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Research Article

Comparison of liquid chromatography-high-resolution tandem mass spectrometry (MS^2) and multi-stage mass spectrometry (MS^3) for screening toxic natural products

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ABSTRACT

Background: Liquid chromatography-high-resolution mass spectrometry (LC-HR-MS) has emerged as a powerful analytical technology for compound screening in clinical toxicology. To evaluate the potential of LC-HR-MS^3 in detecting toxic natural products, a spectral library of 85 natural products (79 alkaloids) that contains both MS^2 and MS^3 mass spectra was constructed and used to identify the natural products. Samples were analyzed using an LC-HR-MS^3 method and the generated data were matched to the spectral library to identify the natural products.
Methods: To test the performance of the LC-HR-MS^3 method in different sample matrices, the 85 natural product standards were divided into three groups to separate structural isomers and avoid ion suppression effects caused by co-elution of multiple analytes. The grouped analytes were spiked into drug-free serum and drug-free urine to produce contrived clinical samples.
Results: The compound identification results of the 85 natural products in urine and serum samples were obtained. The match scores using both MS^2 and MS^3 mass spectra and those using only MS^2 mass spectra were compared at 10 different analyte concentrations. The two types of data analysis provided identical identification results for the majority of the analytes (96% in serum, 92% in urine), whereas, for the remaining analytes, the MS^2-MS^3 tree data analysis had better performance in identifying them at lower concentrations.
Conclusion: This study shows that in comparison to LC-HR-MS (MS^2), LC-HR-MS^3 can increase the performance in identification of a small group of the toxic natural products tested in serum and urine specimens.

1. Introduction

Liquid chromatography-high-resolution mass spectrometry (LC-HR-MS) has emerged as a powerful analytical technology for compound screening in clinical toxicology. It is capable of determining the accurate mass and corresponding molecular formula of an analyte. Following a full scan, specific molecular ions can be selected for fragmentation, and the corresponding product ions can be monitored allowing for tandem mass spectrometry (MS/MS or MS^2). This methodology shows excellent performance in the identification of compounds in complex biological matrices.[1–3] For some instrument platforms, additional generations of product ions can be produced via fragmentation, often referred to as multi-stage mass spectrometry (MS^n).[4] MS^3 product ions can be generated from an MS^2 product ion. In MS^3 analysis, as illustrated in Fig. 1, the fragmentation patterns of precursor ions and those of MS^2 product ions can be matched to a spectral library that contains both MS^2 and MS^3 mass spectra for compound identification, providing structural information and confidence in the detection of targets.[5–9] LC-HR-MS^3 could potentially enhance the specificity or detection limit for identifying compounds of clinical interest,[10] specifically in clinical toxicology applications.

Natural products are chemical substances produced by living organisms, mainly plants.[11] Many natural products have biological effects on humans and animals, and some of them are used as pharmaceuticals.[12,13] A comprehensive screening method for toxic natural products is necessary to aid in their identification and to better

Abbreviations: LC-HR-MS, liquid chromatography-high-resolution mass spectrometry; MS/MS or MS^2, tandem mass spectrometry; MS^n, multi-stage mass spectrometry; DDA, data-dependent acquisition; MeOH, methanol; CAN, acetonitrile; HCD, high-energy C-trap dissociation.

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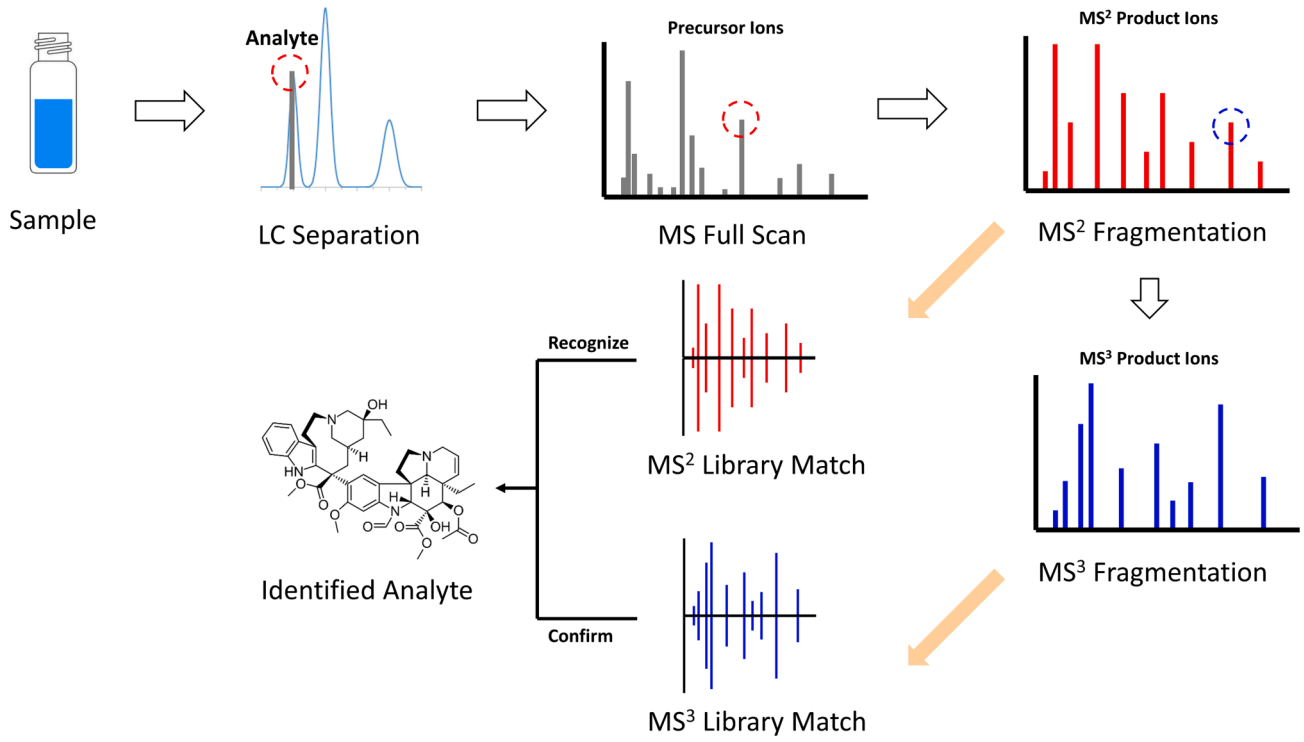

Fig. 1. Data acquisition and compound identification workflow of the LC-HR-MS^3 method.

understand their potential toxic effects in clinical toxicology cases. However, the large number of toxic natural products, including many structural analogs, makes it challenging to develop a comprehensive set of conventional immunoassays to screen them. It is particular due to the fact that an immunoassay lacks a wide coverage of targets and has limited specificity to structural analogs.

Previously we described an LC-HR-MS (MS^2) method for the identification of toxic natural products.[3,14] It is currently unknown whether utilizing LC-HR-MS^3 provides enhanced performance for identifying small molecules in clinical toxicology cases in comparison to MS^2. To evaluate the potential of LC-HR-MS^3 in detecting toxic natural products, a spectral library of 85 natural products containing both MS^2 and MS^3 mass spectra was constructed using a quadrupole-linear-ion-trap-Orbitrap tandem mass spectrometer. The spectral library focused on alkaloids (79 out of 85 natural products), which represent a major and commonly encountered group of toxic natural products.[15,16] As clinical toxicology tests are most often performed using blood and urine samples,[17] the study used serum and urine sample matrices spiked with the analytes. Samples were analyzed using an LC-HR-MS^3 data-dependent acquisition (DDA) method with the same fragmentation conditions as the spectral library construction. The generated data were matched to the spectral library to identify the natural products in two data processing mechanisms: [1] matching both MS^2 and MS^3 mass spectra to the spectral library, and [2] matching only MS^2 mass spectra to the spectral library.

2. Materials and methods

2.1. Chemicals and reagents

Among the 85 natural product standards, 9 (bufotenine, cathinone, coniine, cotinine, dihydrokavain, ephedrine, mitragynine, pseudoephedrine, yangonin) in either dry powder or solution state were purchased from Cerilliant (Round Rock, TX), and all the other 76 natural product standards in dry powder state were purchased from ChemFaces (Wuhan, China). The complete list of the 85 natural products can be found in Table 1. LC-MS grade methanol (MeOH), acetonitrile (ACN), and water were purchased from Fisher Scientific (Waltham, MA). Pooled drug-free serum was purchased from Bio-Rad (Hercules, CA), and pooled drug-free urine was purchased from UTAK Laboratories (Valencia, CA).

2.2. Sample preparation

Natural product standards were dissolved in 1:1 ACN: dimethyl sulfoxide to 0.50 mg/mL and then diluted in a sample diluent (1:1:2 mixture of MeOH, ACN, and 5.0 mM ammonium formate in water, added with 0.05 % formic acid) to 1.0 μg/mL for spectral library construction.

To test the performance of the LC-HR-MS^3 method in different sample matrices, the 85 natural product standards were divided into three groups to separate structural isomers and avoid ion suppression effects caused by co-elution of multiple analytes, as indicated in Table 1. The grouped analytes were spiked into drug-free serum and drug-free urine to produce contrived clinical samples. A series of serum samples were made at analyte concentrations of 0.50, 1.0, 2.5, 5.0, 10, 25, 50, 100, 250, and 500 ng/mL, and a series of urine samples were made at analyte concentrations of 1.0, 2.5, 5.0, 10, 25, 50, 100, 250, 500, and 1000 ng/mL.

For serum samples, 125 μL serum was mixed with 375 μL acetonitrile. The sample was centrifuged at 13000 rpm for 10 min, and 400 μL supernatant was taken and dried under nitrogen flow at 37 °C. The sample was reconstituted in 50 μL sample diluent. For urine samples, 100 μL urine sample was mixed with 400 μL sample diluent.

2.3. LC-HR-MS^3 method

The LC-HR-MS^3 method utilized an Orbitrap ID-X Tribrid mass spectrometer coupled with a Vanquish UHPLC (Thermo Fisher Scientific, San Jose, CA). LC separation was carried out using an Accucore C18 column (2.1 mm × 100 mm, 2.6 μm particle) with gradient elution (mobile phase A: 5 mM ammonium formate in water with 0.05 % formic acid; mobile phase B: MeOH: ACN 1:1 with 0.05 % formic acid). The

Table 1

The compound identification results of the 85 natural products in urine and serum samples using the LC-HR-MS^3 analysis. If a compound at a certain concentration was identified by both the MS^2-MS^3 tree data analysis and MS^2 data analysis, the corresponding cell is marked green. The cell is marked yellow if a compound was identified by only the MS^2-MS^3 tree data analysis, or it is blank if a compound was identified by neither of them. The difference between the match scores (MS^2-MS^3 tree data analysis minus MS^2 data analysis) is written in each cell (blank if the compound was not identified).

Natural Product	CAS	Typical Natural Source	Biological Activity	Compound Type	Molecular Formula	Analyte Group	Concentration in Serum (ng/ml)									Concentration in Urine (ng/ml)								
							500	250	100	50	25	10	5	2.5	1	0.5	1000	500	250	100	50	25	10	5	2.5	1
Aconitine	302-27-2	Aconitum species	Cardiovascular Toxicity / Neurotoxicity / Gastrointestinal Toxicity	Alkaloid	C34H47NO11	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Ajmaline	4360-12-7	Rauvolfia vertcillata	Cardiovascular Toxicity / Neurotoxicity	Alkaloid	C20H26N2O2	1	0	0	0	0	0	0	0				0	0	0	0	0					
Allomatrine	641-39-4	Sophora alopecuroides	Neurotoxicity / Cardiovascular Toxicity	Alkaloid	C15H24NO2	3	0	0	0	0	0	0	0	0	0		0	0	0	0	0	0	0	0		
Anagyrine	486-89-5	Cytisus scoparius (Common Broom) / Lupinus species (Lupine)	Neurotoxicity / Cardiovascular Toxicity	Alkaloid	C15H20N2O	3	-4	-2	-2	-5	-7	0	0	0			7	0	0	0	0	0				
Arecoline	63-75-2	Areca catechu	Cardiovascular Toxicity / Neurotoxicity / Gastrointestinal Toxicity	Alkaloid	C8H13NO2	2	1										3	6	6	18	40					
Atropine	51-55-8	Atropa belladonna	Neurotoxicity / Cardiovascular Toxicity	Alkaloid	C17H23NO3	1	-1										0	-1	0	0	-1	-1	-1	0	0	0
Berberine	2086-83-1	Coptis chinensis (Chinese Goldthread); Corydalis yanhusuo; Hydrastis canadensis (Goldenseal) / Berberis species	Gastrointestinal Toxicity / Cardiovascular Toxicity / Neurotoxicity / Hepatotoxicity	Alkaloid	C20H18NO4	1	0	0	0	0	0	0	0													
Boldine	476-70-0	Litsea glutinosa	Hepatotoxicity	Alkaloid	C19H21NO4	1	-1	-3	-1	0	0	-3	0	3	2		0	0	-3	-1	-1	1	4	8	10	
Brucine	357-57-3	Strychnos nux-vomica	Neurotoxicity / Rhabdomyolysis / Nephrotoxicity	Alkaloid	C23H26N2O4	1	0	0	0	0	0	0	0	0			0	0	0	0	0	0	0	0	0	
Bufotenine	487-93-4	Bufo gargarizans (Asiatic Toad)	Gastrointestinal Toxicity / Cardiovascular Toxicity / Neurotoxicity	Alkaloid	C12H16N2O	3	0	-1	-1	0	-1	0	-3				0	0	-1	0	0	2	0			
Camptothecin	7689-03-4	Camptotheca acuminata	Hematologic Toxicity / Gastrointestinal Toxicity	Alkaloid	C20H16N2O4	3	0	0	0	0	0						0	0	0	0	0	0	0			
Catharanthine	2468-21-5	Catharanthus roseus (Rose Periwinkle)	Neurotoxicity / Hematologic Toxicity / Gastrointestinal Toxicity / Hair Loss	Alkaloid	C21H24N2O2	1	0	2	1	17	15	17	2	14	12	15	2	1	1	10	16	14	18	26	38	
Cathinone	71031-15-7	Catha edulis (Khat)	Cardiovascular Toxicity / Neurotoxicity	Alkaloid	C9H11NO	1	0	0	0	0							0	0	0	0						
Cannabidiol	13956-29-1	Cannabis sativa	Inactive Constituent of Cannabis sativa	Terpenoid	C21H30O2	3	0	0	28	0							31	0								
Cannabinol	521-35-7	Cannabis sativa	Constituent of Cannabis sativa	Terpenoid	C21H26O2	1	0	0	0								0	0								
Cephalotaxine	24316-19-6	Cephalotaxus fortunei	No Reported Toxicity	Alkaloid	C18H21NO4	2	0	0	0	0	0	0					0	0	0	0	0	0				
Chelerythrine	34316-15-9	Chelidonium majus	Hepatotoxicity	Alkaloid	C21H18NO4	2	0																			
Chelidonine	476-32-4	Chelidonium majus	Hepatotoxicity	Alkaloid	C20H19NO5	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Cinchonine	118-10-5	Cinchona ledgeriana	Cardiovascular Toxicity / Neurotoxicity (presumably / found with quinine)	Alkaloid	C19H22N2O	2	0	0	0	0	0	0	0	0	0		0	0	0	0						
Cinnamoylcocaine	521-67-5	Erythroxylum coca	Toxicity Not Described	Alkaloid	C19H23NO4	2	0	0	0	0	0						0	0	0	0	0	0	0			
Colchicine	64-86-8	Colchicum autumnale (Autumn Crocus)	Gastrointestinal Toxicity / Hematologic Toxicity / Cardiovascular Toxicity	Alkaloid	C22H25NO6	1	0	0	0	0	0						0	0	0							
Coniine	3238-60-6	Conium maculatum (Poison Hemlock); Oenanthe javanica (Java Waterdropwort)	Neurotoxicity / Cardiovascular Toxicity	Alkaloid	C8H17N	3	0	0	0	0	0	0					0	0	0	21	0	0	0			
Coptisine	3486-66-6	Chelidonium majus; Corydalis yanhusuo	Cardiovascular Toxicity / Gastrointestinal Toxicity / Hepatotoxicity	Alkaloid	C19H14NO4	2	0	0	0																	
Corydaline	518-69-4	Corydalis ambigua; Corydalis yanhusuo	CYP2C19 and CYP2C9 Inhibitor	Alkaloid	C22H27NO4	1	-1	0	-2	0	0	-1	0	0	2	-2	-1	0	0	-1	0	1	0	0	10	18
(+)-Corynoline	18797-79-0	Corydalis ambigua; Corydalis incisa	CYP3A4 Inhibitor	Alkaloid	C21H21NO5	3	0	-1	-1	0	0	0	0	0	0	0	-1	0	0	0	0	0	0	1	0	0
Cotinine	486-56-6	Nicotiana tabacum (Tabacco)	Nicotine Metabolite	Alkaloid	C10H12N2O	1	0	0	-1	0	0	0					0	0	0	0	0	0				
Crotaline	315-22-0	Crotalaria pallida	Hepatotoxicity	Alkaloid	C16H23NO6	2	-1	-1	-1	0	0	0	0	3			-1	0	0	0	0	0	0	0		
Cytisine	485-35-8	Genista tinctoria (Dyer's Greenweed)	Neurotoxicity / Cardiovascular Toxicity / Gastrointestinal Toxicity	Alkaloid	C11H14N2O	2	0	0	0	0	0	0	46				0	0	0	0	0	6	0			
Dihydrocapsaicin	19408-84-5	Capsicum annuum (Chili Peppers)	Neurotoxicity / Irritant	Alkaloid / Capsaicinoid	C18H29NO3	3	-2	-1	-1	-2	-4	-5	0	0			-1	0	-3	0	0	0	0	0		
Dihydrokavain	587-63-3	Piper methysticum (Kava)	Hepatotoxicity	Lactone	C14H16O3	1	-1	-1	0	0	-1	0	0	0	0	0	0	0	-1	0	0	0	0	0	0	0
Dihydrosanguinarine	3606-45-9	Chelidonium majus	Cardiovascular Toxicity	Alkaloid	C20H15NO4	2											0	0								
Ephedrine	299-42-3	Ephedra species	Cardiovascular Toxicity / Neurotoxicity / Hepatotoxicity	Alkaloid	C10H15NO	1	-1	-1	-1	0	0	0	1	2			-1	-1	0	0	1	1	4			
Evodiamine	518-17-2	Tetradium ruticarpum	Hepatotoxicity / Cardiovascular Toxicity	Alkaloid	C19H17N3O	3	-1	-1	-1	-1	-1	0	-1	0	0	0	-1	-1	-1	-1	-1	0	-1	-1	-1	-1
Galantamine	357-70-0	Lycoris radiata	Neurotoxicity / Cardiovascular Toxicity	Alkaloid	C17H21NO3	2	-2	-2	-1	-1	-1	-2	-2	-2			-3	-3	-2	6	1					
Glaucine	475-81-0	Corydalis yanhusuo	Neurotoxicity	Alkaloid	C21H25NO4	1	0	0	0	0	0	0	0	13	14	14	0	0	0	0	0	0	0	13	15	10
Harmalidine	109794-97-0	Peganum harmala (Wild Rue)	Neurotoxicity / Hematologic Toxicity / Cardiovascular Toxicity	Alkaloid	C16H18N2O	3	0	0	6	0	0						0	0	0	0	0	12				
Harmaline	304-21-2	Peganum harmala (Wild Rue)	Neurotoxicity / Hematologic Toxicity / Cardiovascular Toxicity	Alkaloid	C13H14N2O	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Harmane	486-84-0	Peganum harmala (Wild Rue)	Neurotoxicity / Hematologic Toxicity / Cardiovascular Toxicity	Alkaloid	C12H10N2	3	0	-1	0	0	0	0	0				0	0								
Harmine	442-51-3	Peganum harmala (Wild Rue)	Neurotoxicity / Hematologic Toxicity / Cardiovascular Toxicity	Alkaloid	C13H12N2O	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	4	4
Hordenine	539-15-1	Dendrolobium triangulare	Toxicity Not Described	Alkaloid	C10H15NO	2	0	0	0	0	0	1	4	5			0	1	3	13						
Hyoscyamine	101-31-5	Atropa belladonna	Neurotoxicity / Cardiovascular Toxicity	Alkaloid	C17H23NO3	3	0	0	-1	0	-1	-1	0	-1	-1	2	-1	0	-1	-1	-1	-1	-1	0	0	0
Hypaconitine	6900-87-4	Aconitum species	Neurotoxicity / Cardiovascular Toxicity / Gastrointestinal Toxicity	Alkaloid	C33H45NO10	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Ibogaine	83-74-9	Ervatamia hirta	Cardiovascular Toxicity / Neurotoxicity	Alkaloid	C20H26N2O	2	0	0	0	0	0	0	0	0			0	0	0	6	5					
Lappaconitine	32854-75-4	Aconitum species	Cardiovascular Toxicity / Neurotoxicity / Gastrointestinal Toxicity	Alkaloid	C32H44N2O8	3	1	6	1	0	0	0	0	1	0	0	2	1	0	0	6	0	0	1	0	0
Leonurine	24697-74-3	Leonurus heterophyllus	Neurotoxicity	Alkaloid	C14H21N3O5	3	0	0	-1	1	-1	-1	0	0	2	1	0	-1	-1	0	-1	0	-1	0	-4	0
Lobelin	90-69-7	Lobelia sessilifolia	Neurotoxicity / Cardiovascular Toxicity	Alkaloid	C22H27NO2	1	0	0	0	19	0	0	19	20	21	21	16	14	0	19	20	20	19	20	19	
Lupanine	550-90-3	Lupinus species (Lupine)	Neurotoxicity / Cardiovascular Toxicity	Alkaloid	C15H24N2O	1	0	0	0	0	0	0	0				0	0	0	0	0	0				
Lycorine	476-28-8	Lycoris radiata	Gastrointestinal Toxicity	Alkaloid	C16H17NO4	2	0	0	0	0	0	0	0	0			0	0	0	0	0	6				
Matrine	519-02-8	Sophora japonica	Neurotoxicity	Alkaloid	C15H24N2O	2	0	0	0	0	0	0	0	0			0	0	0	0	0	0	0	0	0	
Mesaconitine	2752-64-9	Aconitum species	Neurotoxicity / Cardiovascular Toxicity / Gastrointestinal Toxicity	Alkaloid	C33H45NO11	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
N-Methylcytisine	486-86-2	Sophora flavescens	Cardiovascular Toxicity / Neurotoxicity / Gastrointestinal Toxicity	Alkaloid	C12H16N2O	1	0	0	0	0	0	0	0				0	0	0	0	0	0				
Methylscopolleterine	18747-42-7	Sedum sarmentosum	Neurotoxicity / Gastrointestinal Toxicity	Alkaloid	C9H17NO	2	0										2	4	11	11	30	41				
Mitragynine	4098-40-2	Mitragyna speciosa (Kratom)	Neurotoxicity / Cardiovascular Toxicity	Alkaloid	C23H30N2O4	1	0	0	0	0	-2	0	0	0	0	0	0	-2	-1	0	0	0	0	2	2	0
Neferine	2292-16-2	Nelumbo nucifera (Lotus)	Cardiovascular Toxicity	Alkaloid	C38H44N2O6	3											0	0	0	0	0					
Nonivamide	2444-46-4	Capsicum annuum (Chili Peppers)	Irritant	Alkaloid / Capsaicinoid	C17H27NO3	3	-2	0	0	0	0	0	0	6			0	0	0	0	0	0	0	0		
Nuciferine	475-83-2	Nelumbo nucifera (Lotus)	Neurotoxicity	Alkaloid	C19H21NO2	3	0	0	0	0	0	0	49	49	0	1	0	0	0	0	49	0	0	0	0	1	0
Oliquindox	23696-28-8	Cyprinus carpio (Common Carp)	Toxicity Not Described	Quinoxaline Derivative	C12H13N3O4	3	0	0	0	0	0															
Oxymatrine	16837-52-8	Sophora flavescens	Neurotoxicity	Alkaloid	C15H24N2O2	2	0	0	0	0	0	0	0	0	0		0	0	0	0	0	0	0			
Palmatine	3486-67-7	Coptis chinensis (Chinese Goldthread) / Corydalis yanhusuo / Berberis species	Cardiovascular Toxicity / Gastrointestinal Toxicity	Alkaloid	C21H22NO4	2	0	0	0	0	0	0	0	0	0		0	0	0							
Peimine	23496-41-5	Fritillaria thunbergii	Toxicity Not Described	Alkaloid	C27H45NO3	3	0	-13	-12	-13	0	0	0	1	1	4	-11	-11	0	0	0	0	0	0	1	1
Protopine	130-86-9	Corydalis yanhusuo	Neurotoxicity / Gastrointestinal Toxicity	Alkaloid	C20H19NO5	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Pseudoephedrine	90-82-4	Ephedra species	Cardiovascular Toxicity / Neurotoxicity / Hepatotoxicity	Alkaloid	C10H15NO	3	-1	0	-1	-1	-1						-1	-1	-1	-1	0					
Quinine	130-95-0	Cinchona ledgeriana	Cardiovascular Toxicity / Gastrointestinal Toxicity / Hematologic Toxicity / Metabolic Toxicity	Alkaloid	C20H24N2O2	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0				
Reserpine	50-55-5	Rauvolfia species	Neurotoxicity / Cardiovascular Toxicity	Alkaloid	C33H40N2O9	2	0	0	0	0	0	1	0	0	4	1	0	0	0	0	0	0	0	0	0	-10
Reserpinine	24815-24-5	Rauvolfia species	Cardiovascular Toxicity / Neurotoxicity / Gastrointestinal Toxicity	Alkaloid	C35H42N2O9	2	1	-1	0	0	-1	-1	15	32	31		0	0	0	32	0	0				
Rhynchophylline	76-66-4	Uncaria rhynchophylla	Cardiovascular Toxicity / Neurotoxicity	Alkaloid	C22H28N2O4	3	0	0	-1	-1	-1	-1	0	0	1	1	-1	0	-1	-1	0	0	-1	-2	-2	0
Rutaecarpine	84-26-4	Tetradium ruticarpum	Cardiovascular Toxicity / Hematologic Toxicity [x53]	Alkaloid	C18H13N3O	3	0	0	0	0	0	0	0	0	0		0	0	0	0	0	0	0	0	0	0
Sanguinarine	2447-54-3	Chelidonium majus (Nipplewort)	Cardiovascular Toxicity / Hepatotoxicity	Alkaloid	C20H14NO4	2	0	0	0	0	0	0					0	0								
Scopolamine	51-34-3	Atropa belladonna	Cardiovascular Toxicity / Neurotoxicity	Alkaloid	C17H21NO4	1	-1	0	-2	0	0	0	19	19	19	1	1	2	-4	0	19	19	19			
Scopoline	487-27-4	Atropa belladonna	Neurotoxicity / Gastrointestinal Toxicity / Cardiovascular Toxicity	Alkaloid	C8H13NO2	3	0	0	0	0							0	0	0	0	0	0	0	0	0	0
α-Solanine	20562-02-1	Solanum nigrum	Gastrointestinal Toxicity / Neurotoxicity / Cardiovascular Toxicity	Alkaloid / Glycoside	C45H73NO15	1	0	0	0	0	0	0	0	0	0		0	0	0	0	0	0	0	0	0	
Solasodine	126-17-0	Solanum nigrum	Neurotoxicity	Alkaloid	C27H43NO2	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
(-)-Sparteine	90-39-1	Lupinus species (Lupine)	Neurotoxicity / Cardiovascular Toxicity	Alkaloid	C15H26N2	2	0	0	0	0	0	0	0	0												
Strychnine	57-24-9	Strychnos nux-vomica	Neurotoxicity / Cardiovascular Toxicity / Rhabdomyolysis / Nephrotoxicity	Alkaloid	C21H22N2O2	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
Tetrahydroberberine	522-97-4	Phellodendron chinense / Corydalis yanhusuo / Hydrastis canadensis (Goldenseal) / Berberis species	Neurotoxicity	Alkaloid	C20H21O4N	1	-3	-1	-2	-1	-1	-3	-1	-1	0	11	-1	-1	-1	-1	-1	-1	-1	-1	-2	0
Tetrahydropalmatine	2934-97-6	Corydalis yanhusuo	Gastrointestinal Toxicity / Neurotoxicity / Hepatotoxicity / Cardiovascular Toxicity	Alkaloid	C21H25NO4	2	-2	0	0	-9	0	-10	8	9	12	12	0	-4	0	-5	0	0	9	10	11	5
Tetrandrine	518-34-3	Stephania tetrandra	Potential Cardiovascular Toxicity	Alkaloid	C38H42N2O6	2											0									
Tetrahydrocannabinol	1972-08-3	Cannabis sativa	Neurotoxicity / Cardiovascular Toxicity	Terpenoid	C21H30O2	1	0	9	0	0	0						0	0	0	0						
Veratramine	60-70-8	Veratrum nigrum	Cardiovascular Toxicity / Neurotoxicity	Alkaloid	C27H39NO2	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Vincamine	1617-90-9	Vinca minor (Lesser Periwinkle)	Neurotoxicity / Cardiovascular Toxicity / Gastrointestinal Toxicity	Alkaloid	C21H26N2O3	3	0	0	0	0	0	0	0	0			0	0	0	0	0					
Vincristine	57-22-7	Catharanthus roseus (Rose Periwinkle)	Neurotoxicity / Hematologic Toxicity / Gastrointestinal Toxicity / Hair Loss	Alkaloid	C46H56N4O10	1	0	0	0	0	0	0					0	0	0	0	0					
Vindoline	2182-14-1	Catharanthus roseus (Rose Periwinkle)	Hematologic Toxicity / Gastrointestinal Toxicity / Neurotoxicity	Alkaloid	C25H32N2O6	1	-4	-4	0	0	0	0	-1	0	0	0	-5	0	0	0	0	0	0	13	20	48
Yangonin	500-62-9	Piper methysticum (Kava)	Hepatotoxicity	Lactone	C15H14O4	1	0	0	0	0	0	1	17	0	0		0	-1	0	0	0	1	0	1	0	0
Yohimbine	146-48-5	Corynante yohimbe	Neurotoxicity / Cardiovascular Toxicity	Alkaloid	C21H26N2O3	2	1	11	3	3	4	-1	10	1	0		1	7	-1	4	14	12	0	0	0	
Yunaconitine	70578-24-4	Aconitum species	Cardiovascular Toxicity / Neurotoxicity / Gastrointestinal Toxicity	Alkaloid	C35H49NO11	2	0	0	0	-1	0	0	0	0	1	0	0	0	0	1	0	0	0	0	-2	0

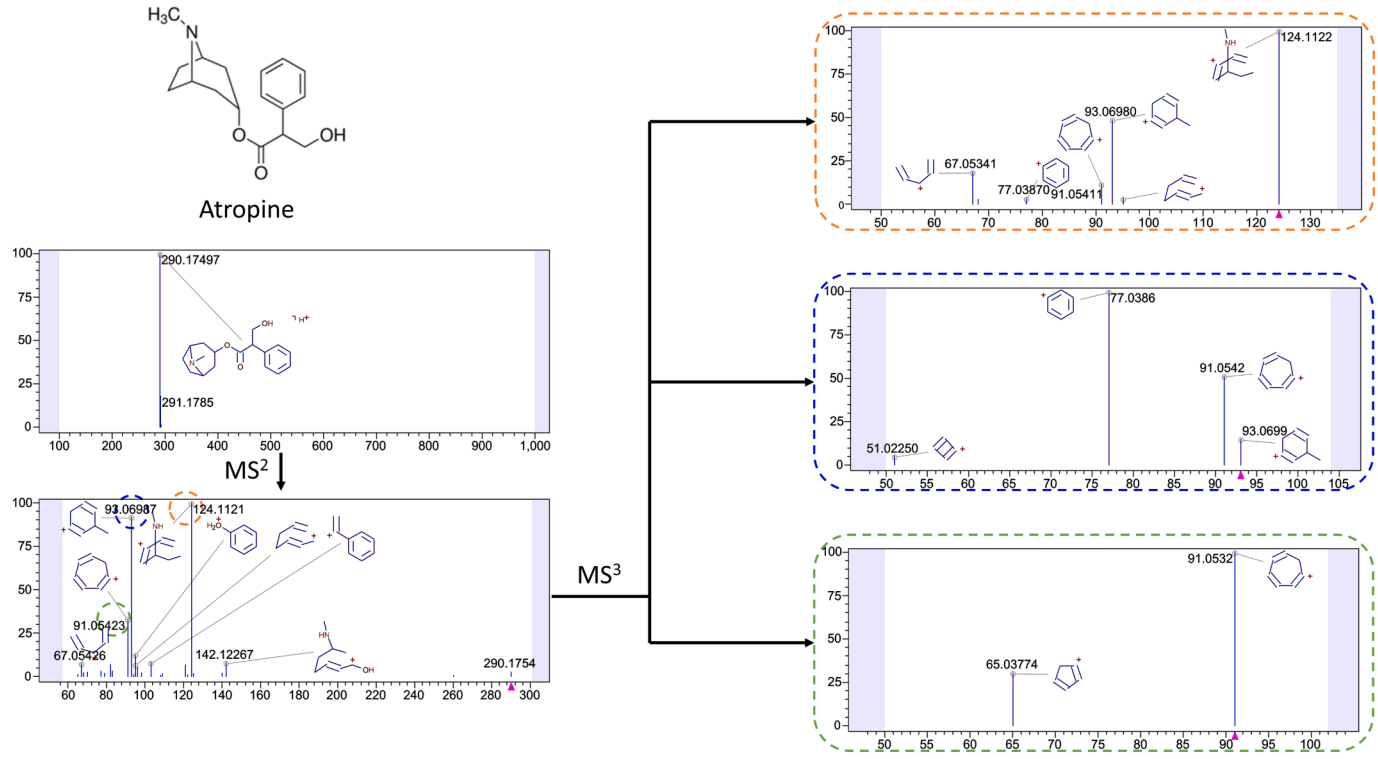

Fig. 2. The MS^2 and MS^3 mass spectra acquired from the precursor ion of atropine in the LC-HR-MS^3 analysis. The colored circles denote the corresponding MS^2 fragment and MS^3 mass spectrum.

column temperature was set at 35 °C. The sample injection volume was set at 5 μL, and the autosampler temperature at 10 °C. For MS analysis, ESI positive ion mode was selected, the spray voltage was set at 3.4 kV, the ion-transfer capillary temperature at 300 °C, sheath gas flow rate at 40, aux gas flow rate at 10, aux gas heater temperature at 375 °C, RF lens level 45.

DDA was implemented in scan cycles, each of which consisted of the following steps: (1) a full-scan with a scan range *m/z* 100–1000 at 120 K resolution, (2) higher-energy C-trap dissociation (HCD) fragmentation of a precursor ion followed by MS^2 product-ion scan with auto mass range at 30 K resolution, and (3) HCD fragmentation of a MS^2 product ion followed by MS^3 product scan with auto mass range at 7.5 K resolution. In step (2), the top 10 abundant precursor ions in a full-scan were selected with an isolation window of 1.5 *m/z* and assisted collision energy optimization was employed to automatically pick a normalized HCD energy value from 20, 35, 45, 55, and 65. The assisted collision energy optimization was implemented in the linear ion trap in the mass spectrometer, which had a higher scan speed than the Orbitrap mass analyzer. An inclusion list with the mass-to-charge ratios of all the analytes was referenced during the full-scan to increase the detection capability for the compounds of interest. The inclusion list can be expanded if more compounds are added to the spectral library, but it should be noted that if more than 10 analytes co-elute in the LC-HR-MS^3 method, only the 10 most abundant analytes will be selected from a full-scan for fragmentation. In step (3), the top three MS^2 product ions were selected with an isolation window of 2 *m/z* and a normalized HCD energy of 30 was used. To ensure high mass accuracy, EASY-IC internal mass calibration was employed.

2.4. Data analysis

The data analysis for the LC-HR-MS^3 results was performed in Mass Frontier software (Thermo Fisher Scientific, San Jose, CA). The results of the LC-HR-MS^3 were analyzed in two data processing mechanisms for comparison. In the MS^2-MS^3 tree data analysis, data were searched against the spectral library constructed using the 85 natural product standards. Extracted ion chromatograms were generated using joint component detection (JCD) mechanism with built-in mass tolerance based on the mass accuracy of the data. The retention time window was set at ± 0.1 min for candidate ion extraction.[18] A candidate ion was scored by matching the precursor ion mass, MS^2 mass spectrum, and MS^3 mass spectrum to the corresponding data for a particular compound in the spectral library. In the MS^2 data analysis, the candidate ion was also scored by matching only the precursor ion mass and MS^2 mass spectrum to the spectral library. The match scores were generated by proprietary fitting algorithms. A compound was called positive if the match score was over a threshold of 80, which was determined by systematically analyzing the match scores of known analytes.

3. Results and discussion

When implementing the LC-HR-MS^3 method, MS^2 and MS^3 mass spectra were generated using the Orbitrap ID-X Tribrid mass spectrometer. The MS^2 and MS^3 mass spectra acquired from one precursor ion are organized in a tree structure in the data analysis software, as shown in Fig. 2. Based on the dissociation reaction mechanisms in mass spectrometry, the in-silico fragmentation prediction tool in the software can annotate the mass peaks in a mass spectrum with predicted chemical structures of product ions. This function can be used to interpret specific features in mass spectra.

Using the LC-HR-MS^3 method, the compound identification results of the 85 natural products in urine and serum samples were obtained, as shown in Table 1. The match scores using both MS^2 and MS^3 mass spectra (MS^2-MS^3 tree data analysis) and those using only MS^2 mass spectra (MS^2 data analysis) were compared at 10 different analyte concentrations. Additional information about the natural products, including natural sources and biological activities, is also provided in Table 1.(3) Using a match score of 80 as the threshold for compound identification, if a compound at a certain concentration was identified by both the MS^2-MS^3 tree data analysis and MS^2 data analysis, the

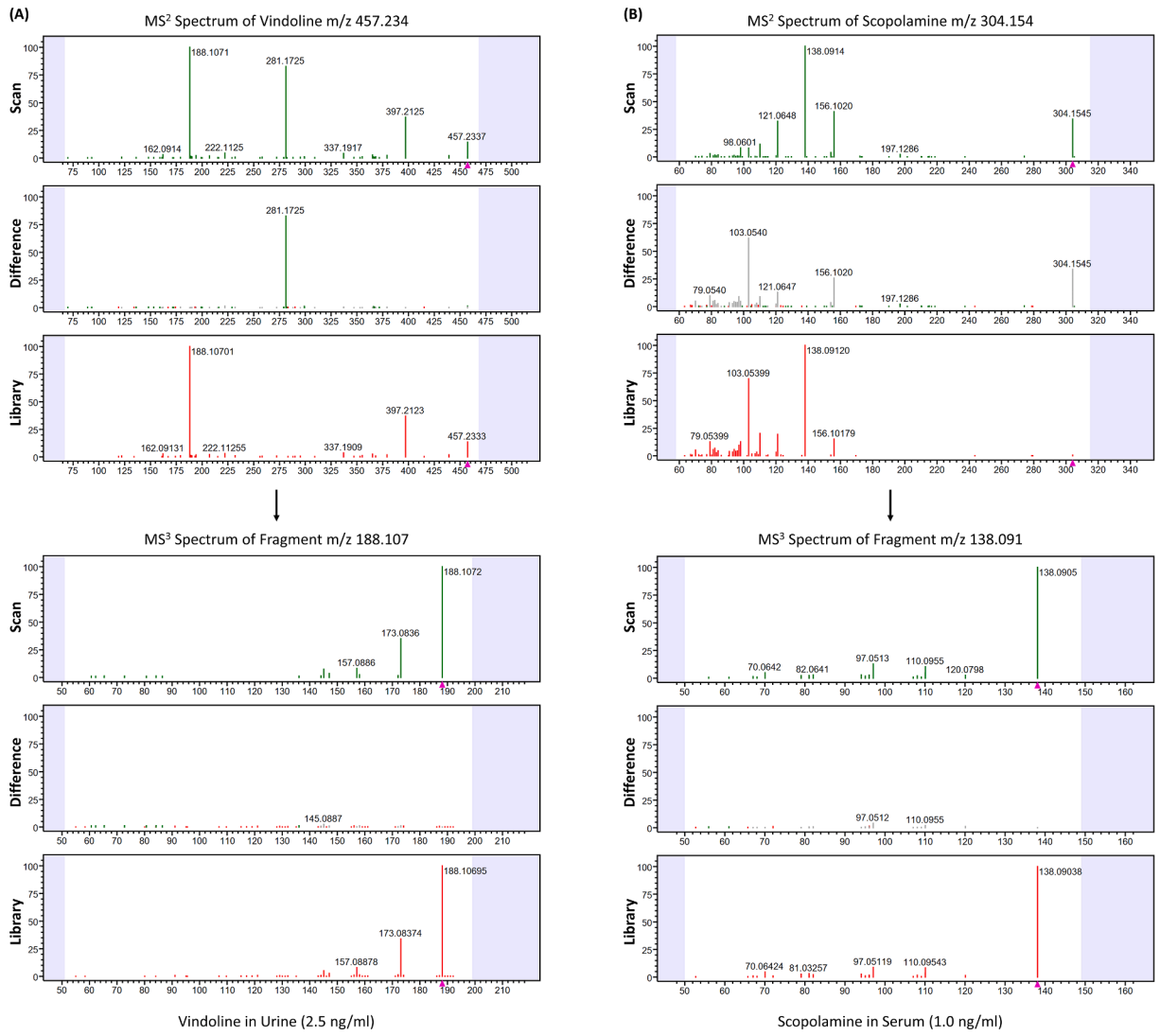

Fig. 3. Two examples showing that the MS^2 mass spectra contained significant background noise while the MS^3 mass spectra did not. (Left) For natural product vindoline in urine (2.5 ng/ml), the MS^2 mass spectrum of the precursor ion of *m/z* 457.234 and the MS^3 mass spectrum of the MS^2 fragment of *m/z* 188.107. (Right) For natural product scopolamine in serum (1.0 ng/ml), the MS^2 mass spectrum of the precursor ion of *m/z* 304.154 and the MS^3 mass spectrum of the MS^2 fragment of *m/z* 138.091.

corresponding cell is marked green. The cell is marked yellow if a compound was identified by only the MS^2-MS^3 tree data analysis, or it is blank if a compound was identified by neither of them. Five compounds in serum (cannabidiol, cytisine, lobeline, nuciferine, reserpinine) and 11 compounds in urine (arecoline, boldine, catharanthine, cannabidiol, coniine, lobeline, methylisopelletierine, nuciferine, nonivamide, reserpinine, vindoline) at certain concentrations were identified by only the MS^2-MS^3 tree data analysis. No compound was identified by only MS^2 data analysis. The difference between the match scores (MS^2-MS^3 tree data analysis minus MS^2 data analysis) is written in each cell (blank if the compound was not identified). In addition, there were three compounds in serum (dihydrosanguinarine, neferine, tetrandrine) and five compounds in urine (berberine, chelerythrine, coptisine, olaquindox, sparteine) not identified at any concentration, probably due to matrix effect in the screening method. This issue is not uncommon in compound screening and can potentially be solved by establishing specific analytical methods with optimized sample preparation protocols. Besides these compounds, Table 1 shows that the two types of data analysis provided identical identification results for the majority of the analytes (96 % in serum, 92 % in urine), whereas, for the remaining analytes, the MS^2-MS^3 tree data analysis had better performance in identifying them at lower concentrations. In other words, at higher concentrations none of the analytes required adding MS^3 mass spectra to identify them, but adding MS^3 mass spectra extended the identification to lower concentrations for a small number of analytes (three compounds in serum, six compounds in urine). In the latter cases, the difference can solely be attributed to the employment of MS^3 spectral matching, where the two-level fragmentation in MS^3 analysis provided in-depth structural information of analytes to increase the match score. On the other hand, as the contribution of MS^3 mass spectra was limited for the majority of the analytes, it was indicated that in general the one-level fragmentation in MS^2 analysis could provide sufficient specificity in compound identification.

There were some examples when the MS^3 mass spectra allowed for enhanced identification when MS^2 mass spectra contained significant background noise. Two examples are shown in Fig. 3. For the natural product vindoline in urine (2.5 ng/ml), the MS^2 mass spectrum of the precursor ion of *m/z* 457.234 contained a significant background peak at *m/z* 281.172 which resulted in a lower match score, while the MS^3 mass spectrum of the MS^2 product ion of *m/z* 188.107 provided a good match score to the spectral library. For the natural product scopolamine in serum (1.0 ng/ml), the MS^2 mass spectrum of the precursor ion of *m/z*

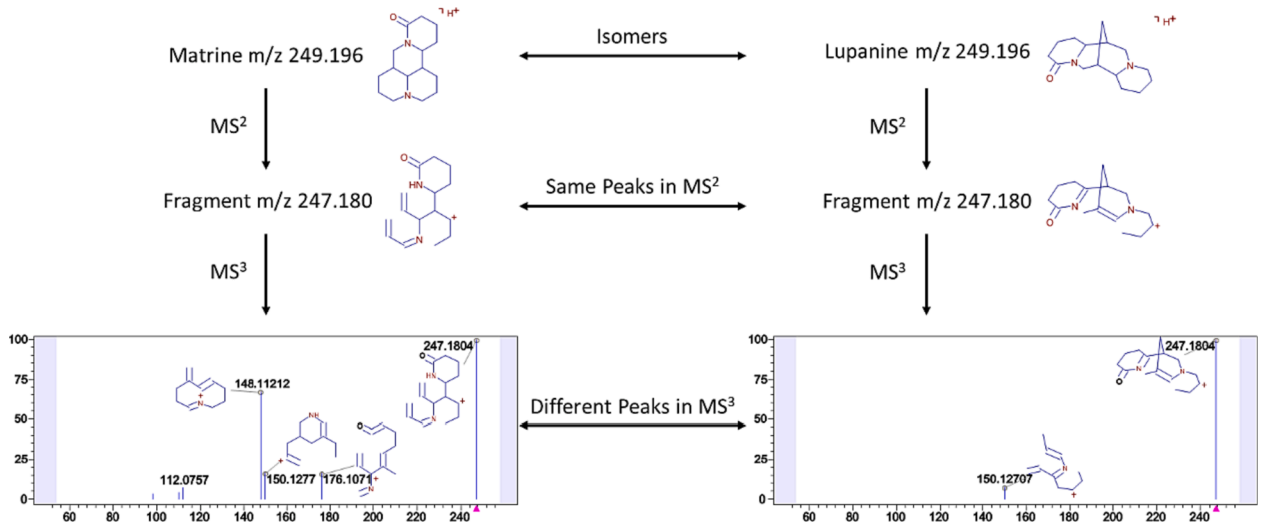

Fig. 4. Differentiation of matrine and lupanine using the MS^3 mass spectra of the MS^2 fragment of *m/z* 247.180.

304.154 had multiple background peaks which resulted in a lower match score, while the MS^3 mass spectrum of the MS^2 product ion of *m/z* 138.091 matched well to the spectral library. The background peaks in these examples might be fragment ions from other co-eluting substances that formed precursor ions at the same nominal *m/z* as the analyte. This observation help explain the differences between the match scores (MS^2-MS^3 tree data analysis minus MS^2 data analysis), which were 20 for vindoline and 19 for scopolamine.

The employment of MS^3 spectra in data analysis also increased the depth of structural characterization and enhanced the differentiation of structural isomers. For example, matrine and lupanine are structural isomers and analogs, and the MS^2 mass spectra of the two analytes contained mass peaks at the same *m/z* values; a single mass peak may correspond to different product ions with various structures but an identical chemical formula. By checking the MS^3 mass spectra of the MS^2 product ion at *m/z* 247.180, different MS^3 fragmentation patterns were observed, and the two analytes could be differentiated, as shown in Fig. 4. The software interpreted that the MS^2 product ions at *m/z* 247.180 in the two MS^2 mass spectra actually had different structures, as denoted in the graphs in Fig. 4. The MS^3 fragmentation successfully revealed the structural difference between the two MS^2 product ions with the same *m/z* value. Although in this study matrine and lupanine were separated into different groups for LC-HR-MS^3 analysis, this example demonstrated the effectiveness of MS^3 fragmentation patterns in differentiating structural isomers. Given that structural isomers may co-exist in actual samples, the use of MS^3 can be particularly beneficial in solving clinical toxicology cases.

Since the mass spectrometer is able to acquire further fragmentation patterns from MS^3 product ions, the use of MS^4 mass spectra for compound identification was tested on some natural products but no benefit was identified. This is likely due to the following facts: (1) MS^3 product ions mostly represent simple structures at low *m/z* and further fragmentation did not provide additional value for compound identification; and (2) the scan cycle time in DDA was significantly increased to include MS^4 fragmentation, which might reduce the data quality of low-concentration analytes, i.e., missing the chromatographic peak summit or collecting fewer ions for MS^n analysis. Thus, in the current experiment settings, it is unnecessary to employ MS^4 or higher-stage mass spectra for compound screening.

In clinical toxicology cases, accurate identification of toxins significantly aids in patient management and decreases the need for additional diagnostic evaluations. The potential of MS^n has been largely unexplored for the identification of toxins in clinical cases. This study shows that in comparison to LC-HR-MS (MS^2), LC-HR-MS^3 can increase the performance in identification of a small group of the toxic natural products tested in serum and urine specimens. Further investigations are warranted to fully understand the potential advantages of utilizing MS^3 mass spectra for compound identification in clinical toxicology, such as testing other types of toxic natural products and synthetic compounds.

In a broader scope of clinical applications, it is clear that the depth of structural characterization would enrich the information content of clinical markers and significantly enhance the performance of clinical tests. Successful application of MS^3 has been reported in clinical research, i.e., tissue imaging and pharmacokinetics.[19,20] In addition, MS^n can be particularly useful for analyzing clinical specimens in which metabolites are not included in the spectral library or co-eluting substances are present. By providing in-depth structural information about analytes, MS^n has the potential to complement the current mainstream tandem mass spectrometry and enhance the performance of mass spectrometry in clinical applications.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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