DEFENCE R&D DÉFENSE

The Identification of Mid-Spectrum Agents in Sand Samples

Results from the Third NATO International Training Exercise

J.R. Hancock, P.A. D'Agostino, L.R. Provost and C.L. Chenier
Defence Research Establishment Suffield

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Abstract

In February 2001, the NATO Land Group 7 subgroup on Sampling and Identification of Biological and Chemical Agents (SIBCA) conducted the third international training exercise on the identification of mid-spectrum agents. The objective of this training exercise was to evaluate the participating national laboratories' ability to identify specific mid-spectrum agents (MSAs) spiked onto sand samples. The compounds were selected from a list of mid-spectrum agents compiled by Canada (host nation) and distributed as unknowns to the laboratories. Six national laboratories, one each from France, Germany, Italy, Netherlands, Sweden and the United States, participated in the exercise.

The level of identification (provisional, confirmed or unambiguous) obtained by each of the laboratories was determined using the identification criteria for mid-spectrum agents developed by the SIBCA subgroup. All the participating laboratories were able to identify, at the provisional level, Ochratoxin A as the MSA in the first sand sample. Five of the six laboratories were able to identify, at the provisional level, Ile-Ser-Bradykinin as the MSA in the second sand sample. Germany, The Netherlands and Sweden through further experiments were able to identify Ile-Ser-Bradykinin at the unambiguous level. The results from this exercise have revealed areas in which the identification criteria may be improved.

Résumé

En février 2001, le sous-groupe du Groupe Terrestre 7 de l'OTAN spécialisé en échantillonnage et identification des agents biologiques et chimiques (SIBCA) a conduit son troisième exercice d'entraînement international d'identification des agents de spectre moyen. L'objectif de ces exercices d'entraînement était d'évaluer la capacité des laboratoires nationaux participants à identifier des agents de spectre moyen (MSA) spécifiques ensemencés dans des échantillons de sable. Les composés ont été sélectionnés à partir d'une liste d'agents de spectre moyen compilée par le Canada (pays d'accueil) et distribués en tant que substance inconnue dans les laboratoires. Six laboratoires ont participé à cet exercice dont un laboratoire dans chacun des pays suivants, France, Allemagne, Italie, les Pays-Bas, la Suède et les États-Unis.

Le niveau d'identification (préliminaire, de confirmation ou sans ambiguïté) obtenu par chacun des laboratoires a été déterminé en utilisant les critères d'identification des agents de spectre moyen mis au point par le sous-groupe SIBCA. Dans le premier échantillon de sable, tous les laboratoires participants ont été capables de déterminer au niveau préliminaire que l'ochratoxine A était l'agent de spectre moyen Dans le deuxième échantillon, cinq des six laboratoires ont été en mesure de déterminer au niveau préliminaire que l'agent Ile-Ser-Bradykinine était l'agent de spectre moyen. En prolongeant l'expérimentation, l'Allemagne, les Pays-Bas et la Suède ont pu identifier sans ambiguïté l'agent Ile-Ser-Bradykinine. Les résultats de ces exercices révèlent que le domaine des critères d'identification pourrait être sujets à des améliorations.

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Executive summary

Introduction: The Canadian Forces (CF) may be called on to perform peacekeeping or peacemaking operations in regions of the world where there is a significant threat of chemical/biological warfare agent use. To operate effectively in these theatres the CF must be able to detect and identify chemical and biological agent(s). Mass spectrometry (MS) is a powerful analytical technique for the identification of both known and unknown compounds and Defence Research Establishment Suffield (DRES), in conjunction with its NATO allies, is currently investigating this instrumental technique in fulfilment of NATO agent detection and identification requirements.

Results: In February 2001, the NATO Land Group/7 subgroup on Sampling and Identification of Biological and Chemical Agents (SIBCA) conducted the third international training exercise on the identification of mid-spectrum agents. This exercise was open to those NATO and Partnership for Peace (PfP) nations which regularly attend the sub-group meeting. The objective of this training exercise was to evaluate the participating national laboratories' ability to identify specific mid-spectrum agents (MSAs) spiked onto sand samples. The compounds were selected from a list of mid-spectrum agents compiled by Canada (host nation) and distributed as unknowns to the laboratories. Six national laboratories, one each from France, Germany, Italy, Netherlands, Sweden and the United States, participated in the exercise.

The level of identification (provisional, confirmed or unambiguous) obtained by each of the laboratories was determined using the identification criteria for mid-spectrum agents developed by the SIBCA subgroup. Three levels of identification have been defined with each level indicating an increasing level of certainty. Unambiguous identification provides the highest level of certainty required for the development of strategic and political positions. All the participating laboratories were able to identify, at the provisional level, Ochratoxin A as the MSA in the first sand sample. Five of the six laboratories were able to identify, at the provisional level, Ile-Ser-Bradykinin as the MSA in the second sand sample. Germany, The Netherlands and Sweden through further experiments were able to identify Ile-Ser-Bradykinin at the unambiguous level. The results from this exercise have revealed areas in which the identification criteria may be improved.

Significance of Results: The CF may be deployed in regions of the world where there is a significant threat of chemical/biological warfare agent use. Identification of the agent is of importance since the results of such analyses would facilitate the dissemination of technical advice to in-theater field commanders and medical personnel and contribute to the development of strategic and political positions regarding future Canadian military operations.

Future Goals: The CB threat spectrum includes chemical and biological warfare agents and toxins of biological origin in the "mid-spectrum" between these agents. The identification research effort has been focused on the detection and identification of these toxins of biological origin. Use of these warfare agents could easily go unconfirmed, as analytical methods have not been fully developed for their identification. DRES is now actively addressing this deficiency through the application and development of MS methods for the identification of these agents.

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Sommaire

Introduction : Les Forces canadiennes (FC) risquent d'être appelées à exécuter des opérations de maintien de la paix et de pacification dans les régions du monde où il existe une menace importante d'utilisation d'agents chimiques et biologiques. Pour opérer de manière efficace dans ces théâtres, les FC doivent être en mesure de détecter et d'identifier les agents chimiques et biologiques. La spectrométrie de masse est une technique analytique puissante d'identification des composés à la fois connus et inconnus. Dans le but de répondre aux exigences de détection et d'identification de l'OTAN, le Centre de recherches pour la défense, Suffield (CRDS) en conjonction avec ses alliés de l'OTAN, est actuellement en train d'étudier cette technique instrumentale.

Résultats : En février 2001, le sous-groupe du Groupe Terrien 7 de l'OTAN spécialisé en échantillonnage et identification des agents biologiques et chimiques (SIBCA) a conduit son troisième exercice d'entraînement international d'identification des agents de spectre moyen. Cet exercice était offert à toutes les nations membres de l'OTAN et des Partenariats pour la paix (PPP) qui participent régulièrement aux réunions des sous-groupes. L'objectif de ces exercices d'entraînement était d'évaluer la capacité des laboratoires nationaux participants à identifier des agents de spectre moyen spécifiques ensemencés dans des échantillons de sable. Les composés ont été sélectionnés à partir d'une liste d'agents de spectre moyen compilée par le Canada (pays d'accueil) et distribués en tant que substance inconnue dans les laboratoires. Six laboratoires dont un laboratoire dans chacun des pays suivants, France, Allemagne, Italie, les Pays-Bas, la Suède et les États-Unis ont participé à cet exercice.

Les niveaux d'identification (préliminaire, de confirmation ou sans ambiguïté) obtenu par chacun des laboratoires ont été déterminés en utilisant les critères d'identification des agents de spectre moyen mis au point par le sous-groupe SIBCA. Trois niveaux d'identification, chaque niveau indiquant un niveau accru de certitude, ont été identifiés. L'identification sans ambiguïté fournit le plus haut niveau de certitude requis pour le développement des positions stratégiques et politiques. Dans le premier échantillon de sable, tous les laboratoires participants ont été capables de déterminer au niveau préliminaire que l'ochratoxine A était l'agent de spectre moyen. Dans le deuxième échantillon, cinq parmi les six laboratoires ont été capables de déterminer au niveau préliminaire que l'agent Ile-Ser-Bradykinine était l'agent de spectre moyen. En prolongeant l'expérimentation, l'Allemagne, les Pays-Bas et la Suède ont été capable d'identifier sans ambiguïté l'agent Ile-Ser-Bradykinine. Les résultats de ces exercices révèlent que le domaine des critères d'identification pourrait être sujets à des améliorations.

La portée des résultats : les FC peuvent être déployées dans des régions du monde où il existe une menace importante d'utilisation d'agents chimiques et biologiques. L'identification de ces agents est de grande importance puisque les résultats de telles analyses ont le potentiel de faciliter la dissémination de conseils techniques aux commandants des théâtres d'opérations et au personnel médical. Ces résultats peuvent aussi contribuer au développement des positions stratégiques et politiques des futures opérations de l'armée canadienne.

Les buts futurs : le spectre de la menace CB inclut les agents de guerre chimiques et biologiques et les toxines d'origine biologique présentes, entre ces agents, dans le spectre moyen. Les efforts de recherches dans le domaine de l'identification ont été concentrés sur la détection et l'identification de ces toxines d'origine biologique, cependant l'utilisation de ces agents de guerre pourrait facilement rester non confirmés puisque les méthodes d'analyse

n'ont pas encore été complètement mises au point pour l'identification. Le CRDS adresse à présent cette déficience dans le domaine d'identification de ces agents en développant des méthodes d'application et de mise au point de la spectrométrie de masse .

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Introduction

NATO may be called upon to deploy military forces in support of peacekeeping or battlefield operations in regions of the world where there is a significant threat of the use of chemical/biological warfare (CBW) agents. To operate effectively in these theatres, NATO forces must be able to detect and identify CBW agents.

In 1996 and again in 1999, the NATO AC/225 Land Group 7 sub-group on Sampling and Identification of Biological and Chemical Agents (SIBCA) conducted international training exercises on the identification of mid-spectrum agents (MSA). The objective of these exercises, hosted by Canada, was to evaluate the participating national laboratories' ability to identify proteinaceous compounds. In 1996, the compounds were synthetic peptides while in 1999 compounds were selected from the SIBCA list of mid-spectrum agents. In both exercises the compounds were distributed as neat unknowns to the laboratories (1,2).

In February 2001, the SIBCA sub-group held a third international training exercise. This exercise was open to those NATO and Partnership for Peace (PfP) nations which regularly attend the sub-group meeting. Participating nations included: Canada(host), France, Germany, Italy, Netherlands, Sweden and the U.S.A. Results from the previous two training exercises had demonstrated that nations were capable of identifying MSAs when the samples were distributed as lyophilized powders. It was the decision of the sub-group that the third training exercise should require the laboratories to identify the MSA in a more complex matrix. Canada as host nation compiled a list of MSAs (Annex I), from which they selected two MSAs for the training exercise. Canada was responsible for preparing the spiked samples, distributing the samples to the participating nations and preparing a summary report from the individual reports submitted by each nation. The samples were distributed to the laboratories as unknowns spiked onto sand. Laboratories were given the month of February to complete the exercise, with the provision that they limit their analysis to a total of five working days.

Expermental

Preparation of Ochratoxin A Spiked Sand

Ochratoxin A spiked sand was prepared by adding five milligrams of Ochratoxin A (Sigma) to 10 grams of Ottawa sand (EMscience). The Ochratoxin A and sand were then mixed, using a vortex mixer, for approximately one minute. Finally one gram of the mixture was removed and mixed with a further 24 grams of Ottawa sand. The final sample of 25 grams contained Ochratoxin A at the 0.02 mg/mL level. The sample was subdivided into 12 x 2 g samples and labelled MSA01/001.

Preparation of Ile-Ser-Bradykinin Spiked Sand

Ile-Ser-Bradykinin spiked sand was prepared by adding 5 mg of Ile-Ser-Bradykinin (Sigma) to 5 grams of Ottawa sand. Polyethylene Glycol 600 (PEG600) spiked sand was prepared by adding a solution of PEG 600 (Aldrich) in methanol (31.25 grams in 5 mL) to 12.5 grams of Ottawa sand. The resulting slurry was allowed to stand for 10 minutes and the methanol was removed under a stream of helium. This procedure was repeated with two more batches of sand and produced an Ottawa sand stock of 37.5 grams containing PEG 600 at a concentration of 2.5 mg/g.

Twenty grams of PEG 600 spiked sand was combined with the 5 grams of Ile-Ser-Bradykinin spiked sand and mixed on a vortex mixer. The final sample of 25 grams contained Ile-Ser-Bradykinin at the 0.2 mg/mL level and PEG 600 at the 2.0 mg/mL level. The sample was subdivided into 12 x 2 g samples and labelled MSA01/002.

Sample Analysis

In order to ensure the MSAs were stable on the sand and could be recovered for analysis by the participating nations, Canada analyzed the samples immediately after spiking and again after storage at room temperature for 30 days. The recovery for Ochratoxin A and Ile-Ser-Bradykinin was comparable for both the one and thirty day samples. Extraction of the MSA was carried out by adding one milliter of water to one gram of sample and extracting in an ultrasonic bath for 10 minutes. The sample was then centrifuged at 2000 rpm for 15 minutes. The supernatant, which was clear and colourless, was removed and analyzed by LC-MS.

LC separations were performed with an Applied Biosystems Model 140B dual syringe pump (Foster City, CA) equipped with a Micro-Tech Scientific 150 mm x 0.32 ID Zorbax C18 SB (5 µm) packed fused-silica capillary column (Sunnyvale, CA) and a Rheodyne 8125 injector with a 5 µL sample loop (Cotati, CA). The mobile phase solvent compositions were prepared as follows: Solvent A (0.1% trifluoroacetic acid (TFA) in water) and Solvent B (0.1% TFA in acetonitrile/water, 95:5). Chromatographic separations were performed using a 1% to 75% B gradient over 30 minutes. Rapid analyses were made through the column under isocratic conditions (75% B, 15 µL/min) which ensured minimal interaction of the sample with the column. In order to minimize dead volume effects and ensure reproducible mixing, the mobile phase was delivered at 200 µL/min and split prior to the injector such that the flow through the column was approximately 15 µL/min.

The Micromass LCT time-of-flight (TOF) mass spectrometer was equipped with a Z-spray electrospray interface. The electrospray capillary was operated at 3.2 kV and the sampling cone voltage was either 20 or 40 volts. Nitrogen desolvation gas was introduced into the interface (80°C) at a flow rate of 480 L/h. Nitrogen nebulizer gas was introduced at a flow rate of 66 L/h. ESI data were acquired from 700 to 70 Da (1 sec) in the continuum mode with a resolution of 5000 (50% valley definition). For accurate mass measurement, a mixture of PEG200/PEG600 (0.03 mg/mL) was used for recalibration in the same analytical run as the analytes of interest.

Results

Sample Selection

Samples selected for the initial international training exercises (1996, 1999) were distributed to the participating laboratories as one milligram of lyophilized powder. Sample preparation was limited to reconstituting the sample in an appropriate solvent. For the third international training exercise, it was the decision of the SIBCA sub-group to increase the difficulty of the exercise by spiking the mid-spectrum agent into an environmental matrix.

Soil was initially selected as the environmental matrix as DRES had a number of well characterized soil types available. Recovery experiments performed as part of the sample selection process involved spiking proteinaceous mid-spectrum agents onto three different soil types (sandy loam, sandy clay loam and loamy sand) at the 1 mg/g level. Using a standard aqueous extraction with ultrasonification, no mid-spectrum agents or related decomposition products could be recovered from any of the three soils. Attempts to increase the recovery by disrupting the analyte/matrix interaction through changing the extraction conditions (e.g. pH) were not successful. A search for a less retentive matrix lead to the selection of Ottawa standard sand (EM Science), from which it was possible to recovery the MSAs.

Selection of the MSAs was dependent on a number of factors: recovery of the MSA from the matrix, stability on the matrix and issues related to the transportation of dangerous goods. Ochratoxin A was chosen as a representative non-proteinaceous MSA and Ile-Ser-Bradykinin as a representative proteinaceous MSA. Both these compounds when spiked onto sand could be recovered even after storage at room temperature for 30 days. Of the two, only Ochratoxin A is classified as a dangerous good for transportation purposes. At the spike level (0.02mg/g) used for this training exercise, it is not restricted under the International Air Transportation Regulations, an important factor in distributing the samples to participating laboratories.

The first sample MSA01/001 contained Ochratoxin A spiked onto sand at the 0.02 mg/g level. This spike level was 50 times lower than that used in the previous training exercises and was designed to provide a moderate sensitivity challenge. The second sample MSA01/002 contained Ile-Ser-Bradykinin spiked onto sand at the 0.2 mg/g level. The presence of the MSA was masked with the addition of polyethylene glycol 600 (PEG 600) at the 2mg/g level. PEG 600 is a homologous series of glycols with an average molecular mass of 600 daltons. This sample was designed to challenge the participating laboratories ability to identify the presence of a MSA in the presence of a complex background.

Samples were sent by courier to the participating laboratories on the 25th January 2001. Participating nations were to complete their analysis in the month of February. Within that month they were allowed to spend up to five working days analyzing the samples. Canada as the host country analyzed the samples prior to the training exercise as part of the sample selection process. Canada also analyzed each of the spiked training exercise samples immediately before they were shipped and again at the end of February 2001. Results from these analysis confirmed that Ochratoxin A and Ile-Ser-Bradykinin were still present, in the training exercise samples, at the original spike level after 30 days. Participating nations submitted their reports to Canada in March 2001 describing the sample handling and analysis of the two samples. Canada reviewed the data and based on the NATO Criteria for Identification of Mid-spectrum Agents (Annex II), assigned one of three levels (provisional, confirmed or unambiguous) of identification. Table 1 indicates the level of identification for each country. Detailed descriptions of the information submitted and the level of identification for each sample is presented in the following sections.

Table 1. *Level of Identification Achieved by Participating Laboratories*

MSA01/001	Participating Laboratories						
	FR	GE	IT	NL	SW	US	CA[a]
Provisional Identification	✓	✓	✓	✓	✓	✓	✓
Confirmed Identification		✓			✓		✓
Unambiguous Identification							
MSA01/002							
Provisional Identification	✓	✓		✓	✓	✓	✓
Confirmed Identification							
Unambiguous Identification		✓		✓[b]	✓		

[a] Canada as the host country analyzed these samples prior to the training exercise as part of the sample selection process.

[b] Identification based on one rather than two sets of chromatographic data.

Sample MSA01/001

Sample MSA01/001, contained the non-proteinaceous MSA, Ochratoxin A spiked onto sand (Figure 1). Participating laboratories utilized a variety of solvent systems including: pure aqueous, mixtures of organic/aqueous and pure organic solvents to extract Ochratoxin A from the sand matrix. In some cases these solvents were used either sequentially or applied to separate aliquots of the sample. Hand agitation or ultrasonic extraction was followed by centrifugation and analysis of the supernatant.

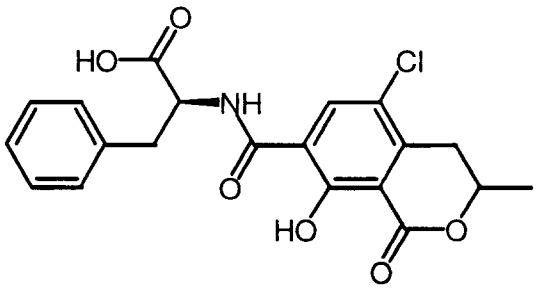

Figure 1. *Ochratoxin A*

Monoisotopic molecular mass:	403.08227 Da
Average molecular mass:	403.81976 Da
CAS Number:	303-47-9

Provisional Identification

Most participating laboratories determined the molecular mass of the unknown in MSA01/001 using atmospheric pressure ionization (API) mass spectrometry. Matrix assisted laser desorption ionization (MALDI) was used by the United States while, electrospray ionization (ESI) was used by the remaining six laboratories. Figure 2 illustrates the mass spectrum, of the molecular ion region, acquired by the United States during the analysis of MSA01/ 001. The mass spectrum contained a protonated molecular ion ($[M+H]^+$) at m/z 404 from which the molecular mass was determined. The isotopic pattern associated with the presence of naturally occurring ^{37}Cl was present in the isotopic cluster for both the $[M+H]^+$ and the sodium adduct of the molecular ion ($[M+Na]^+$) at m/z 426. Most laboratories commented that the presence of isotope pattern and ion intensities for the ^{37}Cl isotope in Ochratoxin A aided in their identification. Once the laboratories obtained the molecular masses for the sample they were able to identify the sample as Ochratoxin A from the list of MSAs provided by Canada. Table 2 lists the monoisotopic molecular masses reported by each country. Mass errors were typically less than 20 millimass units (mmu) with ESI, while a significantly larger mass error was reported using MALDI. Based on the identification criteria, a MSA is considered provisionally identified when the molecular mass matches that of a known MSA.

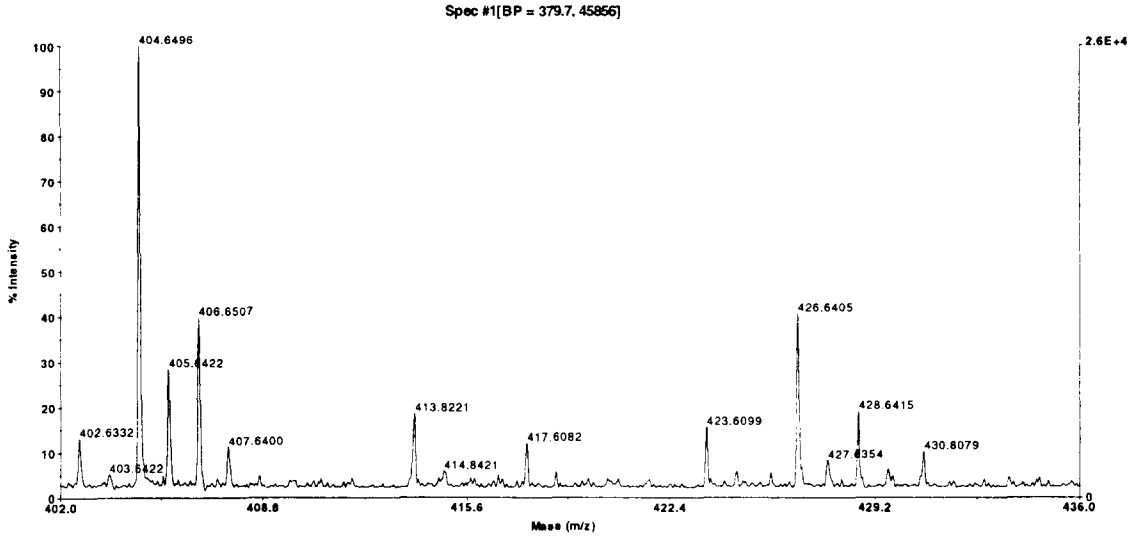

Figure 2. *MALDI-TOF mass spectrum of molecular ion region acquired by the United States during analysis of a chloroform extract of MSA01/001.*

Table 2. *Monoisotopic Molecular Masses Determined for MSA01/001 (Ochratoxin A)*

Country	Instrument	Observed Monoisotopic Molecular Mass[b]	Error mmu
France	Bruker Esquire 3000 Ion Trap	403.06	22
Germany	PE Biosystems Q Star	403.083	1
Italy	Micromass ZMD	403.1 ± 0.2	18
Netherlands	Micromass Q-TOF	403.1	18
Sweden	Micromass Autospec OA-TOF	403.0812	1
U.S.	Applied Biosystems Voyager MALDI-TOF	403.6418	560
Canada[a]	Micromass LCT	403.082 ± 0.001	1

[a] host country
[b] theoretical monoisotopic molecular mass: 403.08227 DA

Confirmed Identification

For a non-proteinaceous mid-spectrum agent such as Ochratoxin A, confirmed identification can be obtained, either by acquiring chromatographic retention data under two different experimental conditions or by obtaining a positive response from an immunological assay in addition to matching the molecular mass of the unknown to that of a known MSA. Germany, using a commercially available ELISA for Ochratoxin A (with 90% cross reactivity for Ochratoxin B), was able to obtain a positive response for an aqueous extract of MSA01/001, thereby confirming the identification of Ochratoxin A. Canada, Germany and Sweden acquired chromatographic retention data for MSA01/001 and compared it to retention data for an Ochratoxin A reference. Figures 3 and 4 illustrate the chromatograms obtained by Sweden during the analysis of an extract of MSA01/001 and an Ochratoxin A reference. The chromatographic retention data for the extract and the reference agreed within experimental error confirming the identification of Ochratoxin A by Sweden.

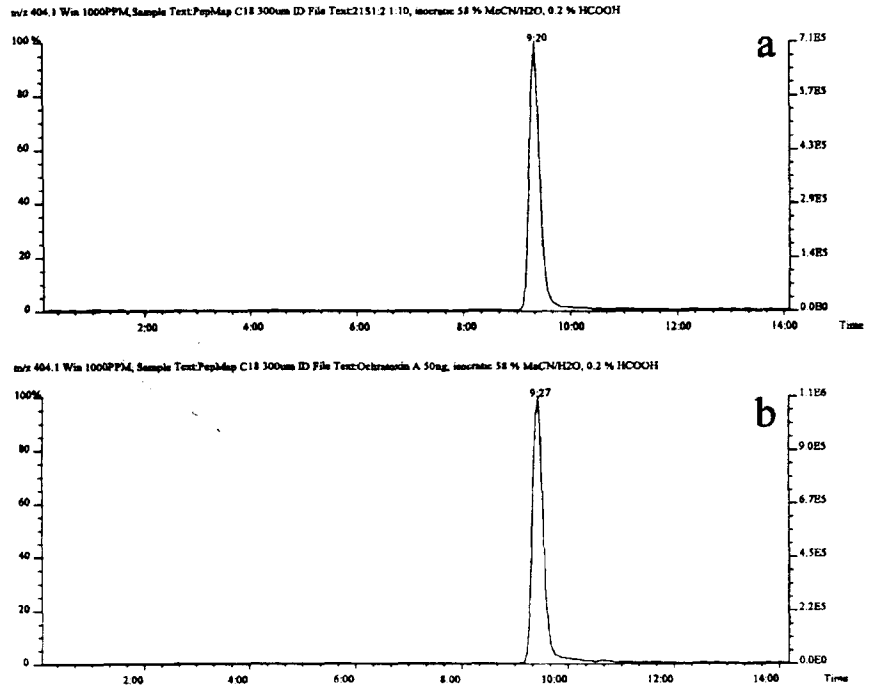

Figure 3. *LC-ESI-MS reconstructed ion chromatograms (m/z 404.1) acquired by Sweden during isocratic analysis (38% ACN/Water + 0.2% formic acid) of a) extract of MSA01/001 and b) Ochratoxin A reference.*

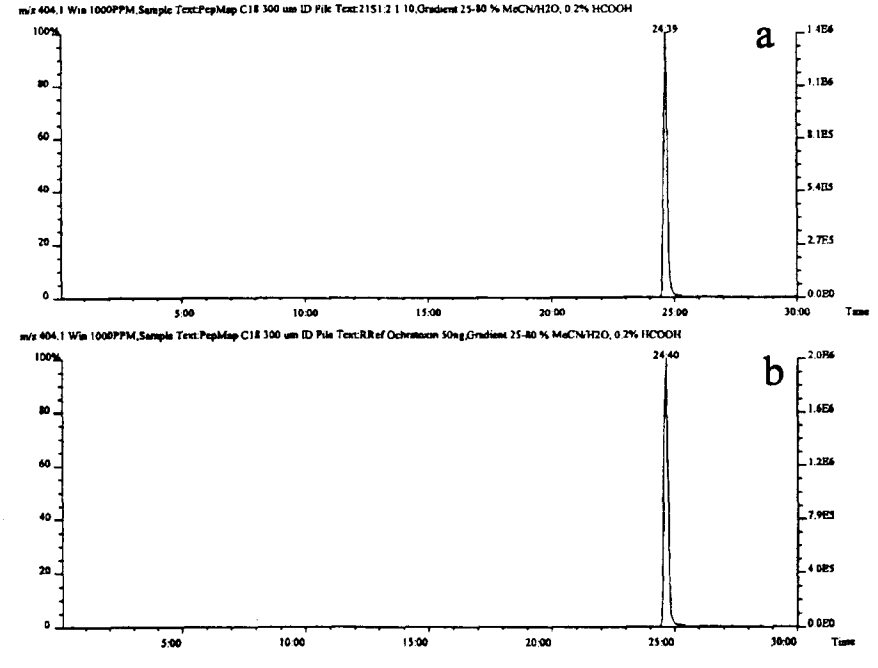

Figure 4. *LC-ESI-MS reconstructed ion chromatograms (m/z 404.1) acquired by Sweden during gradient analysis (25-80% ACN/Water + 0.2% formic acid) of a) extract of MSA01/001 and b) Ochratoxin A reference.*

Unambiguous Identification

While none of the participating laboratories were able to unambiguously identify Ochratoxin A in MSA01/001, a number of laboratories provided additional information that supported their identification. Sweden acquired electrospray spectra in both the positive and negative ion modes for MSA01/001 and compared them to spectra acquired for an Ochratoxin A reference. Germany derivatized a dichloromethane extract of MSA01/001 and an Ochratoxin A reference with diazomethane. These samples when analyzed by GC-MS using electron impact ionization resulted in two similar EI mass spectra. Figure 5 illustrates MS/MS data acquired by The Netherlands for an Ochratoxin A reference and an extract of MSA01/001. These spectra clearly match and using the criteria for identification would be considered spectrometric data from one of the required two techniques for unambiguous identification.

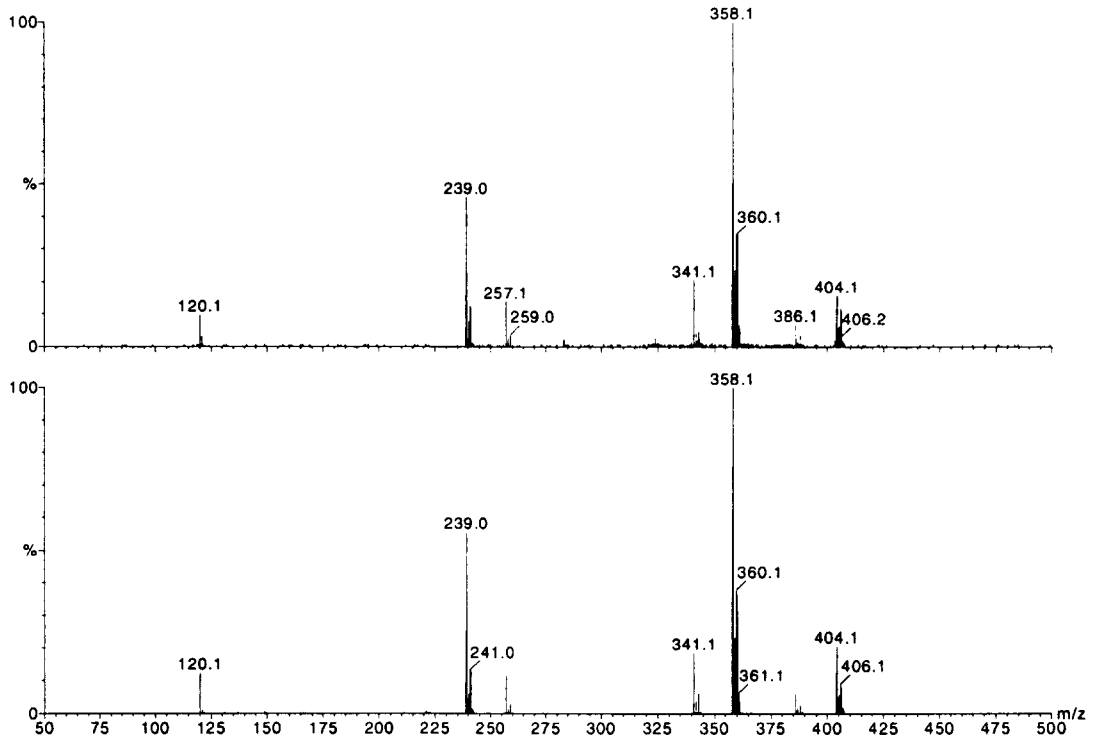

Figure 5. *LC-ESI-MS/MS product ion spectrum of precursor ion m/z 404.1 acquired by The Netherlands during the analysis of an Ochratoxin A reference (top) and an extract of MSA01/001 (bottom). Collisional gas: argon, collision energy: 16 eV.*

Sample MSA01/002

The second sample labelled "MSA01/002" contained Ile-Ser-Bradykinin spiked onto sand at the 0.2mg/g level. In addition the sand was contaminated with PEG 600 at the 2mg/g level. The amino acid sequence of Ile-Ser- Bradykinin is illustrated in Figure 6.

Ile-Ser-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg

Figure 6. *Primary Amino Acid Sequence for Ile-Ser-Bradykinin*

Monoisotopic molecular mass:	1259.6774 Da
Average molecular mass:	1260.46657 Da
CAS number:	86030-63-9

The presence of PEG 600 at ten times the concentration of Ile-Ser-Bradykinin was intended to mask the MSA during chromatographic analysis. It has been widely reported that the retention characteristics of reverse phase columns vary from manufacturer to manufacturer and even from lot to lot from a single manufacturer. Such differences were observed during this training exercise where The Netherlands using a C18 column observed Ile-Ser-Bradykinin eluting near the last PEG peak, while Sweden using a different C18 column had it elute prior to the first PEG peak. Sample handling also influenced the extraction of the PEG's and Ile-Ser-Bradykinin. France extracted one fraction of MSA01/002 with a mixture of acetonitrile/water (80/20) followed by an aqueous extraction. The acetonitrile/water extract contained primarily PEG's with a small amount of Ile-Ser-Bradykinin. The aqueous extract, illustrated in Figure 7, contained a small amount of PEG and a much higher percentage of Ile-Ser-Bradykinin.

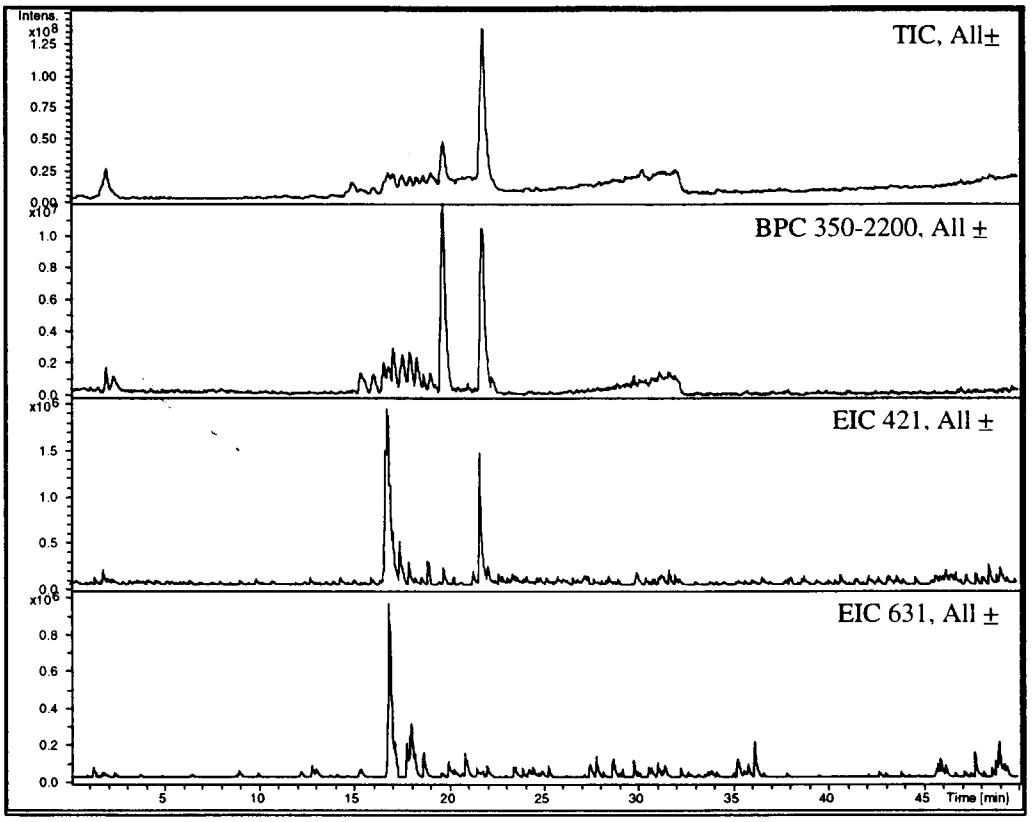

Figure 7. *LC/MS chromatograms: a) total ion current, b) base peak chromatogram, c) extracted ion chromatogram for [M+3H]$^{3+}$ ion (421 Da) and d) extracted ion chromatogram for [M+2H]$^{2+}$ ion (631 Da) acquired by France during the analysis of a sample extract of MSA01/002 with a C8 column. Numbered peak (1) is Ile-Ser-Bradykinin.*

Provisional Identification

The participating laboratories determined the molecular mass of the unknown in MSA01/002 using ESI-MS and MALDI-MS. MALDI-MS was used by both The Netherlands and the United States while, ESI was used by the remaining five laboratories. Figure 8 illustrates the mass spectrum acquired by Germany under nanoelectrospray conditions during the analysis of MSA01/002. The mass spectrum contained a $[M+H]^+$ ion at m/z 1260.7 and a $[M+2H]^{2+}$ ion at m/z 630.9 from which the molecular mass was calculated. By infusing the sample directly into the electrospray interface and monitoring ions up to approximately 3000 Da, Germany was able to observe the protonated molecular ion for Ile-Ser-Bradykinin free of the interference of the PEG 600 envelope. Once the laboratories obtained the molecular mass they were able to identify, the sample as Ile-Ser-Bradykinin. Table 3 lists the monoisotopic molecular masses obtained by each country. At higher molecular mass, errors are generally reported in parts per million (ppm). Sweden, using a high resolution magnetic sector instrument with an off axis time of flight mass spectrometer, reported the most accurate data for Ile-Ser-Bradykinin with a 2 ppm error. Based on the identification criteria, a MSA is considered provisionally identified when the molecular mass matches that of a known MSA.

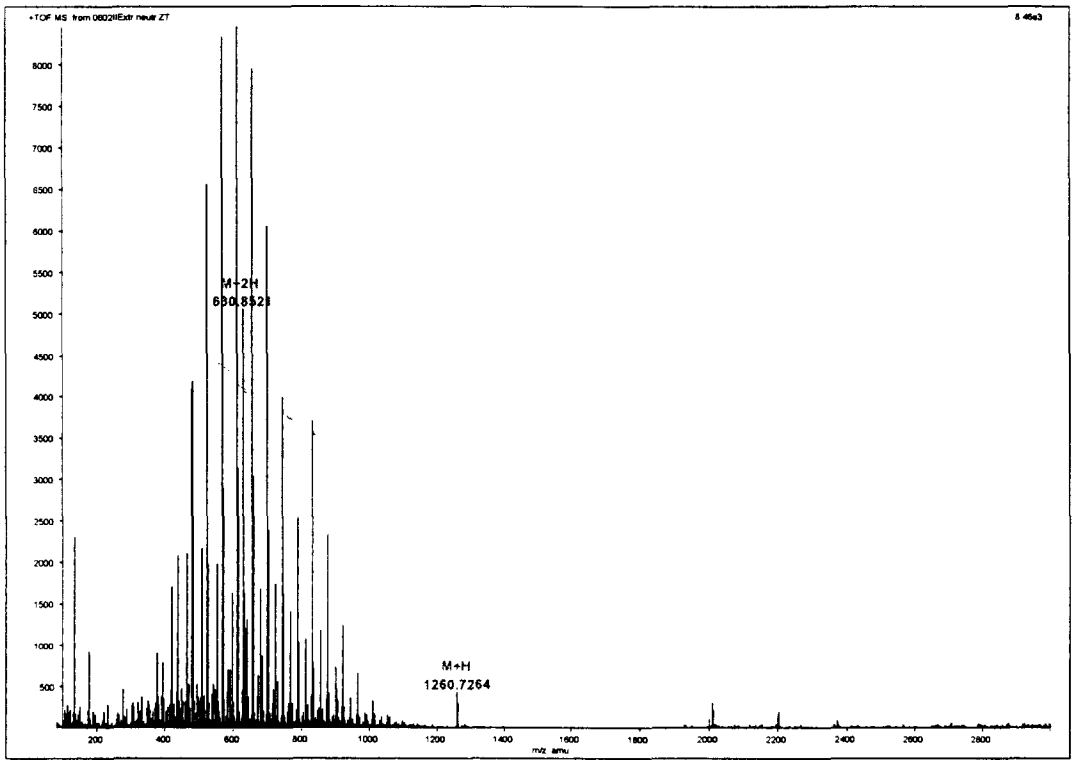

Figure 8. *NanoElectrospray mass spectrum acquired by Germany during the infusion of sample MSA01/002. $[M+H]^+$ and $[M+2H]^{2+}$ ions for Ile-Ser-Bradykinin were observed at m/z 1260.7 and 630.9 respectively.*

Table 3. *Monoisotopic Molecular Masses Determined for MSA01/002 (Ile-Ser-Bradykinin)*

Country	Instrument	Observed Monoisotopic Molecular Mass[b]	Error ppm
France	Bruker Esquire 3000 Ion Trap	1259.75	58
Germany	PE Biosystems Q-Star	1259.72	34
Italy	Micromass ZMD	528.4 ± 0.2	
Netherlands	Bruker Biflex III TOF	1260.2	380
Sweden	Micromass Autospec OA-TOF	1259.675	2
U.S.	Applied Biosystems Voyager MALDI-TOF	1259.6323	36
Canada[a]	Micromass LCT	1259.689 ± 0.005	9

[a] host country
[b] theoretical monoisotopic molecular mass: 1259.67749 Da

Confirmed Identification

None of the participating laboratories confirmed the identification of Ile-Ser-Bradykinin in MSA01/002, however three countries achieved unambiguous identification. This apparent discrepancy is more a function of how the criteria for identification were written rather than the data provided by the laboratories. Confirmed identification of a proteinaceous mid-spectrum agent requires the molecular mass and corresponding mass map of the enzymatic digestion products to match that of a known mid-spectrum agent. None of the laboratories submitted this type of data. When the identification criteria were written, it was thought unambiguous identification required obtaining the complete amino acid sequence for the MSA. As the molecular mass of a proteinaceous MSA increases the ability to acquire sequence data using ESI-MS gradually diminishes. Eventually, it is necessary to enzymatically digest the MSA and obtain molecular masses (mass map) and sequence data from the smaller fragments. During this training exercise, laboratories were able to identify Ile-Ser-Bradykinin at the unambiguous level by obtaining the complete amino acid sequence without the need for enzymatic digestion.

Unambiguous Identification

Three of the participating laboratories: Germany, The Netherlands and Sweden were able to unambiguously identify Ile-Ser-Bradykinin in MSA01/002. Once they had provisionally identified Ile-Ser-Bradykinin, these countries purchased reference samples of Ile-Ser-Bradykinin for comparison purposes. Initially, they demonstrated that the chromatographic retention data acquired for extracts of MSA01/002 and the reference agreed within experimental error. Figure 9 illustrates the ESI-MS/MS spectrum acquired by Germany for the doubly charged (m/z 630.8) precursor ion. While no complete series (e.g. y-series) of ions were observed, from the overlap of the observed a-, b-, y- and z-series ions the amino acid sequence of Ile-Ser-Bradykinin could be completely assigned. The same fragmentation pattern and sequence information was acquired for the Ile-Ser-Bradykinin reference thereby unambiguously identifying Ile-Ser-Bradykinin by Germany. Similar data was reported by both Sweden and The Netherlands.

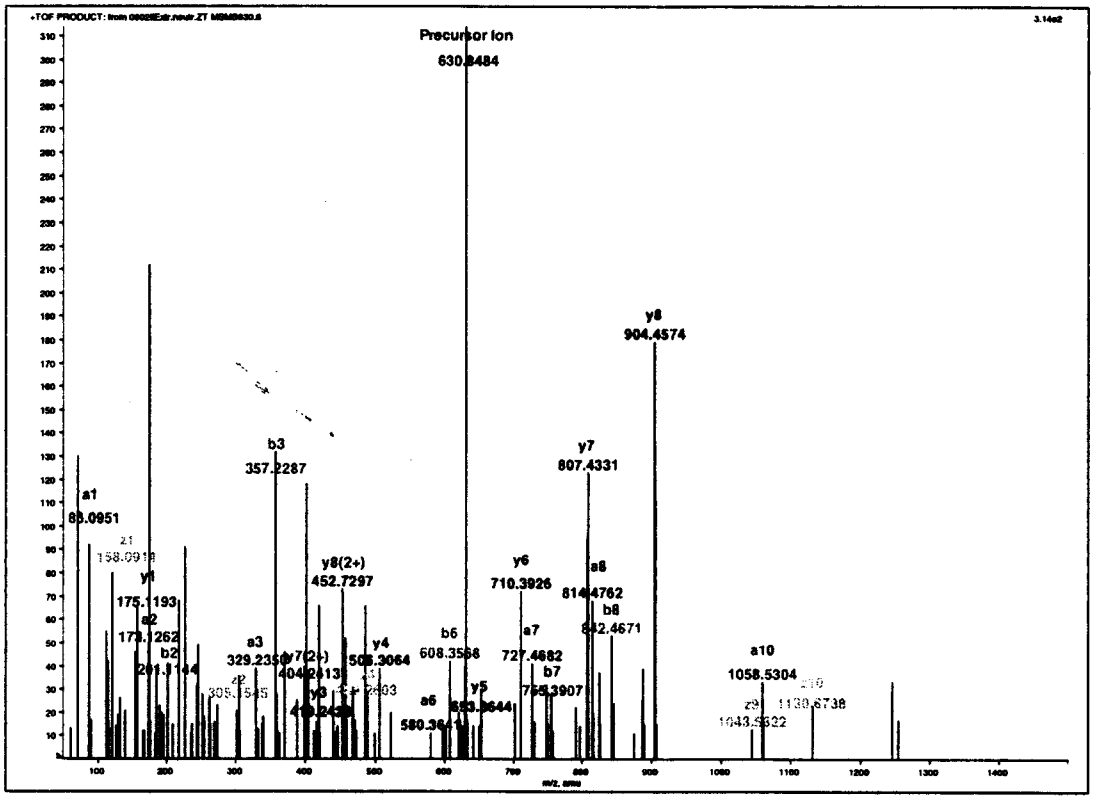

Figure 9. MS/MS spectrum of obtained by Germany for the doubly charged precursor ion (m/z 630.8) of Ile-Ser-Bradykinin.

Discussion

The results from this international training exercise demonstrated that the participating laboratories continue to improve their identification techniques, with 3 laboratories achieving unambiguously identification of Ile-Ser-Bradykinin. Some laboratories indicated problems with obtaining reference samples for confirmed and unambiguous identification within the timeframe of the exercise. While laboratories were allowed five working days to carry out their analysis, they were free to work on the samples anytime during the month of February. Some countries choose to spend one or two days provisionally identifying the MSAs, they then ordered the reference materials from commercial suppliers, resuming their analysis after they had obtained the reference materials. Given the range of compounds that could be used as MSAs, no one country would likely be able to keep all possible reference materials on hand. The requirement for comparing the data acquired for an unknown to that from a reference compound suggests that the list of MSAs may need to be reduced.

When the SIBCA subgroup developed the identification criteria for mid-spectrum agents in the mid 1990's it was with the intention that the criteria should be reviewed as experience in the sample handling and analysis of MSAs matured. In light of the information acquired during three international training exercises, it may be time for the subgroup to re-examine the identification criteria.

An example of where changes in the criteria would be useful was illustrated during the analysis of Ochratoxin A. A number of laboratories compared the ESI-MS spectra for MSA 01/001 and a reference sample of Ochratoxin A under CAD conditions. These spectra, while clearly similar, on their own do not satisfy any of the levels of identification in the identification criteria for mid-spectrum agents. However, within the criteria for identification of chemical agents, such data would constitute confirmed identification. It is recommended that the SIBCA sub-group consider altering the MSA criteria to ensure consistency between the different criteria.

The fact that three countries; Germany, The Netherlands and Sweden were able to identify Ile-Ser-Bradykinin at the unambiguous level without initially confirming the identification indicates that the criteria need to be revised. When the SIBCRA subgroup developed the identification criteria for MSAs, the group believed that unambiguous identification required obtaining the complete amino acid sequence for the MSA. Having been able to completely sequence Ile-Ser-Bradykinin, it was felt that these laboratories had achieved unambiguous identification even though that had not met the strict definition described in the criteria. It should be noted that Ile-Ser-Bradykinin is a relatively low molecular mass MSA and it may not prove feasible to obtain complete amino acid sequences from large proteinaceous MSAs even after enzymatic digestion.

Conclusions

The third NATO international training exercise on the identification of mid-spectrum agents was held in February 2001. Participating nations included: Canada(host), France, Germany, Italy, Netherlands, Sweden and the U.S.A.. Ochratoxin A a non-proteinaceous MSA and Ile-Ser-Bradykinin a proteinaceous MSA were spiked onto sand and distributed as unknowns to the participating laboratories. The results from this international training exercise demonstrate that the participating laboratories continue to improve their identification techniques, with three laboratories identifying Ochratoxin A at the confirmed level and three laboratories identifying Ile-Ser-Bradykinin at the unambiguous level. Based on the type and quality of data provided by the laboratories, it is Canada's recommendation that the SIBCA subgroup consider updating the NATO identification criteria for mid-spectrum agents. While selecting the matrix (sand) for this exercise, it was observed that the MSAs could not be recovered from the soil types available at DRES, indicating that further research in extraction and sample preparation will be needed.

References

1. J.R. Hancock and P.A. D'Agostino, *NATO Panel VII/SICA International Training Exercise on the Identification of Peptides*, Suffield Memorandum No 1481, September 1996, UNCLASSIFIED, 26 pages.

2. J.R. Hancock and P.A. D'Agostino, *NATO AC/225 LG/7 Second International Training Exercise on the Identification of Mid-Spectrum Agents*, DRES Technical Memorandum No 2000-013, March 2000, UNCLASSIFIED, 22 pages.

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Annex I

List of Candidate Mid-Spectrum Agents

Compound	Monoisotopic MW	Average MW	CAS Number
Fumitremorgin C	379.18959	379.46048	118974-02-0
Gliotoxin G	389.98364	390.52353	53348-47-3
Gonyautoxin II	395.08593	395.35229	60508-89-6
Gonyautoxin VI	395.08593	395.35340	82810-44-4
Fumitremorgin F	402.13281	402.41073	61897-89-0
Fumitremorgin J	402.13281	402.41073	66212-51-9
Ochratoxin A	403.08227	403.81976	303-47-9
Gonyautoxin IV	411.08085	411.35280	64296-26-0
Batrachotoxinin A	417.25152	417.54744	194587-37-5
Fumitremorgin E	418.12772	418.41006	61897-87-8
Ochratoxin C	431.11357	431.87374	4865-85-4
Fumitremorgin G	432.14337	432.43705	61897-91-4
Fumitremorgin I	502.18523	502.52838	66180-23-2
Verrucarin A	502.22028	502.56311	3148-09-2
Roridin H	512.24102	512.60181	29953-50-2
Roridin E	514.25667	514.61776	16891-85-3
Roridin J	528.23593	528.60113	74072-83-6
Satratoxin H	528.23593	528.59940	53126-64-0
Satratoxin G	544.23085	544.60046	53126-63-9
Tryptoquivaline A	546.21145	546.58168	55387-45-6
Penitrem D	567.33486	567.77135	78213-64-6
Fumitremorgin A	579.29445	579.69576	12626-18-5
Penitrem B	583.32977	583.77068	11076-67-8
Substance P (6-11)	740.36796	740.92585	51165-07-2
Bufotoxin	756.43095	756.94034	464-81-3
"Phalloidin, dethio-"	758.35990	758.83177	35173-40-1
Okadaic acid; OA	804.46599	805.01922	78111-17-8
Brevetoxin PbTx-1	866.48164	867.09036	98112-41-5
Brevetoxin PbTx-3	896.49221	897.11667	85079-48-7
$-Amanitin	919.33819	919.96830	21150-22-1
Neuropeptide Ro II	919.41882	919.99552	113800-65-0
Bradykinin	1059.56138	1060.22812	5979-11-3
Neuropeptide FF	1080.58689	1081.29011	99566-27-5
Ile-Ser-Bradykinin	1259.67749	1260.46657	86030-63-9
Met-Lys-Bradykinin	1318.69685	1319.60035	550-19-6
Substance P	1346.72815	1347.65433	33507-63-0
α-Conotoxin SI"	1352.51341	1353.63198	133605-58-0
α-Conotoxin GI (reduced)	1356.54471	1357.66389	115797-06-3
Bombesin	1618.81507	1619.87554	31362-50-2
Conotoxin GIA	1625.63196	1626.88456	78301-21-0
Neuropeptide D	1763.83261	1764.87238	111461-48-4
Neuropeptide F2	1944.92669	1946.37769	112667-34-2
Apamin; Bee Venom	2025.88661	2027.36779	24345-16-2

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Annex II

Identification Criteria for Mid-Spectrum Agents

Three levels of identification have been defined as follows to indicate the increasing level of certainty associated with each.

PROVISIONAL IDENTIFICATION: A mid-spectrum agent may be considered provisionally identified when one of the following criteria has been met:

I The chromatographic retention data acquired for the mid-spectrum agent under two different experimental conditions matches that of known mid-spectrum agent data; or

II The molecular mass of the mid-spectrum agent, determined by MS, matches that of known mid-spectrum agent data; or

III A specific immunological assay registers a positive response.

CONFIRMED IDENTIFICATION: The identification of a mid-spectrum agent is confirmed when any two of the three criteria for provisional identification are met or:

I In the case of proteinaceous mid-spectrum agents, the molecular mass and corresponding mass map of the enzymatic digestion products (with a minimum of three products) matches that of known mid-spectrum agent data.

UNAMBIGUOUS IDENTIFICATION: Unambiguous identification provides the highest level of certainty required for the development of strategic and political positions. The identification of a mid-spectrum agent is unambiguous when the following conditions are met:

For non-proteinaceous mid-spectrum agents

I The chromatographic retention data acquired for the mid-spectrum agent and spectra acquired using two different spectrometric techniques (MS, NMR or IR) match those for authentic reference standards acquired under identical experimental conditions. If the molecular ion is not present in the mass spectrum, techniques such as chemical ionization must be carried out to confirm the molecular mass.

For proteinaceous mid-spectrum agents:

I The chromatographic retention data acquired for the mid-spectrum agent under two different experimental conditions matches that of an authentic reference standard acquired under identical experimental conditions or a specific immunological assay registers a positive response; and

II The molecular mass and corresponding mass map of the enzymatic digestion products (with a minimum of three products) matches that for an authentic reference standard acquired under identical experimental conditions; and

III Sequence data for the mid-spectrum agent matches that for an authentic reference standard acquired under identical experimental conditions.

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Hancock, James R., D'Agostino, Paul A., Provost, Lionel R. and Chenier, Claude L.

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In February 2001, the NATO Land Group7 subgroup on Sampling and Identification of Biological and Chemical Agents (SIBCA) conducted the third international training exercise on the identification of mid-spectrum agents. The objective of this training exercise was to evaluate the participating national laboratories' ability to identify specific mid-spectrum agents (MSAs) spiked onto soil samples. The compounds were selected from a list of mid-spectrum agents compiled by Canada (host nation) and distributed as unknowns to the laboratories. Seven national laboratories, one each from Canada, France, Germany, Italy, Netherlands, Sweden and the United States, participated in the exercise.

The level of identification (provisional, confirmed or unambiguous) obtained by each of the laboratories was determined using the identification criteria for mid-spectrum agents developed by the SIBCA subgroup. All the participating laboratories were able to provisionally identify the MSA in the first soil sample as Ochratoxin A. Six of the seven laboratories were able to provisionally identify the MSA in the second soil sample as Ile-Ser-Bradykinin. Germany, The Netherlands and Sweden through further experiments were able to unambiguously identify Ile-Ser-Bradykinin. The results from this exercise have served to illustrate areas in which the identification criteria may be improved.

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Ile-Ser-Bradykinin
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