American Journal of Analytical Chemistry, 2011, 2, 632-637
doi:10.4236/ajac.2011.25072 Published Online September 2011 (http://www.SciRP.org/journal/ajac)

Determination of Amitraz in the Honey Samples by Dispersive Liquid-Liquid Microextraction Followed by Gas Chromatography—Flame Ionization Detection

Mostafa Bashiri-Juybari[1], Ali Mehdinia[*], Ali Jabbari[1], Yadollah Yamini[3]
[1]*Department of Chemistry, Faculty of Sciences, K. N. Toosi University of Technology, Tehran, Iran*
[2]*Department of Marine Living Resources, Iranian National Institute for Oceanography, Tehran, Iran*
[3]*Department of Chemistry, Tarbiat Modares University, Tehran, Iran*
E-mail: [*]*mehdinia@inio.ac.ir*
Received June 15, 2011; *revised July* 2, 2011; *accepted August* 3, 2011

Abstract

Dispersive liquid-liquid microextraction (DLLME) followed by gas chromatography–flame ionization detection (GC-FID), as a simple, rapid and efficient method, was developed for the determination of amitraz in honey samples. This method involves the use of an appropriate mixture of the extraction and disperser solvents for the formation of a cloudy solution in 5.0 mL aqueous sample containing amitraz. After extraction, phase separation was performed by centrifugation and the concentrated amitraz in the sedimented phase was determined by gas chromatography—flame ionization detection (GC-FID). Some important parameters such as the type and volume of extraction and disperser solvents, and the effect of pH and salt on the extraction recovery of amitraz were investigated. Under the optimum conditions (13 μL of carbon tetrachloride as an extraction solvent, 1 mL of acetonitrile as a disperser solvent, no salt addition and pH 6) preconcentration factor and the extraction recovery were 955 and 95.5%, respectively. The linear range was 0.01 - 1.0 $mg{\cdot}kg^{-1}$ and the limit of detection was 0.0015 $mg{\cdot}kg^{-1}$. The relative standard deviation (RSD, n = 4) for 0.1 $mg{\cdot}kg^{-1}$ of amitraz was 3.2%. The recoveries of amitraz from honey samples at the spiking levels of 0.1 $mg{\cdot}kg^{-1}$ were 78.8 and 98.2%. The results indicated that DLLME is an efficient technique for the extraction of amitraz in honey samples.

Keywords: Dispersive Liquid-Liquid Microextraction, Amitraz, Honey Sample

1. Introduction

Amitraz (N′-2,4-(dimethylphenyl)-N-[(2-4-di-amitmethyl-phenyl)imino] methyl methanimid-amide) is a member of formamidine pesticide family. It is widely applied on beehives to control the beehive parasite Varroa lacobsoni destructor which endangers beekeeping all over the world [1]. Therefore, it can contaminate honey. Amitraz produces behavioral, physiological and biochemical effects in humans [2]. The most characteristic symptoms are the central nervous and respiratory systems depression, bradycardia, hypotension and convulsions [3-5]. Maximum residual limit in honey was set as 0.01 $mg{\cdot}kg^{-1}$ in Germany and Italy and 0.2 $mg{\cdot}kg^{-1}$ for European Union [6]. For these reasons, the development of accurate and sensitive methods for the determination of amitraz in honey samples is necessary.

Several instrumental techniques have been applied for the determination of amitraz; these include high performance liquid chromatography (HPLC) with UV detection [7], gas chromatography with electron capture [8] and thermionic specific [9] detectors, cyclic voltammetry [10] and ultra-high-pressure liquid chromatography–quadrupole time-of-flight mass spectrometry [11]. Low concentration and matrix interference are two problems in detecting amitraz. Therefore, in spite of developments in modern analytical instruments, extraction and preconcentration processes are needed for the determination of amitraz.

In recent years, several pretreatment techniques have been proposed for the extraction of amitraz such as solid phase extraction (SPE) [12], solid phase microextraction (SPME) [9] and headspace solvent microextraction (HSME) [13].

Rezaee *et al.* have developed dispersive liquid–liquid microextraction (DLLME) for the first time as a simple and rapid microextraction method, which was initially applied for the extraction of polycyclic aromatic hydrocarbons (PAHs) from water samples [14]. The method consists of two steps: 1) Injection of an appropriate mixture of extraction and disperser solvents into the aqueous samples, containing the analyte(s). In this step, the extraction solvent is dispersed into the aqueous sample as very fine droplets and the analytes are enriched into it. Owing to the considerably large surface area between the extraction solvent and the aqueous sample, the equilibrium state is achieved quickly and thus the extraction is independent of time. This is the most important advantage of the DLLME method. 2) Centrifugation of cloudy solution. After centrifugation, the determination of the analyte(s) in the sedimented phase can be performed by instrumental analysis. Up to now, DLLME has been successfully applied to the extraction of several families of organic and inorganic species [15-18].

In this study, DLLME followed by gas chromatography–flame ionization detector (GC-FID) has been investigated for the determination of amitraz in honey samples. The effects of various experimental parameters, such as the type and volume of extraction and dispersive solvent, pH of sample solution and salt effect were studied and optimized. The optimized method was applied to determine amitraz in honey in order to evaluate the application of this method to real samples.

2. Experimental

2.1. Reagents and Standards

All the reagents and standards were of analytical grade unless otherwise stated, and all dilutions were made with twice distilled water. Stock standard (100 $mg \cdot L^{-1}$) of amitraz was obtained by dissolving appropriate amounts of analytical standards of amitraz (Fluka, Milwaukee, WI, USA) in acetonitrile and stored in a refrigerator at 4˚C. Other chemicals, such as carbon tetrachloride, carbon disulfide, chloroform, chlorobenzene, methanol, acetone, acetonitrile, HNO_3 (>90%), and NaOH (>99%) were purchased from Merck (Darmstadt, Germany).

The honey samples were obtained from Tabriz (Azarbayeja, Iran) and Juybar (Mazandaran, Iran).

2.2. Instrumentation

A gas chromatograph (Shimadzu GC-14B) equipped with a split/splitless injector system and a flame ionization detector was applied for the separation and determination of amitraz. Ultra-pure helium (99.9999%, Air products, UK) that was passed through a molecular sieve and oxygen trap (Crs, USA), was used as the carrier gas at a constant flow of 3 $mL \cdot min^{-1}$. The injection port was held at 260˚C and operated in the splitless mode for 1 min. Then the split valve was opened and a split ratio of 1:10 was applied. The separation was carried out on a DB-5 (25 m × 0.32 mm × 0.25 μm film thickness) from SGE (Victoria, Australia) capillary column. The oven temperature was held at 120˚C for 2 min, then increased to 270˚C at the rate of 20˚C·min^{-1} and finally held at 270˚C for 7 min. The total time for one GC run was about 20 min. The FID oven temperature was maintained at 280˚C. Hydrogen gas was generated by hydrogen generator (OPGU-2200s, Shimadzu) and used for FID at flow rate of 40 $mL \cdot min^{-1}$. The flow rate of zero air (99.999%, Air products, UK) was 400 $mL \cdot min^{-1}$ for FID. The model 2010D Centurion Scientific Centrifuges (West Sussex, UK) was applied for the separation of the sedimented phase from the sample solution.

2.3. Dispersive Liquid-Liquid Microextraction Procedure

A 5.0 mL of twice distilled water was placed in a 10 mL screw cap glass test tube with conic bottom and spiked at the level of 0.1 $mg \cdot kg^{-1}$ of amitraz. One mL of acetonitrile (as disperser solvent) containing 13.0 μL of CCl_4 (as extraction solvent) was rapidly injected into a sample solution by 1.0 mL syringe, then, the mixture was gently shaken. A cloudy solution (water, acetonitrile and carbon tetrachloride) was formed. The cloudy state was stable for a long time. The mixture was centrifuged for 1.5 min at 6000 rpm and the dispersed fine particles of the extraction phase were sedimented in the bottom of the conical test tube. Finally, 2.0 μL of the sedimented phase was injected into the GC for analysis. The volume of the sedimented phase was about 5.0 μL which was measured using a 10 μL microsyringe.

For the determination of amitraz in honey samples, 0.05 g of the honey samples was dissolved in 5 mL of twice distilled water and a homogenized solution was produced. Then, the DLLME procedure was done similarly to the aqueous samples.

3. Results and Discussion

In order to obtain a high recovery and preconcentration factor, the effect of different parameters such as type and volume of the extraction and disperser solvents and salt addition on the extraction recovery (*ER*) were examined and the optimal conditions were obtained. The preconcentration factor (*PF*) and extraction recovery were calculated based on the following equations:

$$PF = C_{sed} / C_0 \quad (1)$$

where, C_{sed} and C_0 are the concentration of the analyte in the sedimented phase and initial concentration of the analyte in the aqueous sample, respectively.

$$ER\% = \frac{C_{sed} \times V_{sed}}{C_0 \times V_{aq}} \times 100 = PF \times \frac{V_{sed}}{V_{aq}} \times 100 \quad (2)$$

where, *ER%*, V_{sed} and V_{aq} are the extraction recovery and volumes of the sedimented and aqueous sample, respectively. C_{sed} was calculated from the related calibration curve, obtained by direct injection of amitraz standard solutions into the extraction solvent with the concentrations in the range of 10 - 100 mg·L^{-1}.

3.1. Selection of Extraction Solvent

The suitable extraction solvent should have some properties such as (a) its density should be higher than that of water, (b) it should have extraction capability of the desired compound, and (c) it should have a good gas chromatographic behavior. Carbon disulfide, carbon tetrachloride, chloroform and chlorobenzene were tested as extraction solvents. A series of sample solutions containing 100 µg·L^{-1} of amitraz were prepared. Acetonitrile (1.0 mL) of containing different volumes of the extraction solvents (12.0, 13.0, 25.6 and 45.0 µL of C_6H_5Cl, CCl_4, CS_2 and $CHCl_3$, respectively) was rapidly injected into the sample solutions to achieve 5.0 µL volume of sedimented phase. The average extraction recoveries using different extraction solvents are shown in **Figure 1**. The results revealed that CCl_4 has the highest extraction recovery in comparison with C_6H_5Cl, CS_2 and $CHCl_3$. Thereby; CCl_4 was selected as the extraction solvent in the subsequent experiments.

3.2. Selection of Disperser Solvent

Miscibility of disperser solvent with extraction solvent and aqueous phase is the main factor used for its selection. Thereby, acetone, acetonitrile and methanol were selected as disperser solvents. A series of sample solutions containing 100 µg·L^{-1} of amitraz were prepared and extracted using 1.0 mL of each disperser solvent containing 13.0 µL of CCl_4. The extraction recoveries obtained from acetone, acetonitrile and methanol were 76.5%, 96.4% and 77.7%, respectively. According to the results, acetonitrile has the higher extraction recovery and better gas chromatographic behavior in comparison with the other disperser solvents. Thus, acetonitrile was used as disperser solvent in the subsequent experiments.

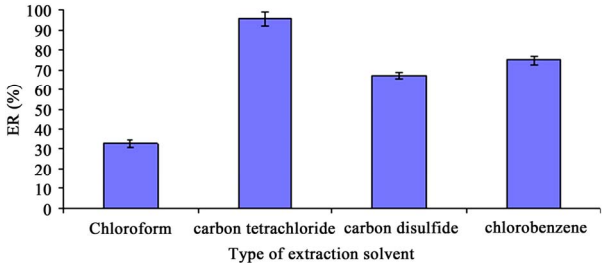

Figure 1. Effect of type of extraction solvent on the extraction recovery of amitraz. Extraction conditions: water sample volume, 5.0 mL; disperser solvent (acetonitrile) volume, 1.0 mL; extraction solvent volumes, 45.0 µL $CHCl_3$, 12.0 µL C_6H_5Cl, 13.0 µL CCl_4 and 25.6 µL CS_2; concentration of amitraz, 0.1 mg·kg^{-1}.

3.3. Effect of Extraction Solvent Volume

To examine the effect of extraction solvent volume on the extraction recovery, solutions containing different volumes of CCl_4 were used in DLLME procedure. The experimental conditions included the use of 1.0 mL acetonitrile containing different volumes of CCl_4 (13.0, 18.0, 23.0 and 28.0 µL). According to **Figure 2**, the volumes of the sedimented phase were changed from 5.0 to 14.0 µL by increasing the volume of CCl_4 from 13.0 to 28.0 µL. As the volume of the sedimented phase increases, the *PF* decreases due to the dilution of sedimented phase (**Figure 2**). Thereby, the highest sensitivity was achieved by using 13.0 µL of CCl_4.

3.4. Effect of Disperser Solvent Volume

Variation of disperser solvent volume causes a change in the volume of the sedimented phase; hence, it is necessary to consider the influence of disperser solvent volume on the extraction efficiency. In order to achieve a

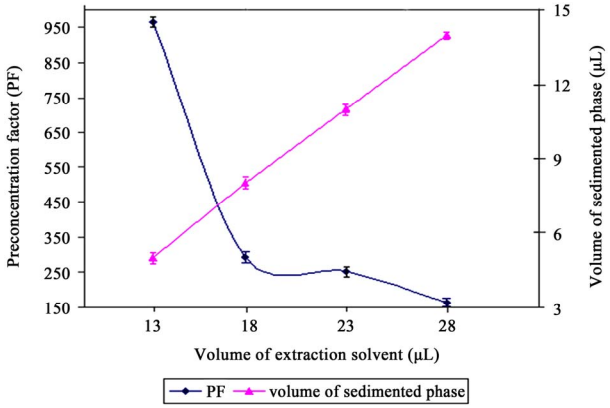

Figure 2. Effect of the extraction solvent (CCl_4) volume on the sedimented phase volume and preconcentration factor of amitraz. Extraction conditions: water sample volume, 5.0 mL; disperser solvent (acetonitrile) volume, 1.0 mL; extraction solvent (CCl_4) volumes, 13.0, 18.0, 23.0, 28.0 µL; concentration of amitraz, 0.1 mg·kg^{-1}.

constant volume of the sedimented phase, the volumes of acetonitrile (disperser solvent) and CCl_4 (extraction solvent) were changed, simultaneously. The experimental conditions were fixed and included the use of different volumes of acetonitrile (0.5, 1.0, 1.5 and 2.0 mL) containing 11.0, 13.0, 17.0 and 21.0 µL of CCl_4, respectively. Under these conditions, the volume of sedimented phase remained constant (5.0 ± 0.3 µL). As shown in **Figure 3**, the extraction recovery enhances by increasing of the acetonitrile volume up to 1.0 mL and then decreases by further increasing of acetonitrile volume. It seems that at low volume of acetonitrile cloudy state is not well pronounced and the extraction recovery decreases. On the other hand, at high volumes of acetonitrile the solubility of amitraz in water increases, and the extraction recovery decreases. Therefore 1.0 mL of acetonitrile was chosen as the optimum volume in the further works.

3.5. Effect of Ionic Strength

To investigate the influence of ionic strength on the extraction recovery of amitraz, different amounts of NaCl (0% - 10% w/v) were added to the solutions, whereas other experimental conditions were kept constant. The volume of the sedimented phase increased from 5 to 11 µL by increasing of the amount of NaCl from 0% to 10% w/v, because of the decreasing solubility of the extraction solvent in the aqueous phase. According to **Figure 4**, the preconcentration factor decreases as the volume of sedimented phase increases. Therefore, all of the extraction experiments were carried out without salt addition.

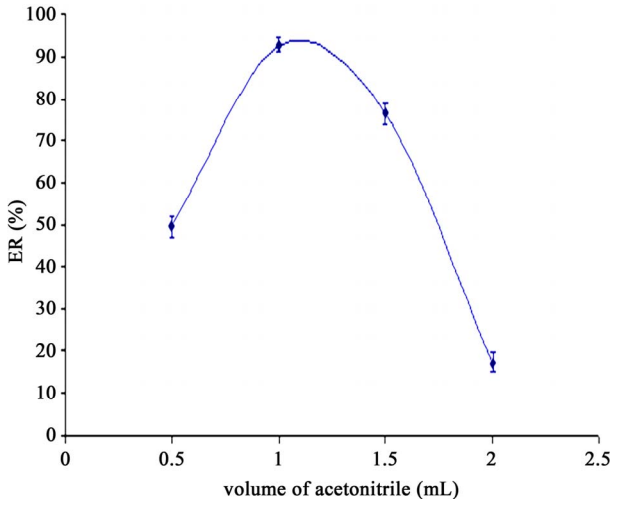

Figure 3. Effect of the disperser solvent (acetonitrile) volume on the extraction recovery of amitraz. Extraction conditions: water sample volume, 5.0 mL; disperser solvent (acetonitrile) volumes, 0.50, 1.0, 1.5 and 2.0 mL; extraction solvent (CCl_4) volumes, 11.0, 13.0, 17.0 and 21.0 µL; concentration of amitraz, 0.1 mg·kg^{-1}.

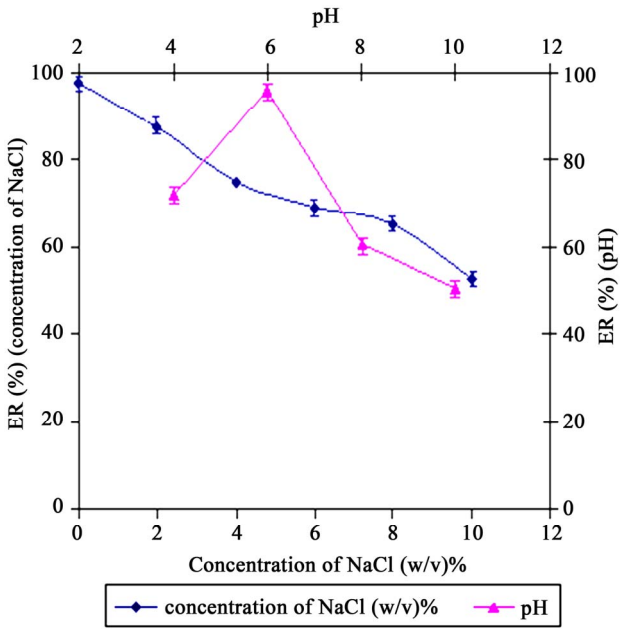

Figure 4. Effect of salt addition and pH on the extraction recovery of amitraz. Extraction conditions: water sample volume, 5.0 mL; disperser solvent (acetonitrile) volume, 1.0 mL; extraction solvent (CCl4) volume, 13.0 µL; concentration of amitraz, 0.1 mg·kg^{-1}.

3.6. Influence of pH

The effect of pH on the extraction recovery of amitraz was studied in the range of 4.0 - 10.0, using ammonium acetate solution and step wise addition of NaOH. As shown in **Figure 4**, the highest extraction recovery was obtained at pH of 6.0. It is due to the lowest hydrolysis of amitraz at pH 6.0.

3.7. Analytical Performance of the Method

Linearity of the method was over the range of 0.01 - 1.0 mg·kg^{-1}(with nine standards, r^2 = 0.998). The ER% and PF of the method were 95.5% and 955, respectively at spike level of 0.1 mg·kg^{-1}. The relative standard deviation (RSD, n = 4) at the concentration level of 0.1 mg·kg^{-1} of amitraz was 3.2%. The limit of detection (LOD), based on the signal-to-noise ratio (S/N) of 3 was 0.0015 mg·kg^{-1}. **Table 1** comprises the figures of merit of proposed extraction method with the other extraction methods of amitraz. As shown, DLLME have shorter extraction time and lower RSD and LOD value with acceptable linear range (LR) compared with the other extraction methods.

3.8. Honey Samples Analysis

In order to test the applicability of the proposed method

to real samples, two honey samples were extracted and analyzed. The results showed that the analyzed samples were free of amitraz. To study the matrix effect on the extraction recovery of amitraz in the honey samples, 0.05 g of the honey samples was dissolved in 5 mL of the twice distilled water and a homogenized solution was produced. Both the honey samples were spiked with the amitraz standard solution at 0.1 $mg \cdot kg^{-1}$ concentration level to assess the recovery values. The obtained relative recoveries were 78.8% and 98.2%. The results showed that the matrix had little effect on the DLLME of amitraz. **Figure 5** shows GC-FID chromatograms of a honey sample (a) before and (b) after being spiked of the honey with amitraz at 0.1 $mg \cdot kg^{-1}$ level.

4. Conclusions

A rapid and sensitive method for the extraction and determination of amitraz in honey samples by applying DLLME-GC-FID was developed. The experimental results showed that the present method provides high extraction recovery and preconcentration factor within a short time. The extraction and determination of amitraz from the honey samples by applying the proposed method was satisfactory. The newly developed microextraction technique (DLLME-GC-FID) has distinct advantages over the conventional methods in terms of short time of extraction, low volume of the solvents required and low detection limits. Further, the proposed sample preparation procedure is much simpler than the conventional liquid-liquid extraction (LLE) and solid phase extraction (SPE) methods.

Table 1. Comparison of DLLME-GC-FID with other methods for determination of amitraz.

Methods	LOD[b] ($mg \cdot kg^{-1}$)	LR[c] ($mg \cdot kg^{-1}$)	RSD[d] (%)	Extraction time (min)	Sample volume (mL)
HSME[a]-GC-TSD[13]	0.01	0.1 - 10	10	10	5
SPME-GC-ITD[9]	0.001	0.005 - 0.1	11.1	30	10
DLLME-GC-FID	0.0015	0.025 - 1	3.2	≤ 5	10

[a]Headspace solvent microextraction; [b]Limit of detection for S/N=3; [c]Linear range; [d]Relative standard deviation.

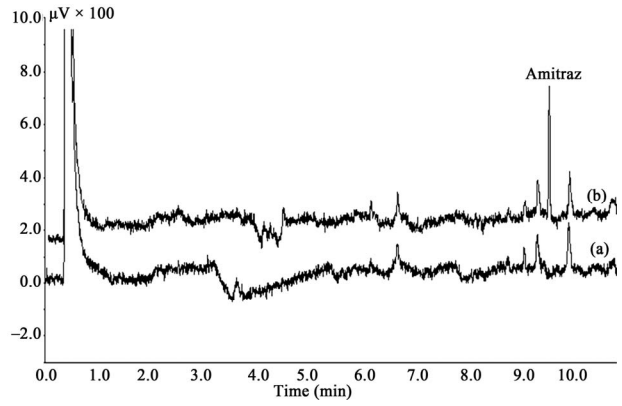

Figure 5. DLLME-GC-FID chromatograms of the honey sample under optimum conditions (a) before and (b) after spiking with 0.1 mg·kg-1 of amitraz.

5. References

[1] R. Rial-Otero, E. M. Gaspar, I. Moura and J. L. Capelo, "Chromatographic-Based Methods for Pesticide Determination in Honey: an Overview," *Talanta*, Vol. 71, No. 2, 2007, pp. 503-514. doi:10.1016/j.talanta.2006.05.033

[2] Y. Demirel, A. Yilmaz, S. Gursoy, K. Kaygusuz and C. Mimaroglu, "Acute Amitraz Intoxication: Retrospective Analysis of 45 Cases," *Human & Experimental Toxicology*, Vol. 25, No. 10, 2007, pp. 613-617.

[3] F. M. Young, M. F. Menadue and T. C. Lavranos, "Effects of the Insecticide Amitraz, an Alpha 2-Adrenergic Receptor Agonist, on Human Luteinized Granulosa Cells," *Human Reproduction*, Vol. 20, No. 11, 2005, pp. 3018-3025.

[4] E. Elinav, Y. Shapira, Y. Ofran, A. H. Hassan and I. Z. Ben-Dov, "Near-Fatal Amitraz Intoxication: The Overlooked Pesticide," *Basic & Clinical Pharmacology & Toxicol*ogy, Vol. 97, No. 3, 2005, pp. 185-187.

[5] H. L. Yilmaz and D. R. Yildizdas, "Amitraz Poisoning, an Emerging Problem: Epidemiology, Clinical Features, Management, and Preventive Strategies," *Arcives of Diesease in Childhood*, Vol. 88, 2003, pp. 130-134. doi:10.1136/adc.88.2.130

[6] M. caldow, R. J. Fussell, F. Smith and M. Sharman, "Development and Validation of an Analytical Method for Total Amitraz in Fruit and Honey with Quantification by Gas Chromatography-Mass Spectrometry," *Food Additives and Contaminants*: *Analysis*, *Surveillance*, *Evaluation*, *Control*, Vol. 24, No. 3, 2007, pp. 280-284.

[7] E. Corta, A. Bakkali, L. A. Berrueta, B. Gallo and F. Vicente, "Kinetics and Mechanism of Amitraz Hydrolysis In Aqueous Media by HPLC and GC-MS," *Talanta*, Vol. 48, No. 1, 1999, pp. 189-199. doi:10.1016/S0039-9140(98)00237-9

[8] H. Yua, Y. Taoa, T. Leb, D. Chena, A. Ishsana, Y. Liua, Y. Wanga and Z. Yuan, "Simultaneous Determination of Amitraz and Its Metabolite Residue in Food Animal Tissues by Gas Chromatography-Electron Capture Detector and Gas Chromatography—Mass Spectrometry with Accelerated Solvent Extraction," *Journal of Chromatography B*, Vol. 878, No. 21, 2010, pp. 1746-1752.

[9] M. E. C. Queiroz, C. A. A. Valadão, A. Farias, D. Carvalho and F. M. Lanças, "Determination of Amitraz in Canine Plasma by Solid-Phase Microextraction-Gas Chromatography with Thermionic Specific Detection," *Journal of Chromatography B*, Vol. 794, No. 2, 2003, pp. 337-342.

[10] R. Brimecombe and J. Limson, "Voltammetric Analysis of the Acaricide Amitraz and its Degradant, 2,4-Dimethylaniline," *Talanta*, Vol. 71, No. 2, 2007, pp. 1298-1303.

[11] Y. Pico, M. Farre, N. Tokman and D. Barcelo, "Rapid and Sensitive Ultra-High-Pressure Liquid Chromatography-Quadrupole Time-of-Flight Mass Spectrometry for the Quantification of Amitraz and Identification of Its Degradation Products in Fruits," *Journal of Chromatography A*, Vol. 1203, No. 1, 2008, pp. 36-46.

[12] A. Economou, H. Botitsi, S. Antoniou and D. Tsipi, "Determination of Multi-Class Pesticides in Wines by Solid-Phase Extraction and Liquid Chromatography-Tandem Mass Spectrometry," *Journal of Chromatogaphy A*, Vol. 1216, No. 31, 2009, pp. 5856-5867.

[13] M. Shamsipur, J. Hassan, J. Salar-Amoli and Y. Yamini, "Headspace Solvent Microextraction-Gas Chromatographic Thermionic Specific Detector Determination of Amitraz in Honey after HYDROLYSIS to 2,4-Dimethylaniline," *Journal of Food Composition and Analysis*, Vol. 21, No. 3, 2008, pp. 264-270.

[14] M. Rezaee, Y. Assadi, M. R. Milani Hosseini, E. Aghaee, F. Ahmadi and S. Berijani, "Determination of Organic Compounds in Water Using Dispersive Liquid-Liquid Microextraction," *Journal of Chromatography A*, Vol. 1116, No. 31, 2006, pp. 1-9. doi:10.1016/j.chroma.2006.03.007

[15] N. Shokoufi and A. Hamdamali, "Laser Induced-Thermal Lens Spectrometry in cOMBINATION with Dispersive Liquid-Liquid Microextraction for Trace Analysis," *Analytica Chimica Acta*, Vol. 681, No. 1-2, 2010, pp. 56-62. doi:10.1016/j.aca.2010.09.021

[16] S. C. Cunha and J. O. Fernandes, "Quantification of Free and total Bisphenol A and Bisphenol B in Human Urine by Dispersive Liquid-Liquid Microextraction (DLLME) and Heart-Cutting Multidimensional Gas Chromatography-Mass Spectrometry (MD-GC/MS)," *Talanta*, Vol. 83, No. 1, 2010, pp. 117-125.

[17] L. Kocúrová, I. S. Balogh, J. Şkrlíková, J. Posta and V. Andruch, "A Novel Approach in Dispersive Liquid- Liquid Microextraction Based on the Use of an Auxiliary Solvent for Adjustment of Density: UV-VIS Spectrophotometric and Graphite Furnace Atomic Absorption Spectrometric Determination of Gold Based on Ion Pair Formation," *Talanta*, Vol. 82, No. 5, 2010, pp. 1958-1964.

[18] N. M. Najafi, H. Tavakoli, R. Alizadeh and S. Seidi, "Speciation and Determination of Ultra Trace Amounts of Inorganic Tellurium in Environmental Water Samples by Dispersive Liquid-Liquid Microextraction and Electrothermal Atomic Absorption Spectrometry," *Analytica Chimica Acta*, Vol. 670, No. 1-2, 2010, pp. 18-23.

[19] J. Leníček, M. Sekyra, A. R. Novotná, E. Vášová, D. Titěra and V. Veselý, "Solid Phase Microextraction and Gas Chromatography with Ion Trap Detector (GC-ITD) Analysis of Amitraz Residues in Beeswax after Hydrolysis to 2,4-Dimethylaniline," *Analytica Chimica Acta*, Vol. 571, No. 1, 2006, pp. 40-44.

