Journal of Food and Drug Analysis, Vol. 12, No. 3, 2004, Pages 212-216 藥物食品分析 第十二卷 第三期

Solid-Phase Extraction and GC-MSD Determination of Amitraz and Metabolites in Urine

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(Received: November 14, 2003; Accepted: April 15, 2004)

ABSTRACT

Urine samples were collected from seven farmers before and after applying 20% amitraz EC to pear and citrus fields. Amitraz and its metabolites including BTS-27919, BTS-27271, and 2,4-dimethylaniline were eluted with *n*-hexane, dichloromethane and methanol in sequence from SPE-C18 cartridge by loading 5 mL of urine before GC-MSD determination. The mean recoveries of amitraz, BTS-27919, BTS-27271 and 2,4-dimethylaniline were 75.7 ± 4.2 , 81.3 ± 2.4 , 87.7 ± 3.7 and 83.3 ± 1.8%, respectively. Neither amitraz nor metabolites were detected in any of the seven farmers' urine samples.

Key words: Amitraz, metabolites, urine, GC-MSD

INTRODUCTION

Amitraz (*N*-methylbis-(2,4-xylyiminomethyl)-amine) is a non-systemic acaricide and insecticide. Its mode of action probably involves an interaction with octopamine receptors in the nervous system of ticks for increasing nervous activity. Amitraz is used to control all stage of *tetranychid* and eriophyid mites, pear suckers, scale insects, mealybugs, whitefly, aphids and eggs and the first instar larvae of *lepidoptera* on pome fruit, citrus fruit, cotton, stone fruit, bush fruit, strawberries, hops, cucurbits, aubergines, capsicums, tomatoes, ornamentals and some other crops. Amitraz is also used as an animal ectoparasiticide to control ticks, mites and lice on cattle, dogs, goats, pigs and sheep[1]. In Taiwan, 20% amitraz EC is applied to citrus and pears fields to control red citrus mites and two-spotted spider mites.

Amitraz is rapidly metabolized in several species, including humans, to form six metabolites which are excreted primarily in urine. The EPA has established a reference dose (RfD) for amitraz of 0.002 mg/kg/day, based on results that from chronic oral toxicity study in dogs. Amitraz rapidly degrades in the environment, into two primary transformation products BTS-27271 (*N*-(2,4-dimethylphenyl)-*N*'-methylformamidine) and BTS-27919 (2,4-dimethylphenylformamide)[2]. Its metabolites rapid degradation in the environment are similar to that parent amitraz, and are not a matter of concern in ground or surface waters, although BTS-27271 and BTS-27919 have been shown to persistent moderately in aquatic and terrestrial environments[3].

The residues of amitraz in fruit and soil samples was analyzed by Hornish *et al*. (1984), using a series of process including base-hydrolysis of amitraz and its metabolites to 2,4-dimethylaniline, steam distillation/continuous extraction, acid/base partition clean-up, derivation of heptafluorobutyranilide and then quantitated by gas chromatography with electron capture detector. The sensitivity of amitraz was 0.05 ppm and the average recovery were 77 ± 10% within 0.03-1.0 ppm range[4]. This method by gas chromatography was analyzed only for total amitraz, and the sample preparation and derivatization spent a long time. However, several studies applied this method to analyze the amitraz in fruits and honey at that time[5,6].

A method for analyze amitraz and its major metabolites, BTS-27271 and BTS-27919, in pears was developed by NOR-AM chemical company (1992). The compounds were extracted by blending the sample with acetone/sodium carbonate, and the extracts was partitioned to petroleum ether/dichloromethane. Amitraz and metabolites were finally detected by gas chromatography using a nitrogen/phosphorus detector. The mean recoveries rates of amitraz, BTS-27271, and BTS-27919 were 85.1 ± 8.5%, 73.9 ± 11.2%, 95.1 ± 13.9%, respectively. The limit of detection was 0.05 ppm for amitraz, BTS-27271 and BTS-27919[7]. However, these two studies did not analyze amitraz and metabolites in the urine of occupationally exposed persons.

The goal of this study is to develop a rapid and efficient analytical method for determining the residues of amitraz and metabolites in persons who exposure in amitraz in fields. Solid phase extraction (SPE) was used to isolate the amitraz and metabolites from urine samples, to reduce the amount of solvent used, and GC-MSD was then used to detect amitraz and its metabolites simultaneously without any derivative procedures.

MATERIALS AND METHODS

I. *Reagents and Materials*

Amitraz and its metabolites, BTS-27271 and BTS-

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27919, were obtained from Nissan Cooperation (Taiwan Branch). 2,4-dimethylaniline was purchased from Dr. Ethrenstorfer (Augsburg, Germany). Acetone, *n*-hexane, acetonitrile and methanol were purchased from Merck (Darmstadt, Germany). Dichloromethane was purchased from Mallinckradt Chrom. All the solvents were LC grade.

The SPE-C18 cartridge was obtained from J&W scientific (folsom, USA) and 20% amitraz EC pesticide was purchased from Nissan Cooperation (Taiwan Branch).

II. *GC-MSD Apparatus and Conditions*

A Hewlett-Packard 6890 series gas chromatograph, equipped with a 5972 mass selective detector, was used. The column was a Hewlett-Packard 5 MS (25 m × 0.2 mm). The carrier gas was helium, and the system was maintained in constant flow mode with a pressure of 15 KPa at 220˚C. Injection volume was 1.0 μL in split mode. The injector temperature was 220˚C, and the transfer line to the MSD system was set at 280˚C. The initial temperature of oven was 60˚C, which was held for 2 min, following injection, the temperature was increased at 10˚C/min to 270˚C, at which it was held for 5 min.

The temperature of the ion source and the quadrupole were set on 230˚C and 150˚C, respectively, ionization was performed in electron impact mode (EI) at 70 eV. Detection was in the selected ion mode (SIM), the monitored ion were as follows. (1) 0-5 min: 121 and 106 m/z (2,4-dimethylaniline), (2) 5-8 min: 120, 149 and 106 m/z (BTS-27919), (3) 8-10 min: 162, 132 and 120 m/z (BTS-27271). (4) 12 min: 293, 121 and 132 m/z (amitraz).

III. *Preparation of the Calibration Curve*

Amitraz and its metabolites were prepared at concentrations of 0.1, 0.2, 0.5, 1.0, 2.0, 5.0, 8.0 and 10 μg/mL, and injected to the instrument three times of each concentration. Calibration curve was performed by plotting the peak area of each compounds versus concentration.

IV. *Cleaning Up Using Solid-phase Extraction*

Five mL urine samples were loaded onto an SPE cartridge (C18, 500 mg, with a volume of 3 mL), which had been pre-conditioned with 10 mL of water. The cartridge then eluted with different solvents to fractionate amitraz and metabolites. The first fraction with 10 mL of *n*-hexane was 2,4-dimethylaniline, the second fraction with 15 mL of dichloromethane was amitraz and BTS-27919, and the third fraction with 15 mL of methanol was BTS-27271. All the fractions were collected, and the final volume of each fraction was made up to 1mL by dissolving in acetone, and was determination by GC-MSD.

V. *Recovery Test*

Five mL of urine samples from unexposed people were spiked with amitraz and metabolites at concentration of 0.05, 0.1 and 1.0 μg/mL in triplicate. Chemicals spiked urine samples were cleaned up and analyzed by the method mentioned above, the recovery were calculated before the urine samples were analyzed.

VI. *Stability of Amitraz in Urine during Storage*

One μg/mL of amitraz was added to 5 mL of urine sample of unexposed subjects, stored for 0, 4, 8, 16, 20 and 24 hours and 2, 3, 4, 5, 6, 7 days at 25˚C, and for 0, 3, 7, 10, 14, 18 and 21 days at -20˚C in triplicate, to examine the stability of amitraz in urine.

RESULTS AND DISCUSSION

I. *Calibration Curve and Gas Chromatography*

Calibration graphs of abundance, y, versus concentration (μg/mL), x, obtained from amitraz, BTS-27919, BTS-27271 and 2,4-dimethylaniline are shown in Figure 1. The regression equations of the curve and their correlation coefficients were also calculated, and the R^2 were between 0.976 (2,4-dimethylaniline) and 0.999 (BTS-27919).

Figure 2 shows the total ion chromatograms and the mass spectra of amitraz and its metabolites. According to the total ion chromatogram, the retention time (RT) was as follows: 2,4-dimethylaniline (RT = 8.49 min), BTS-27919

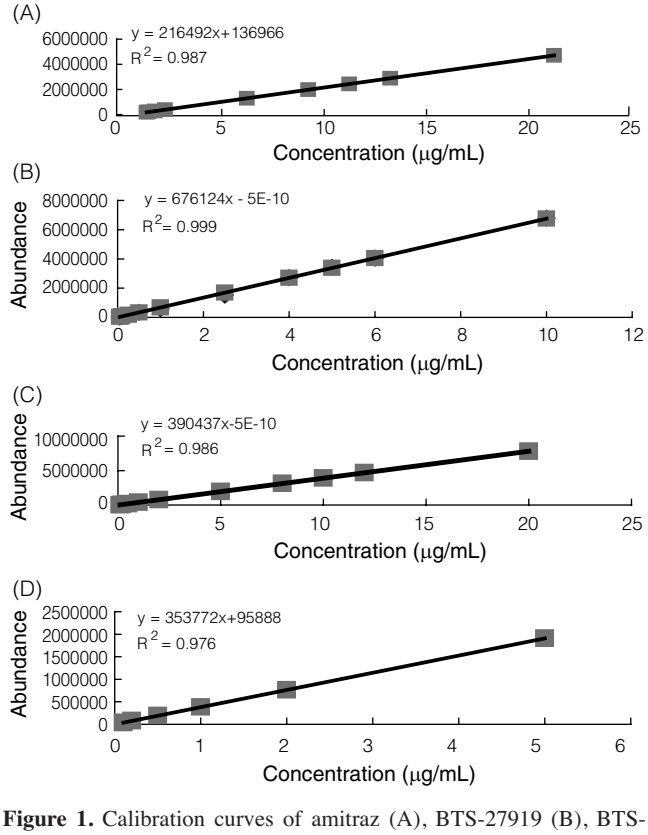

Figure 1. Calibration curves of amitraz (A), BTS-27919 (B), BTS-27271 (C), and 2,4-dimethylaniline (D) on GC-MSD.

(RT = 12.9 min), BTS-27271 (RT = 13.53 min) and amitraz (RT = 21.62 min). The parent ions in the mass spectra for amitraz, BTS-27271, BTS-27919 and 2,4-dimethylaniline, appeared at 293, 162, 149 and 121 m/z, respectively, reflecting the molecular weights of these compounds.

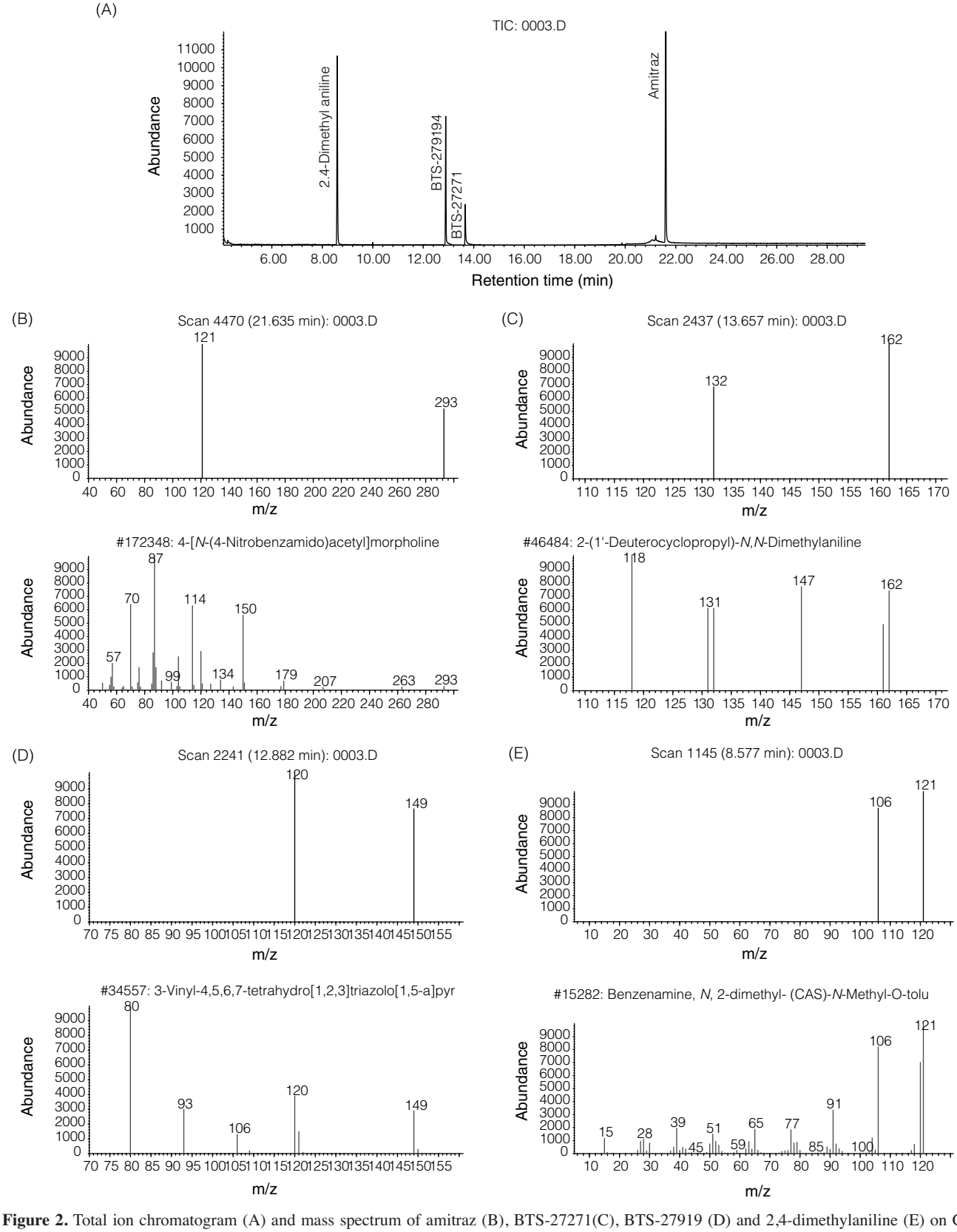

Figure 2. Total ion chromatogram (A) and mass spectrum of amitraz (B), BTS-27271(C), BTS-27919 (D) and 2,4-dimethylaniline (E) on GC-MSD.

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II. *Recovery and Detection Limits of Amitraz and Metabolites in Urine*

In this study, various amounts of amitraz and its metabolites (0.05, 0.1 and 1.0 μg/mL) were spiked into 5 mL of urine samples, after clean up by the SPE procedures and determine by GC/MS to estimate the recovery. The recovery of each compound at three concentrations as follows: 2,4-dimethylaniline were 75 ± 2.4, 86 ± 1.6, and 89 ± 1.5%, BTS-27271 were 73 ± 1.6, 84 ± 2.4, and 87 ± 3.3%, BTS-27919 were 82 ± 3.0, 87 ± 3.7 and 94 ± 4.5%, and amitraz were 72 ± 4.8, 66 ± 3.3, and 89 ± 4.5%, respectively, and the detection limits of amitraz and metabolites were between 0.024 and 0.0024 ng/mL (Table 1). The average of the recovery was over 80% except amitraz, since the parent compound amitraz is less polar compound compared with BTS-27919, when the non-polar C18 was used, it could be not completely eluted from C18; on the contrary, BTS-27919, a medium polar metabolite, can be eluted perfectly from C18 when dichloromethane as the eluting solvent.

III. *Analytical Methods and Results of the Urine Samples*

The formulated 20% amitraz EC was applied to citrus and orange fields at a rate of 2.0 L/ha, and urine samples were collected from the seven farmers 24 hr after exposure, to determine the amounts of amitraz and metabolites simultaneously. The toxicity of amitraz is clearly very important, especially in mammals[8]. The metabolism pathway of amitraz in rats were BTS-27271, BTS-27919 and BTS-24868 (2,4-dimethylaniline)[9]. Notably, the latter are number analog chemical of aniline, a carcinogenic compounds[10].

The urine samples contained many large molecules such as proteins and lipids. Therefore non-polar C18 was chosen, the solvents used to elute the compounds here was by means of the polarity of solvents. For instance, hexane a non-polar solvent was used to elute the non-polar 2,4-dimethylaniline, the medium polarity dichloromethane was used to co-elute the amitraz and BTS-27919, and the more polar methanol was used to elute the more polar compound BTS-27271. After that, amitraz and metabolites were separated from the urine samples before GC-MSD determination.

The results of the urine samples after analysis showed that the urine of the seven farmers contained neither amitraz nor the metabolites. Thougsinthusk *et al.* [11] estimated dermal absorption using the exponential saturation model. The percentage of residual amitraz in the test animals was 8.67%, after 10 hr of dermal exposure (during which the dermal absorption rate was 13.8%). In this study, amitraz was applied to the citrus and pear trees, at a rate of 2.0 L/ha by the auto-pumping sprayer. For the direct measurement of exposure, we had placed absorbent pads at various points on the farmer's body and allowed him to go about his usual spray operations (wear gloves and mask). Urine samples were collected before, during and after exposure (within 24 hr) to determine amitraz and metabolites; however, the time of amitraz spraying was only 1 hr, so the real exposure to amitraz of the farmers is less than that in the Thougsinthusk's model. The urine samples from the farmers did not show detectable residues of amitraz or metabolites.

IV. *Stability of Amitraz in Urine during Storage*

A known amount (1.0 μg/mL) of amitraz was spiked into 5 mL un-exposed urine samples, which were stored at 25˚C and -20˚C. The stability of amitraz over time was investigated. When stored at -20˚C, the concentration of amitraz was found not to change over time (Data not shown). But when stored at 25˚C, the concentration fell to 0.5 μg/mL, after 20 hr (Figure 3). Therefore, the half-life of amitraz was 20 hr at 25˚C. After seven days, amitraz was not detectable in the urine at 25˚C implying that the stability of amitraz in urine depends on the temperature

Table 1. Recovery and detection limit of amitraz, BTS-27919, BTS-27271, and 2,4-dimethylaniline in urine

Pesticide	Recovery (%)			Detection limit
	μg/mL	Recovery	CV (%)	(ng/mL)
Amitraz	0.05	72.0 ± 4 .8	6.6	
	0.1	66.0 ± 3.3	5.0	
	1.0	89.0 ± 5.0	5.0	
	Mean	75.7 ± 4.2	5.5	0.026
BTS-27271	0.05	73.0 ± 1.6	2.2	
	0.1	84.0 ± 2.4	2.8	
	1.0	87.0 ± 3.3	3.8	
	Mean	81.0 ± 2.4	2.9	0.0024
BTS-27919	0.05	82.0 ± 3.0	3.6	
	0.1	87.0 ± 3.7	4.3	
	1.0	94.0 ± 4.5	4.8	
	Mean	87.7 ± 3.7	4.2	0.0023
2,4-Dimethylaniline	0.05	75.0 ± 2.4	3.1	
	0.1	86.0 ± 1.6	1.9	
	1.0	89.0 ± 1.5	1.7	
	Mean	83.3 ± 1.8	2.2	0.024

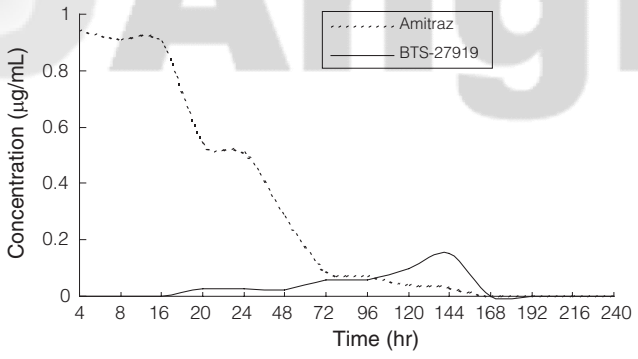

Figure 3. The degradation of amitraz in urine during storage at 25°C.

of storage. At 25°C the amounts of amitraz and metabolites in urine during storage were found to be correlated. The amount of amitraz fell as the metabolite BTS-27919 at 25°C. Corta's work[12] on the kinetics and mechanism of amitraz hydrolysis in solution stated that the process is dependent on pH. When the pH of the medium was below 2, most of the metabolites were 2,4-dimethylaniline, but when the pH of the medium was between 3 and 6, the metabolites were BTS-27271 and BTS-27919. In alkaline solution (pH = 8), the metabolites were only BTS-27919. The pH of the urine in this study was between 6 and 8, favoring the formation of BTS-27919. The results herein clearly agree with the results of Corta's investigation. Based on these results we stored the urine samples at -20°C before analysis.

CONCLUSIONS

Developing a fast and efficient method for detecting amitraz and its metabolites in urine is important, especially for biological monitoring when occupational exposures to amitraz occurs in fields. This study developed a method of combining SPE and GC-MSD, and the highest recovery was achieved when C18 cartridge was preconditioned with water, and then solvent fractions were used to elute amitraz and its metabolites in order. The average recovery of amitraz, BTS-27919, BTS-27271 and 2,4-dimethylaniline were 75.7 ± 4.2%, 87.7 ± 3.7%, 81.0 ± 2.4% and 83.3 ± 1.8%, respectively.

Methods based on GC-MSD were described which allow the determination of low-levels of the substances of interest in urine. BTS-27919 (0.0023 ng/mL) and BTS-27271 (0.0024 ng/mL) were more sensitive biomarkers of amitraz than indicated in the literature[4,5,6,7]. We recommend the method adopted herein, not only because other analytical methods are unsuitable for application in human biomonitoring studies, but also because the present method that combines SPE and GC-MSD is more rapid and sensitive.

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