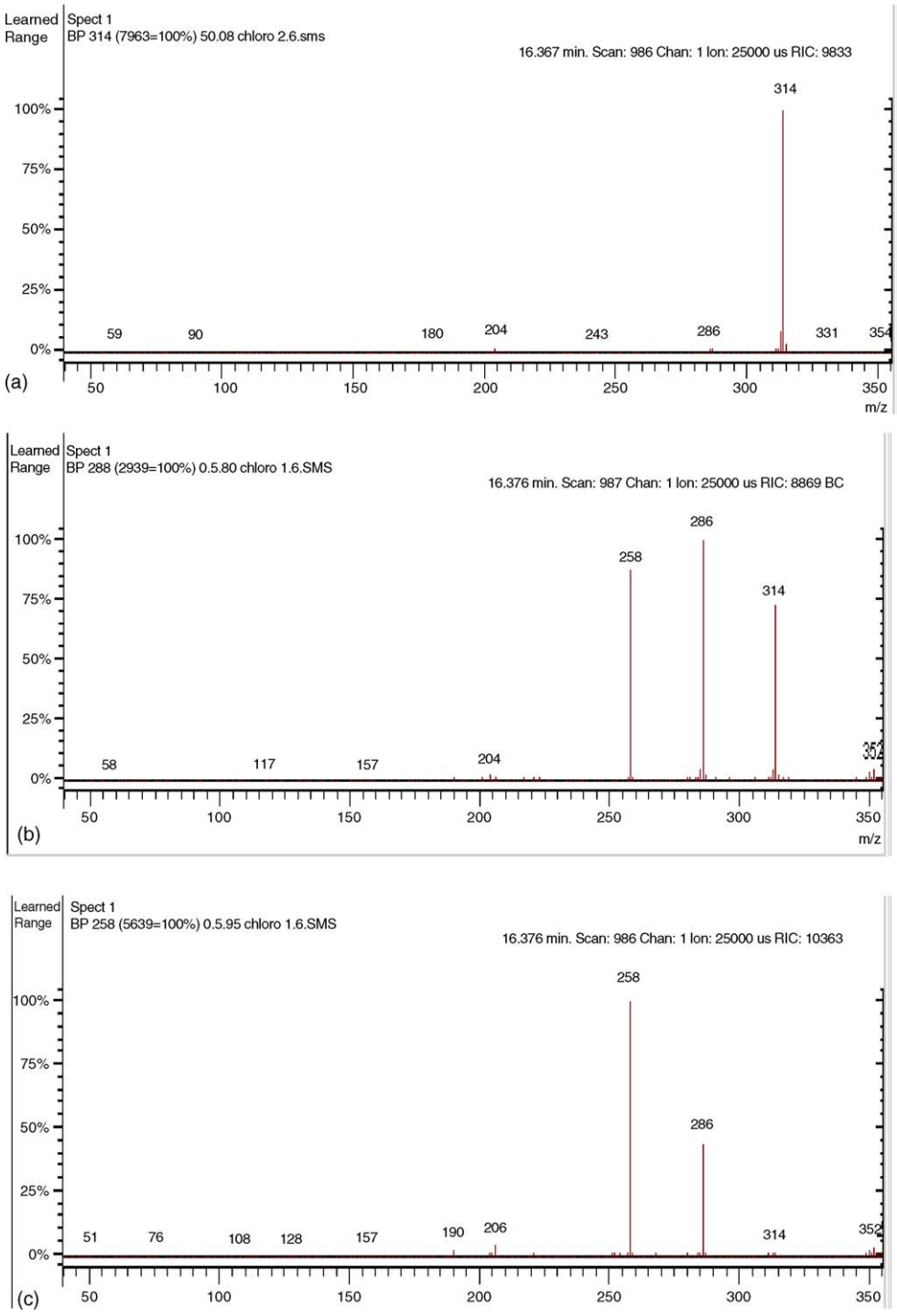

Fig. 2. (a) MS/MS Spectra recorded for chlorpyrifos on 70 V. (b) MS/MS Spectra recorded for chlorpyrifos on 80 V. (c) MS/MS Spectra recorded for chlorpyrifos on 95 V.

m/*z* at 258 (V) was formed due to elimination of two molecule of ethyl alcohol from $[M+1]^+$. The elimination occurred due to homolytic fission with free radical rearrangement and also may be hydride ion transfer with rearrangement (scheme III). The phosphorus (P) atom contains vacant d-orbital hence it formed dπ–Pπ bond with oxygen atom as per the reported bonding [21]. Due to formation of dπ–Pπ bonding the electron density is maximized on P atom. Hence the elimination of ethyl alcohol occurred. The *m*/*z* 198 is due to expulsion of complete side chain from *m*/*z* 350 leading the formation of TCP (VI) a stable metabolite of chlorpyrifos. The formation of TCP is due to the homolytic fission (scheme IV).

We are concluding from fragmentation schemes that the one stable new structure may be formed at *m*/*z* 258 (scheme I, structure III and scheme II, structure IV). Similarly the benzyne like very unstable structure may also be formed.

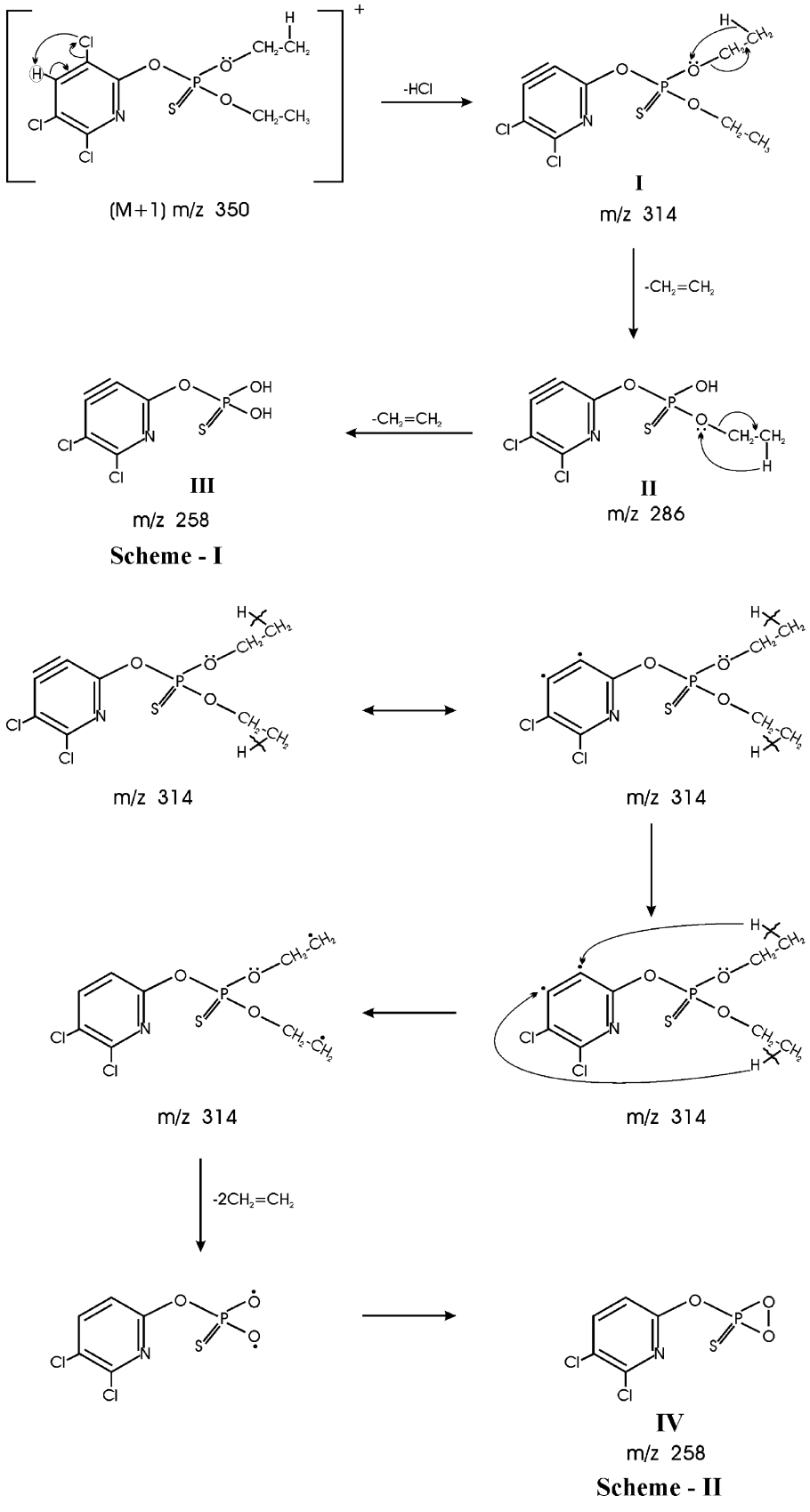

Fig. 3. Illustration of ion formations of chlorpyrifos.

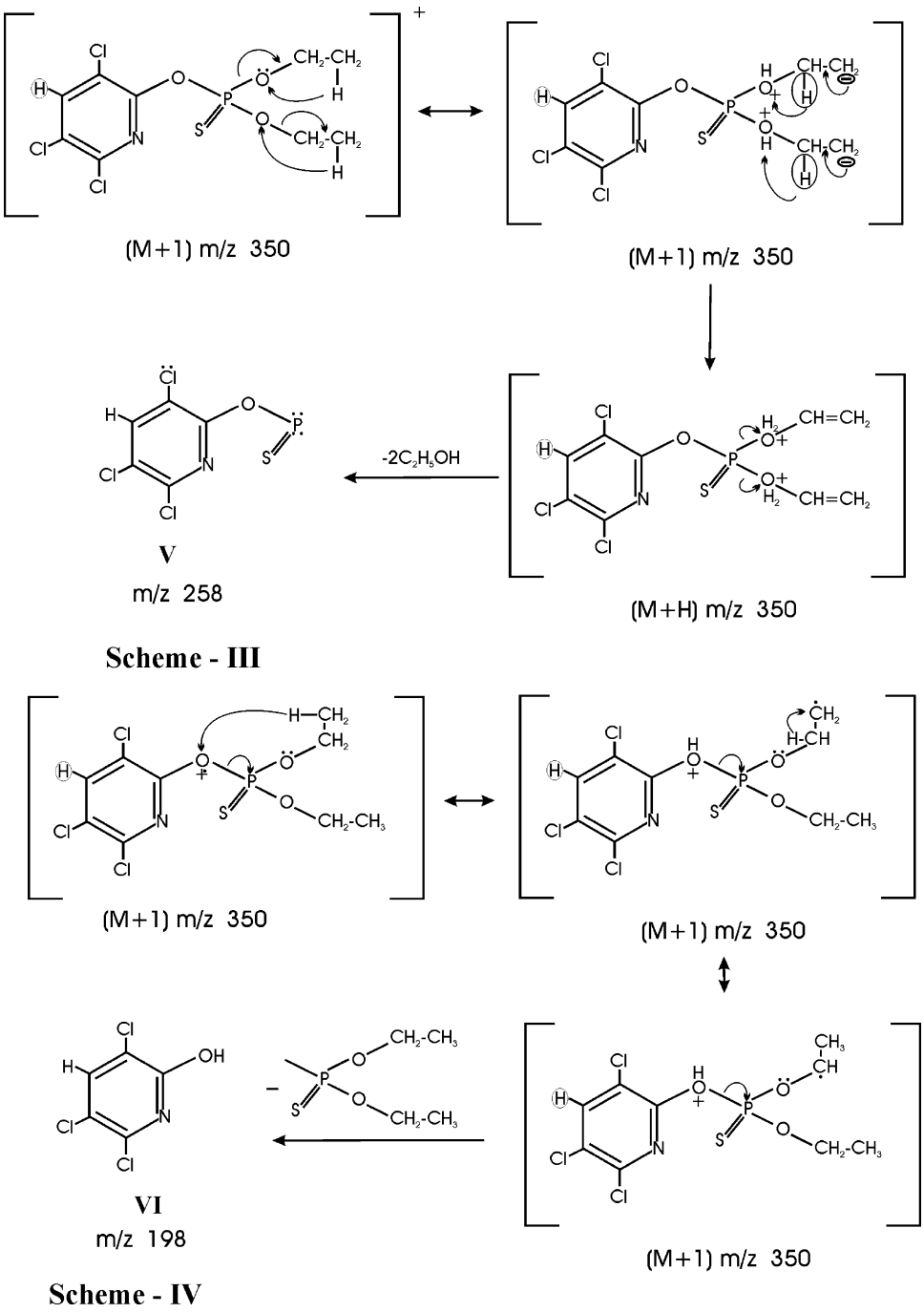

Fig. 3. (*Continued*).

3.2. Quantification of chlorpyrifos in blood samples

Slight modifications were made in the reported method [17] for the extraction of chlorpyrifos from blood and these modifications include 0.2 ml blood instead of 0.5 ml blood for analysis of chlorpyrifos, the quantity of solvent used for extraction is three times more than reported method for better extraction, use of low speed rotorac for extraction and time required for mixing of analyte with vortex.

The MS/MS spectra recorded on different collision dissociation energy (30–100 V) and the *S/N* ratio were calculated by software on each condition. The obtained *S/N*; were 7; 10; 13; 14; 17; 21; 24; on used 30; 40; 50; 60; 70; 80; 95 V dissociation energy, respectively, on injection of 1 μl from 100 ng/ml concentration of chlorpyrifos (Fig. 4). Maximum sensitivity was observed on 95 V. The increased sensitivity and signal of this method were due to the complete elimination of blood matrices and the stable ion produced at *m/z* 258 (Fig. 3, scheme I, structure III and scheme II, structure IV). Therefore, the *m/z* 258 (base peak) was used for quantification of chlorpyrifos in blood samples. Similarly the *S/N*: 3; 6; 37; 59; were obtained on 95 V, on injecting the 1 μl of different concentrations (1–1000 ng/ml)

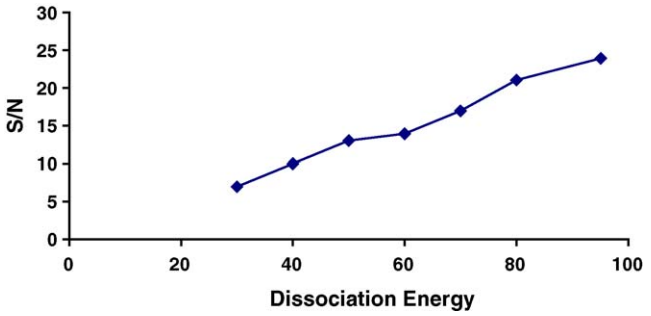

Fig. 4. Effect of different dissociation energy (30–100 V) on sensitivity, signals and detection limit of this method for chlorpyrifos in MS/MS mode injecting on 1 μl volume of 100 ng/ml concentration.

of chlorpyrifos and the correlation diagram was plotted between concentrations versus *S/N* ratios, showed significant linear correlation $R^2 = 0.98$. Calibration curves were constructed for the chlorpyrifos based on their optimized MS/MS conditions. External standard quantification was performed on the characteristic fragment ion at *m/z* 258 (on 95 V) produced from the parent ion. A five-point calibration curve was constructed using area count plotted against concentrations 0.1, 0.2, 1, 2 and 20 ng/ml. A correlation coefficient of $r > 0.99$ with an equation of $Y = 387.78x + 125.1$ was obtained. The human control blood was spiked with standard of chlorpyrifos in the concentration range 0.1–20 ng/ml. The mixed solvent of methanol and hexane resulted in absolute extraction efficiency of 96% for chlorpyrifos from blood. The lowest spike level was 0.1 ng/ml blood for chlorpyrifos and the detection limit for this compound was 0.1 ng/ml with *S/N* ratio 2. The chromatograms of spiked, blank blood, study sample and standard is shown in Fig. 5(a)–(d). The retention time of chlorpyrifos was at 16.367 min with 0.21% relative standard deviation (R.S.D.). To determine the percentage recovery the control human blood was spiked with 0.1–20 ng/ml of chlorpyrifos triplicate and analyzed. The recoveries of this analytical method were 95.33–107.67%. The precision of this method for chlorpyrifos was found excellent with coefficients of variation ranging from 1.58% to 3.53%. Inter-day variability of five separate analyses is shown in Table 1. The concentration ranges from 0.1 to 20 ng/ml given average

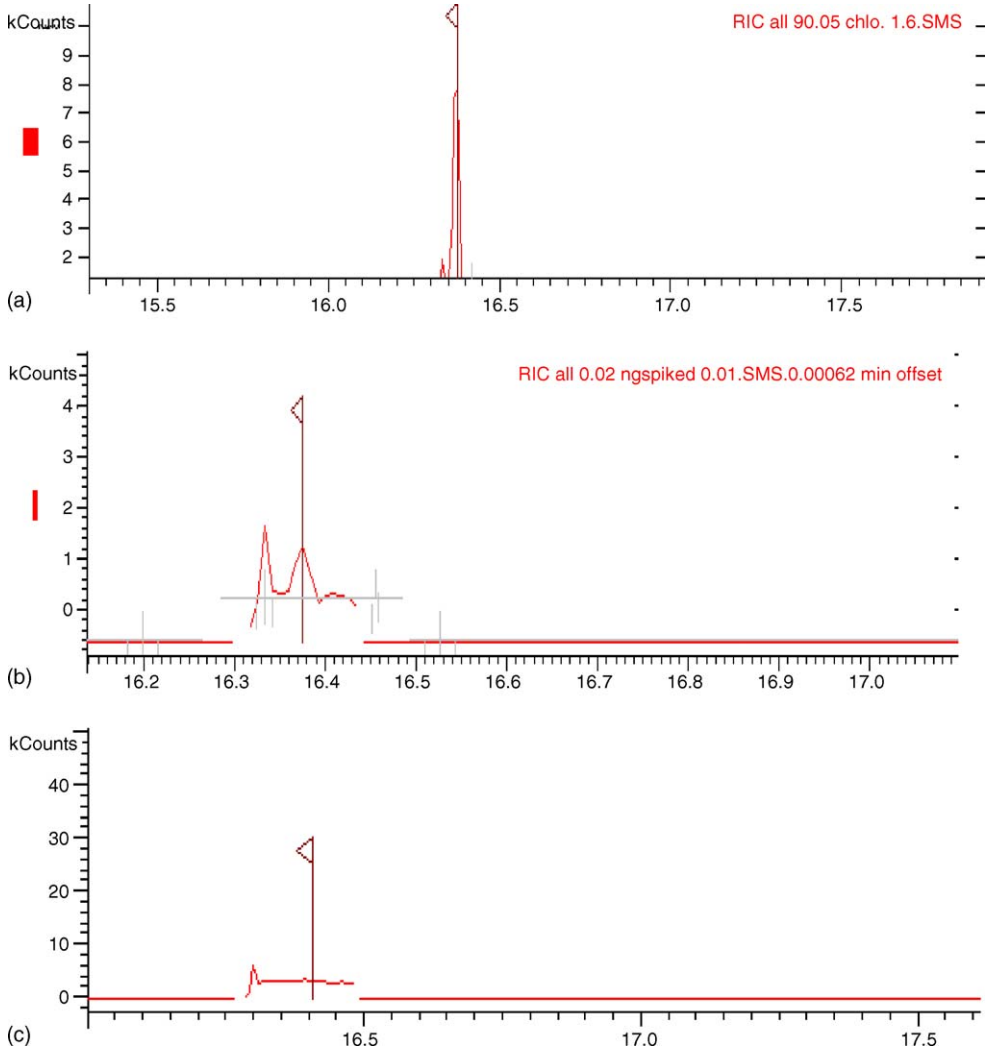

Fig. 5. (a) Chromatograms of chlorpyrifos standard with retention time 16.376. (b) Chromatograms from an extract of human blood fortified with 0.1 ng/ml concentration of chlorpyrifos. (c) Chromatogram of blood control.

Table 1
Inter-day recovery of chlorpyrifos form fortified human blood samples

Concentration (ng/ml)	Recovery (%)		*n*
	Mean ± S.D.	CV	
0.1	96.01 ± 2.72	2.72	5
0.2	103.40 ± 3.05	3.05	5
2	98.49 ± 1.77	1.85	5
20	107.50 ± 1.69	1.57	5

S.D.: standard deviation; CV: coefficient of variation; *n*: number of samples.

recoveries range 96.01–107.50%. The precision of this analyse was good with 1.57–2.72% R.S.D. The blood samples containing 0.1 ng/ml yielded a base peak for chlorpyrifos at *m/z* 258 with *S/N*; 2 (Fig. 6). Based on these data the limit of quantification (LOQ) for chlorpyrifos was set 0.1 ng/ml with *S/N*; 2. Due to unavailability of the environmental and occupational chlorpyrifos exposed samples this method was applied on poisoning cases. The chlorpyrifos concentration in human blood of poisoning case is shown (Fig. 7). The concentration was 3300, 3000, 2200, 1000, 600 and 300 ng/ml on day 1, 3, 6, 8, 10 and 12 of exposure. The result also revealed that the 90.9% reduction in concentration of chlorpyrifos was observed on 12th day of poisoning. There was a continuous reduction of the chlorpyrifos until its concentration below detection limit on the 14th day after exposure. The results revealed that there was a drastic reduction in the chlorpyrifos concentration on 14th day of exposure. This may be due to the transformation of this compound in to its metabolite Oxon and TCP. This type of observation was

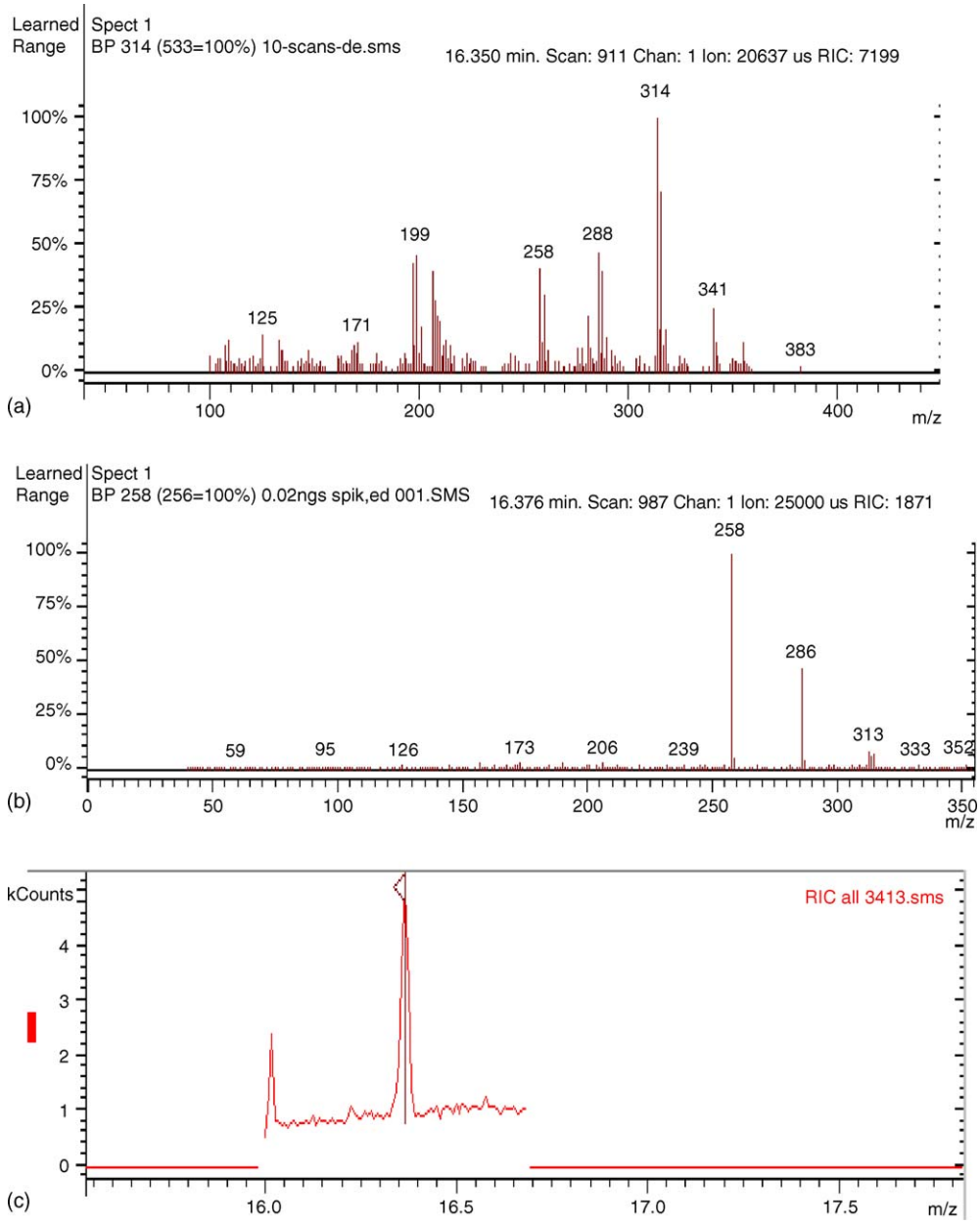

Fig. 6. (a) MS spectrum in full scan mode of chlorpyrifos with ions *m/z* 350, 286, 260, 258, 199,198. (b) MS/MS Spectrum of an extract of human blood fortified with 0.1 ng/ml concentration of chlorpyrifos. (c) The GC chromatogram of study sample of poisoning case with retention time 16.376.

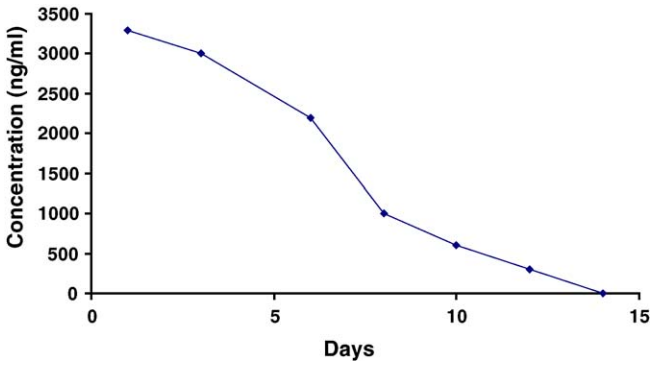

Fig. 7. Chlorpyrifos concentrations profiles in blood sample for poisoning case taken at different days interval.

also supported by fragmentation schemes with ion formation (Fig. 3).

Some of the reported methods for the quantification of chlorpyrifos are summarized for comparison (Table 2). The table showed that the method reported by different workers has high detection limit with less percent recovery. Very few methods have been used for Chlorpyrifos quantification at ppt levels in biological samples. Most of the analysis for organophosphorus compounds like chlorpyrifos are conducted with flame photometric detection (FPD) [6,7] and electron capture detector (ECD) [11]. However, these methods do not allow the structural confirmation that can be obtained with this analytical method using GC–MS/MS. One of the limitations to the quantification using SIM mode is the unequivocal identification of analyte in human blood samples due to endogenous matrix interferences with less fragmentation. Analysis in MS/MS mode of the blood samples resulted in elimination of the interfering peaks and the broad background chemical noise seen in SIM mode [17]. Our method provides better detection limit than reported method [17]. The analysis of chlorpyrifos using high-resolution gas chromatography mass spectrometry [22], solid-phase organophosphorous compounds with tandem mass spectrometry [23] and full-scan gas chromatography mass spectrometry [24] is reported for the quantification of chlorpyrifos in human plasma, serum and water samples, respectively. However, these methods do not provide the ion formation with new structure with respect to each peak obtained in mass spectra and isolated ion, which is formed at *m/z* 286 and 258 with three principle ion criteria. The all methods reported in Table 2, do not establish the relationship between dissociation energy with ion formations (Fig. 1) and *S/N* ratio (Fig. 4). But our method established a direct relationship between dissociation energy with ion formations and Peak signal. Therefore, the MS/MS analytical method for structural confirmation, ion formation and quantification at ppt level for chlorpyrifos was developed.

Table 2
Analytical methods for quantification of chlorpyrifos

Sample	Isolation method	Analytical method	Detection limit	Reference number
Blood	Extraction with acetone and hexane followed by clean-up using silica gel	GC/FPD	No data	[9]
Plasma	Extraction using hexane	GC/ECD	50 ng/ml	[11]
Serum	Extraction with hexane	GC/MS	No data	[16]
Blood	Extracted with ethanol and hexane mixture	NCI-GC–MS	1 ng/ml	[17]
Serum	Solid-phase extraction	GC–MS/MS	0.5 ng/ml	[23]
Serum	Solid-phase extraction	HR-GC–MS/MS	Pg/g	[22]

4. Conclusion

A EI-GC–MS/MS method was developed for the qualitative and quantification of chlorpyrifos at ppt level. The sensitivity, signal and low-level detection limit for chlorpyrifos was increased unambiguously using EI-GC–MS/MS spectrometer. The structural confirmation was also determined unambiguously using this method. The MS/MS spectra were recorded on different dissociation energy (30–100 V), and one parent ion at *m/z* 314 and two daughter ion at *m/z* 286 and 258 have been isolated, on 95 V, the *m/z* 258 behaved as a base peak with new stable structure obtained during fragmentation and on this energy level, the sensitivity, signal and detection limit of this analytical method were increased for chlorpyrifos. Therefore, *m/z* 258 was used for quantification of chlorpyrifos in human blood. The percentage recovery for chlorpyrifos was 95.33–107.67% with an excellent precision. The calibration curve for quantification was linear with coefficient correlation $r > 0.99$. Similarly the linear plot was also obtained between concentrations versus *S/N* ratio for chlorpyrifos ($R^2 = 0.99$). Even some laboratories may desire to use GC–FPD. It has been shown that EI-MS/MS analysis provides much better sensitivity and selectivity for the detection of chlorpyrifos in human blood than GC–MS-CI. Therefore, a change in dissociation energy may result in significantly higher detection limits for the analytes.

This analytical method may be useful unambiguously to qualitative and quantitative estimation of chlorpyrifos in human blood at levels that are very important for biological relevance.

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