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Effect of dissociation energy: Signal to noise ratio on ion formation and sensitivity of analytical method for quantification and confirmation of triazofos in blood samples using gas chromatography–mass spectrometer (GC–MS/MS)

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ABSTRACT

The paper describes the effect of dissociation energy on ion formation and sensitivity of triazofos in blood samples. Six millilitres hexane was used for the extraction of triazofos from 2 mL serum samples. The extract was reconstituted in 1 mL hexane and analyzed by GC–MS/MS in electron impact MS/MS mode. The structure, ion formations, nature of base peak and fragmentation schemes were correlated with the different dissociation energies. The new ion was obtained at mass to charge ratio 161 (100%), which was the characteristic ion peak of triazofos. On using different exciting amplitudes different behaviours of fragmentation schemes were obtained. The effect of dissociation energy on sensitivity of the analyte was also demonstrated. The mass spectra recorded at different exciting amplitudes <50 V in between 50–60 V and >60 V correspond to the m/z 161 (100%), 77 (100%) and 119 (100%), respectively. The maximum sensitivity of analyte in blood sample was obtained on using 50 V dissociation energy. Additionally, the effect of current on sensitivity of the method was also demonstrated. In all conditions the new characteristic ion at m/z 161 was obtained and used for quantification of triazofos in blood samples with maximum sensitivity. The limit of detection and quantification was 0.351 and 1.17 ng mL^{-1}, respectively, with 99% accuracy. The observed correlation coefficient was 0.995. The inter-day percentage recoveries from 83.9% to 111% were obtained below 9.38 percentage RSD. This present method gives combined picture of confirmation and quantification of triazofos 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 triazofos in poisoning emergency cases.

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1. Introduction

Triazophos [O,O-diethyl-O-1-phenyl-1H-1,2,4-triazol-3-yl phosphorothioate] is an organophosphorous pesticides, commonly used on various crops such as cotton and rice to control aphids, fruits borers, leave hoppers and cut worms. It is moderately toxic and broad spectrum, non-systemic pesticide [1,2] and is used throughout the world. A solid-phase micro-extraction (SPME) with gas chromatography (GC) using a flame-photometric detector in phosphorous mode was used for the estimation of organophosphorus pesticides (diazinon, fenitrothion, fenthion, quinalphos, triazophos, phosalon and pyrazophos) in fruit (pears) and fruit juice samples [3]. The photo-catalytic degradation of triazofos in aqueous TiO_2 suspension has been studied in a photo reactor operating with simulated solar radiation [4]. Seventeen degradation products were identified using high-performance liquid chromatography with ultraviolet detector (HPLC-UV), liquid chromatography with mass spectrophotometer (LC–MS/MS), gas chromatography with mass spectrophotometer (GC–MS/MS) and ion chromatography (IC), and by comparing retention times and spectra of authentic standards [4]. On the basis of the observed transformation products, two routes were proposed, one based on the initial oxidative cleavage of phosphorous and sulphur (P–S) bond and phosphorus and oxygen (P–O), and the other on initial cleavage of the ester P–O bonds. This result is important for the solar treatment of pesticide-contaminated waters. Additionally, kinetics and products of photo-fenton degradation of triazofos were studied to support the photo-degradation hypothesis. [5]. The GC–MS in MS/MS study of chlorypyrifos in blood samples in poisoning cases was reported recently [6]. Three multivariate calibration methods, partial least squares (PLS-1 and PLS-2) and principal component regression were applied to the simultaneous determination of the five pesticides including triazofos by high-performance liquid chromatography with diode array detection [7]. The animal study showed the effect of cholinesterase

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of triazofos and toxicity test in serum samples was studied and the highest sensitivity was obtained with AChE and the lowest sensitivity with horse serum BuChE. The limit of detection (LOD) values for investigated pesticides correlate with acute toxicities expressed as LD50 (oral, rat). The presented FIA system could serve as an alternative screening test to evaluate the toxicity of different environmental samples, new cholinesterase inhibiting pesticides or other products (e.g. nerve gases) [8].

A simple, rapid and sensitive sample pretreatment technique, dispersive liquid–liquid micro-extraction (DLLME) coupled with high-performance liquid chromatography-fluorescence detection (HPLC-FLD), has been reported to determine carbamate (carbaryl) and organophosphorus (triazofos) pesticide residues in water and fruit juice samples [9]. Gas chromatography (GC) with nitrogen–phosphorus detection (NPD) and mass spectrometry (MS), in full scan and tandem (MS–MS) modes have been used to determine eleven pesticides in aqueous samples [10]. In this method pre-concentration of 500-mL water with C_{18} cartridges was used for the determination of pesticides at ng L^{-1} levels. The best precision and sensitivity were obtained by using GC-NPD and GC–MS–MS. The obtained results were compared with NPD and the advantage of MS–MS over NPD for highly selective quantitative determination of pesticides in complex matrices. Pesticide residues in fruit and vegetables were determined by the gas chromatography/tandem mass spectrometry (GC/MS/MS) [11]. Electron impact (EI)/MS/MS and chemical ionization (CI)/MS/MS were developed for the determination of eighty compounds, including organochlorine, organophosphorus, organonitrogen, and pyrethroids in fruit and vegetable samples [11]. A gas chromatography with pulsed flame-photometric detection (GC–PFPD) was used for the determination of 24 organophosphorus (OP) pesticides in vegetables. In this method pesticides were extracted with dichloromethane. The recovery was between 73% and 110% with precision >15%. The limit of detection was typically <0.01 mg kg^{-1}, much lower than the maximum residue levels stipulated by European legislation. Three pesticides were detected and confirmed in vegetable samples [12].

A multi-residue method was developed for determining fifty-five organophosphorus and organochlorinated compounds and pyrethroids commonly used in crop protection. Pesticide residues are extracted from samples with a mixture of ethyl acetate and sodium sulphate, obtaining a final pre-concentration of 1 mg sample [13]. An optimized HPLC method for the analysis of selected pesticides (atrazine, diuron, dichloran, methiocarb, folpet, triazophos, vinclozolin, tetradifon and carbophenothion) was developed for analysis [14]. Several methods [15–28] were reported by different researchers for the analysis of pesticides in different matrixes. They however, are not always useful for confirmation and quantification of pesticides in blood samples. None of the methods gives the combined picture of confirmation, ion formation, and effect of dissociation energy on fragmentation schemes, sensitivity detection limit and S/N ratio for the quantification and confirmation of triazofos in blood samples. Therefore our aim was to develop and demonstrate the effect of dissociation energy (DE), current on ions formation and sensitivity of analytical method for the treatment of poisoning patient in emergency cases.

2. Experimental

2.1. Materials

Standard of triazifos (certified reference material) of highest purity grade was purchased from M/s Sigma–Aldrich. The highest purity grade solvent was used and checked for any residue contamination before use.

2.2. Blood samples

Blood samples were collected from the high tech laboratory, B.J. Medical College and Hospital, Asarwa, Ahmedabad 380016, India. The blood sample (6–8 mL) was centrifuged at 2000 rpm for 10 min. Serum was separated and stored at −80 °C until analysis.

2.3. Extraction of triazofos from blood samples

Two millilitres of the serum sample was taken in 15 mL stopper centrifuge tube and 6 mL n-hexane was added. The contents were mixed on roto-rac machine (indigenously fabricated) for 2 h. Five millilitres portion of n-hexane was taken in another tube and purged gently under stream of purified nitrogen. The analyte was reconstituted in 1 mL n-hexane for GC–mass analysis. The recovery experiments were also conducted with the range of 5–250 ng mL^{-1} concentration.

2.4. GC–MS study

The mass spectrometric study was carried out using Varian Saturn 2000 gas chromatography–mass (GC–MS/MS) spectrophotometer (Varian Pvt. Ltd., USA) equipped with Varian CP-3800 gas chromatography and MS/MS ion trap mass detector. The data system contains National Institute of Standards and Technology (NIST) library with more than one hundred and fifty compounds [6]. A Varian MS workstation version 5.2 was used for the system control and data acquisition. The GC–MS method was used for analysis with the slight modification of our early reported method [6]. The DB-5 MS capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness Varian Pvt. Ltd., USA) was used for the separation of triazofos. 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 injector temperature was set at 280 °C and 1.0 μL sample was injected in the splitless mode. Samples were analyzed using the following temperature programming: initial temperature of 50 °C holds for 3 min, increased by 10 °C min^{-1} to 270 °C hold for 10 min. The total running time is 35 min.

2.5. MS in scan mode

The MS spectra firstly recorded in full scan electron impact (EI) mode with the mass range 40–500 Da (μ) of 0.90 s/scan with zero thresholds. The emission current of the ionization filament was set 30 μM-generating electrons with the 70 eV energy. The trap temperature, manifold and transfer line temperature were 180, 40 and 270 °C, respectively. The used electron multiplier voltage was 1500–1600 V for scanning spectra.

2.6. MS/MS study

The MS method was first attempted in GC–MS with electron impact auto ionization in full scan mode, the interfering peaks from blood matrices, peak tailing, peak broadening, and poor sensitivity complicated this method at ppb levels. Therefore, a more sensitive MS/MS method was developed with the slight modification of our early reported method [6] for confirmation and to increase the sensitivity for low-level quantification of triazofos in blood samples. The samples were analyzed in electron impact auto mode with MS/MS ion preparation by using mass range 40–400 μ with background mass of 40 μ and scan rate equal to 0.90 s/scan. The axial modulation voltage was 4 V. The parent ion storage was m/z 161 μ and excitation storage level was 50 V. The isolation window for the spectra was 3 m/z. The non-resonant MS/MS parameters used for selective fragmentation of parent ion analysis. The segment starting time for triazofos was 23.8 min and ending time was

25.05 min. The spectra of triazofos compound were recorded on different DE (20–80 V), injecting similar concentration of analyte to demonstrate the effect of DE on relative abundance of molecular ions as well as fragment ions in MS/MS. Similarly, the MS/MS spectra of this compound were recorded on different DE (20–80 V) on injecting same concentration to improve the signal to noise ratio and level of detection of this analytical method. Calibration curves were plotted between area count and different concentration ranges from 100 to 1000 ng mL^{-1} and the signal to noise ratio (S/N) was also constructed against different concentrations.

2.7. Limit of detection (LOD)

For this study, the point at which the measured value was considered reliable was when it was larger than the uncertainty associated with it, also called the LOD. In this method, the analytical LOD was calculated as per the earlier reported method [29,30].

2.8. Limit of quantification (LOQ)

For this study, the lower level where measurements became quantitatively meaningful was called the LOQ and was calculated as per the earlier reported method [29,30].

2.9. Percentage recovery

The recoveries of the method were determined by spiking blood samples free of pesticides with different known concentrations of reference standards. The recovery of each pesticide was calculated at each of the known concentration levels by comparing the measured concentrations with the spiked concentrations, as per the reported method [29]. A ratio of 1.00 indicated 100% recovery. The percentage recovery was determined using 5–250 ng mL^{-1} concentrations.

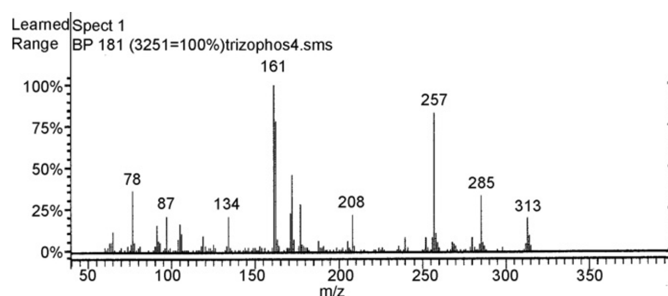

Fig. 1. MS spectrum in full scan mode of triazofos with ions m/z 313, 285, 257, 208, 161, 134, and 78.

3. Results and discussions

3.1. Structure reactivity

The full scan GC–MS spectra (Fig. 1) show ions at m/z 313, 285, 257, 208, 161, 134, 97 and 78 in positive ionization mode [M^+] and these ions were also confirmed by NIST library search. The selected molecular ion, and selected product ion scan were performed and different collision dissociation energies were applied in MS/MS mode to obtain different fragmentation patterns. The ion formation of study sample is shown in Fig. 2. The m/z 285 (III) was obtained due to the elimination of ethylene ($-CH_2{=}CH_2-$) molecule from parent ion molecule m/z 313 (II), because the oxy-

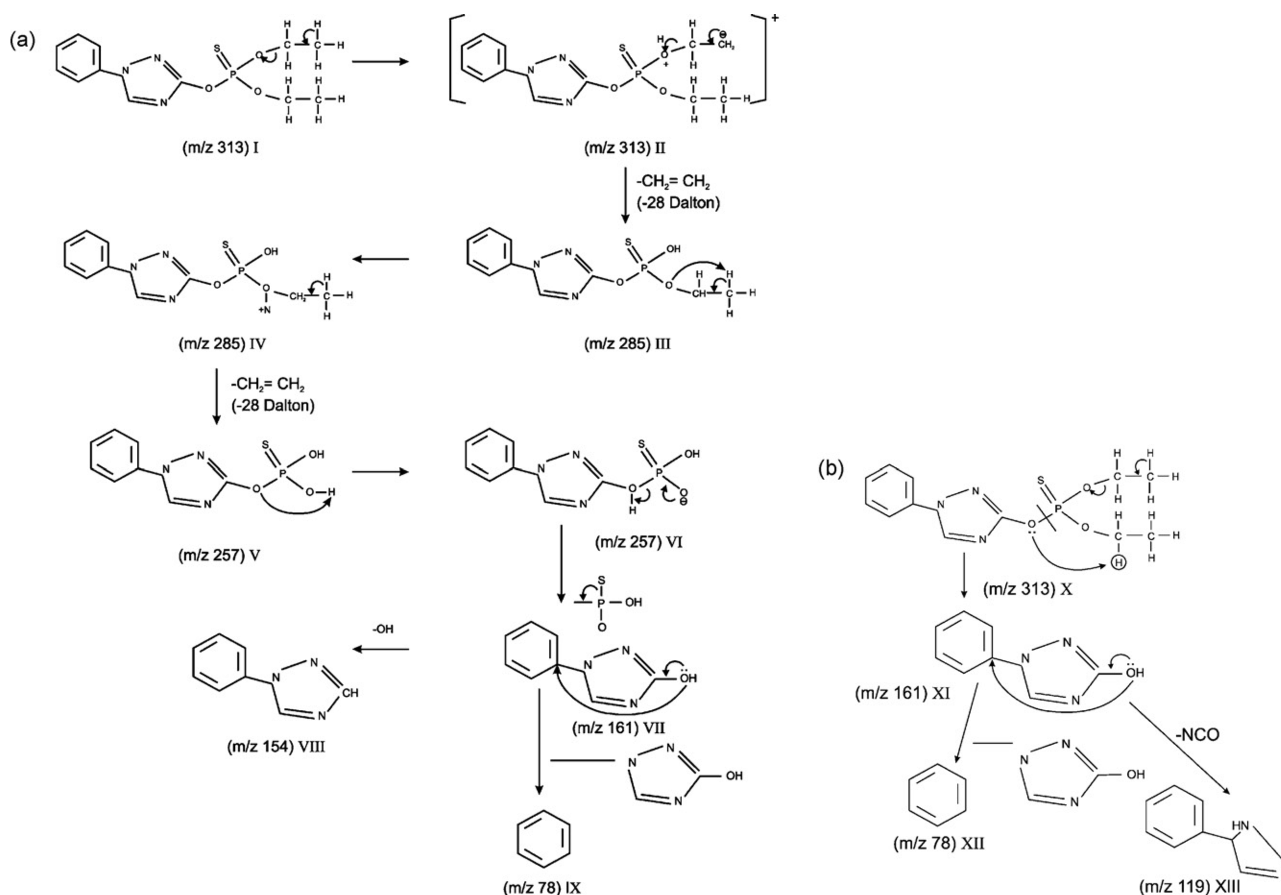

Fig. 2. Illustration of ion formations of triazofos.

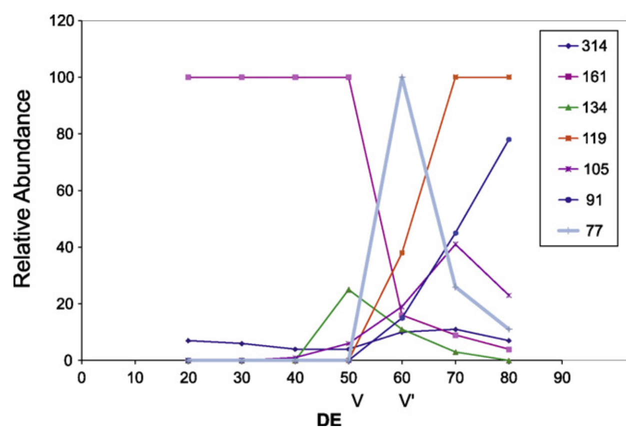

Fig. 3. Effect of the different dissociation energies (20–80) on ion formation of triazofos recorded using GC–MS/MS spectrometer in MS/MS mode. At low dissociation energy (<50 V), the base peak obtained at m/z 161 used for quantification. In between 50 and 60 V, the m/z at 77 behaved as a base peak. The peak at m/z 119 represents base peak at 70 V.

gen atom donates the lone pair to hydrogen atom by remote charge mechanism. Additionally, the three nitrogen atoms are present in pyridine ring, which deactivate the whole molecule and therefore ethylene molecule removed from the parent ion molecule. Similarly, other ethylene molecule was removed from m/z 285 (IV) leading to the formation of structure V at m/z 257. The triazofos possesses a sufficient long chain to permit transfer of β-hydrogen namely loss due to hydrogen rearrangement mechanism. Similar pattern noted previously with chlorpyrifos and phenolate ion [6]. The stable VII structure was formed at m/z 161 due to the removal of –OPSOH group from structure VI. This new structure has been isolated due to structure reactivity and ion reaction mechanism of triazofos; till one reported. Additionally, a new stable structure benzene was formed at m/z 78 (IX) due to the expulsion of complete pyridine ring (–$C_2N_3OH_2$) from m/z 161 (VII), till noone reported the formation of benzene ring from triazofos due to ion mechanism. The loss of –N–CO group from m/z 161 (X) with rearrangement through a three-member ring intermediate is formed at m/z 119 (XII) (scheme 2, Fig. 2). The scheme 2 (Fig. 2) also demonstrated the formation of stable structure at m/z 119 and 78.

From this study we conclude that due to reaction ion mechanism two stable structures may be formed at m/z 161 and 78, which may play vital role in triazofos toxicity in human.

3.2. Qualitative analysis of triazofos

The different behaviours of spectral pattern recorded on different DE are illustrated in Fig. 3. These results reveal three regions in the MS/MS spectra of triazofos in relation to percentage relative abundance of base peak and fragment ion peaks. In region 1 (V), DE, lower than <50 V, a base peak at m/z 161 (100%) was obtained. In region 2, the range between V and V′ (50–60 V) the m/z 77 (100%) behaves as a base peak with several fragment ions. In region III, the condition is greater than >V′ (>70 V), the m/z at 119 (100%) behaves as a base peak with several fragment ions. The MS/MS spectra of triazofos in study samples have shown in Fig. 4(a)–(c) to understand the effect of DE on fragmentation schemes of triazofos. In Fig. 4a the base peak is obtained at m/z 161 [M^-−153] (100%) with fragmented ion peak at m/z 160 (16%), 314 (7%), 258, 178, 134, 105, 91, 77 and 161 were also isolated on used energy in between 20 and 50 V. The spectra recorded on 60 V illustrated in Fig. 4(b) suggest that the m/z 77 (100%) behaved as a base peak with several fragment ions peak at m/z 314 (10%), 161 (16%), 162 (50%), 134 (11%), 119 (38%), 105 (19%), 91(15%), 160 (29%) were

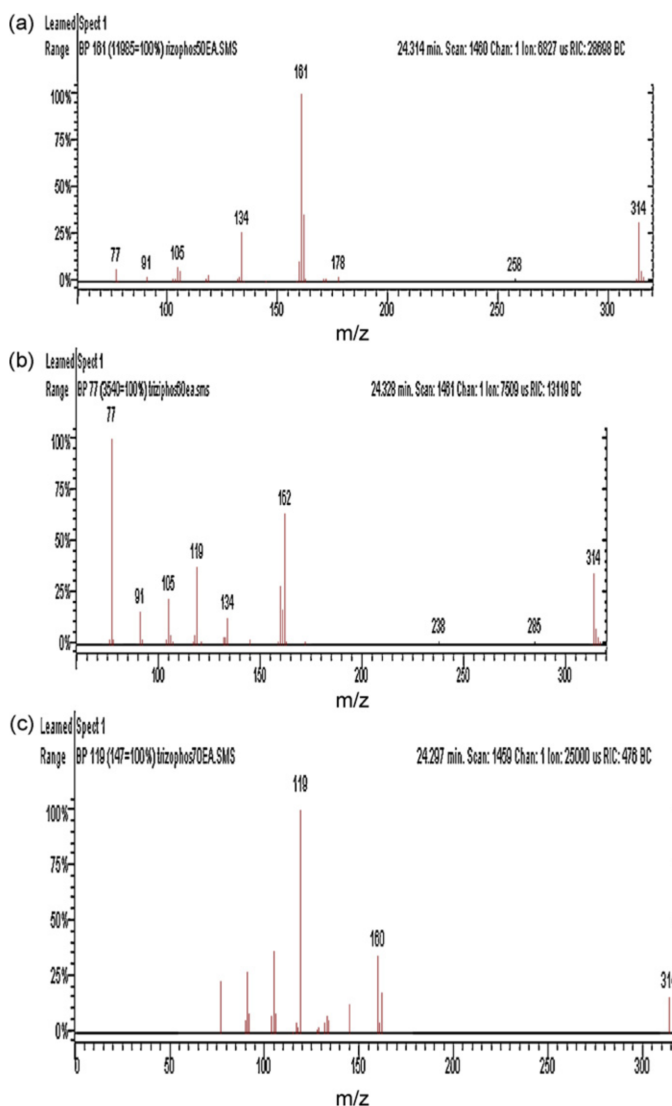

Fig. 4. (a) MS/MS spectra recorded for triazofos on 50 V. (b) MS/MS spectra recorded for triazofos on 60 V. (c) MS/MS spectra recorded for triazofos on 70 V.

also obtained. The spectra recorded <60 V (Fig. 4c) the base peak at m/z 119 (100%) is obtained. Besides base peak several fragment ion peaks were also obtained at m/z 314 (11%), 160 (46%), 161 (4%), 162 (7%), 132 (13%), 105 (41%), 77 (26%), and 91 (45%). This study clearly explains the effect of DE on fragmentation behaviour of triazofos compound.

From this study we conclude that the MS/MS recorded at 70 V in region III is very useful for structural confirmation of triazofos while the region II is very useful for quantification and qualitative estimation of triazofos. In the region I, the spectra recorded on 50 V is very useful for quantification of triazofos. Similar type of study has been reported earlier by us [6].

The 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 = 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 the significance of the acquisition.

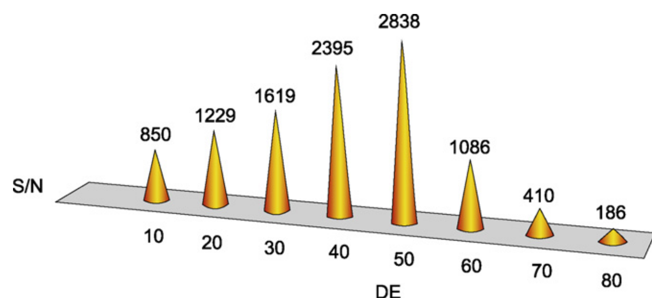

Fig. 5. Effect of different dissociation energies (10–80 V) on sensitivity, signals and detection limit of this method for triazofos in MS/MS mode injecting on 1 μL volume of 500 ng mL^{-1} concentration.

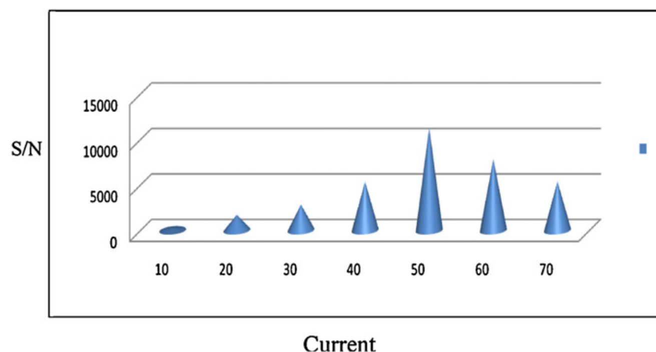

Fig. 6. Effect of different currents (20–70 A) on sensitivity, signals and detection limit of this method for triazofos in MS/MS mode injecting on 1 μL volume of 500 ng mL^{-1} concentration.

3.3. *Quantification of triazophos in blood samples*

Hexane was used for the extraction of triazofos from blood samples. The different MS/MS spectral patterns were obtained on using different DE (20–80 V), and different current and the S/N ratio were also calculated by software in each condition. The obtained S/N were 850, 1229, 1619, 2395, 2838, 1086, 410, 186 on used 10:20:30:40:50:60:70:80 V dissociation energy, respectively, on injection of 1 μL from 500 ng mL^{-1} concentration of triazofos (Fig. 5). Additionally, the current was plotted against S/N ratio (Fig. 6). The result showed that the maximum sensitivity was observed on 50 V on maintaining similar condition with the injection of similar concentration. The DE and current play a vital role to eliminate the matrix in blood samples, therefore the sensitivity of the method is drastically increased with the isolation of very stable ion at *m*/*z* 161 (100%) on 50 V. Nineteen blood samples whose endogenous pesticide concentrations were well-characterized were used to evaluate recoveries. The inter-day percentage recoveries were obtained in between 87.94 and 105.6 (Table 1). The precision of this method for triazofos was found excellent with RSD ranging from 4.41 to 9.91. The lowest spike level was 5 μg L^{-1} and LOD and LOQ were 0.350 and 1.17 ng mL^{-1}, respectively, with 99% accuracy [31]. A five point calibration curve was plotted between area count versus spiked

Table 1
Inter-day percentage recovery of triazofos.

Spiked levels (μg L^{-1})	Mean recovery (%)	RSD (%)	Ranges	*N*
5	95.472	7.09	89.09–104	4
75	102.88	4.41	96.9–108	5
100	105.6	9.91	90–111	5
250	87.94	6.12	83.9–94.8	5

N = number of replicate. RSD = relative standard deviation.

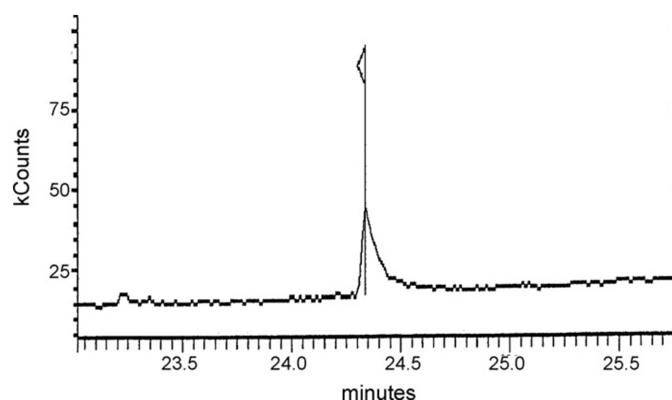

Fig. 7. Chromatogram from an extract of human blood fortified with 5 ng mL^{-1}.

concentrations 100, 250, 500, 750 and 1000 ng mL^{-1}, respectively. A correlation coefficient of $r > 0.995$ was obtained with 99% accuracy. The chromatogram of spiked blood sample has shown in Fig. 7. The retention time of the compound was 24.338 min.

From this study we conclude that the sensitivity is drastically increased due to the use of different DE and current. The signal to noise ratio depends upon DE and current. Additionally, it may be concluded that the 50 V DE and 50 A current is optimum for the quantification of triazofos in blood samples.

4. Conclusion

A GC–MS/MS method was developed for the qualitative and quantification of triazofos at ng mL^{-1} concentration for the treatment of human poisoning in emergency case. The sensitivity of this method depends upon DE and current. The structural confirmation was also determined unambiguously using this method at lower level concentration. The MS/MS spectra were recorded in three different regions of DE (20–80 V) and one parent ion two precursor ions have been isolated. On 50 V *m*/*z* 161 (100) was isolated which was used for quantification, besides these ions the other ions at *m*/*z* 314, 258, 228, 134, 105 and 77 were used for confirmation. Similarly, on 60 and 70 V, the *m*/*z* 119 (100) and 77 (100) were isolated. The vital point of this method is the formation of two new structures at *m*/*z* 161 and 78, reported first time. These structures may cause toxicity in human. The LOD and LOQ of this method were achieved at ng mL^{-1} level. The achieved percentage recovery varied from 87.94% to 105.6% at good precision. The calibration curve for quantification was linear with coefficient correlation $r > 0.995$. This study clearly demonstrated that the spectra obtained at 50 V DE were the best for quantification and confirmation. Additionally, the DE affects sensitivity of this method.

This analytical method is very important for biological relevance because it gives unambiguously combined picture of confirmation and quantification of triazofos in biological samples for critical medical care practice.

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