

Analysis of pesticide residues in cucumber using highly sensitive ultra-performance liquid chromatography – tandem mass spectrometry

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ABSTRACT

In this study the determination of pesticide residues namely, malathion in cucumber collected from local markets in Riyadh city has been carried out. Samples of treated cucumbers were collected and analyzed during a 14 days period for obtaining the residue amount of malathion. A liquid–liquid sampling technique has been applied to ultra-performance liquid chromatography/tandem mass spectrometry for analysis of residual malathion in cucumber. Multiple reactions monitoring for two transitions was acquired while the transition with higher sensitivity used for quantification and the transition with lower sensitivity used for confirmation analysis of malathion. The proposed method has been validated by establishing performance parameters such as percent recovery, precision, linear ranges, limits of detection (0.0042 µg/ml) and limits of quantification (0.014 µg/ml). The obtained recoveries of malathion in fresh cucumber samples of 10 replicates were found to be in the range between 96.9 and 101.2% (%RSD < 5.2) for two fortification levels (0.02 and 0.1 mg/kg). The intra-day and inter-day RSD values were obtained between 1.7 and 2.1%. The extracted cucumber samples have shown different level of contamination upto certain time interval (7 days) and after that no residues between 11-14 days were detected.

Keywords: Pesticide residue; Ultra Performance Liquid Chromatography; MS Detector; Malathion

INTRODUCTION

Next to tomatoes, cabbage, and onions, cucumbers are the most widely cultivated vegetable in the planet. Cucumbers are scientifically known as *Cucumis sativus* and belong to the same botanical family as melons and squashes. Although, they have not received as much attention as other vegetables in terms of health benefits, but this widely-cultivated food provides a unique combination of nutrients, including antioxidant (vitamin C, beta-carotene, and manganese), anti-inflammatory and anti-cancer (Yang et al, 2006; Hedges and Lister; Wordpress). In addition, cucumbers contain numerous flavonoid antioxidants, including quercetin, apigenin, luteolin, and kaempferol (<http://www.whfoods.com/genpage.php?tname=foodspice&dbid=42>).

Now a day, plenty of chemical compounds are being used in the field of manufacturing agricultural commodities, thanks to their enormous demand in modern agriculture. Pesticides are considered as essential groups of chemicals that were developed and produced to control the agricultural pests. On one side great success of pesticides in agricultural applications has led to an increase crop production while on the other side the improper use of pesticides result in the contamination of vegetable, fruits and other crops. A large number of organophosphorous pesticides have been used as protectants to agricultural crops. Among them, malathion (O, O-dimethyl dithiophosphate of diethyl mercaptosuccinate) (M) is highly demanding. This is usually a broad spectrum, non-systemic organophosphorous insecticide and has globally been used over 30 years on a wide variety of crop sites including agriculture, nurseries, home and garden, and public health (Tawab et al, 2006). Some residential and agricultural uses of this pesticide in food and beverages can rather have high application rates and resulting exposure to human health (Council Directive 90/642/EEC, 1990). The applications of malathion in public health programs are also the significant route to human exposure (Jury et al, 1987; Jury and Sposito, 1985).

The organophosphates pesticides are able to inhibit insect acetyl cholinesterase and strongly interfere with neural transmission in other organisms, including humans. Therefore, they represent a potential hazard for the environment and human health, which demands the continuous assessment and monitoring of these pesticides (Council Directive 80/778/EEC, 1980). Toxic symptoms results from malathion exposure are breathing problems, headache, nausea and dizziness, while the high exposure can produce fatal poisoning in human health (WHO/FAO, 1977; Mccarroll et al, 2000; Abou-Donia, 1992). On addition, when pesticides are properly utilized and adequately monitored, there is a negligible risk for the consumer's health.

The required rates of application of malathion are varied under different agricultural and climatic conditions from country to country and even between different regions of the same country. Therefore, pesticide control is necessary for both the economic and health reasons. In the recent time, cucumber, which is susceptible to insect and

various disease attacks, has been widely studied for its pesticide residues. Among them, many studies have reported that the main pesticide residues are organophosphorous pesticides (Mansour et al, 2009; Pan et al, 2002). Although boiling, frying, roasting and blanching lead to a significant reduction of pesticide residues in food stuffs (Chavarri et al, 2005; El-Behissy et al, 2001; Nagayama, 1996; Radwan et al, 2005; Randhawa et al, 2007; Soliman, 2001; Zabik et al, 2000) but cucumber is most often eaten raw in salads and in cold soups. Therefore, there is a little chance of complete removal of pesticides from raw cucumber before consumption. Thus a highly sensitive method is required for the trace level quantitation of malathion residue in cucumber.

The Commissions of the Food and Agriculture Organization (FAO) and World Health Organization (WHO) have been established maximal residue limits (MRLs) of pesticides in a variety of foods. In Europe, the maximum admissible levels of pesticide residues in foodstuffs of animal or vegetal origin are defined according to the criteria proposed by the European Council (Council Directive 97/41/EC, 1997), the limits depends on the type of pesticides and foods. But the prevention of toxicological risks of human health, due to contaminated food consumption is primary goal in food safety policy (Miller, 1987). Therefore, in the studied method an attempt has been made to determine the pesticide residues in M sprayed cucumber samples collected from the local markets in Riyadh city. The developed UPLC-MS/MS method is rapid, sensitive and reproducible. The method was successfully applied to the determination of M residues present in the sprayed raw cucumber.

EXPERIMENTAL

Materials and method: Malathion standard, HPLC grade acetonitrile, celite, sodium chloride, petroleum ether, anhydrous sodium sulfate and florisil were purchased from Sigma-Aldrich (St. Louis, MO, USA). Methanol and ethyl acetate were purchased from BDH chemicals Ltd (Poole, England). Formic acid for mobile phase preparation was obtained from Panreac (Barcelona, Spain). Water was purified through a Milli-Q water purification system (Millipore Corporation, Bedford, MA, USA). The stock solution of 10µg/ml of malathion standard was prepared in HPLC grade acetonitrile and stored at 4 °C in the refrigerator. All other working standard solutions were prepared immediately before use by diluting the stock solution with the mobile phase Acetonitrile: 0.1% aqueous solution of formic acid (50:50, v/v).

INSTRUMENTATION

Ultra-performance liquid chromatography (UPLC): Chromatographic separation and identification of M was achieved in less than 1 min. The method has been evaluated for analysis accuracy of the analyte evaluated in cucumber samples. The chromatographic separation of standard M was performed using an Acquity UPLC system (Waters, Manchester, UK). The system was consisting of binary solvent manager, sample manager and a column thermostat equipped with bridged ethyl hybrid (BEH) C₁₈ column (50mm × 2.1mm i.d, 1.7µm particle size) (Waters,

Mildford, MA, USA). The smaller size column particles are able to provide improved peak resolution and high pressure with high liner velocity of the mobile phase (Zou et al, 2013). All injected solutions were stored in the auto-sampler at 4 °C. The partial loop with needle overfill mode was set up to inject the samples. Acetonitrile was used as a strong wash, and 5% acetonitrile in water was used as a weak wash solvent. The various composition of different mobile phase such as methanol/water, acetonitrile/ water with 0.1% formic acid in isocratic elution was used to get best peak separation of M. The column temperature was also varied to see the effect on the chromatographic separation. The sample temperature was kept at 10 °C and the volume injected for the analysis was 1 µL.

Mass spectrometry: Mass spectrometry (MS) was carried out on a Micromass Quattro Premier tandem mass spectrometer fitted with an ESI interface and controlled by MassLynx V4.1 software (Waters, Manchester, UK). An Oerlikon rotary pump, model Sogevac SV40 BI (France) provided the primary vacuum to the mass spectrometer. MS ion source was used both positive and negative electrospray ionization. The parameters affecting the ion transmission were optimized by infusing a standard solution of analyte. High-purity nitrogen (99.99% purity) was produced using a Peak Scientific NM30LA nitrogen generator (Inchinann, UK) and used as nebulizing, desolvation and cone gas, while high purity argon (99.9999%) was used as collision gas. Data acquisition was carried out with MassLynx V4.1 software.

Sample Analysis and storage: Five samples of cucumber were harvested after M spraying at the following intervals; one day, four days, seven days, eleven days and fourteen days. Each sample was chopped and divided into four subsamples (100 g each) which were stored in individual polyethylene bags at -24 °C until extraction was carried out.

Extraction Procedure: In order to clean up and real sample preparations, extraction procedure was used in this study similar to that previously reported in the literature (Islam et al, 2009) and briefly consisted of mixing 100 g of chopped sample of cucumber with 10 g celite and 200 mL acetonitrile. The mixture was then homogenized using a mixture grinder and then filtrated through Buchner funnel. The filtrate was put into the separating funnel (SF) of 1000 ml and 100 ml petroleum ether was added into it following by shaking for 2 min. After that 10 ml saturated sodium chloride solution and 600 ml of milli-Q H₂O was added and shaken horizontally for 1 min and allowed the mixture to stand for some time. The lower aqueous layer of the SF was removed while the upper organic solvent which containing the analyte was washed with 100 ml milli-Q water twice. The organic layer was transferred into a clean flask and 15 gm anhydrous sodium sulfate was added to remove any aqueous traces. Then it was filtered and the filtrate was concentrated using rotary evaporator (Buchi). The cleanup step of the extracts was

performed by using florisil column. The column was prepared by putting a piece of glass wool in an empty chromatographic glass column, then 10 – 15 gram of activated florisil (130 °C, 6 hours) was placed onto the glass wool which was kept inside the column. Additionally, 1 gm of anhydrous sodium sulfate was spread on the surface of the activated florisil. The column conditioning was performed by adding 40-50ml of petroleum ether. After that the concentrated extract was added to the column and elution was performed with 200ml of a mixture of petroleum ether and diethyl ether (50:50; v/v) with a flow rate 5 ml/min. After these clean up steps, again the collected extracts were concentrated and then injected into UPLC–MS/MS system. To avoid obstruction during the UPLC analysis the extracted samples were filtered with 0.22 µm PVDF syringe filter prior to injection.

Validation Study: The following method parameters were determined to validate the quality of the proposed method: linearity, recovery, precision, Limits of Detection (LOD) and Limits of Quantification (LOQ). The recovery steps were performed by spiking M solutions at two different fortification levels to fresh cucumber samples that had not been treated with M pesticides. Both fortification levels were performed in 10 replicates to validate the method. The performance parameters of the proposed analytical method such as precision (intra-day and inter day), lower limits, and linear ranges were also determined. Analysis of samples was carried out by injecting 1 µL of the sample into the UPLC-MS/MS system.

RESULTS AND DISCUSSION

Chromatographic conditions: The development of a high throughput analytical technique for the analysis of M in cucumber using reversed phase UPLC is of high awareness. The most important benefits of the UPLC columns, where particle size is <2.0 µm, is that the efficiency does not drop when increasing the flow rates (Zou et al, 2013; Wabaidur et al, 2013). Isocratic elution by means of single eluent such as water, methanol and acetonitrile, and a mixture of two eluents (water/methanol, water/methanol containing 0.1% formic acid, acetonitrile/water, and acetonitrile/water containing 0.1% formic acid) of different composition were compared to optimize the mobile phase. The best chromatographic separation was achieved using a binary mobile phase consisting of a mixture of acetonitrile and 0.1% aqueous solution of formic acid (75:25, v/v), while the analytical column was kept at room temperature. The optimal flow rate was chosen at 0.4 ml/min and the analysis of M was achieved in less than 1 min. Figure 1 shows the UPLC–MS/MS chromatogram of M (1 µg/ml) standards in multiple reaction monitoring (MRM) acquisition modes.

Optimization of ESI-MS/MS conditions: The ESI-MS/MS conditions were optimized by infusing standards of 1 µg/ml of M in both positive and negative electrospray ionization (ESI) modes to dissolved efficiently the aqueous/organic

mobile phase and provide the maximum analyte response. In negative ESI mode, no highly abundant analytes signal was appeared under different ion source parameters. On the other hand, in the positive ESI mode a highly abundant analyte signal was appeared at m/z 331. Hence, positive ESI mode was chosen for mass spectrometric detection. On addition, the narrow chromatographic peaks (5 s width) required a fast scanning analyzer to define the peaks with enough points which was provided by triple quadrupole mass detector used in this work. The optimized MS/MS parameters used for the analysis of M were as follows: capillary voltage, 3.0 kV; cone voltage, 22 V; source temperature, 120 °C; desolvation temperature, 300 °C; desolvation gas flow rate, 600 L/h; cone gas flow rate, 60 L/h; collision gas flow, 0.10 ml/min. MRM for two transitions of M was acquired while the most sensitive transition ($331 > 127$) was used for quantification and the other one ($331 > 99$) for confirmation analysis (Fig. 2b). The Optimized MS/MS parameters such as, precursor and product ions, cone voltages and collision energies, dwell time are shown in Table 1.

Method validation: The performance of the developed analytical method was evaluated by determining the quality parameters, such as, System suitability, linearity, LOD, LOQ, repeatability (run-to-run precision), reproducibility (day-to-day precision) and recovery.

Malathion Standard Calibration Curve and quantitation : The recommendation of ICH guidelines (Q2 R1) considers system suitability as an important parameter for the analytical methods. The system suitability test confirms that the performance of the reagents, column and the instrument are appropriate for the analysis purpose. Briscoe et al 2007, in his article recommended signal response, signal stability and carry over as crucial factor to evaluate the system suitability. To establish the system suitability of the developed method, six different samples of malathion standard solution ($1.0 \mu\text{g ml}^{-1}$) were injected and the peak response was recorded. The obtained data revealed that the RSD (%) of the peak area of six samples of malathion was found to be less than 2 % which indicate good performance of the system. Other parameter such as carryover was also checked by injecting six blank injections, where no carryover was observed which also indicate the passing of the system suitability test.

The linearity of the proposed method was determined by investigating the detection signals of the analyte as a function of their concentration, with the aid of a regression line by the method of least squares. The calibration curves were constructed by plotting the peak area against M concentration and it was linear over the range of 0.01 – 1.0 $\mu\text{g/ml}$. The correlation coefficient (r^2) obtained was 0.999 ($n=3$). The LOD and LOQ values of the developed method were calculated. Usually, LOD of the quantitative analysis indicates the lowest level of the analyte that can be measured with definable statistical certainty in a sample and defined as the concentration that give a signal equivalent to three times the signal to noise on

analysis. While, LOQ was calculated from the concentration of the analytes that provided signals equal to ten times the signal to noise on analysis (Wabaidur et al, 2013). The calculated LOD (signal-to-noise ratio, 3:1) and LOQ (signal-to-noise ratio, 10:1) values of the analyzed compound were found to be 0.0042 and 0.014 µg/ml, respectively. Precision of the proposed method were evaluated by performing intra and inter-day validation. The intra and inter-day precision were obtained by determining the concentrations of spiked M in cucumber samples in six replicates for three different concentration levels. The intra and inter-day precision were calculated by repeating the assay method three times (six replicates of each concentration levels) on the same day and on three consecutive days (six replicates of each concentration levels each day), respectively, and the obtained results are presented in table 2. High repeatability and reproducibility with RSD value lower than 2.5% was achieved. The result confirms that liquid-liquid extraction in combination with UPLC-MS/MS can be used in the routine analysis of M in sprayed cucumber sample. All the obtained results are presented in table 2.

To assess the efficacy of liquid – liquid extraction, recovery studies at two different spiking level of M were performed. Initially, 500 g of fresh chopped cucumber samples that had not been treated with M were spiked with known concentration of pure standard M to achieve the final concentrations of M in samples 0.02 mg/kg and 0.1 mg/kg. After that the spiked cucumber samples were extracted and analyzed under optimal experimental conditions. The method was validated by extracting and analyzing 10 replicates of each recovery assay and 10 blank samples of cucumber. In samples the LOD and LOQ for target analyte was found 0.008 and 0.025 µg/mL respectively. The recovery rates were obtained in the range between 96.9 and 101.2%. The relative standard deviation (RSD) of recovery rates (n = 10) was lower than 5.2% in both cases. The data indicates that the employed extraction procedure was efficient for extracting M from sprayed cucumber samples.

Analytical application: Extracts of cucumber samples typically contain many compounds which produce interfering compounds at the retention time of the interest. But, this proposed UPLC-MS/MS positive ion electrospray method for estimation of residual M was provided good selectivity and sensitivity and enabling the detection of the target compound in the cucumber extract. The selectivity offered by MS/MS method enabled the detection of the M ion m/z 331 (Fig. 2a). The subsequent quantitative diminution analysis was performed using the most sensitive transition (m/z 127), while the qualitative analysis was confirmed using the less sensitive transition (m/z 99) (Fig 2b). The chromatograms did not show any interference, as no detectable matrix peak was found in the retention time of M. The recoveries of studied compound were calculated from the regression slope of the added concentration versus the measured concentration of M standard. The recoveries were found to be in the range of 89–98%, depending on the types of

extracted samples after certain time periods. Table 3 shows the M amount and estimated recovery rates.

Diminution of Pesticide Residues Levels with Time : The highest residue levels of M in cucumbers were found in the samples taken just after one day of the pesticide application (63.1 $\mu\text{g/kg}$). Malathion was also detected in the samples taken after four days of the application (19.6 $\mu\text{g/kg}$). Traces of M (2.3 $\mu\text{g/kg}$) was found in samples taken after seven days of the applications. But the total losses of pesticide in the experimental data were found between days 11 and 14. The loss parameters of M with times are shown in figure 4. Therefore, from the obtained results (Fig. 4) it was concluded that the malathion residues present in the cucumber extracts are below the MRLs reported by FAO/WHO (1993).

CONCLUSIONS

A simple, reliable and fast UPLC-MS/MS method for residual malathion determination in cucumber has been established. All the performance parameters confirm the reliability of the developed method. The maximum residual malathion was found after 1 day extracted samples (63.1 $\mu\text{g/kg} \pm 0.80$, $n = 4$) and most importantly the obtained values for each analysed samples are below MRLs (200 $\mu\text{g/kg}$) of M (FAO/WHO, 1993). The obtained LOD and LOQ are 0.0042 and 0.014 $\mu\text{g/ml}$, respectively. Therefore, it can be concluded that our method will be helpful for monitoring of M in food samples in order to protect the human life for the indiscriminate use of such pesticides. Additionally, the developed UPLC-MS/MS method is suitable, rapid and precise to this purpose.

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Table 1. Data acquisition parameters of MRM transitions for M used in UPLC-MS/MS^a.

Analyte	Precursor ion [M+H] ⁺ (<i>m/z</i>)	Quantification transition			Confirmation transition		
		Product ion (<i>m/z</i>)	Cone voltage	Collision energy	Product ion (<i>m/z</i>)	Cone voltage	Collision energy
Malathion	331	127	22 V	18 eV	99	20 V	22 eV

^aDwell time was 0.025 s in all cases; Ionization mode: ESI+

Table 2. Quality parameters of the proposed UPLC-MS/MS method.

Analyte	LOD ^a (ppm)	LOQ ^b (ppm)	Intra-day precision (RSD%) ^c	Inter-day precision (RSD%) ^c
Malathion	0.0042	0.014	1.7	2.1

^a Limit of detection was calculated at a signal-to-noise ratio of 3.

^b Limit of quantification was determined at a signal-to-noise ratio of 10.

^c Relative standard deviation (n = 6).

Table 3. Malathion residues level in sprayed cucumber samples extracted after certain time period and estimated recovery rates.

M extracted after (Days)	M \pm SD ($\mu\text{g/kg}$) ^a	Recovery (%)
1	63.1 \pm 0.80	98
4	19.6 \pm 0.39	93
7	2.3 \pm 0.09	92
11	nd	89
14	nd	90

a, mean of three measurements; SD, Standard deviation obtained from addition standard calibration curve; nd, not detected

Figure captions:

Figure 1. UPLC–MS/MS chromatogram of 1 μ L injected malathion (1 μ g/ml) standards in MRM mode.

Figure 2. (a) UPLC–MS/MS mass spectra of precursor ions of malathion (1 μ g/ml) (m/z 331) in MRM mode, (b) UPLC–MS/MS spectra of product ions (m/z 127 and m/z 99) of malathion in MRM mode.

Figure 3. UPLC–MS/MS chromatogram of cucumber extracts (extracted after 1-day of the spray of malathion).

Figure 4. Diminution of malathion in cucumber with time.

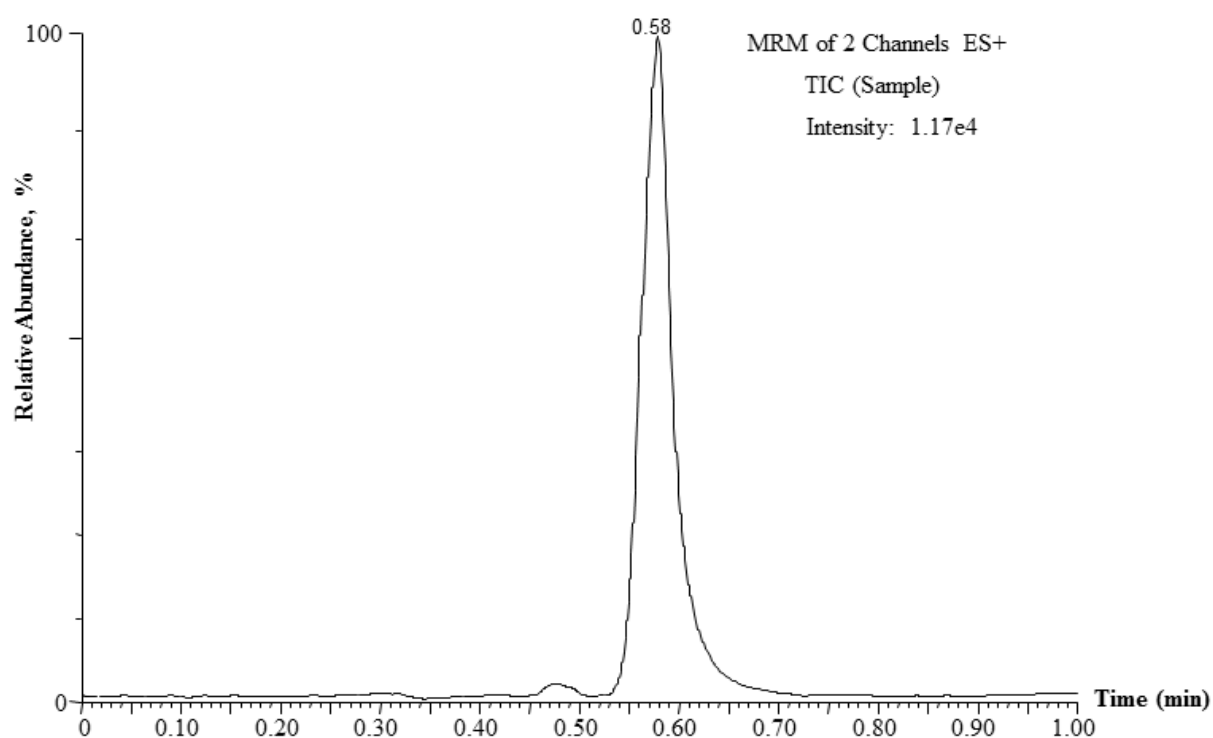


Fig. 1

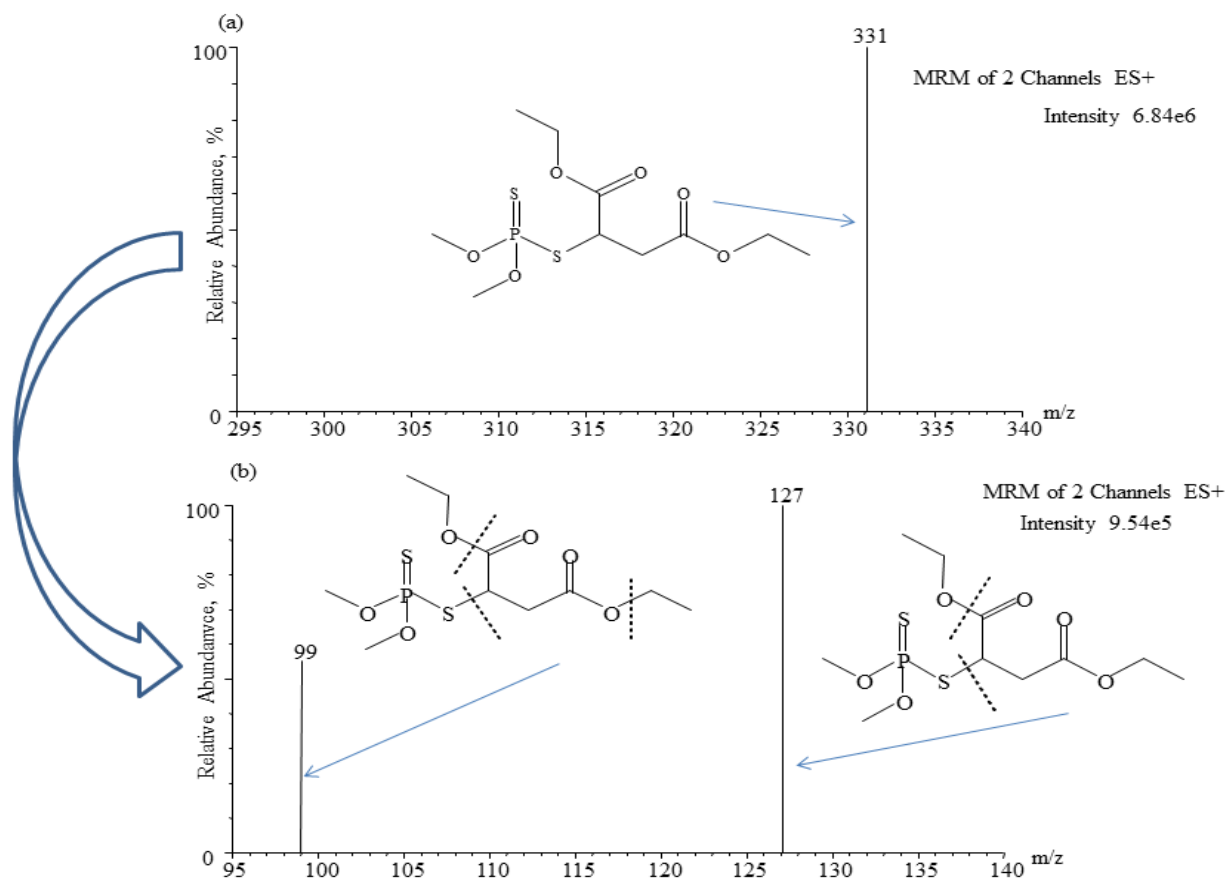


Fig. 2

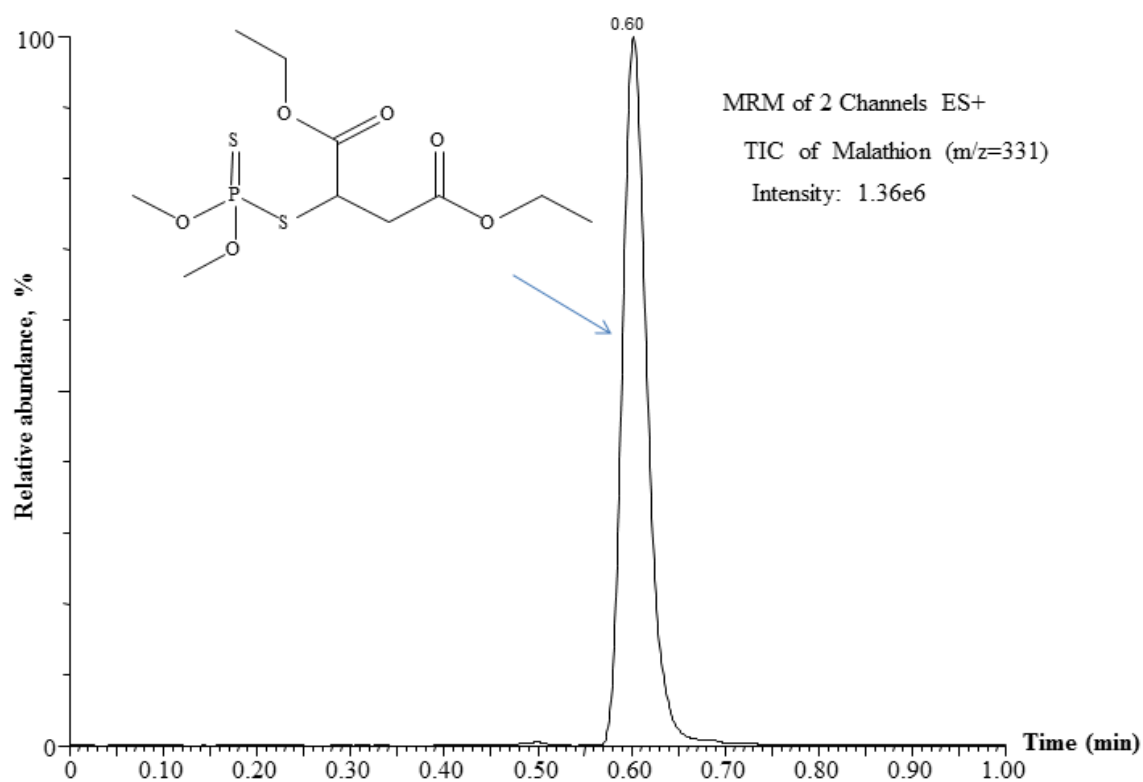


Fig. 3

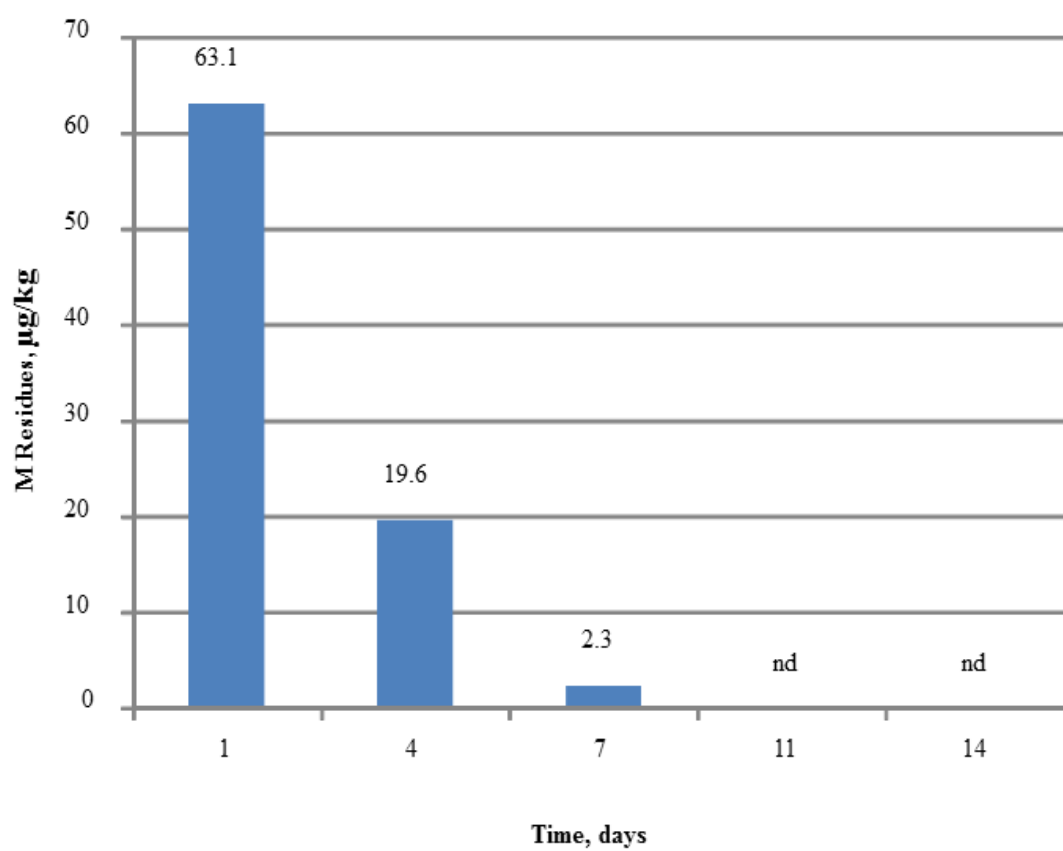


Fig. 4