— **Minireview** —

Mass Spectrometric Strategy for the Determination of Natural and Synthetic Organic Toxins

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Organic toxic substances, consisting of natural and synthetic toxins, are diverse in structure and toxicity. In poisoning cases, such toxins can be determined using a variety of analytical methods. Mass spectrometry is superior for obtaining structural information. Low molecular weight (MW) lipophilic toxins can be analyzed by gas chromatography-mass spectrometry (GC-MS), as exemplified by chemical warfare agents. Low MW polar toxins and metabolites of lipophilic toxins can be also determined by GC-MS after conversion to volatile derivatives. Liquid chromatography (LC)-MS with electrospray ionization (ESI) or atmospheric pressure chemical ionization can be used in the direct analysis for polar low MW toxins, even though the chromatographic peak resolution is low, as exemplified by saxitoxin. Intermediate MW (around 1000) polar toxins can be analyzed by matrix-assisted laser desorption ionization (MALDI)-time-of-flight (TOF)-MS and LC-ESI/MS, and the associated MS/MS technique, which permits structural information, such as the peptide sequence to be obtained. High MW proteinous toxins can be also analyzed by LC-MS and MALDI-TOF-MS, as exemplified by staphylococcal enterotoxin B. However, only an approximate MW estimation can be obtained for ricin, because of the difficulty of obtaining accurate MW values, due to the presence of multiple monoisotopic molecular ion peaks and heterogeneity as the result of posttranslational modification. Instead, LC-MS/MS analysis after proteolytic digestion is typically used in the identification of digested intermediate MW peptides and the subsequent sequencing of the ricin protein.

Key words ——— mass spectrometry, toxin, identification

INTRODUCTION

Organic toxic substances consist of both natural and synthetic toxins. Synthetic toxins are, in most cases, low molecular weight (MW) compounds with a somewhat lipophilic nature: toxic gases, volatile organic solvents, drugs, anesthetics and agricultural chemicals. In contrast, natural toxins are diverse in the origins (animal, plant, microorganism), structures (alkaloid, steroid, heterocyclic, guanidium, polycyclic polyether, peptide, protein) and target organs (nervous system, blood, organ), and their toxicity is generally high. Most toxins that are of interest to forensic toxicology are low MW compounds, and one of the main analytical methods for their determination is gas chromatography-mass spectrometry (GC-MS).[1] Other toxins, low and intermediate MW (MW: around 1000) polar toxins, are typically targets of hygiene and clinical chemistry, and these toxins are mainly examined by means of bioassay, immunoassay and simple electrophoresis or chromatography.[2] However, considering the recent situations involving chemical[3] and biological[4] terrorism, the threat of terrorism using proteinous toxins,[5] ricin, botulinum toxin and staphylococcal enterotoxin B (SEB), is now emerging. As a result, it is necessary to establish analytical methods for identifying such high MW toxins. Given the current progress in instrumental analytical methodology, analytical targets are gradually expanding. Here, we propose new forensic methodology for the identification of natural and synthetic toxins using MS technology.

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LOW MOLECULAR WEIGHT TOXINS

Low MW lipophilic toxins (MW: up to 600) can be extracted with an organic solvent, and amenable to GC-MS analysis. We previously reported on nerve gases and mustard gas[6] all of which are regulated as chemical warfare agents (CWA). Figure 1 shows that the peaks for these toxins are well separated on a gas chromatogram. Capillary GC provides for high chromatographic peak resolution with high detection sensitivity (Table 1). Electron ionization (EI) and chemical ionization (CI) with methane both show characteristic fragmentation patterns, thus providing structural information and a protonated molecular ion, respectively.

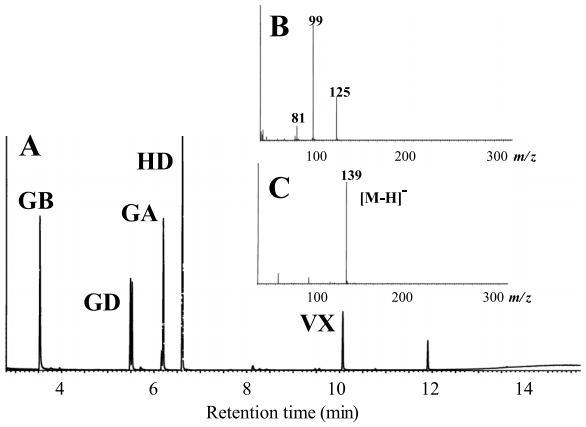


Fig. 1. GC-MS of Chemical Warfare Agents

GC: DB-5MS (0.25 mm × 30 m, 0.25 μm film thickness), 40°C (1 min) – 20°C/min – 290°C (5 min), He 0.7 ml/min, split (250°C) 50 : 1, 1 μl injection; MS: quadrupole, EI (70 μA, 60 eV, positive, *m*/*z* 40–320), CI (methane, 50 μA, 150 eV, negative, *m*/*z* 55–320). A: Total ion chromatogram of a mixture of sarin [GB, $CH_3P(=O)(-F)OCH(CH_3)_2$], soman [GD, $CH_3P(=O)(-F)OCHCH_3C(CH_3)_3$], tabun [GA, $C_2H_5OP(=O)(-CN)N(CH_3)_2$], mustard gas [HD, $S(CHCHCl)_2$] and VX [$CH_3P(=O)(-OC_2H_5)SCH_2CH_2N(CH(CH_3)_2)_2$] (each 250 μg/ml); B: EI-MS (GB); C: CI-MS (GB).

Some low MW lipophilic toxins are decomposed in the environment and metabolized in the body, being converted to polar compounds which are not amenable to GC-MS analysis. These metabolites can be converted to volatile compounds using various derivatizing agents (alkylation, esterification, silylation).[7] Nerve gases are readily hydrolyzed[8] to produce alkylmethylphosphonic acids and methylphosphonic acid, and can be determined by GC-MS after *tert*-butyldimethylsilylation.[9,10] High chromatographic peak resolution and high detection sensitivity can be achieved using capillary GC (Table 1).

Liquid chromatography (LC)-MS has emerged as an analytical tool for target compounds that can not be determined by GC-MS, and has been extensively utilized in various research and practical fields. Capsaicin (*N*-(4-hydroxy-3-methoxybenzyl)-8-methylnon-*trans*-6-enamide), a pepper extract, possesses lachrymating effects, and is used as the main ingredient in self-defense sprays, which have been encountered in foul smell incidents.[11] Capsaicin can be directly detected by GC-MS,[6] but the sensitivity of detection is not good (Table 1), because of the adsorptive characteristics. LC-MS[12] provides for reasonable chromatographic peak resolution and better detection sensitivity (Table 1). Saxitoxin (STX) is a typical polar mussel paralytic toxin, and is registered as a CWA. STX is usually measured by bioassay[13] or LC,[14] but can also be analyzed by LC-MS.[15] STX is retained weakly on a reversed phase stationary phase (Fig. 2), offering low chromatographic peak resolution (Table 1) and possible severe chromatographic interference by other compo-

Table 1. Analytical Parameters of Toxins

	MW	GC-MS			LC-MS	
		LOD (EI+)[a]	LOD (CI−)[a]	N	LOD (ESI)	N
sarin	140	0.3 (*m*/*z* 99)	7 (*m*/*z* 139)	50000	3 (+, *m*/*z* 141)[a]	5000
isopropyl methylphosphonic acid	138	0.5 (*m*/*z* 157)		400000	0.08 (−, *m*/*z* 137)	300
capsaicin	305	5 (*m*/*z* 137)	200 (*m*/*z* 304)	200000	0.001 (+, *m*/*z* 306 SIM)	20000
saxitoxin	299				3 (+, *m*/*z* 300)	3000
angiotensin I	1296				0.002 (+, *m*/*z* 649)	300000
staphylococcal enterotoxin B	28400				3 (+, TIC)	40000
ricin	62600				6 (+, TIC)	5000

The limit of detection [LOD, S/N = 3 on the extracted ion chromatogram (mass number shown in parenthesis), total ion chromatogram (TIC written in parenthesis)] or selected ion chromatogram (SIM written in parenthesis), and the number of theoretical plates (N) were calculated from a GC-MS analysis using a DB-5 column (split ratio 50 : 1) with EI or methane CI, LC-MS using capillary ODS column except for capsaicin which was measured using a conventional ODS column. *a*) Polarity: (+) positive; (−) negative.

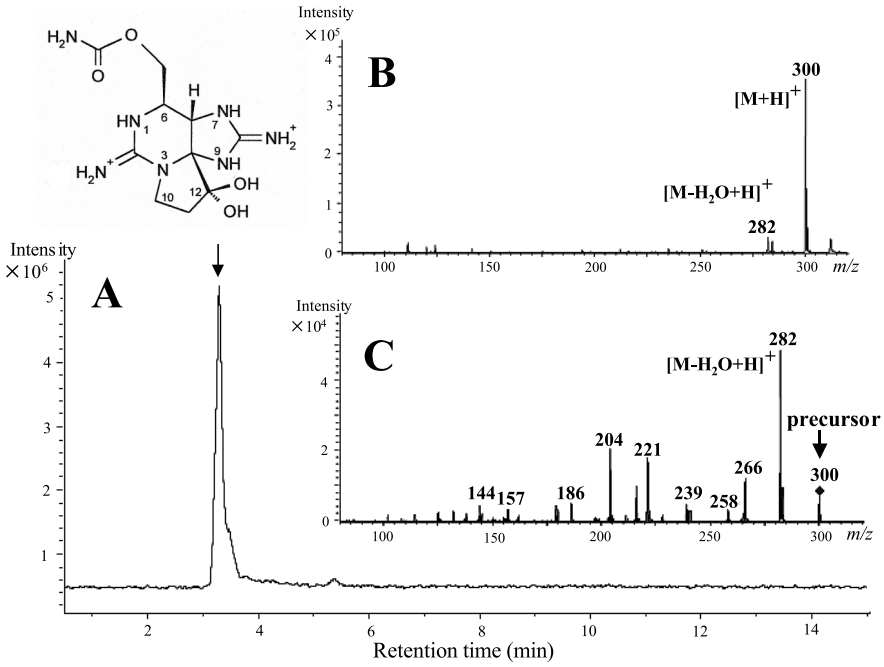


Fig. 2. LC-MS of Saxitoxin

LC: Zorbax SB C18 300A (0.5 × 150 mm, 5 μm), 40°C, 1% acetonitrile in 0.05% TFA – 0.677%/min – 11%, 10 μl/min, 1 μl injection; MS: ion trap, ESI (capillary 3.5 kV, dry gas N_2 300°C, 5 l/min, nebulizer gas 20 psi, positive, *m/z* 80–1800). A: total ion chromatogram of saxitoxin (144 μg/ml); B: ESI-MS (STX); C: MS/MS (precursor ion of *m/z* 300).

nents in the complex sample matrix. MS/MS techniques would be necessary for its quantitative detection and identification.

The hydrolysis products of lipophilic toxins can be analyzed by LC-MS, as reported for CWA.[16,17] In the case of nerve gas hydrolysis products, the chromatographic peak resolution is low (Table 1), and the MS patterns are simple for such highly polar low MW compounds.[18] In comparing the derivatization GC-MS technique, the LC-MS technique is inferior both in terms of the accuracy of the quantification due to interference in the ionization, and in the chromatographic peak resolution. LC-MS can be utilized for the rapid and simultaneous determination of polar and apolar target compounds, and is regarded to be an acceptable screening technology.[18]

INTERMEDIATE MOLECULAR WEIGHT TOXINS

Intermediate MW toxins (MW: around 1000), which include peptidic and polycyclic polyether type toxins are familiar compounds. Angiotensin I (a pressure agent precursor, Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu), regarded to be a surrogate peptidic toxin, can be analyzed by capillary LC-ESI/MS, providing high chromatographic peak resolution and a distinctive mass spectrum (Table 1). Matrix-assisted laser desorption ionization (MALDI)-time-of-flight (TOF)-MS also offers distinctive monoisotopic ion peaks. The MS/MS technique[19] enables exact peptide sequencing for such intermediate MW peptidic toxins.

HIGH MOLECULAR WEIGHT TOXINS

Proteinous toxins are usually determined by conventional methods: bioassay, immunochemistry and electrophoresis.[2] These methods are not amenable to absolute protein identification. For example, immunochemical methods show possible cross reactivity with other proteins. The MS technique may have potential for the identification of proteinous toxins, as applied to low MW toxins. However, the high MW nature of proteins might hamper an unambiguous absolute structural elucidation because of the limitations of MS resolution (the MS resolution of quadruple or ion-trap type analyzer is around 2000) and the presence of multiple monoisotopic molecular ion peaks. In addition, many proteins are

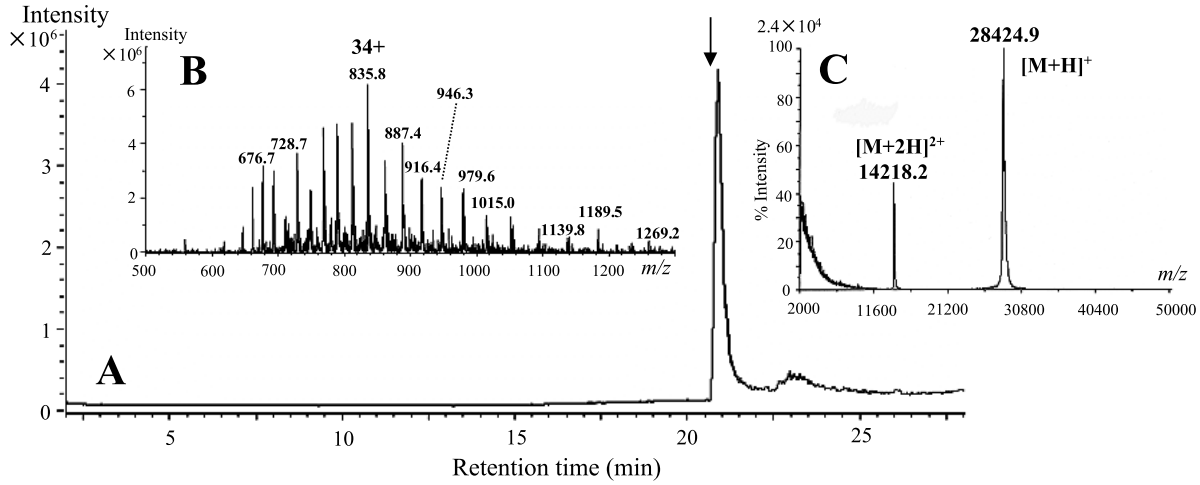


Fig. 3. MS of Staphylococcal Enterotoxin B

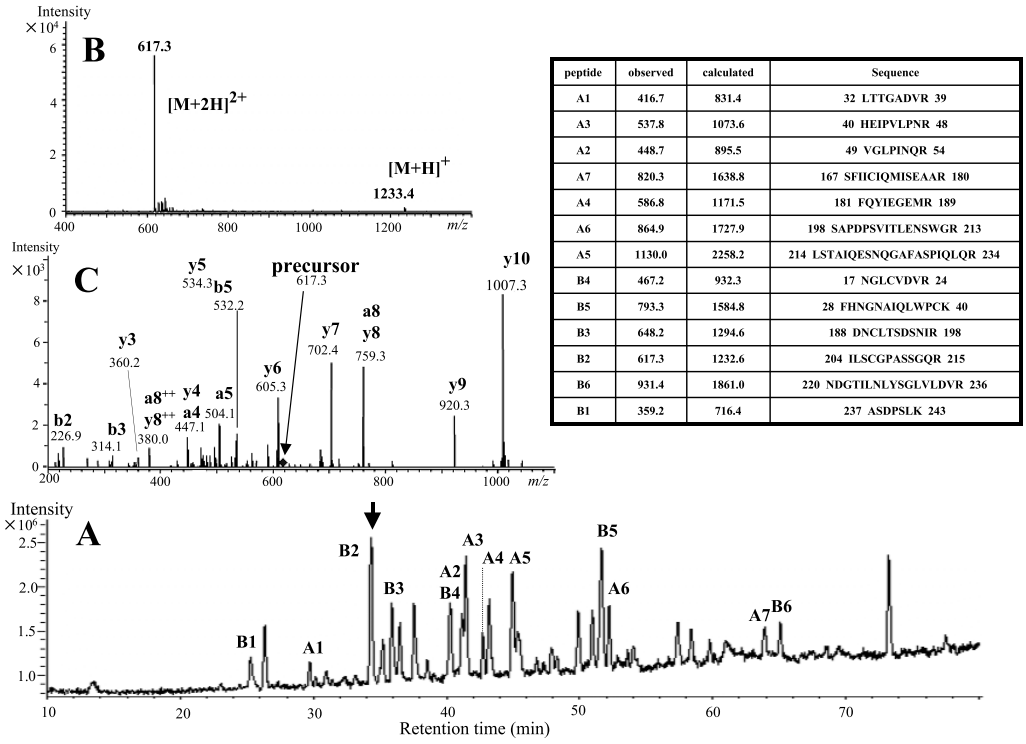
LC: Zorbax SB C18 300A (0.5 × 150 mm, 5 μm), 40°C, 2% (2 min) acetonitrile in 0.1% HCOOH – 3.9%/min – 80%, 20 μl/min, 1 μl injection; MS: ion trap, ESI (capillary 3.5 kV, dry gas N_2 300°C 8 l/min, nebulizer gas 20 psi, positive, *m/z* 80–1800); MALDI-TOF-MS: linear, positive, *m/z* 2000–100000, accelerating 25 kV, delayed extraction 750 nsec, matrix [5 mg/ml sinapinic acid in acetonitrile-water (1 : 3)]. A: total ion chromatogram (25 μg/ml); B: ESI-MS, Deconvolution treatment provided calculated MW of 28366; C: MALDI-TOF-MS (250 μg/ml).

modified posttranslationally. ESI provides multiply charged molecular ions of proteins,[20] and, as a result, the emerging ions approach the region of the instrumental MS range.

SEB[21] is a polypeptide consisted of 239 amino acids, and is usually determined by immunochemical methods.[22] SEB can be analyzed by LC-MS.[23] Our LC-MS results (Fig. 3) showed that one resolved peak on a macroporous capillary octadecylsilica column, which presented a cluster of multiply charged ions centered at *m/z* 836. A computer aided deconvolution analysis gave a measured MW of 28366, which is in good agreement with that calculated from its amino acid sequence (28366). MALDI-TOF-MS analysis also offered a distinctive spectrum giving two peaks (*m/z* 14218, 28425) derived from singly and doubly protonated molecular ions. This MW estimation does not permit the unambiguous exact identification of SEB because of possible existence of other proteins in nature with the same or similar MW. The detection sensitivity of SEB in LC-MS analysis was rather low, compared to low and intermediate MW toxins (Table 1).

Ricin is a A-B heterodimer protein that is produced in the caster bean. Ribotoxin (A subunit) is linked with a galactose-binding lectin (B subunit) via a disulfide bond.[24] Ricin is usually determined by immunochemical methods.[22,25] Ricin can be analyzed by LC-MS and MALDI-TOF-MS.[26] Our result[18] showed, from a direct LC-MS analysis, that ricin was detected as a broad tailing peak, giving broadly distributed complex MS peaks centered at *m/z* 1086. A deconvolution analysis did not give a clear molecular peak. A MALDI-TOF-MS analysis offered singly and doubly protonated molecular ions with rather broad peaks. Molecular heterogeneity derived from the presence of sugar chains and the presence of multiple monoisotopic ions can explain this insufficient identification.

A proteomic approach using MS enables the structural identification of proteins.[27] Proteolytic fragmentation can convert a protein into intermediate MW peptides, and an MS analysis of these peptides permits molecular and structural information to be obtained. Trypsin is, in most cases, used for the proteolytic digestion. We adopted trypsin digestion and iodoacetate alkylation. The digested ricin gave multiple peptide peaks (Fig. 4A), and an ESI mass spectrum was obtained from one peak (designated as B2), giving singly and doubly protonated molecular ions (Fig. 4B). From the MS/MS analysis, a clear peptidic fragmentation pattern (Fig. 4C) was obtained, leading to peptide sequencing. From the other twelve peptidic peaks within about 30 peaks, reference-compatible MW information and peptidic sequences could be obtained (Fig. 4 table). These LC-MS results indicate that it is possible to identify digested peptides derived from ricin A and B subunits, which is compatible with findings reported by Fredriksson *et al.*[28]



peptide	observed	calculated	Sequence
A1	416.7	831.4	32 LTTGADVR 39
A3	537.8	1073.6	40 HEIPVLPNR 48
A2	448.7	895.5	49 VGLPINQR 54
A7	820.3	1638.8	167 SFIICIQMISEAAR 180
A4	586.8	1171.5	181 FQYIEGEMR 189
A6	864.9	1727.9	198 SAPDPSVITLENSWGR 213
A5	1130.0	2258.2	214 LSTAIQESNQGAFASPIQLQR 234
B4	467.2	932.3	17 NGLCVDVR 24
B5	793.3	1584.8	28 FHNGNAIQLWPCK 40
B3	648.2	1294.6	188 DNCLTSDSNIR 198
B2	617.3	1232.6	204 ILSCGPASSGQR 215
B6	931.4	1861.0	220 NDGTILNLYSGLVLDVR 236
B1	359.2	716.4	237 ASDPSLK 243

Fig. 4. LC-MS of Digested Ricin

Digestion; 7 M guanidine-HCl, 0.25% dithiothreithol, 0.6% iodoacetic acid, pH 8.5, 25°C, 2.5 hr, then 5 μg/ml trypsin, pH 8.1, 37°C, 24 hr; LC: Zorbax SB C18 300A (0.5 × 150 mm, 5 μm), 40°C, 1% acetonitrile in 0.05% TFA – 0.667%/min – 70%, 10 μl/min, 1 μl injection; MS: ion trap, ESI (capillary 3 kV, dry gas N_2 300°C, 5 l/min, nebulizer gas 20 psi, positive, *m*/*z* 80–1800). A: total ion chromatogram of a ricin digest (100 μg/ml), each numbered peak can be attributed to a digested peptide fragment derived from the ricin A or B chain; B: ESI-MS (B2, 34.3 min); C: MS/MS (precursor ion of *m*/*z* 617.3). Fragmentation ions, *N*-terminal aldimine ions (a series), *N*-terminal acylium ions (b series) and *C*-terminal peptide ions (y series) are depicted. MS/MS data were evaluated from database searches using Mascot sequence Query (http://www.matrixscience,com/cgi/index.pl?-page-./home.html).

CONCLUSION

Low MW lipophilic toxins can be detected and identified by GC-MS with high chromatographic peak resolution and their distinctive mass spectra. Decomposition products of lipophilic toxins or polar low MW toxins can be also determined by GC-MS if appropriate derivatization is used. LC-MS and MALDI-TOF-MS offer the exclusive detection and identification of polar low and intermediate MW toxins although in some cases it suffers from the limited chromatographic resolution and low detection sensitivity, necessitating an MS/MS analysis. For high MW toxins (proteins), direct LC-MS and MALDI-TOF-MS offer only approximate MW information, but LC-MS analysis combined with proteolytic digestion can be used for peptide sequencing of the digested peptides, leading to the structural identification of the toxin (Fig. 5).

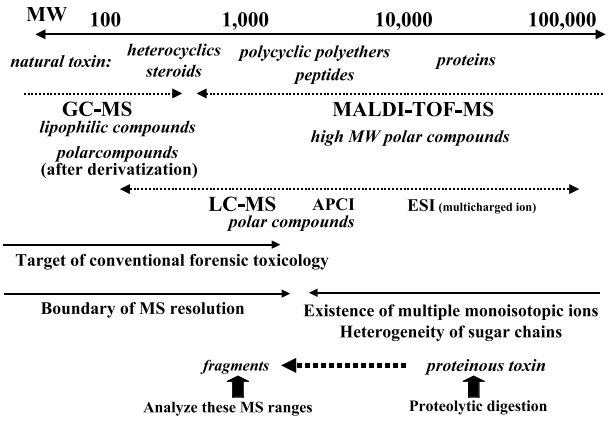


Fig. 5. Molecular Weights of Toxins and Applicable Mass Spectrometric Techniques

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