

# Simple and Sensitive Analysis of Nereistoxin and Its Metabolites in Human Serum Using Headspace Solid-Phase Microextraction and Gas Chromatography–Mass Spectrometry

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## Abstract

A simple method for the analysis of nereistoxin and its metabolites in human serum using headspace solid-phase microextraction (SPME) and gas chromatography–mass spectrometry (GC–MS) is developed. A vial containing a serum sample, 5M sodium hydroxide, and benzylacetone (internal standard) is heated to 70°C, and an SPME fiber is exposed for 30 min in the headspace of the vial. The compounds extracted by the fiber are desorbed by exposing the fiber in the injection port of the GC–MS. The calibration curves show linearity in the range of 0.05–5.0 µg/mL for nereistoxin and *N*-methyl-*N*-(2-methylthio-1-methylthiomethyl)ethylamine, 0.01–5.0 µg/mL for *S*,*S*'-dimethyl dihydronereistoxin, and 0.5–10 µg/mL for 2-methylthio-1-methylthiomethylethylamine in serum. No interferences are found, and the analysis time is 50 min for one sample. In addition, this proposed method is applied to a patient who attempted suicide by ingesting Padan 4<sup>R</sup>, a herbicide. Padan 4<sup>R</sup> contains 4% cartap hydrochloride, which is an analogue of nereistoxin. Nereistoxin and its metabolites are detected in the serum samples collected from the patient during hospitalization. The concentration ranges of nereistoxin in the serum are 0.09–2.69 µg/mL.

## Introduction

Cartap (brand name Padan) was developed from nereistoxin and is widely used for pesticides. Nereistoxin was first isolated from a marine annelid (*Limbriconereis heteropoda* Marenz) (1). The structure of nereistoxin is similar to that of carbamates, but the inhibitory mechanism of nereistoxin is different from that of carbamates. Cartap is hydrolyzed to nereistoxin in insects and humans. Nereistoxin then combines with the acetylcholine receptor of the cholinergic neurons, competing with acetylcholine

(2,3). As a result, the conduction of nerve impulses is obstructed. Several poisoning cases involving cartap are reported every year (4). Detection methods of nereistoxin in soil or human blood using photometric and gas chromatographic (GC) methods have been reported (5–7). However, there is only one report (8) in which the concentrations of nereistoxin and its metabolites in biological materials of a poisoning case were measured. The sample preparation procedures described in the report were laborious and time consuming. A more accurate, simple, and rapid method for analysis of nereistoxin is required in forensic or clinical practices.

A solid-phase microextraction (SPME) method involves the partitioning of analytes between the fiber and sample matrix and integrates sampling, extraction, concentration, and sample introduction (9). In previous studies, the SPME method was applied to the determination and quantitation of nicotine, cotinine, amphetamines, malathion, local anaesthetics, and tetracyclic antidepressants in medico-legal or acute poisoning cases (10–15). In addition, the SPME method has been applied to extract organophosphorus and carbamate pesticides from biological fluids (16,17).

A more simple method using headspace-SPME for the analysis of nereistoxin and its metabolites in serum has been developed. Cartap is quantitatively hydrolyzed to nereistoxin when it is spiked in blood (7). Therefore, nereistoxin and its metabolites were quantitatively analyzed by selected ion monitoring (SIM). This method was applied to a suspected suicide case in which the patient ingested Padan 4<sup>R</sup>.

## Experimental

### Materials

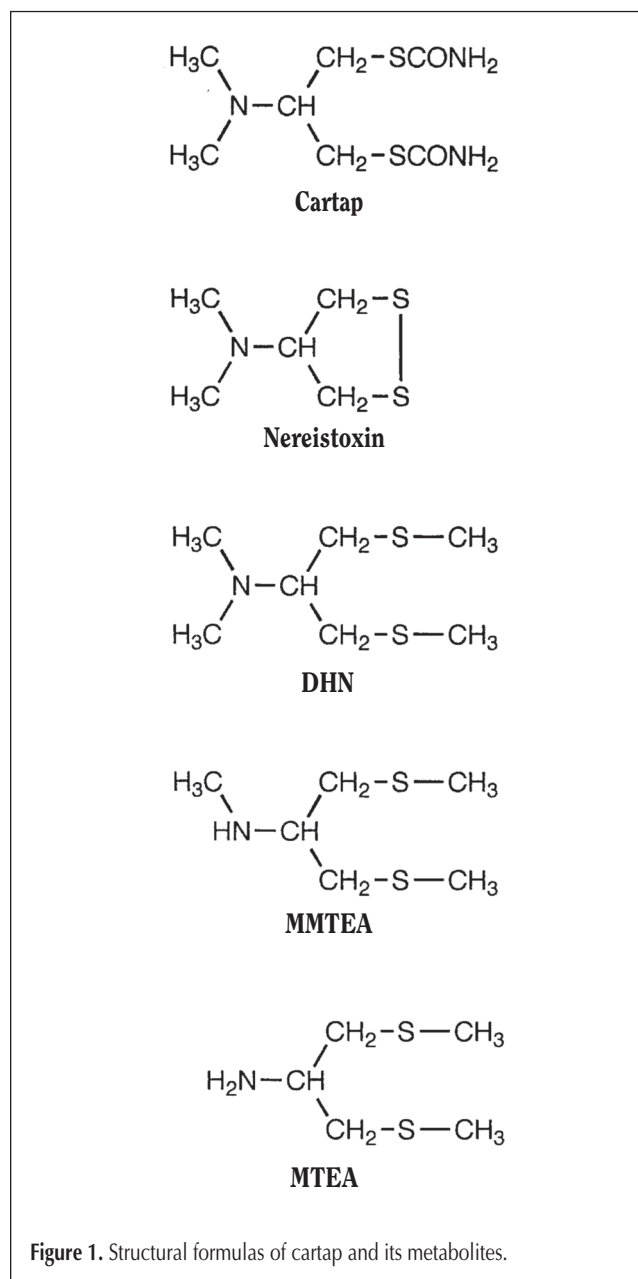
Nereistoxin oxalate and benzylacetone as an internal standard were purchased from Wako Pure Industries (Tokyo,

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Japan). Cartap hydrochloride and its three metabolites, *S,S'*-dimethyl dihydronereistoxin (DHN), *N*-methyl-*N*-(2-methylthio-1-methylthiomethyl)ethylamine (MMTEA), and 2-methylthio-1-methylthiomethylethylamine (MTEA), were supplied from Takeda Chemical Industries (Tokyo, Japan). The structure of each analyte is shown in Figure 1. Other reagents and solvents were purchased at the highest commercial quality.

All aqueous solutions were prepared using deionized water. Stock standard solutions (1.0 mg/mL) of cartap and its three metabolites were dissolved in water and stored at 4°C in a refrigerator. Stock standard solutions were analyzed by direct injection using GC-mass spectrometry (MS) every week and proved to be stable for several months.

A drug-free serum sample collected from a healthy adult male was used to make blank serum samples containing cartap and its metabolites. The drug-free serum sample and serum samples collected from a suspected suicide case were kept



frozen at -20°C until analyzed.

A manual SPME assembly with replaceable extraction fibers coated with polydimethylsiloxane (PDMS, 100 μm), polydimethylsiloxane/divinylbenzene (PDMS/DVB, 65 μm), polyacrylate (PA, 85 μm), and carbowax/divinylbenzene (CW/DVB, 65 μm) was purchased from Supelco (Supelco Japan, Tokyo). The fibers were activated in a GC injection port at 250°C for 1 h prior to use.

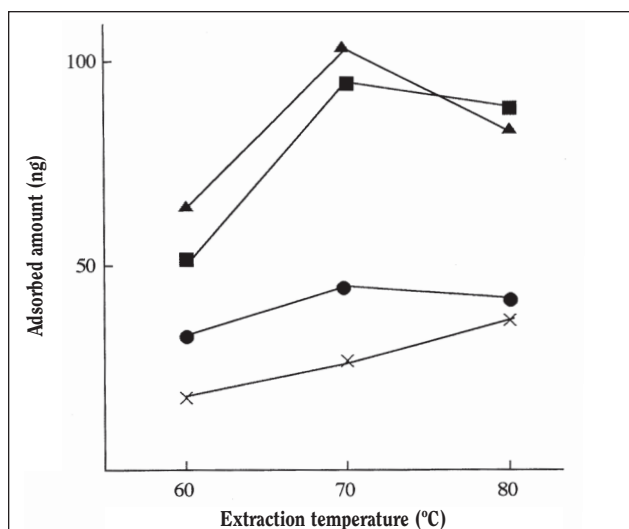
#### GC-MS

The GC-MS was a Shimadzu (Tokyo, Japan) QP-5000 equipped with a fused-silica capillary column (Supelco PTE-5, 30 m × 0.25-mm i.d., 0.25-μm film thickness). The column temperature was set at 50°C for 5 min, and then it was programmed to heat from 50 to 200°C at 15°C/min. The temperatures of the injection port and the interface were set at 250 and 230°C, respectively. Splitless injection mode was used, and the splitter was opened after 5 min. The carrier gas was helium, and its flow rate was set at 1.8 mL/min.

The selected ions monitored were *m/z* 70 and 149 for nereistoxin, *m/z* 61 and 90 for MTEA, *m/z* 61 and 104 for MMTEA, and *m/z* 71 and 118 for DHN. The quantitation ions were *m/z* 70 for nereistoxin, *m/z* 90 for MTEA, *m/z* 104 for MMTEA, *m/z* 118 for DHN, and *m/z* 105 for benzylacetone (internal standard).

#### Sample preparation for analysis

A serum sample (0.5 mL), benzylacetone internal standard (5 μL, 0.1 mg/mL), and sodium hydroxide (1.5 mL, 5M) were placed into a 12-mL vial and quickly sealed with a silicone septum and an aluminum cap. The vial was immediately heated to 70°C in an aluminum block heater (Dry Thermo Unit TAH-1, TAITEC, Saitama, Japan). The needle of the SPME device containing an extraction fiber was inserted through the septum of the vial, and the extraction fiber was exposed to the headspace. After 30 min, the needle was removed from the vial and inserted into the injection port of the GC-MS. The compounds extracted from the fiber were desorbed by exposing the fiber in the GC injection port for 5 min.



**Figure 2.** The effect of temperature on the adsorbed amount of nereistoxin and its metabolites. ●, nereistoxin; s, DHN; □, MMTEA; x, MTEA.

### Analytical data

Four different types of coatings were examined in this study for the extraction of nereistoxin and its metabolites from serum at a concentration of 2.0 µg/mL. Each fiber was exposed for 30 min at 70°C. Extracted amounts were calculated in comparison with the direct injection of these compounds into the GC-MS. The effect of extraction temperature of the targets with PDMS/DVB from serum is shown in Figure 2. A serum sample containing 2.0 µg/mL of nereistoxin, DHN, and MMTEA and 10 µg/mL of MTEA was prepared. The fiber was exposed for 30 min at 3 different temperatures (60, 70, and 80°C) and then analyzed. To determine the effect of exposure time on the adsorbed amount of nereistoxin and its metabolites, the fiber was exposed at 70°C for 7 different time periods (5, 10, 20, 30, 45, 60, and 90 min). Because the volatilization of analytes from biological matrices depends on some basic parameters such as pH or salt concentration, extraction recoveries were examined for various conditions (1.5 mL of 1M NaOH or 5M NaOH and 0.5 g NaCl were tested). Serum samples spiked with nereistoxin and its metabolites each at a concentration of 2.0 µg/mL were prepared and analyzed using the aforementioned procedure.

### Method validation

To determine calibration curves, serum samples spiked with nereistoxin and its three metabolites at concentrations ranging from 0.01 to 20 µg/mL were prepared and analyzed using the aforementioned procedure. The calibration curves were obtained by plotting the peak-area ratio between target compounds and benzylacetone internal standard. Reproducibility was evaluated by analyzing serum samples containing 2 different concentrations (0.2 and 2.0 µg/mL) of nereistoxin and its metabolites on the same day in 5 replicates (intraday reproducibility) and over 6 consecutive days (interday reproducibility).

### Application to a suspected suicide case

An 83-year-old woman attempted suicide by ingesting an unknown amount of Padan 4<sup>R</sup> containing 4% cartap hydrochloride. The patient was hospitalized approximately 3 h after ingesting it. Her pupils were 4.0 mm in diameter upon admission. A gastric lavage was performed, and furosemide was administered as a diuretic. Serum was collected 3 times during hospitalization. The concentrations of nereistoxin and its metabolites in the serum were analyzed.

## Results and Discussion

SPME is based on the extraction of analytes from biological matrices using a fiber coating (9). The extracted analytes are then directly desorbed from the fiber into the injection port of the GC. The amount of analytes extracted by the fiber coating is dependent upon the partition coefficient of the analytes toward the fiber coating. It is necessary to select a suitable fiber that provides a high enough recovery of analytes to allow a sufficiently low detection limit. Figure 3 shows the effect of fiber coatings. The amounts recovered from the PDMS/DVB fiber

were higher than those from the other fibers for all the target analytes. The coefficient of variation values of the PDMS/DVB fiber (1.5–4.4%) were smaller than those of the other fibers (2.3–10.1%). Therefore, the PDMS/DVB fiber was selected and used for the following experiments. Generally, a PDMS-coated fiber is used as the first choice. However, polar compounds are more likely to be adsorbed on polar coatings (18,19). The PDMS fiber showed excellent selectivity for volatiles (20). Nereistoxin and its metabolites are volatile amines (1). Concerning these results and other collected data (19,21,22), however, the PDMS/DVB fiber seems to be suitable for the determination of volatile amines when compared with the PDMS and other examined fibers.

The effect of extraction temperature of the targets with PDMS/DVB from serum is shown in Figure 2. The adsorbed amount of nereistoxin, DHN, and MMTEA were maximized at 70°C, but MTEA increased as the temperature increased. This occurs because adsorption of the analytes into the fiber is an exothermic process, and heating the sample to an elevated temperature decreases the partition coefficients of the analytes in the fiber. Although the adsorbed amount of MTEA was not maximized at 70°C, substantial amounts were extracted into the fiber at 70°C. Therefore, a temperature of 70°C was adopted. SPME theory dictates that because the fiber extraction

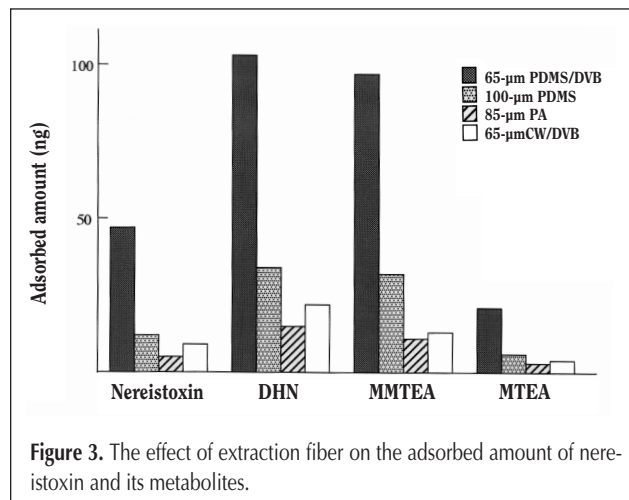


Figure 3. The effect of extraction fiber on the adsorbed amount of nereistoxin and its metabolites.

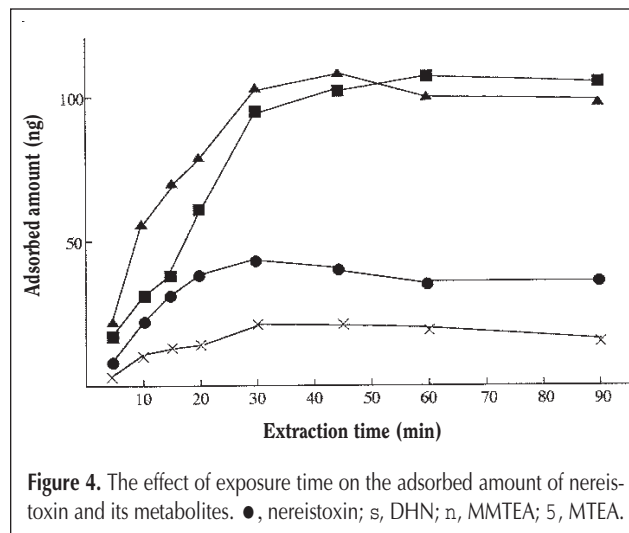


Figure 4. The effect of exposure time on the adsorbed amount of nereistoxin and its metabolites. ●, nereistoxin; s, DHN; n, MMTEA; 5, MTEA.

**Table I. Recovery of Nereistoxin and Metabolites in the Presence of NaOH or NaCl\***

Mixture composition	Mean recovery and standard deviation			
	Nereistoxin	DHN	MMTEA	MTEA
0.5 mL Water–1.5 mL 5N NaOH	285 ± 13.1	254 ± 8.38	148 ± 13.6	17.7 ± 1.24
0.5 mL Serum–1.5 mL water	16.7 ± 0.65	10.6 ± 0.95	3.1 ± 0.55	ND <sup>†</sup>
0.5 mL Serum–1.5 mL 1N NaOH	20.8 ± 0.42	32.9 ± 0.59	14.2 ± 0.53	ND <sup>†</sup>
0.5 mL Serum–1.5 mL 1N NaOH–NaCl	22.0 ± 1.69	40.8 ± 0.90	18.5 ± 0.99	ND <sup>†</sup>
0.5 mL Serum–1.5 mL 5N NaOH	46.7 ± 1.40	103 ± 1.55	97.5 ± 1.95	20.1 ± 0.68
0.5 mL Serum–1.5 mL 5N NaOH–NaCl	41.0 ± 0.66	68.6 ± 0.89	72.8 ± 0.44	38.0 ± 7.82

\* Recoveries were calculated by comparing the peak areas obtained from the extracts of the spiked human serum samples (2.0 µg/mL) with those obtained from directly injected authenticals (500 ng of each drug). The results are averages of 5 duplicated tests.  
<sup>†</sup> ND, not detected.

**Table II. Accuracy, Intraday, and Interday Precision for Analysis of Nereistoxin and Metabolites\***

Target concentration (µg/mL)	Analyte	Intraday		Interday	
		Recovery (%)	CV <sup>§</sup> (%)	Recovery (%)	CV (%)
0.2	Nereistoxin	4.2 ± 0.18	4.3	3.9 ± 0.20	5.1
	DHN	9.9 ± 0.22	2.2	9.8 ± 0.29	3.0
	MMTEA	9.3 ± 0.27	2.9	9.0 ± 0.36	4.1
	MTEA	1.8 ± 0.14	7.7	2.0 ± 0.19	9.5
2.0	Nereistoxin	4.7 ± 0.14	3.0	4.5 ± 0.15	3.3
	DHN	10.3 ± 0.15	1.5	10.0 ± 0.25	2.5
	MMTEA	9.8 ± 0.20	2.0	9.6 ± 0.23	2.4
	MTEA	2.0 ± 0.09	4.4	2.0 ± 0.13	6.6

\* Recoveries were calculated by comparing the peak areas obtained from the extracts of the spiked human serum samples (0.2 and 2.0 µg/mL) with those obtained from directly injected authenticals (50 and 500 ng of each drug). The results are averages of 5 duplicated tests.

<sup>†</sup> Intraday precision analysis was performed on a single day of analysis ( $n = 5$ ).

<sup>‡</sup> Interday precision analysis was performed over 6 consecutive days of analysis ( $n = 12$ ).

<sup>§</sup> CV, coefficient of variation.

**Table III. Quantitation Limit and Linearity of the Method**

Analyte	Limit of detection (µg/mL)	Range of linearity (µg/mL)	Linearity*	Correlation coefficient
Nereistoxin	10	0.05–5.0	$y = 0.252x + 0.0012$	0.9994
DHN	5	0.01–5.0	$y = 1.757x - 0.0512$	0.9992
MMTEA	10	0.05–5.0	$y = 1.595x + 0.0139$	0.9994
MTEA	500	0.5–10	$y = 0.228x - 0.1186$	0.9962

\*  $x$  represents the amount of analytes (µg/mL);  $y$  represents the peak-area ratio.

**Table IV. Concentration of Nereistoxin and Metabolites in the Patient's Serum**

Time* (h)	Concentration (µg/mL)			
	Nereistoxin	DHN	MMTEA	MTEA
0.5	2.69	2.83	0.30	ND <sup>†</sup>
7.0	0.36	2.84	0.24	ND <sup>†</sup>
24	0.09	0.21	0.05	ND <sup>†</sup>

\* Elapsed time after the patient's admission to the hospital.

<sup>†</sup> ND, not detected.

process is exothermic, an increase in sampling temperature will decrease analyte recovery (21,23). As the sampling temperature increases past the maximum adsorption temperature, the partition coefficient of the analytes decreases, and the fiber loses its ability to absorb analytes. In previous experimental findings (11,14,15), however, the elevated extraction temperature increases the amount of analyte recovered. One reason for this is thought to be a difference of the volatilization of each drug. For semi-volatile compounds, the low volatility may slow the mass transfer from the matrix to the headspace, resulting in the need for a higher temperature or a longer extraction time to provide an adequate sample.

The headspace SPME technique is based on the equilibrium of analytes among the involved phases. The kinetics of the mass transport in which analytes move from the aqueous phase, to the headspace, and finally to the fiber, must also be addressed because it determines the sampling time of the headspace-SPME technique (24). In many reports, samples were heated to equilibrium before extraction with the SPME without considering the character of the compounds. The preheat step was certainly effective for volatiles such as alcohol or inflammable chemicals (25–27). However, it was not effective for semi-volatiles (14,15). In preliminary experiments (data not shown), the absorbed amounts of nereistoxin and its metabolites were not dependent on the preheating time. Therefore, the preheating step was omitted. The effect of exposure time on the adsorbed amount of nereistoxin and its metabolites is shown in Figure 4. The adsorbed amount of nereistoxin was maximized at 30 min and slightly decreased after that time. For a longer exposure time, the fiber was heated, and ana-

lytes were released from the fiber to the headspace. Thus, the amount on the fiber decreased. The adsorbed amount of other metabolites was maximized at 30–60 min. The exposure time of 30 min was used because it provided a sufficient amount to analyze and had a small coefficient of variation. Thus, one serum sample could be analyzed in 50 min, including a GC–MS analysis time for 20 min.

Extraction recoveries were examined for various conditions, because the volatilization of analytes from biological matrices depends on some pH or salt concentrations. The recoveries of nereistoxin and its metabolites are shown in Table I. The recoveries of the analytes in the presence of 5M NaOH were higher than those under other conditions. In general, the addition of the salt into the biological matrices decreases solubility of the target analytes, which results in an increase in the amounts of the analytes extracted by the fiber. In the case of 5M NaOH, the addition of NaCl was not effective for improving recovery. Therefore, the analysis was performed using 5M NaOH. The absolute recoveries of nereistoxin, DHN, MMTEA, and MTEA from serum were 2.0–10.3% (Table II). The typical SIM chromatogram of the spiked serum is shown in Figure 5. Sharp and clear peaks of nereistoxin and its metabolites were obtained without the disturbance of endogenous interferences. The intraday and interday coefficients of variation were 1.5–7.6% and 2.5–9.5%, respectively (Table II). Considering the intraday and interday coefficients of variation, the proposed method was found to be reproducible compared with other SPME results.

The calibration curves of the method for nereistoxin and its metabolites in serum are shown in Table III. The calibration curves showed linearity in the range of 0.05–5.0  $\mu\text{g/mL}$  for nereistoxin and MMTEA, 0.01–5.0  $\mu\text{g/mL}$  for DHN, and 0.5–10  $\mu\text{g/mL}$  for MTEA. The correlation coefficients of the calibration curves were 0.9962–0.9996. The limit of detection in serum of

the method was 0.01  $\mu\text{g/mL}$  for nereistoxin and MMTEA, 0.005  $\mu\text{g/mL}$  for DHN, and 0.5  $\mu\text{g/mL}$  for MTEA (signal-to-noise ratio = 3). In a Padan poisoning case (8), the concentration of nereistoxin in serum was found to be 1.14  $\mu\text{g/mL}$ , which can sufficiently be measured by our present method. A toxicological analysis of cartap in human serum obtained from an 83-year-old woman who attempted suicide by ingesting Padan 4<sup>R</sup> was performed using the proposed SPME method. Sharp peaks of nereistoxin and its metabolites were obtained without the disturbance of endogenous interferences. The typical SIM chromatogram of a serum sample upon admission is shown in Figure 5. Nereistoxin and its three metabolites were detected in the serum samples of the patient (Table IV). Nereistoxin was detected in the serum samples collected from the patient during the hospitalization in the concentration range from 2.69 to 0.09  $\mu\text{g/mL}$ .

## Conclusion

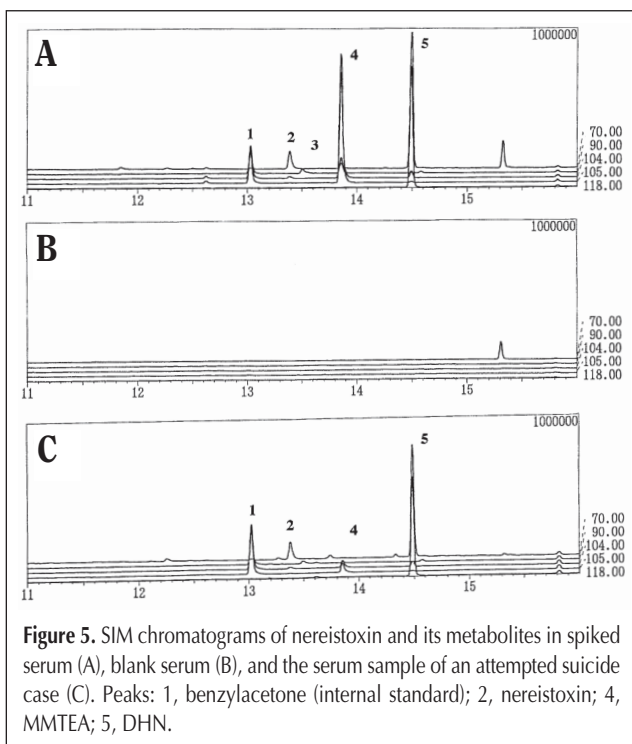
The analytical procedure presented here allows the simultaneous determination of nereistoxin analogues using SPME. The headspace SPME method was more simple and rapid than conventional liquid–liquid and solid-phase extractions, and the background of the SIM chromatogram was very clean. Four types of fiber coatings were evaluated. The PDMS/DVB fiber was found to extract the highest amount of the target compounds from the serum, yielding a detection limit of 0.01  $\mu\text{g/mL}$  for nereistoxin and MMTEA, 0.005  $\mu\text{g/mL}$  for DHN, and 0.5  $\mu\text{g/mL}$  for MTEA. The proposed method was very sensitive because of the high extraction ability of the SPME for vaporized drugs.

## Acknowledgments

The authors would like to thank Dr. N. Fukunaga of Supelco Japan for his technical support, Shimadzu Co. for granting the use of QP-5000, and Takeda Chemical Industries for the supply of cartap and its metabolites.

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**Figure 5.** SIM chromatograms of nereistoxin and its metabolites in spiked serum (A), blank serum (B), and the serum sample of an attempted suicide case (C). Peaks: 1, benzylacetone (internal standard); 2, nereistoxin; 4, MMTEA; 5, DHN.

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Manuscript accepted January 27, 1999.