
Determination of peptaibol trace amounts in marine sediments by liquid chromatography/electrospray ionization-ion trap-mass spectrometry

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Abstract:

Extraction followed by reverse phase liquid chromatography (LC)/electrospray ionization-ion trap-mass spectrometry (ESI-IT-MS) analysis has been successfully developed for the determination of peptaibols, fungal toxic metabolites, in marine sediments. Spiking experiments showed that the mean recovery of target compounds exceeded 85% at a spiking level of 10 ng/g of sediment (wet weight). Detection and quantification limits were 250 and 830 pg/g of sediment, respectively. The method developed constituted the first sensitive assay for quantification of peptaibol trace amounts in a natural environment. A concentration of 5 ng/g in sediment samples collected from Fier d'Ars was found.

Keywords: Fungal peptide metabolites; *Trichoderma* sp.; Marine fungal contamination; Electrospray ionization-ion trap-mass spectrometry (ESI-IT-MS); Matrix matched calibration

1. Introduction

Fungal production of mycotoxins in the marine environment is proposed as a possible cause for episodes of unexplained toxicity observed in shellfish populations during the last decade. Within this framework, numerous strains of toxigenic saprophytic fungi were isolated from shellfish, sediment and seawater samples collected in shellfish farming areas [1]. Among them, different strains of *Trichoderma* sp., grown in marine-like culture conditions, produced peptaibols, peptidic metabolites, which are toxic for different larval models (diptera or crustacean larvae) [2].

Peptaibols constitute a constantly growing family of linear peptide antibiotics of fungal origin. They are characterized by a molecular mass from 500 to 2200 u, an acetyled N-terminus, a C-terminus amino alcohol and a high content of a non proteinogenic amino acid, α -aminoisobutyric acid (Aib or U) [3]. Peptaibols are exclusively produced by filamentous fungi mainly belonging to the genera *Trichoderma*, *Acremonium*, *Paecilomyces*, *Emericellopsis* and *Gliocladium*. They have been classified into subfamilies according to their amino acid chain lengths (ranging from 5 to 20 residues) and their chemical characteristics [4]. These fungal metabolites exhibit a variety of biological activities resulting from their membrane-modifying and pore-forming properties. Thus antibacterial, antifungal and occasionally antiviral and antiparasitic activities have been reported [5-8].

A previous experimental contamination has shown that peptaibols can be accumulated in filter-feeder molluscs (*Mytilus edulis*) when present in sea-water as soluble compounds [9]. The presence of such compounds in the marine environment could lead to health risks for shellfish and their consumers. Different peptaibols were recently detected in sediments in a marine area devoted to shellfish farming (Fier d'Ars, Atlantic coast, France) [10]. These sediment samples displayed high toxicity for mussel larvae in the absence of significant contaminations (metals, PCBs, HAPs, pesticides, antibiotics) or eutrophication [11]. Developing analytical methods allowing the precise determination of these fungal metabolites in the marine environment is therefore of great interest in order to establish a causal relationship between peptaibol concentrations and biological effects. Certain methods, that use radioactivity or capillary electrophoresis coupled with UV and ESI-TOF-MS, have already been described for the quantification of peptaibols isolated from fungal cultures [12,13]. However, they are not sensitive enough for determining trace amounts. The aim of this work is to develop a process for extracting peptaibols from marine sediment matrices and a sensitive assay for the determination of trace amounts by using LC/ESI-IT-MS. The method developed focuses on long-sequence peptaibols, including 18 to 20 amino acid residues, because of their high bioactivity [6] and their predominance in peptaibol family [14].

2. Experimental

2.1. Chemicals

79 Methanol and dichloromethane were purchased from Carlo Erba (Val de Reuil,
80 France) and distilled before use. Ethanol was purchased from APC (Aubervilliers, France).
81 Trifluoroacetic acid (TFA) was obtained from Fluka Chemical (Buchs, Switzerland),
82 hydrochloric acid from Acros organics (Geel, Belgium) and acetic acid from Sigma Aldrich
83 (Saint-Quentin Fallavier, France). For mass spectrometry analysis, HPLC-grade methanol was
84 obtained from Baker (Deventer, Holland). Water was purified to HPLC-grade quality with a
85 Millipore-Q RG ultrapure water system from Millipore (Milford, CT, USA). Alamethicin F50
86 was obtained from Sigma Aldrich (Ref. A4665).

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88 *2.2. Sediment samples*

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90 Sediment samples used for optimizing extraction and purification procedures were
91 collected from La Rochelle (France) in January 2000. They were transported from the site to
92 the laboratory in isothermic containers and frozen at -20°C.

93 Sediment samples used to estimate environmental contamination were collected from
94 different sites on the French Atlantic coast. Surface sediment samples (oxic fraction, 1st cm)
95 were collected from four sites: the Bay of Marennes-Oléron (45° 55' N 1° 13' W), Auray
96 River (47° 38' N 2° 58' W) and the Bay of Veys (49° 22' N 1° 08' W) in June 2004 (in the
97 framework of the French program "MOREST"), and from Fier d'Ars (Ré Island – 46° 13' N
98 1° 29' W) in March 2006. All the samples were transported from the site to the laboratory in
99 isothermic containers and frozen at -20°C until analysis. Each sample (approximately 10 g
100 wet weight) was subjected to extraction, purification and LC/ESI-IT-MS analysis.

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102 *2.3. Optimization of the extraction procedure*

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104 The efficiency of the extraction procedure was checked by recovery experiments. The
105 nature of the extraction solvents was the decisive parameter for which optimization was
106 required. Approximately 10 g wet weight (ww) of sediments were spiked with 100 ng of
107 alamethicin F50 and extracted with 3 × 25 mL of different organic solvents. According to the
108 preliminary experiments, five different mixtures of solvents were selected for definitive tests:
109 (a) dichloromethane/methanol (1:1, v/v), (b) methanol/TFA 0.1% (v/v), (c) ethanol/acetic acid
110 1% (v/v), (d) acetone/acetic acid 1% (v/v) and (e) acetone/hydrochloric acid 0.02% (v/v). At
111 each extraction step, the sample was sonicated for 15 min and centrifuged at 700 g for 5 min.
112 Two procedures were used in order to eliminate salts. The supernatants obtained with
113 mixtures (a), (b) and (c) were evaporated to dryness and redissolved in 50 mL of
114 dichloromethane/methanol/water (2:2:1). The aqueous phase containing salts was washed
115 twice with dichloromethane. The organic phases were then combined and evaporated to
116 dryness. The supernatants obtained with solvent mixtures (d) and (e) were simply filtered and
117 evaporated to dryness (crude extracts).

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119 *2.4. Purification of extracts*

120

121 Purification of crude extracts was performed by vacuum liquid chromatography (VLC)
122 on a diol-silica gel column (10 x 40 mm) (Supelco, Bellefonte, PA, USA). The column was
123 prepared with 2 g of sorbent and rinsed with 10 mL of dichloromethane prior to sample

124 loading. For this step, two deposit modes were investigated. In mode 1, the extract was
125 redissolved and deposited with 3 mL of three successive solvent mixtures in the purification
126 column: dichloromethane/ethanol (100:0, 90:10 and 50:50, v/v). Mode 2 corresponded to a
127 dry deposit. The crude extract was dissolved in 10 mL of dichloromethane/ethanol mixture
128 (50:50, v/v) and mixed with a quarter of the sorbent phase. This mixture was evaporated to
129 dryness and loaded in the column. Elution was performed with 40 mL of successive
130 dichloromethane/ethanol mixtures (100:0, 98:2, 90:10 and 50:50, v/v). The fractions obtained
131 (A, B, C and D, respectively) were evaporated to dryness and redissolved in methanol (500
132 μ L) prior to analysis by using the hyphenated LC/MS technique.

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134 2.5. LC/MS analysis

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136 The samples were analyzed on a modular HPLC system consisting of a Spectrapysics
137 Spectra System P2000 pump, an AS 100XR autosampler (Thermo Separation Products, San
138 Jose, CA, USA) equipped with a Kromasil C-18 5- μ m reverse-phase 2.0 \times 250 mm column
139 (Interchim, Montluçon, France) heated to 40°C and coupled with a Finnigan Matt LCQ™
140 ESI-IT-mass spectrometer (Thermo Separation Products). The mobile phase consisted of a
141 methanol/H₂O (85:15, v/v) mixture delivered at a constant flow rate of 0.2 mL/min (isocratic
142 mode). The sample injection volume was 5 μ L. All mass analyses were performed in positive
143 mode. To ensure optimal detection, perfusion of a methanolic solution of alamethicin F50 (50
144 ng/mL) into the flow of LC using a micrometrically automated 250- μ L syringe (Hamilton,
145 Reno, NV, USA) at a flow rate of 3 μ L/min was performed to optimize the mass spectrometer
146 parameters. The spray voltage was set to 4.50 kV, the capillary temperature to 266°C and the
147 capillary voltage to 42 V. Nitrogen flow rates were 89 and 37 (arbitrary units), respectively,
148 for sheath and auxiliary gas. The parameters of ion optic transmission were adjusted to 55 V
149 for Tube Lens Offset, -3.50 V for Multipole 1 Offset, -6 for Multipole 2 Offset and 400 V for
150 Multipole RF Amplifier (peak to peak).

151 MSⁿ spectra acquisitions were carried out with a collision energy of 32% and an isolation
152 width of 1 u.

153 All spectra acquisitions and reworks were done using LCQ Xcalibur 1.3 software (Thermo
154 Fisher Scientific).

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156 2.6. Calibration and quantification

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158 External and matrix matched calibrations were compared. A commercial solution of
159 alamethicin F50 was used as external standard and characterized by LC/MSⁿ analysis. This
160 product contains four individual components which have been identified as alamethicin
161 F50/5, F50/6a, F50/7 and F50/8b with molecular masses of 1962, 1976, 1976 and 1990 u
162 respectively, according to Kirschbaum *et al.* [15].

163 The two main components, alamethicin F50/5 (m/z 1004.3, t_R 8.8 min) and F50/7 (m/z
164 1011.3, t_R 10.6 min), which represented a constant proportion of 90.5 % in the reference
165 solution, were used for the calibration performed by using LC/ESI-IT-MS. This proportion
166 remained constant after the extraction and purification steps. For external standardization, a
167 calibration curve was prepared using 8 concentrations of alamethicin F50 in methanolic
168 solution (1 to 100 μ g/L). To consider the matrix effects, matrix matched calibration samples

169 were prepared by adding different concentrations of alamethicin F50 to sediment extracts
170 obtained after purification. 100 µL of alamethicin F50 reference solution at 12.5, 25, 50, 100
171 and 200 µg/L were added to 100 µL of each purified fraction C and D. LC/MS analysis of
172 each concentration level was performed 6 times for both external and matrix matched
173 calibrations. The accuracy and precision of the matrix matched calibration method were
174 calculated for each concentration level.

175 The accuracy of the method developed was determined by the analysis of three
176 sediment samples spiked with 100 ng of alamethicin F50 solution. All the percentages of
177 recovery were determined relative to the standard samples.

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179 *2.7. Statistical treatment*

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181 Mann Whitney U-tests were carried out to compare the percentages of recovery of
182 alamethicin F50 and impurity masses obtained during the optimization of the extraction and
183 purification steps. Pearson's correlation was used to test the linearity of the quantification
184 data.

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189 **3. Results and discussion**

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192 *3.1. Selection of extraction conditions of peptaibols from sediments*

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194 To achieve the efficient extraction of the target compounds, recovery experiments with
195 alamethicin F50 spiked sediments were carried out. Five solvent mixtures were evaluated and
196 the results are shown in Table 1. Extraction using mixtures of dichloromethane/methanol,
197 methanol/TFA and ethanol/acetic acid did not provide satisfactory recovery of alamethicin
198 F50, since the values were below 10%. Methanol was generally used in the extraction
199 procedures of peptaibols from fungal cultures (qualitative analysis) [16,17]. In spite of its
200 high eluotropic strength, this solvent was not strong enough to remove peptaibols from a
201 complex sedimentary matrix. Acetone/hydrochloric acid mixture (e) provided a higher
202 recovery of alamethicin F50 with a mean of $64 \pm 9\%$. Satisfactory extraction efficiency ($86 \pm$
203 20%) was obtained using acetone/acetic acid mixture (d) (significant differences with (a), (b)
204 and (c) at the 95% level). An additional extraction test was performed with acetone 100% and
205 resulting in 47% recovery (results not shown), a value lower than those obtained for acidified
206 acetone mixtures. Acid conditions were essential for the extraction of molecules of interest
207 from sedimentary particles. The acetone/acetic acid 1% (v/v) mixture was therefore chosen as
208 the best solvent for further studies.

209

210 **TABLE 1**

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3.2. Purification of analytes

215 The crude extracts thus obtained contained a high level of impurities. Hence it was
216 essential to proceed to further purification steps on extracts to minimize chromatographic
217 interferences and ions suppression. Silica [5,18] and diol-silica gel columns [2,19] were
218 generally used to purify the peptaibols (fungal cultures). In this study, the sediment extracts
219 were purified on diol-silica gel and alamethicin F50 was eluted by fractions C and D
220 (dichloromethane/ethanol 90:10 and 50:50 v/v, respectively). Because of partial dissolution of
221 the extract in dichloromethane, it was necessary to optimize the deposit mode. Thus, two
222 different procedures were tested: mode 1 – solubilization of the extract in three successive
223 solvent mixtures; mode 2 - dry deposit.

224 The recovery of alamethicin F50 was not significantly different depending on modes 1
225 and 2 as shown in Fig. 1a (Mann-Whitney, p-value = 0.042). However, there were
226 significantly fewer impurities eluted when using mode 2 than when eluted with mode 1, as
227 shown in Fig. 1b (Mann-Whitney, p-value = 0.05). Moreover, repeatability was better with
228 mode 2 than with mode 1. The dry deposit mode (mode 2) was therefore chosen for the
229 purification of sediment extracts because of less interference from impurities and better
230 repeatability.

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FIGURE 1

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3.3 LC/MS identification of peptaibols

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237 Analysed under neutral conditions and positive mode by ESI-IT-MS, long-sequence
238 peptaibols mainly appeared as doubly charged sodium adduct ions $[M+2Na]^{2+}$ with a peptidic
239 isotopic profile (Fig. 2a). In LC/MS, their detection was performed through three scan events
240 repeated throughout the chromatographic separation: a total current ion scan (fullscan) from
241 m/z 150 to 2000 and two enhanced resolution scans (zoomscan) from m/z 870 to 890 and from
242 m/z 985 to 1015. An additional analysis in MS² mode was performed during a second run on
243 the sodium adduct ions observed previously. This generated a spectrum containing mainly the
244 a_n and y_n ion series as classically reported by others authors [20], while b_n ions, predominant
245 in the acid medium, were also detectable but in lower abundance [21,22]. Peptide
246 identification was based on the production of N- and C-termini fragments resulting from
247 preferential breaking of the Aib-Pro bond [23-25]. MS² analysis of m/z 1004.3 (alamethicin
248 F50/5) is depicted in Fig. 2b which shows a predominant doubly charged ion $[M-H_2O+2Na]^{2+}$
249 at m/z 995.8 corresponding to a loss of a water molecule on the amino alcohol located at the
250 C-terminus. The N-terminus $[a_{13}+Na]^+$ at m/z 1183.8 and the C-terminus $[y_7+Na]^+$ at m/z
251 796.6 could be easily identified. An Aib residue can be visualized between fragments
252 $[a_{12}+Na]^+$ at m/z 1098.7 and $[a_{13}+Na]^+$ at m/z 1183.8.

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FIGURE 2

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257 *3.4. LC/MS quantification of peptaibols*

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259 External and matrix matched calibrations were compared for peptaibol quantification.
260 To investigate the matrix effect, matrix matched calibration was performed using sediment
261 extracts spiked with the alamethicin F50 reference solution after purification (e.g. for fraction
262 D, Fig. 3a, b, c). Both external and matrix matched calibration curves, obtained by summing
263 the peak areas of the two alamethicin components F50/5 and F50/7, were observed to be
264 linear up to a concentration of 100 µg/L with correlation coefficients higher than 0.98. The
265 comparison of matrix matched calibrations performed with sediments from different origins
266 (La Rochelle and Fiers d'Ars) showed a significant and variable matrix effect with a signal
267 decrease varying from 20 to 52 % compared to the signal of alamethicin F50 in methanolic
268 solution. Matrix matched calibration requires at least two LC/MS runs per analysis: one for
269 the sample extract and one for the sample extract spiked with a known quantity of the
270 reference peptaibol. However, it permitted the correction of signal quenching and taking into
271 account the variability of sedimentary matrices.

272

273 **FIGURE 3**

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275 The analytical method was validated considering the linear range, limit of detection
276 (LOD) and precision. The limit of quantification (LOQ) was determined using the method of
277 Vial and Jardy [26] with a pre-established value of area relative standard deviation (RSD) of
278 10%. For the reference peptaibol in methanolic solutions, LOD and LOQ were respectively
279 0.5 and 1.7 µg/L. For matrix matched calibration samples, the signal intensity of alamethicin
280 F50 was decreased by coeluted substances originating from the sediments. Consequently,
281 LOD and LOQ were increased, reaching respectively 2.5 and 8.3 µg/L, corresponding to a
282 detection of 250 pg/g and a quantification of 830 pg/g of sediment (ww).

283 Intra-day statistics of accuracy and precision were determined for matrix matched
284 calibration method (Table 2). The accuracy, expressed in terms of bias (deviation from true
285 values) was between 29% for the lowest concentration (below LOQ), and 2% for a
286 concentration of 9.4 ng/g of sediment. The precision, given by relative standard deviations,
287 was from 10% for a concentration of 0.6 ng/g to 2% for a concentration of 9.4 ng/g.

288 The whole procedure, from sample treatment to instrumental quantification, provided
289 a satisfactorily accurate result with a recovery of $86 \pm 4\%$ determined using spiked sediment
290 samples at a concentration of 10 ng/g (Fig. 1a).

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294 **TABLE 2**

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297 *3.5. Application to environmental samples*

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The method developed (acetone/acetic acid extraction; dry deposit; LC/MS analysis using three scan events; matrix matched calibration) was applied to natural sediment samples collected from different sites along the French Atlantic coast. Long-sequence peptaibols were identified and quantified in samples collected from Fier d’Ars but they were not observed in sediment samples collected from the Bay of Marennes-Oléron, Auray River and the Bay of Veys.

In the Fier d’Ars samples, after chromatographic separation, four doubly charged ions with a peptidic isotopic profile were observed at m/z 991.2, 991.7, 998.2 and 998.7 (e.g. for m/z 991.7, Fig. 4a, b). The molecular masses and retention times of these compounds are shown in Table 3. To confirm their peptaibolic nature, MS² fragmentation was carried out. Fragmentation profiles were obtained for the two main ions m/z 991.7 and 998.7 and were similar in both cases to the fragmentation pattern of long-sequence peptaibols (e.g. for m/z 991.7, Fig. 4c). An identical N-terminus fragment at m/z 1163.8 was identified for these two peptaibols. Two different C-termini parts were observed, respectively, at m/z 773.5 and 787.5. Peptides with molecular masses of 1937.4 and 1951.4 u and showing these N- and C-termini fragments showed numerous similarities with longibrachins and trichokonins, 20-residue peptaibols isolated from *Trichoderma* species [27-31]. The quantification of peptaibols observed in Fier d’Ars samples allowed establishing a concentration of 5.2 ± 2.1 ng/g of sediment (ww) (n=2).

FIGURE 4

TABLE 3

4. Conclusion

The method described using LC/ESI-IT-MS allows both the identification of peptaibols and, for the first time, their quantification in the pg/g range in complex matrices. LOD and LOQ were respectively 250 and 830 pg/g in marine sediments. Several sediment samples were analysed to evaluate the environmental contamination and the possible implication of these fungal metabolites in toxicity episodes observed in populations of bivalves along the Atlantic coast. The presence of long-sequence peptaibols was shown in sediments collected from Fier d’Ars and trace amounts were determined in these samples. The adaptation of this analytical method to shellfish matrices is under consideration. Further investigations will permit studying the relationship between environmental concentrations and the toxicity of these compounds for marine organisms.

Acknowledgements

341

342 Samples from the Bay of Marennes-Oléron, Auray River and the Bay of Veys were
343 collected in the framework of the French program “MOREST” coordinated by IFREMER.
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345 their technical participation.

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350 **References**

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406 **Figure captions**

407

408

409 **Fig. 1. Influence of the purification mode of crude extracts**

410 **Mode 1:** extract deposited with three successive fractions: dichloromethane/ethanol 100:0, 90:10 and 50:50
411 (v/v);

412 **Mode 2:** dry deposit

413 **(a) Cumulative recovery of alamethicin F50**

414 **(b) Cumulative percentage of eluted impurities**

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417 **Fig. 2. Mass spectra of alamethicin F50/5**

418 **(a) Zoomscan mode**

419 **(b) MS² spectrum of ion at m/z 1004.3 [M+2Na]²⁺**

420 The main fragments corresponding to a_n and y_n ion series are shown.

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422

423 **Fig. 3. LC/ESI-IT-MS analysis of surface sediment samples collected from Fiers d'Ars -**
424 **chromatograms of fraction D spiked with alamethicin F50 reference solution (25 µg/L)**

425 **(a) Total ion current**

426 **(b) Detection on the range [1003.9-1004.9]: peak of alamethicin F50/5**

427 **(c) Detection on the range [1010.9-1011.9]: peak of alamethicin F50/7**

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429

430 **Fig. 4. LC/ESI-IT-MS analysis of surface sediment samples collected from Fiers d'Ars**

431 **(a) Chromatogram (detection on the range [991.2-992.2])**

432 **(b) Mass spectrum corresponding to peak at $t_R = 6.11$ min**

433 **(c) MS² spectrum of ion at m/z 991.7 [M+2Na]²⁺**

434 The main fragments corresponding to a_n and y_n ion series are shown.

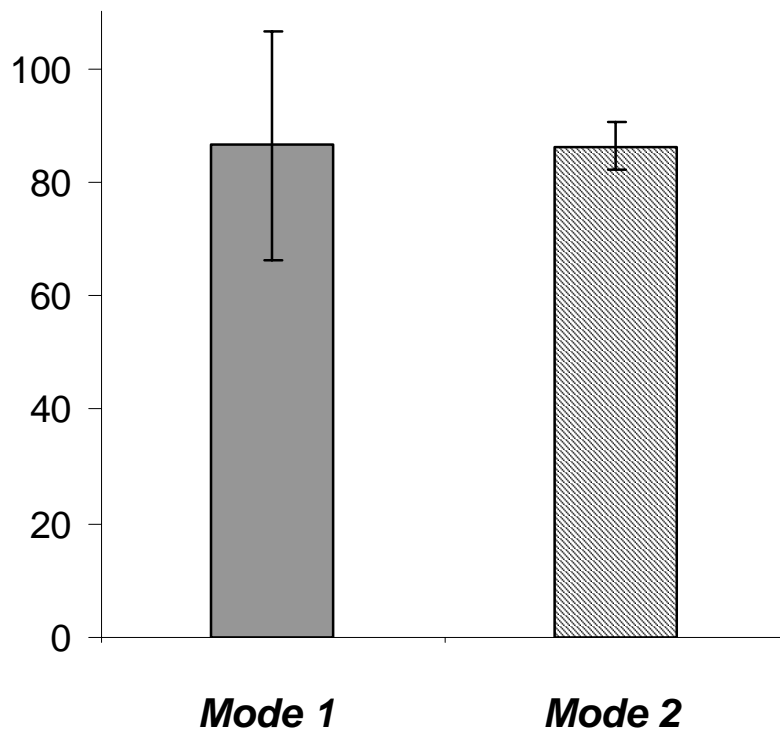
435

436

FIGURE 1

(a)

Cumulative recovery of
alamethicins (%)



(b)

Cumulative percentage of eluted
impurities (%)

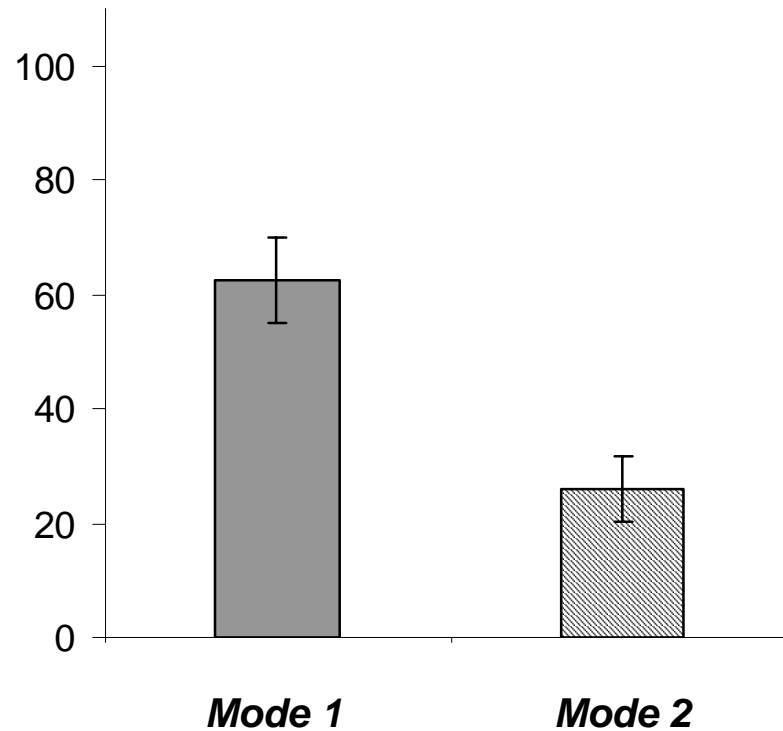


FIGURE 2

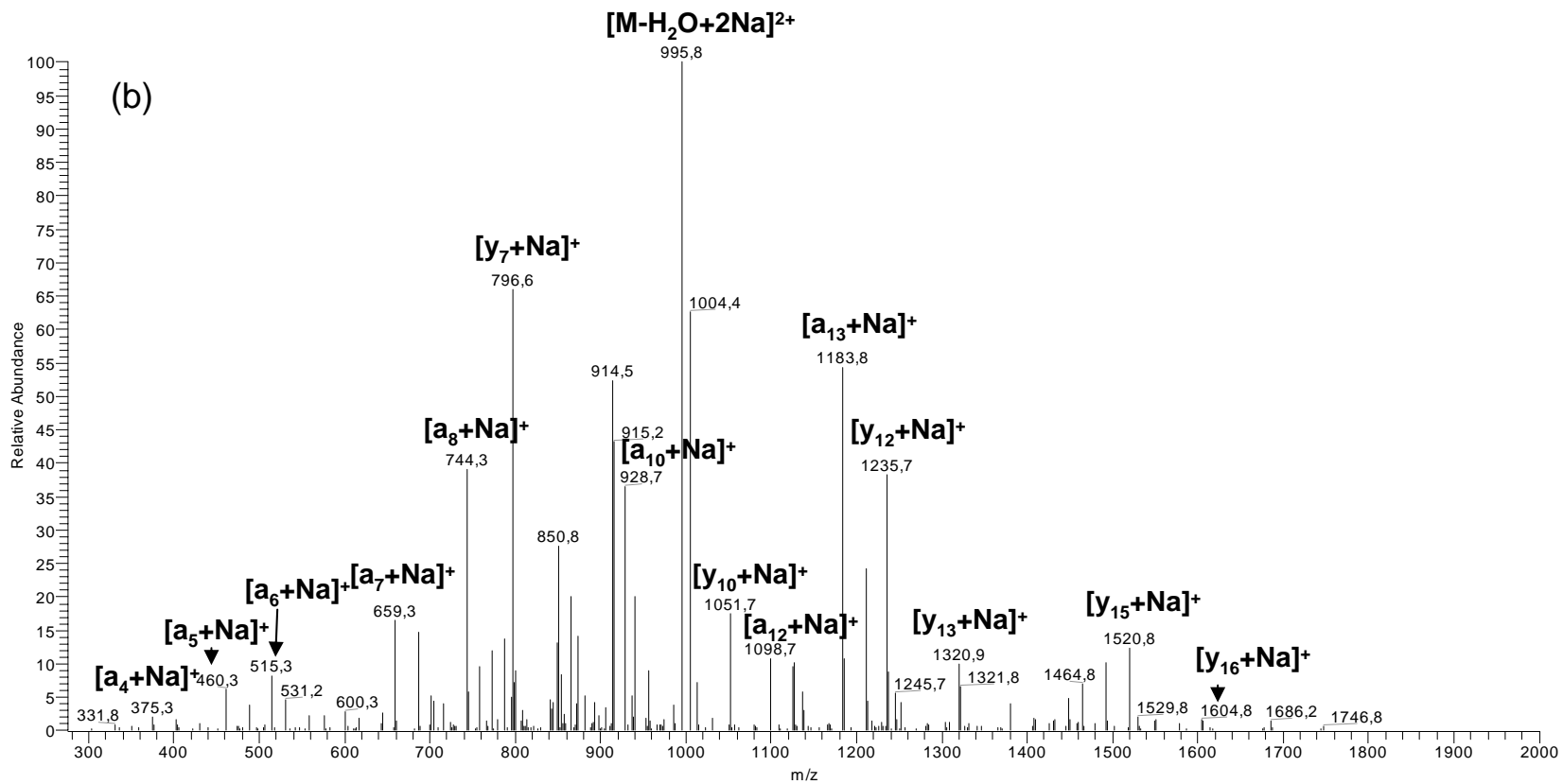
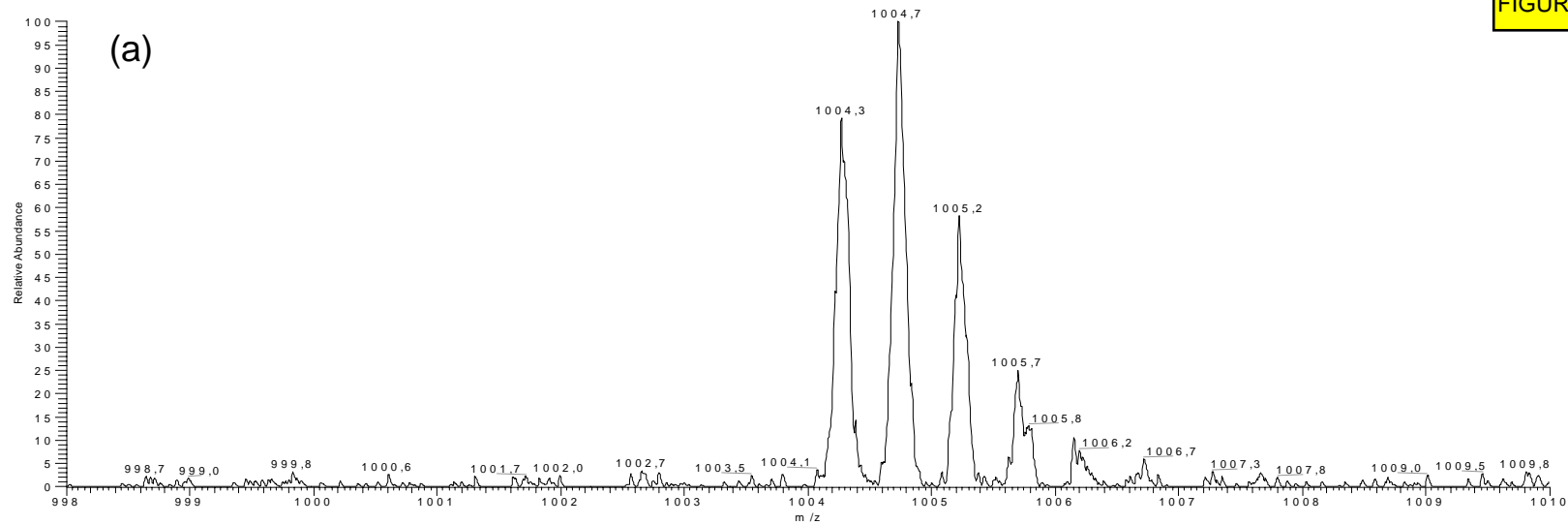
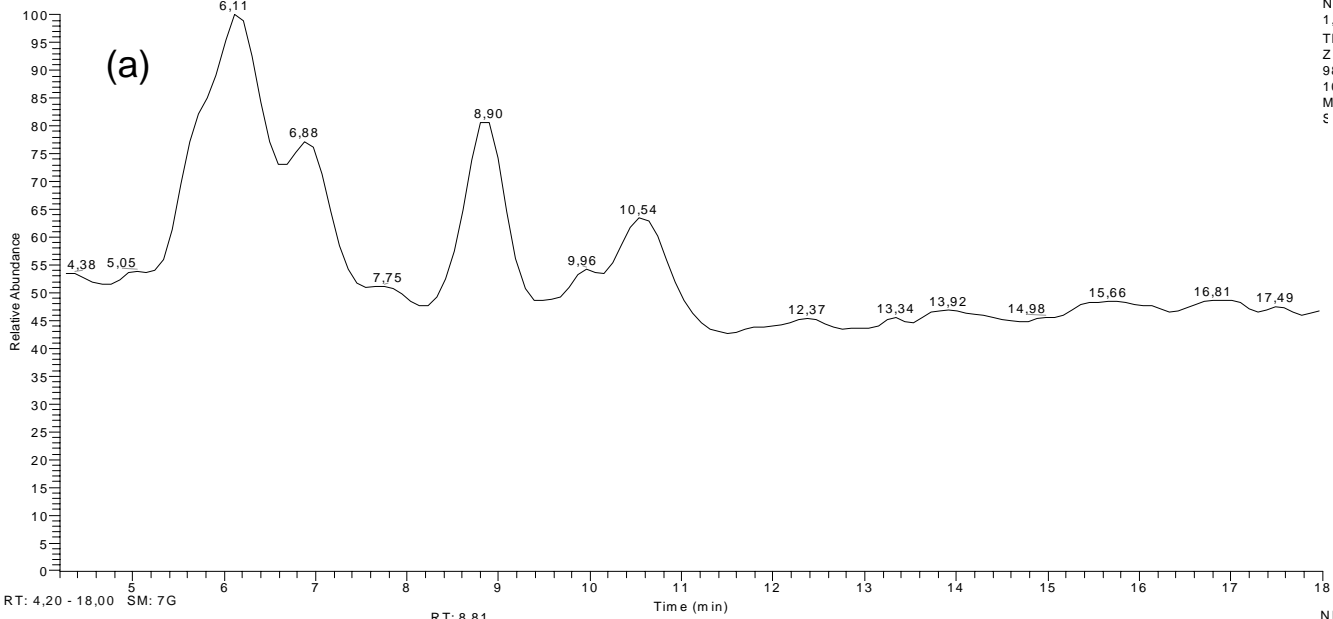


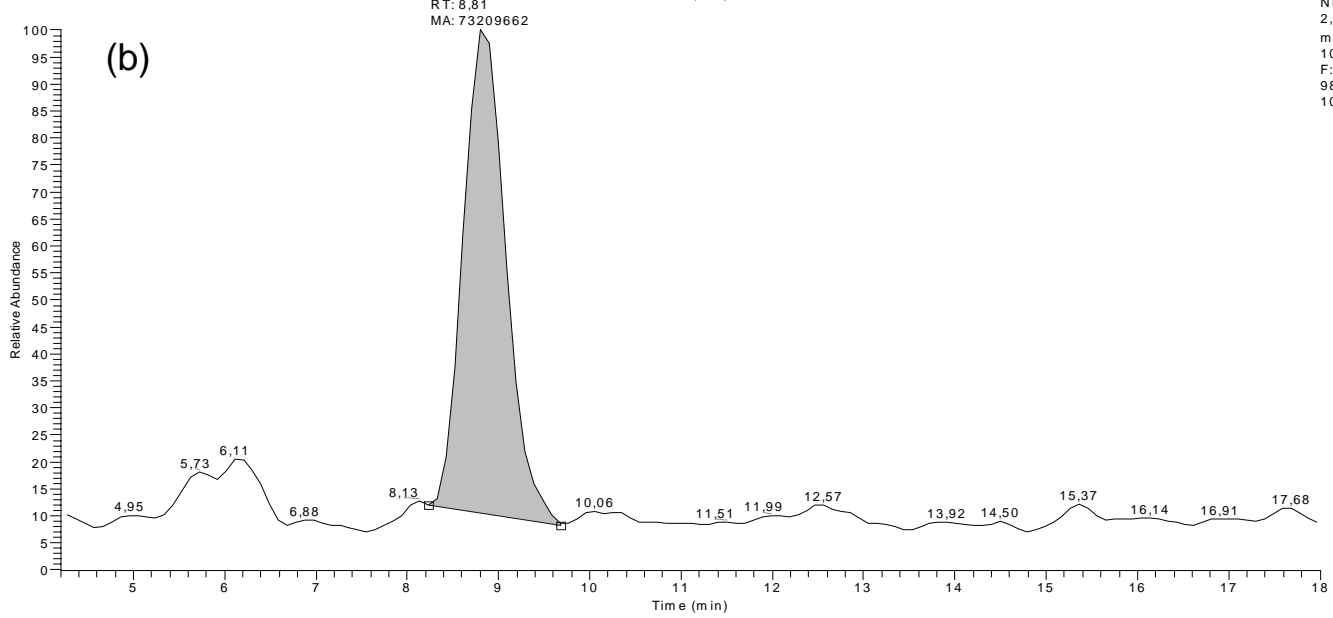
FIGURE 3

RT: 4.20 - 18.00 SM: 7G



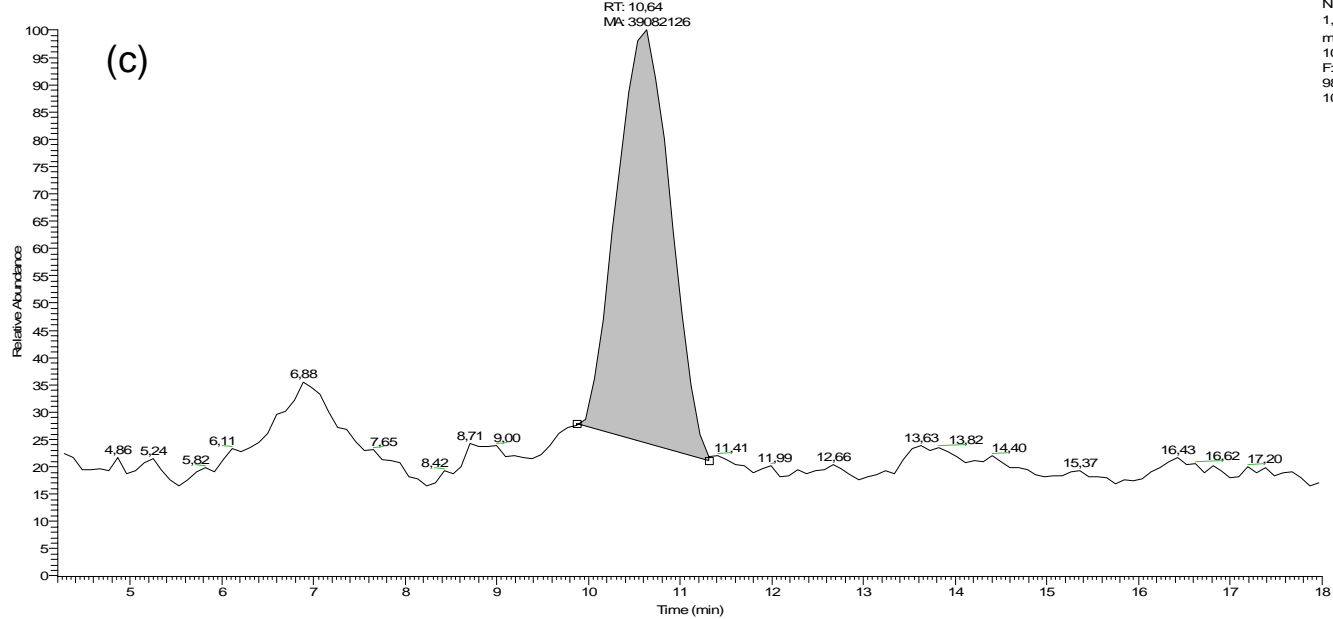
NL:
1,70E7
TIC F: + ESI
Z ms [
985,00-
1015,00]
MS
S

RT: 4.20 - 18.00 SM: 7G



NL:
2,52E6
m/z=
1003,9-1004,9
F: + ESI Z ms [
985,00-
1015,00] MS

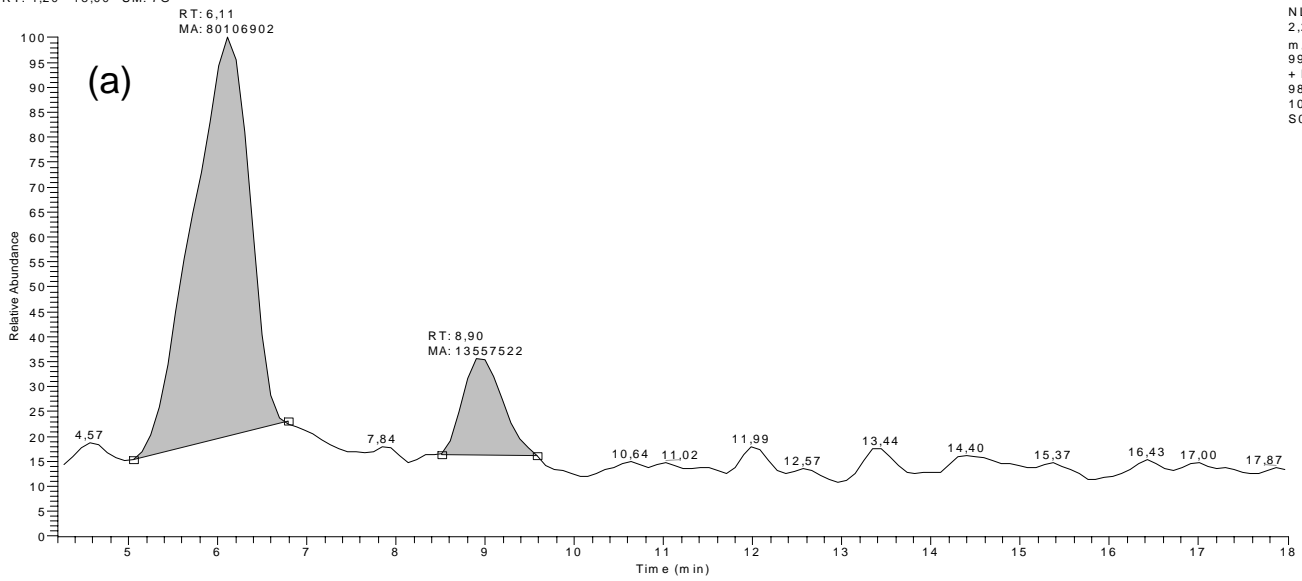
RT: 4.20 - 18.00 SM: 7B



NL:
1,26E6
m/z=
1010,9-1011,9
F: + ESI Z ms [
985,00-
1015,00] MS

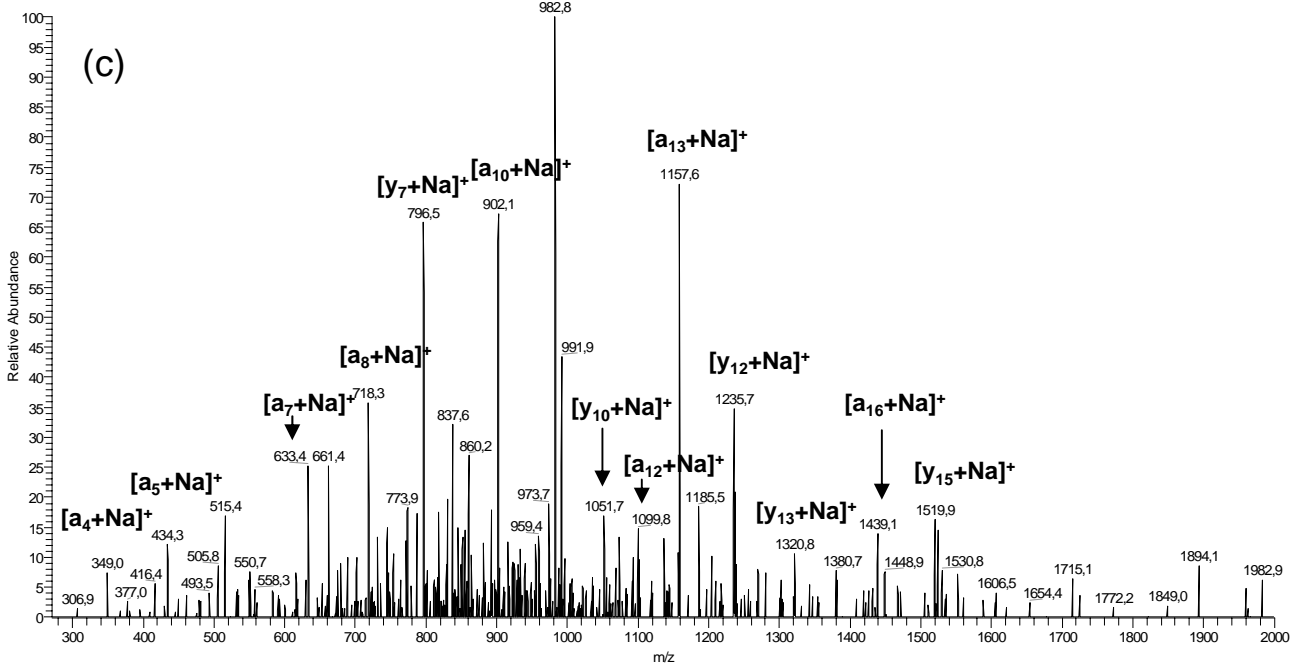
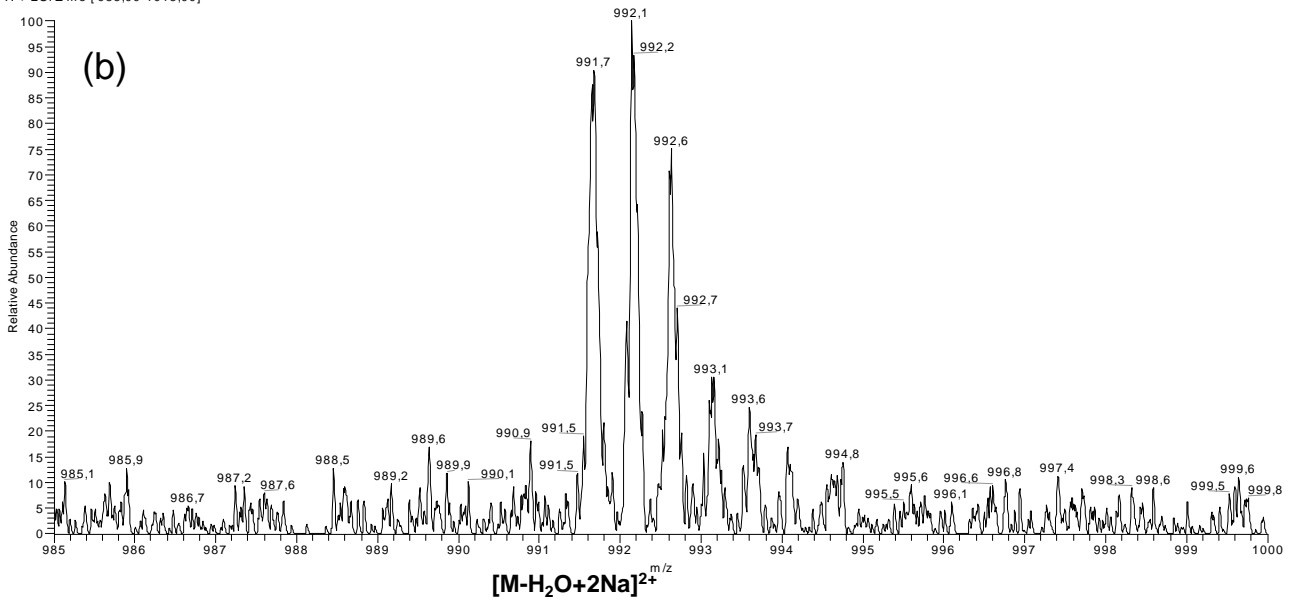
FIGURE 4

RT: 4.20 - 18.00 SM: 7G



NL:
2,25E6
m/z=
991.2-992.2 F:
+ ESIZ ms [
985.00-
1015.00] MS
S0317LP46

T: + ESIZ ms [985.00-1015.00]



1 **TABLES**

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5 **Table 1**

6 **Influence of the solvent mixture on alamethicin F50 extraction from sediments spiked at 10**
7 **ng/g.**

Tested solvents	Mean recovery (%) \pm SD (n=3)
a: dichloromethane / methanol (1:1, v/v)	8.7 \pm 0.0
b: methanol / TFA (0.1%, v/v)	1.9 \pm 0.0
c: ethanol / acetic acid (1%, v/v)	5.8 \pm 0.0
d: acetone / acetic acid (1%, v/v)	86 \pm 20
e: acetone / hydrochloric acid (0.02%, v/v)	64 \pm 9.0

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12 **Table 2**
13 **Matrix matched calibration: repeatability and accuracy.**

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Alamethicin Theoretical (ng/g of sediment)	Mean (ng/g of sediment) ± SD (n=6)	RSD (%)	Bias (%)
0.6	0.8±0.1	10	29
1.2	1.1±0.1	7	-8
2.3	2.2±0.2	9	-4
4.7	3.8±0.2	4	-18
9.4	9.5±0.2	2	2

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19 **Table 3**
20 **Spectral and chromatographic characteristics of peptaibols observed in surface sediment**
21 **samples from Fier d'Ars**

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Observed ions $[M+2Na]^{2+}$ (m/z)	Calculated M (u)	t_R (min)
991.2	1936.4	8.90
991.7	1937.4	6.11
998.2	1950.4	10.06
998.7	1951.4	6.98

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