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Application Note

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Life Science

Analysis of Major Psychoactive Compounds in Nutmeg Using GC-MS/MS

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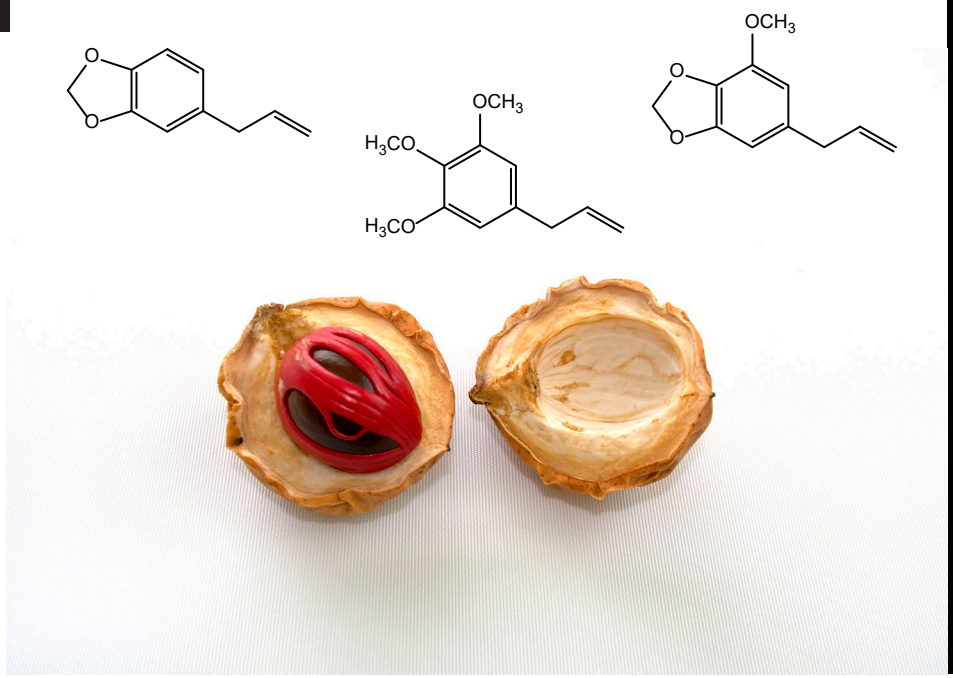

Life Science

■ Abstract

Nutmeg is a spice used in a variety of recipes that can be easily purchased by anyone. Among younger people, however, excessive consumption of nutmeg powder for recreational purposes is increasing, causing hallucinogenic effects. However, there are few reports on toxic or comatose-fatal blood levels and their changes over time in cases of nutmeg poisoning. Therefore, an analytical method using GC-MS/MS was devised and applied to a case of nutmeg poisoning in order to determine the blood concentrations of the main psychoactive substances (safrole, myristicin, and elemicin) in nutmeg and their change over time. The limit of detection (LOD) and limit of quantification (LOQ) of the constructed method were 0.14 to 0.16 ng/mL and 0.5 ng/mL (the lowest point of the calibration curve), respectively. Calibration curves showed good linearity, with R^2 = 0.996 to 0.997 for all substances in the range of 0.5 to 300 ng/mL. The accuracy for QC (low 1 ng/mL, medium 120 ng/mL, and high 240 ng/mL) and LOQ (0.5 ng/mL) was %RSD 2.4 to 11 % for diurnal variation (n = 5) and %RSD 1.5 to 11 % for diurnal variation (6 days). A systematic error of -2.6 to 2.1 % was obtained.

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1. Introduction

Nutmeg, the dried seed of *Myristica fragrans*, has been used as a spice and for medicinal purposes all over the world since ancient times and is widely sold at low prices. Because nutmeg contains safrole, myristicin, and elemicin (Fig. 1) as the major psychoactive compounds, they are sometimes intentionally consumed in large amounts to induce hallucinations or intoxication[8)-10)]. In addition, intoxication by unintentional misuse of nutmeg has recently become a problem. In addition to hallucinogenic effects, too much nutmeg is associated with clinical symptoms such as nausea, vomiting, abdominal pain, agitation, drowsiness, dizziness, tachycardia, blurred vision, dry mouth, and flushing[5), 9), 11)-14)]. However, the majority of nutmeg overdose cases are mild and unlikely to be fatal[14), 15)]. Symptoms of nutmeg poisoning usually begin 2 to 8 hours after ingestion[11), 15), 16)] and subside within a few days[3), 5), 9), 11), 13), 15)-17)]. Only two deaths worldwide have been linked to the consumption of nutmeg[15), 18)].

In a recent study, biological samples were collected from volunteers who ingested nutmeg powder to identify metabolites of major psychoactive substances[19)-23)]. However, although many cases of nutmeg poisoning have been reported to date, there have been no reports evaluating the blood levels and half-lives of major psychoactive substances, such as safrole, myristicin, and elemicin, during poisoning. Therefore, we developed an analytical method for three psychoactive substances using GC-MS/MS, measured the blood levels of these main substances in patients with nutmeg poisoning, and evaluated their changes over time.

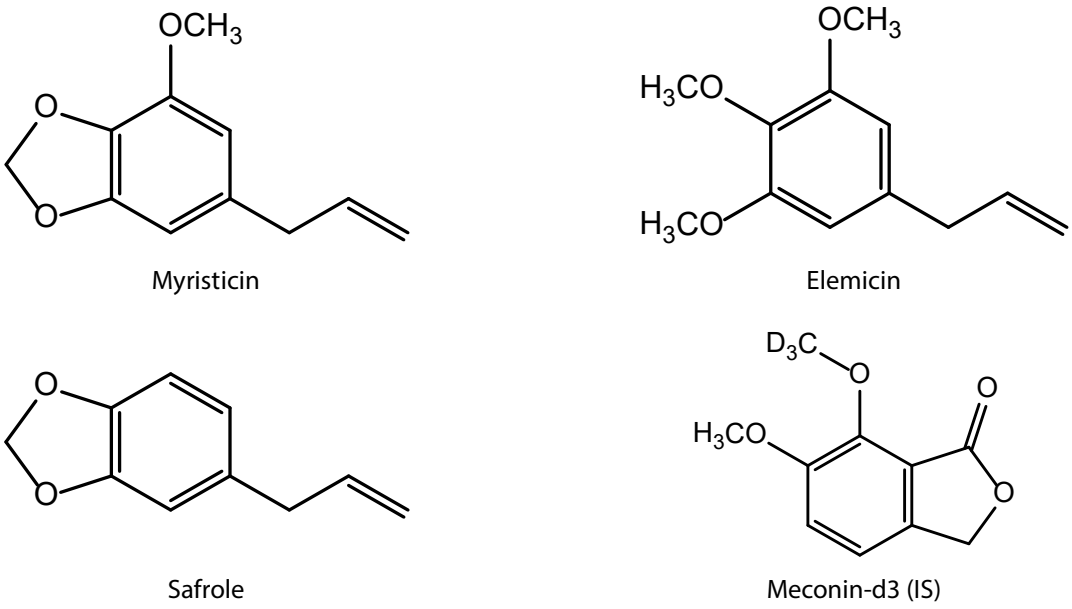

Fig. 1 Structures of Major Psychoactive Compounds in Nutmeg and an Internal Standard

2. Materials and Methods

2.1. Reagents and Instruments

The reagents used in the experiments were ethyl acetate (concentrated 5000 for pesticide and PCB tests), methanol (for LC/MS), ethanol (for LC/MS), safrole (chemical purity > 95 %; FUJIFILM Wako Pure Chemical Corporation); myristicin (chemical purity > 97 %; Merck, Darmstadt, Germany); elemicine (chemical purity > 98 %; Combi-Blocks, San Diego, CA, USA); and five human pooled sera from different batches (Cosmo Bio Inc.). The internal standard used was meconin-d3 (chemical purity > 98 % and isotopic purity > 99.6 %; Toronto Research Chemicals). A MonoSpin® C18 FF cartridge (GL Science) was used to extract the main psychoactive substances from the serum.

2.2. Preparation of Standard Solution

A mixture of standard stock solutions of safrole, myristicin, and elemicin (400 µg/mL) was prepared in ethanol and stored at −30 °C. Mixed working solution aliquots (0.25, 2.5, 5.0, 10, 15, 25, 40, 80, 100, and 150 µg/mL) were prepared by serially diluting the stock solution mixture with deionized water. Calibration standards (0.5, 5, 10, 20, 30, 50, 80, 160, 200, and 300 ng/mL) were prepared by spiking blood serum with appropriate volumes of the working solution mixture. Blood serum quality control (QC) samples were prepared at low (1 ng/mL), medium (120 ng/mL), and high (240 ng/mL) concentrations. An aqueous solution of meconine-d3 (10 ng/mL) was formulated in deionized water as an internal standard and stored at −30 °C.

2.3. Extraction of Psychoactive Compounds

The MonoSpin® C18 FF cartridge was preconditioned with 200 µL of ethyl acetate and centrifuged twice at 2,000 g for 30 s. This process was repeated with 200 µL methanol and followed by 200 µL deionized water. Subsequently, the cartridge was loaded with a mixture solution of 50 µL blood serum and 450 µL meconine-d3 aqueous solution (10 ng/mL), rinsed with 200 µL deionized water, and then centrifuged at 10,000 g for 30 s (three times). The eluate containing safrole, myristicin, and elemicin was recovered by centrifuging 100 µL of ethyl acetate at 2,000 g for 30 s. Then 5 µL of the purified sample was directly injected into the GCMS-TQ™ 8040 system.

2.4. Method Validation

Method validation was performed as described previously[26]. Whole-blood samples (n = 5) were subjected to selectivity tests. Ten-point calibration curves (0.5, 5, 10, 20, 30, 50, 80, 160, 200, and 300 ng/mL) were constructed by plotting the peak-area ratios against the nominal concentrations of meconin-d3 as the calibration standard. The curves were fitted using weighted least-squares linear regression with a weighting factor of 1/x2. The bias and precision of the devised method were computed relative to the calibration range using the QC sample concentrations (1, 120, and 240 ng/mL). Each QC sample was analyzed five times per day for six consecutive days. Bias was calculated as the percentage deviation between the mean values of measured and nominal concentrations. Intra- and inter-day precision values were calculated using one-way analysis of variance and are expressed as relative standard deviation (%RSD). The limit of detection (LOD) and limit of quantitation (LOQ) were determined as LOD = 3.3 × SD/S and LOQ = 10 × SD/S, where SD is the standard deviation of the serum values obtained using the lowest calibrator concentration 0.5 ng/mL and S is the slope of the calibration curve. If the computed LOQ was lower than the lowest calibrator concentration, then the lowest calibrator concentration was deemed the practical LOQ. Using five different samples at two QC concentrations, low (1 ng/mL) and high (240 ng/mL), the recovery, matrix effect, and process efficiency values were determined according to an established method[27]. The stability of processed samples was determined by analyzing the QC samples for 6.5 hours at 26 °C. Three cycles of freezing (at −30 °C for 24 h) and thawing (at room temperature) were used to assess freeze/thaw stability. Long-term stability was evaluated by comparing the QC samples before and after 23 days of storage at −30 °C.

Table 1 GC/MS Analytical Conditions

Analytical conditions	
Instrument	GCMS-TQ™ 8040 NX
Column	SH-I 5Sil MS (30 m × 0.25 mm, 0.25 µm)
Oven temp.	60 °C (1 min) - 320 °C (10 °C/min) - 320 °C (10 min)
Injector temp.	280 °C
Transfer line temp.	280 °C
Injection mode	Splitless
Carrier gas	He
Flow rate	1.8 mL/min
Ionization	EI (70 eV)
Ionsource temp	200 °C
Scan range (Product ion scan)	*m/z* 40 - 220 (dwell time 50 ms)
MRM transition (*m/z*)	Safrole 162 > 104, 131, CE 15 V Myristicin 192 > 161, 91, CE 10 V Elemicin 208 > 193, 177, CE 10 V Meconin-d3 162 > 104, CE 5 V

3. Results and Discussion

3.1. Method Validation Results

Typical MRM chromatograms (for each standard at 120 ng/mL) and mass spectra of safrole, myristicin, elemicin, and internal standard meconine-d3 are shown in Fig. 2, and calibration curves for each target compound are shown in Fig. 3. The validation results for the developed analytical methods are shown in Tables 2 and 3, respectively. The calibration curves for each compound were linear with r^2 = 0.996 to 0.997 over the calibration curve range of 0.5 to 300 ng/mL. The target substance LOD, determined using the SD value of the serum values obtained using the lowest calibrator (n = 30) and calibration curve slope (LOD = 3.3 SD/slope), ranged between 0.14 and 0.16 ng/mL. LOQ (the lowest point on the calibration curve) for each sample was 0.5 ng/mL. The bias and precision of the LOQ and QC samples (1, 120, and 240 ng/mL) are summarized in Table 1. Bias varied from −2.6 % to 2.1 %. Intra- and inter-day %RSD values ranged from 2.4 % to 11 % and 2.5 % to 11 %, respectively. The matrix effect values (calculated using five distinct matrices) corrected by the internal standard (meconine-d3) were acceptable for both the low- (1 ng/mL) and high-concentration (240 ng/mL) QC samples. The QC sample stability results (Table 3) reveal that the target substances were stable under the experimental conditions. These results indicate that this straightforward method yielded acceptable validation parameters.

3.2. Application to Cases of Nutmeg Poisoning

The proposed method was applied to the aforementioned case of nutmeg poisoning. Serum samples were collected at five time points over a period of 93.7 h, beginning at the time of admission (0 h). A representative GC–MS/MS MRM chromatogram of the serum samples extracted from the patient 8 h 40 min after admission demonstrated that the method successfully detected all target substances (Fig. 4).

General screening via LC-QTOF-MS/MS using spectral libraries of > 7950 compounds, including benzodiazepines, antidepressants, pesticides, natural toxins, controlled drugs, and designer drugs, detected no other drugs or poisons in the patient's serum. Quantitative analysis of the target compounds revealed that the compound concentrations decreased over time (Table 4). At admission (ca. 8 h after ingestion), the concentrations of safrole, myristicin, and elemicin were 16.7, 388, and 844 ng/mL, respectively. At that time, the patient presented clinical symptoms of vertigo, photophobia, fatigue, and severe nausea, and the patient was unable to open his eyes for most of the time. Consequently, the patient experienced difficulty in drinking and eating. Vertigo and nausea persisted during follow-up treatments of infusion therapy alone on days 2 and 3 of hospitalization, and the serum levels of safrole, myristicin, and elemicin (45.7 h post-admission) were 2.7, 65.2, and 30.6 ng/mL, respectively. At 93.7 h post-admission, the blood serum levels of each compound were substantially reduced. Despite the persistence of mild vertigo, the physical symptoms were alleviated considerably.

The biological half-life of each psychoactive compound in blood serum was calculated using the quantified concentrations, by plotting them logarithmically against post-admission time (Fig. 5). The biological half-lives of safrole, myristicin, and elemicin were determined to be 19.2, 16.9, and 8.5 h, respectively (Table 4).

Several studies have described the metabolism of the major psychoactive compounds in nutmeg in biological samples[19)-22)]. However, virtually no reports indicating the blood levels, time-course changes, or toxicokinetic parameters of the major nutmeg psychoactives in human serum in nutmeg poisoning cases were discovered, possibly because only two cases of death due to nutmeg poisoning have been documented[15), 18)].

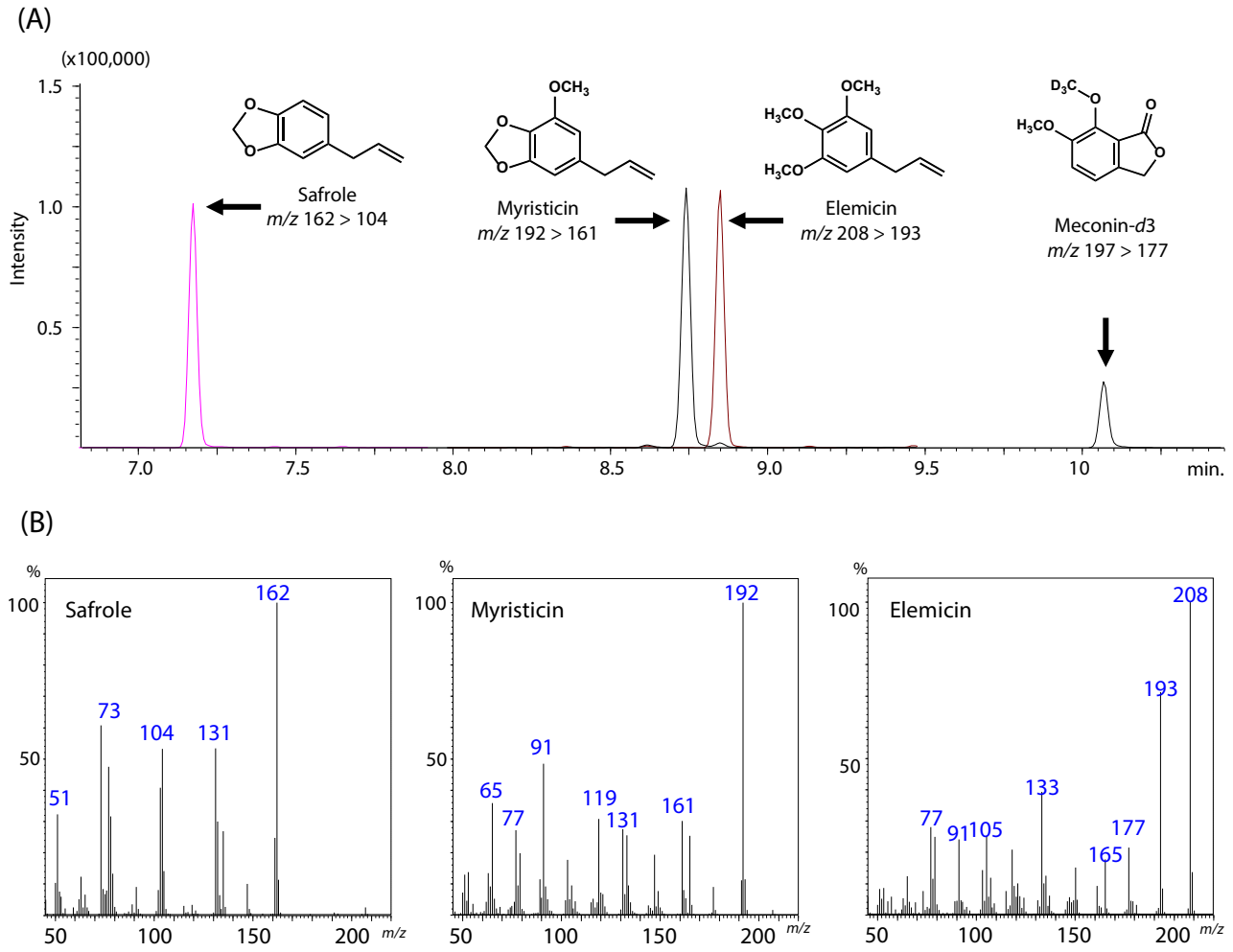

Fig. 2 (A) MRM Chromatogram of Standard (120 ng/mL) (B) EI Mass Spectrum

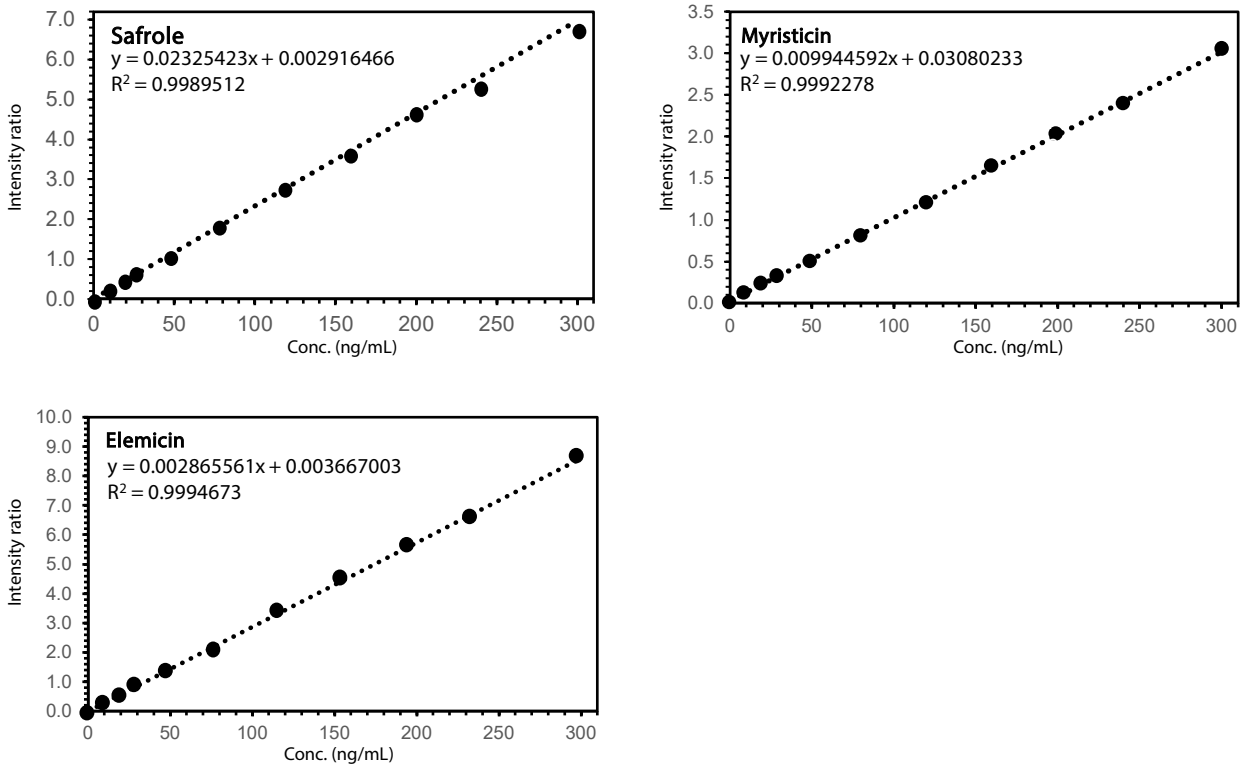

Fig. 3 Calibration Curves

Table 2 Method Validation Results

Calibration range (ng/mL)	0.5 – 300 (0.5, 5, 10, 20, 30, 50, 80, 160, 200, and 300)		
	SAF	**MYL**	**ELE**
r^2	0.996	0.997	0.997

SAF: Safrole, **MYL**: Myristicin, **ELE**: Elemicin *n* = 6

	SAF	**MYL**	**ELE**
LOD (ng/mL)	0.14	0.16	0.15
LOQ (ng/mL)	0.5	0.5	0.5

QCs (ng/mL)	Precision (%RSD)						Bias (%)		
	Intra-day run			Inter-day run					
	SAF	**MYL**	**ELE**	**SAF**	**MYL**	**ELE**	**SAF**	**MYL**	**ELE**
LOQ (0.5)	5.4	11	4.9	7.9	8.7	8.8	-1.8	1.9	-2.2
Low (1)	4.0	7.9	2.7	8.6	5.2	11	1.1	2.1	0.5
Medium (120)	3.8	3.7	3.3	3.8	3.7	3.3	-1.1	-1.3	-0.4
High (240)	3.7	3.0	2.4	3.7	3.7	2.5	-2.6	-0.8	0.0

6 days, n = 5

QCs (ng/mL)	LOW (1)			High (240)		
	SAF	**MYL**	**ELE**	**SAF**	**MYL**	**ELE**
Recovery rate (%)	84.9	95.4	95.4	79.0	88.2	92.0
Matrix effect (%)	109.1	107.9	108.7	101.8	99.8	98.8
Process efficiency (%)	92.3	102.7	103.2	80.2	87.9	90.7

5 days, 5 matrices were used.

Table 3 Long-Term Stability of Target Compounds

	QCs (ng/mL)	Averaged remaining contents					
		SAF		**MYL**		**ELE**	
		Mean (%)	RSD (%)	Mean (%)	RSD (%)	Mean (%)	RSD (%)
Freeze/thaw stability (3 cycles, -30 °C to RT)	1	102.2	3.0	101.2	3.2	97.8	1.8
	120	100.8	5.0	98.8	4.8	97.8	4.4
	240	103.6	2.0	102.8	2.2	101.5	2.1
Long-term stability (43 days, -30 °C)	1	98.8	5.7	99.6	14.4	97.0	9.8
	120	100.3	0.6	99.3	1.6	100.8	1.9
	240	98.9	1.8	98.4	2.3	101.1	2.1

n = 5 (Freeze/thaw stability , Long-term stability)

	QCs (ng/mL)	Averaged remaining contents mean (%)		
		SAF	**MYL**	**ELE**
Processed sample stability (6.5 h, 26 °C)	1	102.5	108.5	99.3
	120	100.2	100.0	99.6
	240	102.1	102.7	101.7

n = 1

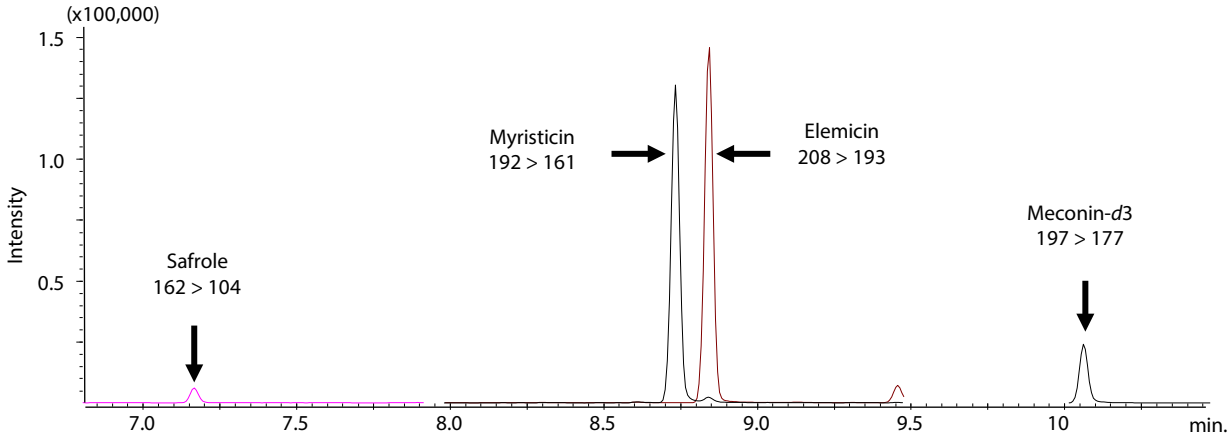

Fig. 4 MRM Chromatogram of Serum Sample

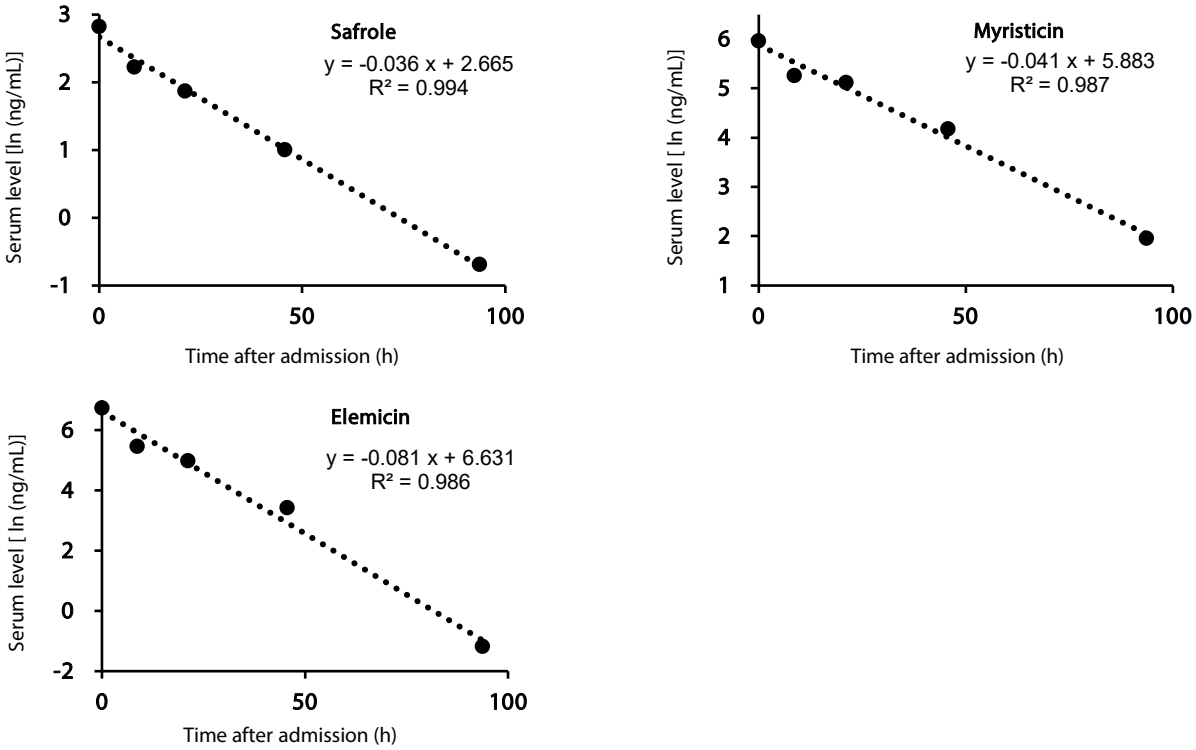

Fig. 5 Correlations between Time after Hospitalization and Serum Concentrations of Target Compounds

Table 4 Serum Concentrations of Target Compounds after Hospitalization

Time after admission (h)	Serum level (ng/mL)		
	Safrole	Myristicin	Elemicin
0	16.7	388	844
8.7	9.2	192	236
21.2	6.5	167	147
45.7	2.7	65.2	30.6
93.7	0.50	7.1	0.30*

* < LOQ, estimated value

Compounds	K_{el}	$T_{1/2}$ (h)
Safrole	-0.036	19
Myristicin	-0.041	17
Elemicin	-0.081	8.5

K_{el}: Elimination rate constant, $T_{1/2}$: Biological half-life

4. Conclusion

Using a MonoSpin® extraction kit in combination with a GC–MS/MS system, a simple method was devised to detect and quantify safrole, myristicin, and elemicin in human serum. This method was validated and applied successfully to a case of nutmeg poisoning. For this patient, the method quantified the time-course changes and half-life of safrole, myristicin, and elemicin in serum. It is believed that this method will help to provide additional blood-concentration data for nutmeg poisoning cases as well as more accurate toxicokinetic parameters.

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This application note is a technical document based on the authors' paper below.

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