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Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry for rapid analysis of eight *Gelsemium elegans Benth* alkaloids in human plasma and urine

Xi Zeng[1,2†], Yu Wang[1,2†], Lin Luo[1], Yina Lu[3] and Zhenlin Xu[1*]

Abstract

Gelsemium elegans Benth alkaloids are the main components of *G. elegans* and can cause acute toxicosis or even death. Although several studies have reported methods for detecting *G. elegans* alkaloids, a high-throughput and environmental-friendly strategy for detection of multiple *G. elegans* alkaloids has not been realized. In this work, a matrix-assisted laser desorption/ionization time-of-flight mass spectrometry method was developed for rapid detection of *G. elegans* alkaloids in human plasma and urine for diagnosis of poisoning. Multiple matrices and crystal spotting methods were evaluated to obtain stable and high peak intensities without "sweet spot". We verified the methodology and obtained excellent results. The matrix effects with different dilutions were compared and good recoveries and a low relative standard deviation were obtained with a 40-fold dilution. This method could shorten the analysis time and greatly reduce the consumption of chemical solvents. Furthermore, it could be applied to quantitative assessment of *G. elegans* alkaloid poisoning incidents.

Keywords Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, *Gelsemium elegans Benth*, High-throughput trial, Alkaloid, Acute poisoning, Biological sample

Introduction

Gelsemium elegans Benth, which is commonly known as Duan Chang Cao and Gou Wen in China, is a plant in the Loganiaceae family that is highly toxic and found throughout China, India, and Thailand [1–3]. This plant reportedly contains more than 150 different monoterpenoid indole alkaloids and iridoids [4]. These compounds can be classified into the following six groups according to their structures: gelsedine-type, gelsemine-type, humantenine-type, koumine-type, sarpagine-type and yohimbine-type [4, 5]. Among these compounds, the primary bioactive and toxic components of *G. elegans* are koumine, gelsemine, humantenine and gelsenicine, which are commonly used as analgesics [6], anti-inflammatories [7], anti-tumor agents [8], and immunomodulators [9] in China.

The morphology of *G. elegans* can appear similar to that of other plants, which can lead to accidental ingestion and acute poisoning or even death [10]. Ingestion of *G. elegans* can cause serious respiratory depression and neurological effects. A lethal case of *G. elegans* poisoning was reported because of the misidentification of *G. elegans* as *Sargentodoxa cuneata* in Guizhou, China [11].

†Xi Zeng and Yu Wang contributed equally to this work.

*Correspondence:
Zhenlin Xu
jallent@163.com
[1] Guangdong Provincial Key Laboratory of Food Quality and Safety, South China Agricultural University, Guangzhou 510642, China
[2] Guangzhou Institute of Food Inspection, Guangzhou 511400, China
[3] Shantou Customs District, Shantou 515041, China

Honey containing toxic compounds from *G. elegans* has also resulted in poisoning [12]. Moreover, products from animals fed *G. elegans* are potentially unsafe for consumers [13].

Koumine and gelsemine are the dominant compounds in *G. elegans*, and gelsenicine is the most toxic (median lethal dose in mice: 0.128 mg/kg) [14]. In *G. elegans* poisoning cases, large quantities are typically consumed and there is often little material remaining to analyze, which means that morphological and biochemical analyses cannot be performed to guide clinical treatment [15]. Furthermore, the *G. elegans* alkaloids are often transferred to other types of foods, making them harder to identify. Consequently, there is an urgent need for a simple, fast, high-throughput, and cost-effective method to determine *G. elegans* alkaloids for food poisoning prevention and clinical diagnosis.

To date, several methods have been developed to determine *G. elegans* alkaloids in biological samples, including hair [16], porcine plasma [17, 18], rats [14], and goat plasma, urine, and feces [4]. These methods mainly use high performance liquid chromatography-tandem (HPLC) [19], (ultra) HPLC tandem mass spectrometry (HPLC–MS/MS) [16], and HPLC coupled with quadrupole time-of-flight (TOF) mass spectrometry (MS) [4]. All available methods involve sample pretreatment and instrumental analysis. The sample preparation processes are complicated and time-consuming, and often need large quantities of biological samples [20]. Instrumental analysis takes 5–40 min for HPLC–MS/MS [4, 14, 17, 18] and even longer for HPLC techniques with single injection of one sample. To date, studies have focused on koumine, gelsemine, gelsenicine, and humantenmine, and this limited range of *G. elegans* alkaloids may not provide sufficient information for clinical treatment and food safety assessment.

Recently, matrix-assisted laser desorption/ionization (MALDI) TOF MS has been widely used in routine detection for clinical microbiology [21] and biomolecules [22, 23] because it is a high-throughput technique with excellent selectivity, sensitivity, and impurity tolerance [24]. With the MALDI technique, compounds are detected by soft desorption and ionization of a crystal composed of matrix and the analyte by irradiation with a laser that has a wavelength that matches the maximum matrix absorption [25]. The sample is spotted on a steel plate that rapidly moved in the x and y directions during laser scanning to enable analysis within approximately 0.3 s per sample [26]. MALDI-TOF MS has been widely used for analysis of macromolecules in biological samples, such as antigenic proteins [27, 28], and heavy chain disease monitoring [24] in plasma. Its detection ability for small molecules is promising but needs refined because of significant matrix interference in the low mass range ($m/z < 700$) [20, 29]. In a previous study, we discovered that certain low mass ranges were free from matrix interference, which enable the detection of small molecules, such as illegal drugs [30]. This research showed that MALDI-TOF MS has potential for routine application in small molecule detection.

In the present study, we investigated multiple matrices and crystal spotting methods for MALDI-TOF–MS determination of eight *G. elegans* alkaloids (Fig. 1). We then evaluated the matrix effect (ME) of *G. elegans* alkaloids in human plasma and urine. Furthermore, we compared detection strategies for *G. elegans* alkaloids and acquired qualitative and quantitative results for method validation. This method improves the efficiency and accuracy of clinical diagnosis, decision-making, and treatment of *G. elegans* poisoning accidents and *G. elegans* contamination of food.

Results and discussion

Matrix selection and MALDI-TOF MS for *G. elegans* alkaloids

Three matrices (α-cyano-4-hydroxycinnamic acid (HCCA), sinapinic acid (SA), and 2,5-dihydroxybenzoic acid (DHB)) were used and all were detected in both positive and negative ion modes. All the matrices exhibited peaks at < 800 Da, with a cluster at < 400 Da in positive ion mode (Fig. S1). When detecting crystals consisted of a single standard compound (100 ng/mL) and matrix (10 mg/mL) in positive ion mode, HCCA was the only matrix that resulted in signals for all the analytes (Fig. S2). Two analytes (sempervirine and koumidine) were detected with SA, and no analytes were detected with DHB. Notably, no *G. elegans* alkaloid was detected in negative ion mode.

In the mass spectra of the eight *G. elegans* alkaloids obtained with HCCA (Fig. 2 and Table S1), all compounds presented as protonated ions ($[M+H]^+$) [25, 31]. Importantly, the peaks of the *G. elegans* alkaloids were distinguishable from the HCCA background peaks, because the signal-to-noise (S/N) ratios of HCCA were less than three at the corresponding *mass-to-charge* ratios (Figs. S1 and 2). This was mainly attributed to HCCA having a low p*K*a (1.17), which facilitates the transfer of protons to alkaline chemicals [32]. Meanwhile, the isotope peaks of all the analytes were clearly detected, and the relative standard deviations (RSDs) of sempervirine, koumidine, koumine, gelsemine, gelsenicine, 11-hydroxygelsenicine, gelsevirine, and humantenine were all lower than 5.0×10^{-5} (Table S1). In previous research, HCCA have been used for small molecule (< 700 Da) detection because of minimal background interference [30,

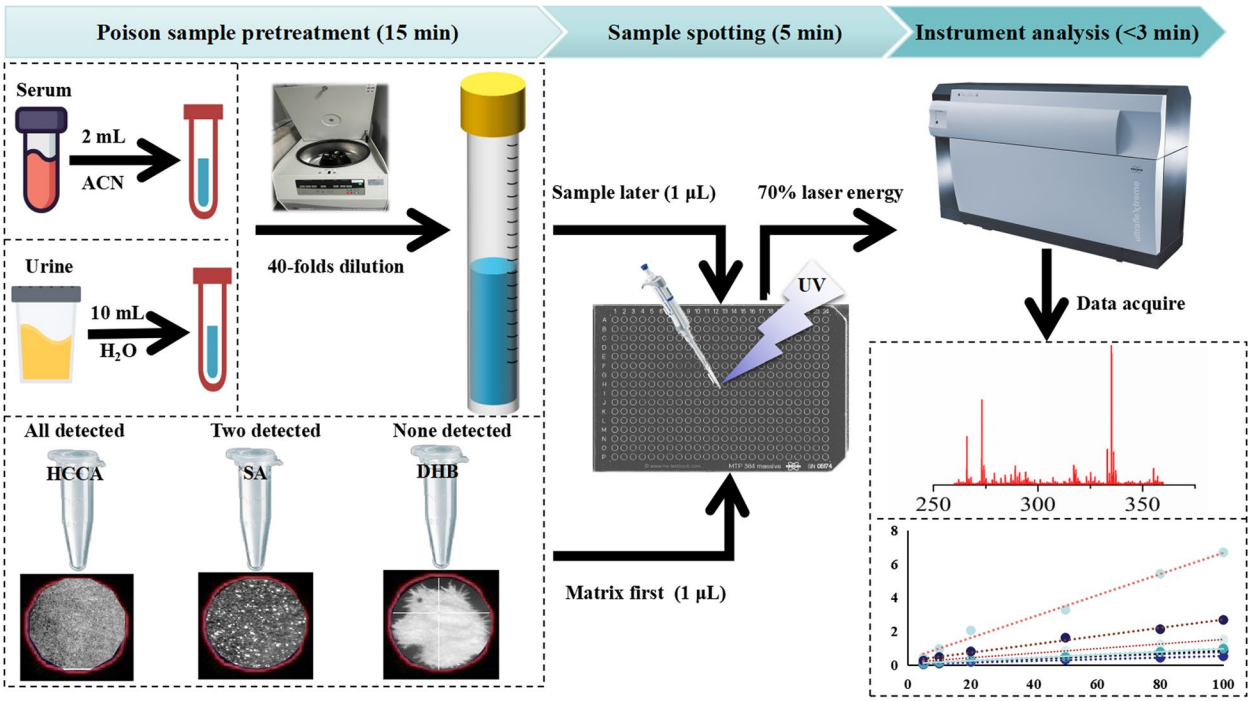

Fig. 1 Schematic illustration of the MALDI-TOF MS method for quantitative detection of eight *G. elegans* alkaloids. ACN, acetonitrile; HCCA, α-Cyano-4-hydroxycinnamic acid; SA, Sinapinic acid; DHB, 2,5-Dihydroxybenzoic acid; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry

33]. According to our results, HCCA was chosen as the matrix for analyzing the *G. elegans* alkaloids in positive ion mode, as well.

Spotting method selection

The crystal formed by the combination of matrix and sample is crucial for acquiring high quality MS signals [32]. In this study, samples were prepared using the mixed standard solution (100 ng/mL) and crystals were performed by four spotting methods for MALDI-TOF MS analysis. All four spotting methods produced peaks for protonated *G. elegans* alkaloids. The peak intensities and S/N ratios of the eight *G. elegans* alkaloids are shown in Fig. 3. Compared with the other three spotting methods, the double layer 1 method consistently gave the highest peak intensities ($>10^5$) and S/N ratios (>25). With the double layer 1 spotting method, all analytes except for sempervirine and koumidine exhibited their highest peak intensities, and all analytes except for sempervirine exhibited their highest S/N ratios. In addition, the peak intensities and S/N ratios were accumulated from 20 single laser shots, and the double layer 1 and mixed spotting methods showed strong stability and repeatability for the individual laser shots (data not shown), which indicated that the MALDI spots were homogenous. By contrast, the "sweet spot" effect was obvious with the sandwich method. The homogeneity of MALDI spots plays a crucial role in the quantification of small molecules [33]. Because it gave the highest peak intensities and S/N ratios for most of the analytes, the double layer 1 spotting method was selected for subsequent experiments.

Laser energy optimization

The signal intensity of an analyte is greatly affected by the laser energy used in MALDI-TOF MS. For samples of the individual standard solutions (100 ng/mL) with HCCA prepared by the double layer 1 spotting method, the laser intensity was varied from 10% to 90% in 10% increments. The peak intensities and S/N ratios of the *G. elegans* alkaloids with increases in the laser energy are shown in Fig. S3. As expected, the peak intensities of the compounds increased with increases in the laser energy. A laser energy of 90% gave the highest peak intensities for all analytes. The S/N ratio increased as the laser energy was increased from 10% to 30%, and then decreased as the laser energy was increased from 40% to 90%. Gelsenicine had the lowest peak intensities (0.2×10^4 to 5×10^4) and sempervirine had the highest peak intensities (1×10^6 to 5×10^6) among the eight *G. elegans* alkaloid standard solutions across the entire laser intensity range. The laser energy influenced the sensitivity and response of each analyte.

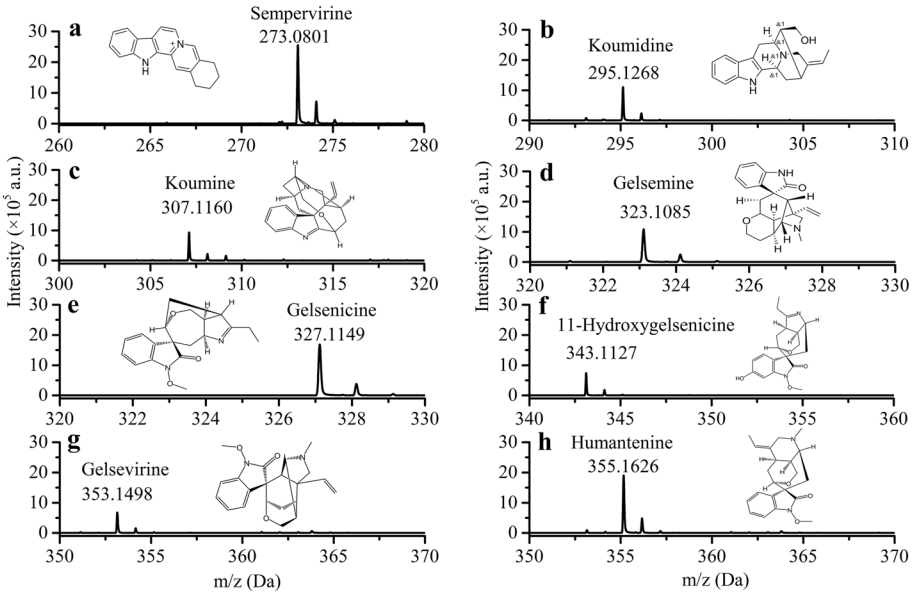

Fig. 2 MALDI-TOF mass spectrometry of *G. elegans* alkaloids standards: (**a**) sempervirine, (**b**) koumidine, (**c**) koumine, (**d**) gelsemine, (**e**) gelsenicine, (**f**) 11-hydroxygelsenicine, (**g**) gelsevirine, and (**h**) humantenine

The mixed standard solution (100 ng/mL) was also used to evaluate the peak intensities and S/N ratio as the laser energy increased (Fig. 4). The peak intensities were only approximately one-tenth of those in corresponding individual standard solutions, but the variation trends in the single and mixed standard solutions were consistent. These results were attributed to dispersion of laser energy by the eight *G. elegans* alkaloids rather than by a single analyte. The S/N ratios of sempervirine and koumidine decreased rapidly after the laser energy reached 70%. Thus, a higher laser energy adversely affected the S/N ratios of the analytes in the mixed standard solution. To obtain better peak intensities while minimizing background noise, a laser energy of 70% was selected for subsequent experiments.

Matrix effect

Human plasma and urine are rich in various organic and inorganic material, and the complexity of these samples can greatly affect the detection efficiency of MALDI-TOF MS. The ME was a crucial factor for quantifying the eight *G. elegans* analytes by MALDI-TOF MS. The length and accessibility of an analytical technique are important for clinical poisoning diagnoses. In this study, dilution was

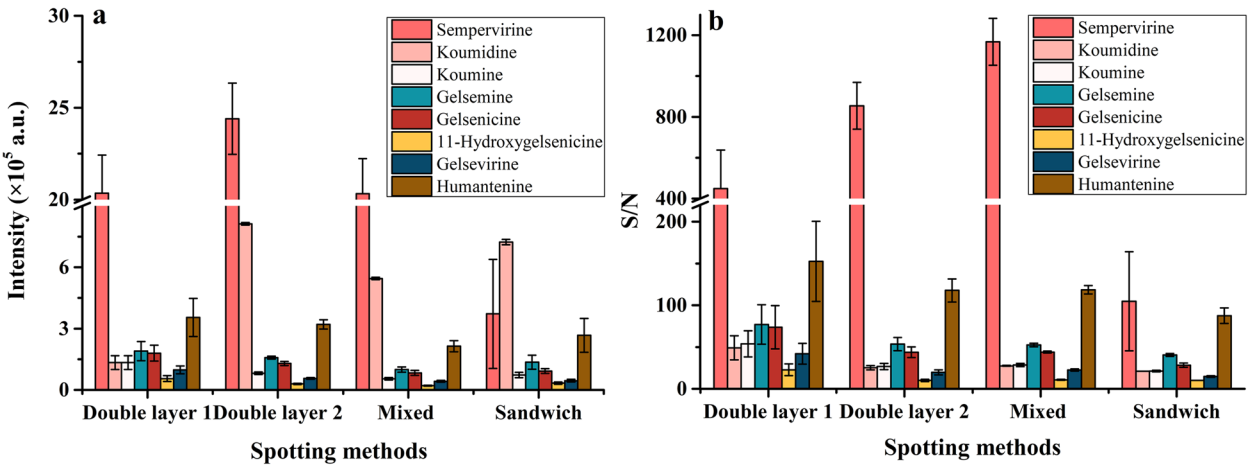

Fig. 3 **a** Peak intensities and (**b**) signal-to-noise (S/N) ratios for a mixed standard of eight *G. elegans* alkaloids with four different spotting methods. Values are means ± standard deviations

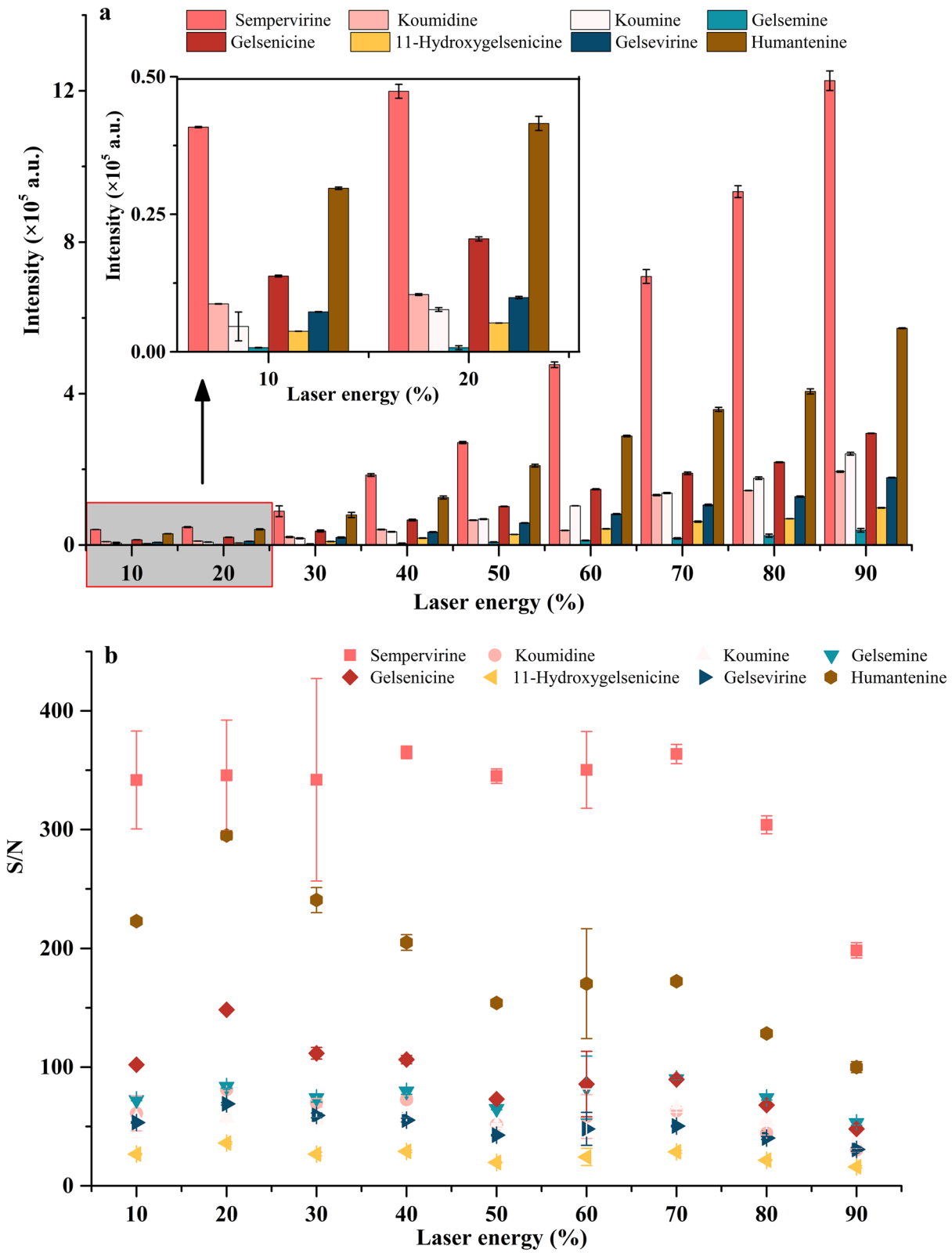

Fig. 4 **a** Peak intensities and (**b**) signal-to-noise (S/N) ratios for a mixed standard of eight *G. elegans* alkaloids at nine different laser intensities. Values are means ± standard deviations

Table 1 Matrix effects of eight *G. elegans* alkaloids in human plasma and urine ($n=3$)

Sample	Spiked levels (μg/g)	Dilution folds	Origin concentration (μg/g)	Matrix effect (%)							
				Sempervirine	Koumidine	Koumine	Gelsemine	Gelsenicine	11-Hydroxygelsenicine	Gelsevirine	Humantenine
Plasma	0.5	10	ND	115.6	87.6	96.4	94.8	98.2	91.4	93.6	84.1
		20	ND	74.0	77.4	76.4	89.3	77.8	72.4	88.1	79.4
		40	ND	90.9	99.8	84.5	81.1	82.1	88.2	84.0	99.2
		50	ND	98.0	84.3	106.9	61.6	92.9	99.3	80.9	103.7
		100	ND	182.1	113.2	91.6	140.6	154.5	153.2	142.9	129.5
	1.0	10	ND	97.3	87.8	133.8	158.3	92.2	80.9	76.5	107.2
		20	ND	76.4	66.6	65.9	83.9	98.2	78.3	69.1	140.7
		40	ND	89.8	88.3	87.5	92.7	86.9	95.2	89.2	83.5
		50	ND	76.8	92.1	64.5	78.2	78.8	79.2	68.1	80.4
		100	ND	115.7	124.5	121.8	101.4	109.4	99.3	93.9	111.5
	4.0	10	ND	27.9	9.8	65.4	56.9	43.7	53.6	59.3	48.1
		20	ND	161.8	22.2	19.3	16.3	14.7	23.2	28.5	99.7
		40	ND	94.9	87.8	87.6	95.8	91.3	90.0	100.7	110.1
		50	ND	95.5	99.2	93.0	96.0	102.2	97.0	89.0	97.1
		100	ND	84.9	80.1	77.2	85.1	73.1	84.9	80.1	77.2
Urine	0.5	10	ND	93.7	72.1	87.4	108.0	64.9	58.1	91.9	74.6
		20	ND	94.4	33.1	53.3	44.2	84.9	101.0	100.9	96.9
		40	ND	81.8	106.5	86.8	80.5	96.3	94.8	90.6	99.5
		50	ND	88.6	152.3	135.4	109.9	113.9	106.9	98.5	96.1
		100	ND	108.6	112.9	126.9	105.1	136.3	146.1	114.0	110.1
	1.0	10	ND	82.8	83.4	106.7	129.9	68.0	81.9	70.6	113.8
		20	ND	97.1	100.4	92.8	99.9	113.0	87.3	138.8	87.1
		40	ND	80.4	91.4	80.9	87.0	81.5	81.4	85.5	92.4
		50	ND	75.1	95.3	91.2	62.7	75.4	76.3	76.8	97.5
		100	ND	96.1	141.3	126.6	101.9	88.2	114.8	78.5	92.1
	4.0	10	ND	31.2	21.9	29.8	22.6	22.6	28.1	27.1	41.1
		20	ND	155.8	27.7	24.1	15.4	10.2	42.3	37.8	23.5
		40	ND	91.2	105.2	99.4	100.8	107.4	100.1	101.1	98.4
		50	ND	80.4	88.4	68.9	78.0	120.9	104.0	100.1	91.6
		100	ND	102.9	84.0	85.5	89.9	87.7	88.0	99.2	87.6

ND not detected

Table 2 Selected publications for *G. elegans* alkaloids detection

Analyte	Biological sample	Method	Pretreatment time (min)	Instrumental analysis time per sample (min)	LOD	References
Gelsemine, koumine and humantenmine	Goat plasma, urine and faeces	HPLC/QqTOF-MS	13–16	40	-	[3]
Gelsemine	Rat liver	HPLC/QqTOF-MS	Over 75	30	-	[9]
11 Gelsemium alkaloids & IS needed	Rat plasma	UPLC-MS/MS	11	5	LLOD < 0.1 ng/mL	[14]
Gelsemine, gelsemium, humantenmine	Porcine plasma, muscle, liver, kidney and urine; goat plasma	2D-LC	15	15	10 ng/mL	[15]
Koumine, gelsemine, and gelsenicine	Hair	UPLC-MS/MS	Over 60	8	1–5 pg/mg	[16]
Gelsemine, koumine and humantenmine	Porcine plasma	LC–MS/MS	Over 50	10	0.10 μg/L	[17]
27 *G. elegans* alkaloids	Pig tissues, urine, and plasma	UPLC-MS/MS	Over 60	15	Below 20 μg/kg	[18]
Koumine, gelsemine & IS needed	Human plasma	HPLC–UV	Over 120	25	0.01–0.05 mg/L	[19]
8 *G. elegans* alkaloids	Human plasma and urine	MALDI-TOF MS	15	Below 3	0.5–5.0 ng/mL	This work

- Not calculated

Table 3 Linear range, linear equation, correlation coefficient (*R*), limit of detection (LOD), and limit of quantification (LOQ) for the eight *G. elegans* alkaloids

Analyte	Linear range (ng/mL)	Calibration curve	*R*	Plasma (ng/mL)		Urine (ng/mL)	
				LOD	LOQ	LOD	LOQ
Sempervirine	5 ~ 100	$y = 628.66x + 3672.4$	0.9951	0.5	1.0	0.5	1.0
Koumidine	5 ~ 100	$y = 69.943x + 557$	0.9952	2.5	8.0	2.0	5.0
Koumine	5 ~ 100	$y = 63.075x + 320.36$	0.9976	2.0	6.0	1.0	3.0
Gelsmine	5 ~ 100	$y = 79.06x + 704.34$	0.9949	4.0	10.0	2.0	5.0
Gelsenicine	5 ~ 100	$y = 135.66x + 1724.9$	0.9930	4.0	10.0	2.0	5.0
11-Hydroxygelsenicine	5 ~ 100	$y = 48.168x + 634.24$	0.9910	5.0	10.0	2.0	6.0
Gelsevirine	5 ~ 100	$y = 88.682x + 868.04$	0.9939	4.0	10.0	3.0	10.0
Humantenine	5 ~ 100	$y = 244.55x + 2584.6$	0.9950	1.0	5.0	2.0	6.0

chosen as the pretreatment approach to evaluate the ME and acquire rapid and accurate results. Spiked human plasma and urine samples were analyzed. The final solution was diluted 10-, 20-, 40-, 50-, or 100-fold using methanol/water (5:5, v/v), and the ME was calculated. Generally, with HPLC–MS/MS, the ME is considered eliminated if the results are within 100% ± 10% [34]. Values above 110% are considered as matrix enhancement, and values below 90% are regarded as matrix suppression [17].

The ME values of the eight *G. elegans* alkaloids are presented in Table 1. At 40-fold dilution, the ME values in human plasma and urine were 81.1%–110.1% and 80.4%–107.4%, respectively, which closely matched the corresponding *G. elegans* alkaloids. Though there was a little matrix suppression observed for gelsemine, gelsenicine, 11-hydroxygelsenicine, and gelsevirine, the values were still within an acceptable range (ME values > 80%). This was different to a previous study that used LC–MS/MS with ME ranged from 88.5% to 107.8%, perhaps because of differences of samples and its pretreatment [14].

For reported HPLC and HPLC–MS/MS methods [14, 19], an internal standard and biological sample matrix standard are often used to eliminate the ME. These process are often complicated and needs extra labor. Moreover, sample processing involves several steps of organic solvent extraction and solid-phase extraction for purification, which is complicated and leads to sample loss. MALDI-TOF MS is considered an efficient alternative to electrospray ionization MS with strong resistance to

Table 4 Recovery results in plasma and urine for the eight *G. elegans* alkaloids ($n=3$)

Analyte	Spiked levels (μg/g)	Mean Recovery ± RSD (%)	
		Plasma	Urine
Sempervirine	0.5	87.9 ± 12.8	90.4 ± 9.5
	1.0	97.1 ± 9.0	102.7 ± 6.6
	4.0	95.0 ± 5.0	91.1 ± 4.2
Koumidine	0.5	86.0 ± 3.8	106.6 ± 3.6
	1.0	92.7 ± 9.7	98.6 ± 8.0
	4.0	87.3 ± 9.2	96.4 ± 12.0
Koumine	0.5	99.9 ± 9.2	103.8 ± 5.6
	1.0	95.7 ± 3.7	99.0 ± 8.1
	4.0	88.0 ± 11.6	103.0 ± 8.2
Gelsemine	0.5	84.2 ± 8.2	88.0 ± 7.7
	1.0	104.7 ± 4.4	95.1 ± 8.5
	4.0	101.9 ± 5.7	101.3 ± 6.4
Gelsenicine	0.5	83.0 ± 12.2	84.1 ± 18.5
	1.0	94.4 ± 0.2	95.1 ± 11.2
	4.0	87.0 ± 7.8	107.7 ± 12.2
11-Hydroxygelsenicine	0.5	82.7 ± 8.6	93.1 ± 17.5
	1.0	92.2 ± 0.1	99.5 ± 2.0
	4.0	69.7 ± 8.0	101.2 ± 1.9
Gelsevirine	0.5	77.4 ± 10.5	103.0 ± 7.4
	1.0	103.6 ± 5.5	97.6 ± 3.8
	4.0	73.1 ± 9.0	91.3 ± 13.3
Humantenine	0.5	90.7 ± 8.9	64.9 ± 7.1
	1.0	100.4 ± 5.2	96.4 ± 9.7
	4.0	107.8 ± 5.7	107.0 ± 4.1

interference from salts and buffer solutions [35]. MALDI tends to produce singly charged ions and provides notable advantages in quantification, particularly for mixtures [36]. However, appropriate dilution of biological samples is required to keep the sample composition constant, which is the best way to minimize the background and eliminate potential interfering peaks. The developed method is compared with previously reported methods in Table 2. The pretreatment protocol for biological samples in the developed method is simpler and more efficient than in other methods.

Selectivity

No significant interference was observed in the MALDI-TOF MS results for the blank samples of human plasma and urine (Fig. S4 and Table 1).

Linear range, limit of detection, and limit of quantification

Calibration curves were constructed showing the concentration (x-axis) of mixed standard concentrations of 10–100 ng/mL, versus the peak area (y-axis) which accumulated of 20 single shots. We ensured that the total MS signals remained unsaturated. The limit of detection (LOD) and limit of quantification (LOQ) were calculated by diluting the lowest concentration spiked sample (10 ng/mL) to a concentration that gave a S/N ratio equal to or greater than three for the LOD and a S/N ratio equal to or greater than 10 for the LOQ. Because the signal intensities varied among the eight *G. elegans* alkaloids, their LODs and LOQs were not the same (Table 3). The regression coefficients (R) of the eight *G. elegans* alkaloids were all > 0.99, which showed that the method had good linearity. The LODs for plasma and urine were 0.5–5.0 ng/mL and 0.5–3.0 ng/mL, respectively. Both plasma and urine had the same LOQ range of 1.0–10.0 ng/mL. Except for humantenine and sempervirine, all analytes had lower LODs in urine than in plasma. Sempervirine had the lowest LOD.

Recovery and precision

According to the ME results, the three spiked levels with eight *G. elegans* alkaloids were diluted 40-fold. The mass spectra of blank human plasma, urine, and the spiked mixture are shown in Fig. S4. The recoveries ranged from 69.7% to 107.8% in plasma and from 64.9% to 107.7% in urine. The RSDs in plasma and urine were 0.1%–12.8% and 1.9%–17.5%, respectively (Table 4). These values were lower than the RSDs observed in other MALDI studies, which reported RSDs of > 50% [33]. The ME, recoveries, and spot-to-spot precision in plasma and urine were within acceptable ranges for the developed method (Tables 1, 2, 3 and 4), which confirmed its validity.

Conclusion

We developed an analytical strategy and performed optimization of the instrumental conditions and validation of the methodology to realize quantitative detection of eight *G. elegans* alkaloids in human plasma and urine. This method expands the application range of MALDI-TOF MS to the low molecular mass range (< 700 Da). After testing different spotting methods with the HCCA matrix, the double layer 1 spotting method was selected as the most effective for elimination of the "sweet spot" phenomenon with *G. elegans* alkaloids. Step-wise dilution of human plasma and urine was an economical approach to avoid the ME. The established method has high-throughput, high sensitivity, and good precision. Furthermore, it only requires a small volume of sample, has low chemical solvent use, and is more environmental friendly than other analytical methods, such as HPLC and HPLC–MS/MS. Because the pretreatment time is rapid (15 min) and the sample volume is low (1 μL), MALDI-TOF MS can be used for routine diagnosis and analysis of *G. elegans* alkaloids with no internal standard addition. Because it takes less than 3 min for instrumental analysis

of *G. elegans* alkaloids, this method could provide vital clinical information in poisoning cases to improve treatment. Further exploration and validation are required for wider potential application in surveillance systems for clinical healthcare and food safety.

Materials and methods

Reagents and instruments

Sempervirine, koumidine, koumine, gelsemine, gelsenicine, 11-hydroxygelsenicine, gelsevirine, and humantenine were purchased from Yuanye Bio-technology Co., Ltd. (Shanghai, China). The chemical information and structure for each analyte are shown in Table S1 and Fig. 2, respectively. HCCA, SA, and DHB were obtained from Bruker Daltonics (Bremen, Germany). Human plasma was provided by Shenzhen Realbio Group Co., Ltd. (Shenzhen, China), and synthetic human urine was obtained from Huzhou InnoReagents Co., Ltd. (Huzhou, China). HPLC-grade methanol and acetonitrile were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Ultra-pure grade water was prepared using a Milli-Q system (Millipore Corporation, MA, USA).

An ultrafleXtreme MALDI-TOF MS and a 384 ground steel plate were obtained from Bruker Daltonics (Bremen, Germany). An ultrasonicator (2600HT) was purchased from Anpel Science Instrument Co. Ltd. (Shanghai, China). A vortex mixer (M3 7610-33CN) was obtained from Thermo Fisher Scientific. A high speed refrigerated centrifuge (Allegra X-30R) was purchased from Beckman Coulter (Brea, CA, USA).

Solutions

A stock standard solution (1.0 mg/mL) was prepared for each of the eight compounds by dissolving it in methanol. These solutions were stored at < − 18 °C in the dark. Single *G. elegans* alkaloid standard or mixed standard with eight *G. elegans* alkaloids intermediate solutions (10 μg/mL) were prepared in methanol and diluted stepwise with methanol/water (5:5, v/v) to obtain working solutions at concentrations of 5, 10, 20, 50, 80, and 100 ng/mL. Matrix solutions (10 mg/mL) were prepared by dissolving 10 mg of HCCA, SA, or DHB in 1.0 mL of methanol/water (5:5, v/v). All the matrix solutions were sonicated for 10 min before use.

MALDI-TOF MS analysis of *G. elegans* alkaloids

Spotting methods

Four different target plate spotting methods were used in this study. The first method (double layer 1) involved preparing a matrix layer by dropping 1 μL of well-mixed matrix solution (HCCA, SA, or DHB) onto a 384 ground steel target plate and air drying. Next, 1.0 μL of standard solution (100 ng/mL) was added to cover the matrix layer and air dried. In the second method (double layer 2), 1 μL of standard solution (100 ng/mL) was spotted onto the target plate and air dried. Next, 1.0 μL of well-mixed matrix solution was added as the second layer and air dried. For the third method (mixed), equal volumes of standard solution and matrix solution were mixed until homogeneous and then 2.0 μL of this mixture was spotted onto the target plate and air dried. In the fourth method (sandwich), 0.5 μL of well-mixed matrix solution was spotted onto the target plate first and air dried. This was followed by layering of 1.0 μL of standard solution and then another 0.5 μL of well-mixed matrix solution.

MALDI-TOF MS conditions

All the sample spots were analyzed in reflected linear positive mode with a 337-nm nitrogen laser. For every spectrum, an average of 200 laser shots were collected with accumulation of 20 laser shots and a frequency of 500 Hz in positive ion mode. The ion source 1 and 2 voltages were 19.00 and 16.55 kV, respectively. The lens voltage was 8.70 kV and the mass range was 100–1000 Da under 70% laser energy.

Sample preparation

Plasma

Plasma samples were prepared following an established method with some modifications [14]. Human plasma (1 g) was added in a 15-mL centrifuge tube and 2.0 mL of acetonitrile was added. The mixture was vortexed for 20 s, ultrasonicated for 10 min, and then centrifuged at 8000 rpm for 5 min. Finally, the supernatant was diluted with a mixture of methanol and water (5:5, v/v) before use.

Urine

Synthetic human urine (1 g) was added in a 15-mL centrifuge tube, and filled with a mixture of methanol and water (5:5, v:v) to 10 mL. The sample was then processed as described above. The Ethics Review Board of the College of Food Science, South China Agricultural University, has approved these experiments and exempted them from the requirement for further ethical approval.

After weighing the samples, they were spiked with the mixed standards at 0.5, 1.0, and 4.0 μg/g and then analyzed as described above.

Matrix Effect

Blank human plasma and synthetic urine were extracted using the above procedure. Analytes were added at 0.5, 1.0, and 4.0 μg/g. The supernatant was diluted with methanol/water (5:5, v/v) to 10-, 20-, 40-, 50-, and 100-fold. Finally, the ME was calculated as follows: ME (%) = corresponding peak area × 100%/pure standard solution peak area.

Method validation

Selectivity

To verify the method's ability to differentiate and quantify analytes in the presence of other compounds in the sample, blank human plasma and synthetic urine samples were evaluated. A blank sample spiked with standards was also tested. The corresponding *mass-to-charge* ratios were compared to ensure that there were no potential impurity peaks within the detection range in both the plasma and urine samples.

Linear range, LOD, and LOQ

A six-point calibration curve was constructed for the concentration range of 5 to 100 ng/mL for the mixed standard of the eight *G. elegans* alkaloids. The LOD and LOQ were calculated as the lowest concentrations of these compounds that could be detected and quantified in real samples with S/N ratios above or equal to 3 (LOD) and 10 (LOQ).

Recovery and precision

The recovery was evaluated by analyzing spiked samples at three different concentrations (0.5, 1.0, and 4.0 μg/g). The precision was assessed using the percentage RSD of spot-to-spot variability, which was calculated from the RSD of three different spots at a specific concentration.

Data analyses

MALDI-TOF MS spectra were manually screened for *G. elegans* alkaloids using flexAnalysis 3.4 software using the accurate mass. All the results are expressed as the mean ± standard deviation of at least three independent experiments and were analyzed using Statistica (version 10, Statsoft).

Abbreviations

DHB	2,5-Dihydroxybenzoic acid
HCCA	α-Cyano-4-hydroxycinnamic acid
LOD	Limit of detection
LOQ	Limit of quantitation
MALDI-TOF MS	Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry
ME	Matrix effect
RSD	Relative standard deviation
SA	Sinapinic acid
S/N	Signal-to-noise

Supplementary Information

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Supplementary Material 1.

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Not applicable.

Authors' contributions

X.Z.: Writing-original draft, Validation, Methodology, Software, Data curation, Visualization, Funding acquisition. Y.W.: Writing-original draft, Methodology, Software, Resources. L.L.: Writing-Review and Editing, Investigation, Supervision. Y.L.: Writing-Review and Editing, Investigation, Validation. Z.X.: Writing-Review and Editing, Methodology, Investigation, Supervision, Funding acquisition. All authors read and approved the final manuscript.

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Data availability

The data used and/or analyzed during this research are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

This research did not involve human subjects, real animals or animal sample testing, collection of personal data that could be linked to an identifiable individual, or the testing of biological samples or any materials that could potentially affect human health or the environment. A formal waiver statement has been provided from the Ethics Review Board of food science college of South China Agricultural University.

Consent for publication

Not applicable.

Competing interests

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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