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

Mostafa Bashiri-Juybari1, Ali Mehdinia*, Ali Jabbari1, Yadollah Yamini3
1Department of Chemistry, Faculty of Sciences, K. N. Toosi University of Technology, Tehran, Iran
2Department of Marine Living Resources, Iranian National Institute for Oceanography, Tehran, Iran
3Department 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·kg⁻¹ and the limit of detection was 0.0015 mg·kg⁻¹. The relative standard deviation (RSD, n = 4) for 0.1 mg·kg⁻¹ of amitraz was 3.2%. The recoveries of amitraz from honey samples at the spiking levels of 0.1 mg·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-amitmethylnitrophenyl)imino] methyl methanimidamide) is a member of formamidine pesticide family. It is widely applied on beehives to control the beehive parasite Varroa jacobseni 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·kg⁻¹ in Germany and Italy and 0.2 mg·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 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, HNO₃ (>90%), and NaOH (>99%) were purchased from Merck (Darmstadt, Germany).

The honey samples were obtained from Tabriz (Azarbayjea, 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·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·min⁻¹. The flow rate of zero air (99.9999%, Air products, UK) was 400 mL·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 kg⁻¹ of amitraz. One mL of acetonitrile (as disperser solvent) containing 13.0 µL of CCl₄ (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:
where, \( C_{\text{sed}} \) and \( C_0 \) are the concentration of the analyte in the sedimented phase and initial concentration of the analyte in the aqueous sample, respectively.

\[
ER\% = \frac{C_{\text{sed}} \times V_{\text{sed}} \times 100}{C_0 \times V_{aq}} = PF \times \frac{V_{\text{sed}}}{V_{aq}} \times 100
\]

where, \( ER\% \), \( V_{\text{sed}} \) and \( V_{aq} \) are the extraction recovery and volumes of the sedimented and aqueous sample, respectively. \( C_{\text{sed}} \) 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\(_6\)H\(_5\)Cl, 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\(_6\)H\(_5\)Cl, 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.

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
constant volume of the sedimented phase, the volumes of acetonitrile (disperser solvent) and CCl₄ (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 CCl₄, 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.

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, r² = 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·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·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.

### 5. References


