TITLE:

Preliminary study for rapid determination of phycotoxins in microalgae whole cells using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOFMS).

SHORT TITLE

MALDI-TOFMS for phycotoxins detection in microalgae whole cells.

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Abstract

Rapid and sensitive methods for identification of several phycotoxins produced by microalgae species such as Yessotoxins (YTXs) for *Protoceratium reticulatum*, Okadaic acid (OA) and Pectenotoxins (PTXs) for *Prorocentrum* spp. and *Dinophysis* spp., Palytoxins (PLTXs) for *Ostreopsis* spp., Ciguatoxins (CTXs) for *Gambierdiscus* spp. or Domoic acid (DA) for *Pseudo-nitzschia* spp. are of great importance to shellfish and fish industry. In this study, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOFMS) was used to detect several phycotoxins in whole cells of some microalgae which are known as toxin producers. To achieve an appropriate MALDI matrix and a sample preparation method, several matrices and solvent mixtures were tested. The most appropriate matrix system for toxin detection was obtained with 10 µg μL^{-1} of DHB in 0.1 % TFA:ACN (3:7, v/v) by mixing the intact cells with the matrix solution directly on the MALDI target (dried-droplet technique). Toxin detection by this procedure is much faster than up-to-now procedures based on solvent extraction and chromatographic separation. This method allowed the rapid detection of main phycotoxins in some dinoflagellate cells of genus *Ostreopsis, Prorocentrum, Protoceratium, Gambierdiscus, Dinophysis* and diatoms from *Pseudo-nitzschia* genus.

Keywords: MALDI-TOFMS; microalgae; whole cells; phycotoxins.

INTRODUCTION

Marine phycotoxins are mainly produced by microalgae, which are consumed by shellfish as part of their natural diet. Thus, toxins are accumulated actively by shellfish during filter feeding. The bioaccumulation of marine phycotoxins by bivalve molluscs poses a health risk and creates serious disruptions for the aquaculture industry. Although for many toxins the producing microalgae are now known (Table 1), their causative organism has not always been clear and often required many years of intense study.[1-3] In the first studies of shellfish poisoning syndromes, it was not clear whether illness was caused by chemical or microbiological agents.[4, 5] Therefore, the different shellfish poisoning syndromes were initially classified based on the symptoms observed in humans after consumption of contaminated shellfish.[6-8]
Nowadays several assays[9-11] and analytical methods[12-14] exist for the detection and determination of phycotoxins in dinoflagellates. Most of these methods involve several extraction steps and frequently, more or less complex purification processes where the toxin presents in the original sample can be lost. In addition, chromatographic separation methods are relatively time-consuming. Therefore, to achieve rapid and sensitive methods for successful identification of phycotoxins in microalgae is of great relevance to shellfish and fish industry. Direct matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOFMS) analysis methods of bacterial or fungi cells[15-17] are widely used for the rapid and accurate identification of microorganisms based on their protein profiles. The speed of MALDI-TOFMS analysis makes this technique especially attractive for many applications.[18] The aim of this work was focused on to achieve a MALDI-TOFMS method for rapid and qualitative detection of phycotoxins in whole cells of different microalgae. This rapid method would be an alternative to classical methods, in order to provide control laboratories a rapid tool mainly for the analyses of microalgae complex field samples which pose difficulties for their morphological and taxonomic interpretation.

EXPERIMENTAL

Chemicals and standards

2,5-Dihydroxybenzoic acid (2,5-DHB), sinapinic acid (SA), α-cyano-4-hydroxy-cinnamic acid (HCCA), 2,3,4-trihydroxy-acetophenone, (2,3,4-THAP) and 2,4,6-trihydroxy-acetophenone (2,4,6-THAP) matrix substances for MALDI-MS were obtained from Bruker Daltonik (Bremen, Germany). Acetone 99.9 % and ammonium dihydrogen phosphate ($(NH_4)H_2PO_4$) were obtained from Sigma-Aldrich (Inc. Germany). Ethanol 96% (EtOH) was obtained from Merck (Darmstadt, Germany). Methanol (MeOH), water with 0.1 % trifluoroacetic acid (TFA) and acetonitrile (ACN) were obtained from Fluka (Inc. Germany). Peptide calibration standard (PSCII) was obtained from Bruker Daltonik (Bremen, Germany). Palytoxin (PLTX) from *Palythoa tuberculosa* (25 ng μL^{-1}, Mw 2680) was provided by Wako Chemicals (Wako Chemical Industries, Ltd., Japan). Yessotoxin (YTX) (17.65 ng μL^{-1}, Mw 1142) was isolated from cultures of *P. reticulatum* (strain GG1AM) by the staff of Toxic Phytoplankton group (Centro Oceanográfico in Vigo, Spain) and previously identified using an YTX standard solution provided by Professor M. Satake (Tohoku University, Sendai, Japan). Pectenotoxin 2 (PTX2) (430 ng μL^{-1}, Mw 859) was provided by Dr. P. Vale (IPIMAR,

Lisboa, Portugal). Okadaic acid (OA) (10 ng μL^{-1}, Mw 805) was extracted and provided by Dr. J. Fernández (IUBO, Tenerife, Spain) and previously identified using a certified standard solution purchased at NCR (Canada). Domoic acid (DA) (180 ng μL^{-1}, Mw 312) certified standard was purchased at NCR (Canada).

Microalgae samples

The most of the microalgae samples used throughout this work were obtained from the Phytoplankton Cultures Collection at the Centro Oceanográfico in Vigo (Spain) (CCVIEO). The species were selected because they produce potent biotoxins. Thus, *Ostreopsis* spp. produce PLTX, *Dinophysis* spp. and *Prorocentrum* spp. produce DSP toxins, *Protoceratium* spp. produce YTX, *Gambierdiscus* spp. produce ciguatoxins (CTXs) and *Pseudo-nitzschia australis* produce DA. Cultures were grown in 100 mL borosilicate glass bottles containing 50 mL of L1 medium without silicates under a irradiance of 150-170 µmol photons $m^{-2}s^{-1}$, on a 12:12 h light:darkness cycle and at a salinity of 34. Growth temperature varies among the different strains analyzed, so *Ostreopsis* genus strains were growth at 25 ±1 ºC; *Dinophysis tripos*, *Protoceratium* spp. and *Prorocentrum* spp. at 18.5 ±1º C; *Gambierdiscus* spp. at 27 ºC ±1ºC and *Pseudo-nitzschia australis* at 16.2 ºC ±1ºC. On the other hand, field samples were collected in four different dates at a fixed station which is a hot spot for *Dinophysis* (Bueu, P2, 42º 21.40´ N, 8º 46.42´ W) in Ría de Pontevedra (Spain).

Preparation of samples

(i) Culture samples. Two kinds of samples have been prepared from each culture, on the one hand a pellet with intact cells and on the other hand a MeOH extract of the cells: In order to obtain the pellet with cells, 10 mL aliquots from each culture were centrifuged at 1500 rpm for 10 minutes, then supernatant was removed and another 10 mL of culture were added over pellet and centrifuged again in the same conditions. The supernatant was removed and the wet pellet with cells was directly analyzed by MALDI-TOFMS. In order to obtain the MeOH extract of the cells, other 20 mL of each culture were filtered through 1.4 µm GF/C glass fibre filters (Whatman, Maidstone, UK). The filters with cells were re-suspended in 1.5 mL of 100 % MeOH (or 50 % MeOH for *Pseudo-nitzschia australis*). Then they were sonicated for 1 min to homogenize the suspension using a sonication probe 4710 of the Ultrasonic Homogenizer (Cole-Parmer, Chicago, IL, USA). Finally they were centrifuged at 7500 rpm for 10 min at 10 ºC. The supernatants were taken and the filters washed again with 1.5 mL of the corresponding solvent. The supernatants were combined, made up to 3 mL with the corresponding solvent and directly analyzed by MALDI-TOFMS.

(ii) Field samples. Dense concentrations of *Dinophysis* spp. obtained from field samples were collected with a submersible pump, with a flow of 138 L min^{-1} during 5 min, moored at 5 m depth. A final water concentrate of 5 L, that previously passed through a set of 20-77 µm superimposed meshes, was carried alive to the laboratory. Once at lab an aliquot of 400 mL was concentrated to 30 mL for toxins analysis. Then, two kinds of samples were prepared as described above for culture samples. In this case 10 mL of concentrate seawater were used to obtain cells and the other 20 mL to prepare the MeOH extract.

Sample preparation for MALDI-TOFMS analysis

During the optimization of the MALDI-TOFMS preparation different matrices, concentrations and solvent mixtures were tested (5 matrix compounds, 4 different concentrations and 2 matrix solvent mixtures). For details of the combinations tested see Table 2. The dried-droplet sample preparation technique was used; approximately a drop of cells or 2 µL MeOH extracts were directly spotted on the MTP AnchorChip TM 800/384 TF MALDI target, Bruker Daltonik (Bremen Germany) and, before sample dry, 2 µL of matrix solution were added and allowed to dry at room temperature. Four spots for each technique were prepared on the MALDI target from the same sample. Before analysis, two of them, were washed with ammonium dihydrogen phosphate 10 mM solution in 0.1 % TFA and the two others were directly analysed.
External mass calibration was performed with a peptide calibration standard (PSCII) for the range 700-3000 (9 mass calibrant points), 0.5 µL of calibrant solution and matrix previously mixed in an eppendorf tube (1:2, v/v) were applied directly on the target and allowed to dry at room temperature.

MALDI-TOFMS analysis

Mass spectra were recorded using an Autoflex III smartbeam MALDI-TOF mass spectrometer Bruker Daltonik (Bremen, Germany) operating in reflector positive or negative ion mode. Ions formed upon irradiation by a smartbeam1 laser using a frequency of 200 Hz. Each mass spectrum was produced by averaging 2500 laser shots collected across the whole sample spot surface by rastering in the range *m/z* 700-4000 (or 200-500 for DA). The laser irradiance was set to 45-50% (relative scale 0-100) arbitrary units according to the corresponding threshold required for the applied matrix systems. Low molecular ion gating was set to 650 Da (or 200 for DA) to remove the ions below this value arising from the matrix and their clusters or other unknown contaminants.
MALDI-TOF/TOFMS was performed on the parent mass selected for each toxin, at ion collision energy of 73 % (relative scale 0-100). Each MS/MS spectrum was acquired by averaging 1500-2000 laser shots collected across the whole sample preparation.

RESULTS AND DISCUSSION

To achieve the goal of rapid detection of phycotoxins directly in dinoflagellate cells an appropriate sample preparation method for MALDI-TOFMS was searched. Five of the most commonly matrices used for MALDI analysis,[16-17] HCCA, DHB, sinapinic acid, 2,3,4-THAP and 2,4,6-THAP, were evaluated. These matrices were dissolved in different solvent mixtures such as acetonitrile, methanol or acetone in pure form or mixed with 0.1% TFA (Table 2). Procedure adjustment was started with the PLTX standard because, among the studied toxins in this work, PLTX shows the most complex ion profile. ESI-MS analysis of PLTX produces multiple charged states (+1, +2 and +3), mixed cationizing species (Na^+, K^+,...) and ion series due to multiple losses of water molecules.[19] This may lead to many uncertainties in ion assignment, even for standard solutions of PLTX. MALDI-TOFMS allows the generation of singly charge

ions. The easier data interpretation caused by the singly charged molecules could make this technique suitable for the detection of PLTX in cells.[19]
To optimize toxin detection in dinoflagellate cells, the strain OS01BR of *Osteopsis ovata* was analyzed in comparison to PLTX standard. Therefore optimal conditions selected for PLTX standard and subsequently *Ostreopsis* samples were applied to analyze other toxins and dinoflagellates.

Palytoxins-ostreocins in *Ostreopsis* strains

The first MALDI matrix system tested with PLTX standard was HCCA in EtOH:acetone (1:1), at concentration [0.3 and 3 μg μL^{-1}] and positive ionization mode, but PLTX ions were not detected. Therefore the HCCA matrix was prepared in acetone 100 % at [3 μg μL^{-1}] and in these conditions some PLTX ions were detected. The second matrix evaluated was 2,5-DHB in 0.1 % TFA:ACN (1:1) or 0.1 % TFA:ACN (3:7) at [5 and 10 μg μL^{-1}]. Despite DHB matrix produces large and randomly distributed sample/matrix crystal layer, toxins were well detected in some of the crystals. Similar MS spectra were obtained for both matrices DHB and HCCA, but higher sensitivity was observed with DHB matrix than HCCA. For 2,3,4-THAP matrix similar sensitivity and spectra than DHB matrix were obtained. With the other matrices tested (table 2) the PlTX standard was not detected and more noise ions were presented, so this matrices were not used in further experiments. The best results were obtained with DHB [10 μg μL^{-1}] in 0.1 % TFA:ACN (3:7) (Fig. 1(a)). The sample preparation method selected was the dried droplet technique. In this procedure 1 μL of PLTX standard in MeOH 50 % and 2 μL of matrix were added to the MALDI target and then dried at room temperature. Inorganic salts, which interfere with the desorption-ionization process, were removed washing the final target spots with 10 mM ammonium dihydrogen phosphate in 0.1 % TFA. In this conditions MALDI-TOFMS spectra of PLTX standard shows a complex ion profile containing as main ion the *m/z* 2701 $[M+Na]^+$ together with other adduct ions at *m/z* 2557, 2665 $[M+H-H_2O]^+$, 2683 $[M+Na-H_2O]^+$, and 2718 $[M+K]^+$ (Fig. 1 (a); Table 3). Samples were also acquired in the negative ion mode, but PLTX ions were not detected.
Optimization of toxin detection in cells was accomplished with the *Ostreopsis ovata* strain OS01BR. The production of ostreocin by this strain was previously reported by LC-FLDMS.[20] Samples analyzed were MeOH extract and cells; each one was mixed with DHB matrix and analyzed by MALDI-TOFMS in the positive ionization mode. The MeOH extracts were prepared following the same method that PLTX standard (2 μL of MeOH extract and 2 μL of matrix), but for the strain OS01BR it was necessary to concentrate the MeOH extract 40x. The main ion detected with DHB matrix in positive mode was the *m/z* 2670 $[M+Na]^+$ being the characteristic *m/z* ion for ostreocin[20] which matches up with ovatotoxin-a described by Ciminiello.[21] For whole cells, the most simple sample preparation method in the MALDI target was selected, a drop of wet pellet of cells was applied directly on the target, following 2 μL of matrix were added and the mixture was allowed to dry at room temperature. We also tried to remove inorganic salts washing the final target spots with ammonium dihydrogen phosphate, but washing removed numerous cells from the target and ion detection drop. Using these conditions we can detect palytoxins from cells and/or MeOH extracts.
The method was applied to analyze other three *Ostreopsis* strains: VGO 1001, VGO 985 and VGO 1049, which results are summarized in Table 4. In all the samples ostreocins were searched first in MeOH extracts and after in cells. In the MeOH extract

of VGO 1049 strain the ion at *m/z* 2670 $[M+Na]^+$, characteristic for ovatotoxin-a,[21] was detected (Fig. 1(b)). This ion was also detected in strain VGO 1001 but it was necessary to concentrate these MeOH extracts 60x, in VGO 985 strain osceocins were not detected. In the case of cells, the ion at *m/z* 2670 $[M+Na]^+$ of ovatotoxin-a was well detected in the VGO 1049 strain (Fig. 1(c)), but was not detected in VGO 1001 and VGO 985 strains (Table 4).
Prominent ions at *m/z* 2701 $[M+Na]^+$ and 2683 $[M+Na-H_2O]^+$ were selected for MALDI-TOF/TOFMS fragmentation of PLTX standard. Main fragment signals detected for the parent ion at *m/z* 2701 were several ions in the *m/z* range 2552-2662 which are due to the lost of H_2O and Na^+. The most interesting fragment ions were obtained at *m/z* 327, 1175, 1416, 1474 and 1548, together with a group of ions in the *m/z* range 2243-2300 (Table 3 and supplementary material S1). Similar fragmentation pattern was obtained for parent ion at *m/z* 2683 (Table 3). Currently only the ion *m/z* 327 is a known fragment.[21]
For *Ostreopsis* strains the parent ion at *m/z* 2670 $[M+Na]^+$ characteristic for ovatotoxin-a was fragmented (Table 4). In MeOH extracts, the same fragmentation pattern that PLTX standard was obtained, the most abundant signal was detected in the range 2558-2613 due to the lost of H_2O and Na^+ and other fragments of interest were obtained at *m/z* 1159, 1233, 1459, 1533, 2306, but the fragment ion at *m/z* 327 was not detected (S1). The same fragments were obtained in cells of the VGO 1049 strain.
Concluding, PLTX and ostreocins were well detected by MALDI-TOFMS in positive ionization mode, with DHB matrix [10 μg μL^{-1}] in 0.1 % TFA:ACN (3:7) and applying one drop of wet cells or 2 μL MeOH extract directly onto the MALDI target. A new fragmentation pattern was also obtained PLTX and ostreocins by MALDI-TOF/TOFMS analysis.

Yessotoxins in *P. reticulatum* strains

Previous to *P. reticulatum* strains analysis the YTX standard was analysed, results were summarized in Table 3. For this toxin negative ionization mode and the same application method that PLTX with DHB matrix were used. The main ions detected were at *m/z* 1061 $[M-SO_3H]^-$ and 1163 $[M+Na-H]^-$ (Fig. 2(a)). Samples were also acquired in positive mode but YTX was no detected.
The same analytical conditions were applied with *P. reticulatum* strains VGO 905 and VGO 764. YTXs were well detected in both, MeOH extracts (Fig. 2(b)) and cells (Fig. 2(c)), for all the samples analyzed. The main ions detected in VGO905 strain were the *m/z* 1061 $[M-SO_3H]^-$ and 1163 $[M+Na-H]^-$ (Table 4) being characteristic ions of YTX.[22] In VGO764 strain the most prominent ions detected were the *m/z* 1075 $[M-SO_3H]^-$ and 1177 $[M+Na-H]^-$ in both MeOH extracts (Fig. 2(d)) and cells (Fig. 2(e)) which are characteristics of homoYTX.[22]
For MALDI-TOF/TOF study (S2) the ions at *m/z* 1061 $[M-SO_3H]^-$ and 1163 $[M+Na-H]^-$ of YTX standard were selected, which generate the typical fragmentation pattern of YTXs.[22] Fragmentation of the same parent ions in the VGO 905 strain yield the same fragments (Table 4 and S2). In the case of VGO 764 strain, which produces homoYTXs,[22] the selected ions were the *m/z* 1076 $[M-SO_3H]^-$ and 1177 $[M+Na-H]^-$. Daughter ions obtained in this case (Table 4 and S2) matched with homoYTX fragmentation pattern.[22]

Concluding, YTXs were well detected in both, MeOH extracts and cells of *P. reticulatum* strains by MALDI-TOF/TOF analysis with DHB matrix in negative ionization mode.

OA and derivatives in *P. belizeanum*, *P. lima* and *Dinophysis* samples

The first approach to OA and analogues detection was achieved with OA and PTX2 standards in positive ionization mode and DHB matrix, results are summarized in Table 3. The OA standard yield as main ion the *m/z* 827 $[M+Na]^+$ and a small peak at *m/z* 843 $[M+K]^+$ (Fig. 3(a)). For PTX2 standard the ions at *m/z* 881 $[M+Na]^+$ and 897 $[M+K]^+$ (Fig. 3(b)) were detected. Analysis was also performed in negative ionization mode, but only at high OA concentration a low intensity peak of the ion at *m/z* 803 $[M-H]^-$ was detected.

For MS/MS analysis (S3), the ions at *m/z* 827 $[M+Na]^+$ for OA and 881 $[M+Na]^+$ for PTX2 standards were fragmented and data summarized in Table 3. Main fragments obtained for *m/z* 827 $[M+Na]^+$ were the expected for OA.[23, 24] Prominent daughter ions detected for PTX2 standard were in agreement with fragmentation of $[M+Na]^+$ ion of PTX2.[25]

Analysis of *P. belizeanum* strain PBMA01 in positive ionization mode with DHB matrix revealed the presence of OA in both, MeOH extract and cells (Table 4). In the MeOH extract the ions at *m/z* 827 $[M+Na]^+$ and 843 $[M+K]^+$ for OA were detected simultaneously with other unknown ions in the range *m/z* 700-1200, this made it difficult to identify OA and derivatives in this *m/z* range (Fig. 4(a)). These ion signals at *m/z* 800 are due to matrix and sample noise. Cells of *P. belizeanum* strain PBMA01 yield the characteristic peaks for OA ions at *m/z* 827 $[M+Na]^+$ and 843 $[M+K]^+$ together with other minor unknown signals at *m/z* 871 and 940 (Fig. 4(b)). It was observed that OA ions were cleaner detected in cells than in MeOH extract (Figs. 4(a) and 4(b)).

In the MALDI-TOF/TOF analysis of both , MeOH extracts and cells of *P. belizeanum* strain, the ion at *m/z* 827 $[M+Na]^+$ was selected, the daughter ions obtained were the same that OA standard 723 and 571 (Table 4 and S4). The prominent ions at *m/z* 800 and 940 were also fragmented but characteristic ions for OAs were not obtained.

In *P. lima* strain PLRN02 a small signal of the ion at *m/z* 827 $[M+Na]^+$ for OA was detected in both, MeOH extract (Fig. 4(c)) and cells (Fig. 4(d)), together with other higher signals characteristics of possible OA derivatives (Table 4). It is, in MeOH extract *m/z* 962 and 980 with similar *m/*z that 7-hydroxy-2,4-dimethyl-hepta-2,4-dienyl okadaate (C9-diolOA)[23, 26] and 5,7-dihydroxy-2,4-dimethylene-heptyl okadaate (C9-triolOA), respectively[24, 26] and in cells *m/z* 996 probably being the ion $[M+Na]^+$ of the 5-hydroperoxy-7-hydroxy-2,4-dimethylene-heptyl okadaate.[26] Fragmentation of *m/z* 827 $[M+Na]^+$ in MeOH extract and cells (S4) yield the same ions than OA standard. Other intense ions were also fragmented; in MeOH extract *m/z* 962 > 214, 343, 589, 723, 787, 826 and 980> 135, 344, 362, 590, 804; in cells the ion at *m/z* 996> 134, 377, 822, 961. These fragmented ions in *P. lima* samples do not generate conventional ions, only for the parent ion at *m/z* 962 a small signal at *m/z* 723 for OA derivatives was detected (S4).

In a *D. tripos* laboratory culture sample (Table 4), the ions at *m/z* 881 $[M+Na]^+$ and 897 $[M+K]^+$, previously found in the PTX2 standard analyzed, were detected in MeOH extract (Fig. 5(a)) and cells (Fig. 5(b)). In this *D. tripos* sample, ions were better detected in cells than in the MeOH extract. Until now, there was only one report of Lee et al (1989) about the presence of toxin in *D. tripos*.[4] In that work DTX1 was detected

in extracts previously fluorescence-labelled with 9-anthryl-diazomethane (ADAM) by fluorometric HPLC analysis. Moreover a small peak at *m/z* 979 $[M+Na]^+$ was also detected in cells being a possible unknown OA-D8+C_2H_4 previously found by Suzuki et al. in *D. acuta*.[27]
Confirmation of PTX2 detection was performed by MALDI-TOF/TOF. Ions at *m/z* 539 and 837 detected in PTX2 standard were also found in MeOH and cells samples (S5). In the other hand the small signal detected for the ion *m/z* 979 did not let a good fragmentation.
In the *Dinophysis* field concentrate sample containing *D. caudata* and *D. acuta* (Table 4), the ions at *m/z* 881 $[M+Na]^+$ and 897 $[M+K]^+$ characteristics for PTX2 were detected. Ions were better detected in MeOH extract (Fig. 6(a)) than in cells (Fig. 6(b)). Other unknown ions in the range *m/z* 800-900, which probably are matrix and sample noise ions, were detected (Figs. 6(a) and 6(b)). The MALDI-TOF/TOF analysis confirmed the detection of PTX2 in MeOH and cells (S6), because fragmentation of *m/z* 881> 539 and 837 (Table 4) gave the same fragmentation pattern than PTX2 standard (Table 3).
In other *Dinophysis* filtrate harvested in different dates and containing *D. acuminata* and D. *caudata* (Table 4; Figs. 6 (c)-6(e)), a small signal of OA and PTX2 ions was only detected in the MeOH extract of field sample 3 (Fig. 6(e)). Due to the low intensity of ions detected it was not possible isolate parent ions for MALDI-TOF/TOF fragmentation.
Summarizing, analysis of OA and derivatives must be approach in positive ionization mode and DHB matrix. Ions of OA and derivatives in samples have similar *m/z* than matrix or sample noise; therefore it is difficult to detect this kind of toxins in samples with low levels of toxins. OAs were detected in both MeOH extract and cells of *P. belizeanum*, *P. lima* strains and *Dinophysis* concentrates. In some samples toxins were better detected in cells than in MeOH extracts.

Ciguatoxins in *Gambierdiscus* strains

The mass of ions detected for *Gambierdiscus* samples was compared with the values reported in the bibliography.[28, 29] In the MeOH extract of *Gambierdiscus* sp. VGO 1022 strain (Table 4), the main ion detected was the *m/z* 1079.6 together with a small peak at *m/z* 1101.5 corresponding with the $[M+H]^+$ and $[M+Na]^+$ ions, respectively for L (or M?)-seco-CTX-4A or -4B.[28] Moreover, the ion at *m/z* 1063.6 $[M+Na]^+$ for L (or M?)-seco-CTX-3C or -3B[28] was also detected (Fig. 7(a)). In samples from this dinoflagellate CTXs were detected in the MeOH extract but not in cells (Fig. 7(b)).
In the MeOH extract of *G. excentricus* VGO 790 strain (Table 4), the main ion detected was at *m/z* 1026.7 which not matches with the mass of any known CTXs,[28, 29] but could be an unknown CTX. Together with this ion other small signals for known CTXs were detected at *m/z* 1079.6 $[M+H]^+$ and 1101.6 $[M+Na]^+$ for L (or M?)-seco-CTX-4A or -4B, the *m/z* 1042.6 $[M+H]^+$ for L (or M?)-seco-CTX-3C or -3B and 1117.6 $[M+Na]^+$ for tetraseco-CTX-3C or -3B (Fig. 7(c)). In the analysis of cells only a low signal of the ions at *m/z* 1042.6 and 1079.6 was detected (Fig. 7(d)).
For MALDI-TOF/TOF analysis of potential CTX in *Gambierdiscus* sp. VGO 1022 strain, the ion at *m/z* 1079.6 $[M+H]^+$ was fragmented. Fragments obtained were at low intensity 132, 490, 948 and 1057 (Table 4)**.** Other detected ions were not fragmented due the low signal obtained for parent ion. For *G. excentricus* the ion at *m/z* 1026

$[M+Na]^+$ was fragmented although the obtained peaks (Table 4) do not matched with bibliography standards.[28]
Concluding, for *Gambierdiscus* spp. possible CTXs were well detected in the MeOH extract but not in cells.

Domoic acid in *Pseudo-nitzchia australis* strain

In the first DA standard analysed (12 ng) in positive ionization mode the main ion detected was at *m/z* 312 $[M+H]^+$,[30] but as it was expected this ion appear among other matrix noise ions, therefore a higher concentration of DA standard was used 180 ng (Table 3) in this case the ion was better detected but also in the middle of matrix noise (Fig. 8(a)). Subsequently in the *Pseudo-nitzchia australis* strain VGO 1045 the DA was not well detected neither MeOH extract nor in cells, in this case the noise ions were incremented with the noise signal of sample (Figs. 8(b) and 8(c)). This result confirms that, as it was expected, the *m/z* 312 $[M+H]^+$ is a very low mass for MALDI-TOF analysis. For DA it would be necessary to optimize a new procedure.
For MALDI-TOF/TOF analysis DA standard at *m/z* 312 $[M+H]^+$ was fragmented detected ions were summarized in Table 3. In the case of potential DA in *Pseudo-nitzchia australis* fragments were not detected due the low signal of the parent ion at *m/z* 312 $[M+H]^+$.

CONCLUSION

The aim of this study was the rapid and qualitative detection of marine phycotoxins in whole cells of various microalgae using MALDI-TOFMS. Results obtained with available standards, MeOH extracts and microalgae cells were successfully for some microalgae and toxin groups. MALDI-TOFMS of microalgae whole cells can be performed yielding an ion spectrum containing in main cases only the ion toxin. It was shown that MALDI-TOFMS analysis is able to detect toxins ions with mass above 800 Da from both, cells and/or MeOH extracts. For toxins under 800 Da such as DA it would be necessary to optimize a new procedure. Sample preparation is very simple and toxin detection by this MALDI-TOFMS procedure is much faster than up-to-now procedures based on solvent extraction and chromatographic separation. This rapid and qualitative detection method is a promising alternative that may allow make quick decisions in routine shellfish monitoring programs, detecting some potentially toxic algae blooms without having to resort to tedious taxonomic determinations and sometimes difficult to contrast.

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Table 1. Toxin groups, human poisoning associated and their microalgal origin.

Toxin group	Lipophilicity	Human Poisoning*	Microorganism associated
Saxitoxin Neosaxitoxin	Hydrophilic	PSP	*Aphanazomenon flosaqua, Anabaena, Oscillatoria* (Cyanobacteria)**
			Alexandrium spp., *Gymodinium catenatum, Pyrodinium bahamense*
Domoic acid	Hydrophilic	ASP	*Pseudo-nitzschia* spp. (Diatom)**
Okadaic acid	Lipophilic	DSP	*Dinophysis* spp., P*rorocentrum* spp.
Pectenotoxin	Lipophilic	-	*Dinophysis* spp.
Brevetoxin	Lipophilic	NSP	*Karenia brevis*
Gymnodimine	Lipophilic	-	*Karenia selliformis*
Spirolids	Lipophilic	-	*Alexandrium ostenfeldii*
Azaspiracid	Lipophilic	AZP	*Azadinium spinosum*
Ciguatoxin Maitotoxin	Lipophilic	CFP	*Gambierdiscus* spp.
Yessotoxin	Amphiphilic	-	*Protoceratium reticulatum, Lingulodinium polyedrum, Gonyaulax spinifera*
Palytoxin	Amphiphilic	Palytoxin poisoning	*Ostreopsis* spp.

*PSP (paralytic shellfish poisoning), ASP (amnesic shellfish poisoning), DSP (diarrhetic shellfish poisoning), NSP (neurotoxic shellfish poisoning), AZP (azaspiracid poisoning), CFP (ciguatera fish poisoning).
**Most phycotoxins are produced by dinoflagellates, except saxitoxin which is also produced by certain cyanobacteria and domoic acid produced by the diatom *Pseudo-nitzschia* spp.

Table 2. Matrix material. Concentrations and solvent mixtures tested in the MALDI-TOFMS analysis.

Matrix tested	Concentration	Solvent
α-cyano-4-hydroxy-cinnamic acid (HCCA)	0.3 and 3 μg μL^{-1}	EtOH:acetone (1:1)
	3 μg μL^{-1}	Acetone 100 %
2,5-dihydroxybenzoic acid (DHB)	5 and 10 μg μL^{-1}	0.1 % TFA:ACN (1:1)
	5 and 10 μg μL^{-1}	0.1 % TFA:ACN (3:7)
2,3,4-trihydroxyacetophenone (2,3,4-THAP)	10 μg μL^{-1}	0.1 % TFA:ACN (1:1)
2,4,6-trihydroxyacetophenone (2,4,6-THAP)	10 μg μL^{-1}	0.1 % TFA:ACN (1:1)
Sinapinic acid (SA)	10 μg μL^{-1}	0.1 % TFA:ACN (1:9)

Table 3. MALDI-TOFMS results for analyzed standards.

Toxin	Teoric MW	MALDI mode	Main MALDI-TOF ions detected	MALDI-TOF/TOF ions detected Parent ion > daughter ions
PLTX	2680	+	2701 $[M+Na]^+$	2701> 327, 1175, 1416, 1474, 1548 2243-2300; **2552-2662**
			2683 $[M+Na-H_2O]^+$	2683> 327, 1175, 1416, 1474, 1548 2243-2300; **2532-2629**
YTX	1142	-	1061 $[M-SO_3H]^-$	1061> 713, **856**, 925
			1163 $[M+Na-H]^-$	1163> 815, **957**, 1026, 1149
OA	805	+	827 $[M+Na]^+$	827> 571, 705, **723**, 781, 807
PTX2	859	+	881 $[M+Na]^+$	881> **538**, 726, **837**
DA	312	+	312 $[M+H]^+$	312> 193, 248, 266

MS values were rounded up. Most abundant daughter ions in each spectrum are denoted by bold values.

Table 4. Microalgae samples, origin country, sample type and MALDI-TOFMS results.

Sample	Origin country	Sample type	MALDI Mode	Main MALDI-TOF ions detected	MALDI-TOF/TOF ions detected [b]	Toxin
Ostreopsis ovata (OS01BR)[a]	Rio de Janeiro, Brasil	MeOH extract	+	2670 $[M+Na]^+$	2670> 1159, 1233, 1459, 1533, 2306, 2558-2628	Ostreocins
		Cells	+	2670 $[M+Na]^+$ traces		Ostreocins
Ostreopsis ovata (VGO 1001)[a]	Lanzarote Island, Spain	MeOH extract	+	2670 $[M+Na]^+$	-	Ostreocins
		Cells	+	-	-	Ostreocins
Ostreopsis siamensis (VGO 985)[a]	Girona, Spain	MeOH extract	+	-	-	Ostreocins
		Cells	+	-	-	Ostreocins
Ostreopsis ovata (VGO 1049)[a]	Llavaneras, Barcelona, Spain	MeOH extract	+	2670 $[M+Na]^+$	2670> 1159, 1233, 1459, 1533, 2306, 2558-2628	Ostreocins
		Cells	+	2670 $[M+Na]^+$		Ostreocins
Protoceratium reticulatum (VGO 905)[a]	Pontevedra, Spain	MeOH extract	-	1061 $[M-SO_3H]^-$, 1163 $[M+Na-H]^-$	1061> 713, 855, 924, 1049	YTXs
		Cells	-	1061 $[M-SO_3H]^-$, 1163 $[M+Na-H]^-$	1163> 815, 957, 1026, 1149	YTXs
Protoceratium reticulatum (VGO 764)[a]	Delta del Ebro, Spain	MeOH extract	-	1075 $[M-SO_3H]^-$, 1177 $[M+Na-H]^-$	1075> 727, 869, 938	homoYTXs
		Cells	-	1075 $[M-SO_3H]^-$, 1177 $[M+Na-H]^-$	1177> 829, 971, 1040	homoYTXs
Prorocentrum belizeanum (PBMA01)[a]	Mayotte Island, France	MeOH extract	+	827 $[M+Na]^+$, 843 $[M+K]^+$, 940?	827> 571, 723, 763, 781, 799	OAs
		Cells	+	827 $[M+Na]^+$, 843 $[M+K]^+$	827> 571, 723, 781	OAs
Prorocentrum lima (PLRN02)[a]	Réunion Island, France	MeOH extract	+	827 $[M+Na]^+$, 962?, 980?	827> 571, 723, 781 962> 214, 343, 589, 787, 826 980> 135, 344, 362, 590, 804	OAs
		Cells	+	827 $[M+Na]^+$, 962?, 996 ?	996> 134, 377, 822, 961	OAs
Dinophysis tripos (not CCVIEO code)	Galicia, Spain	MeOH extract	+	881 $[M+Na]^+$	881> 727, 837, 858	PTXs
		Cells	+	881 $[M+Na]^+$, 897 $[M+K]^+$, 979?	881> 698, 727, 837, 858 979> 821, 934, 962	PTXs
D. caudata + *D. acuta* Field Sample	Bueu, Galicia, Spain	MeOH extract	+	881 $[M+Na]^+$, 897 $[M+K]^+$	881> 184, 539, 743, 837	PTXs
		Cells	+	881 $[M+Na]^+$, 897 $[M+K]^+$	881> 539, 726, 837, 858	PTXs
D. acuminata+D. caudata Field Sample 1	Bueu, Galicia, Spain	MeOH extract	+	-	-	OAs
D. acuminata+D. caudata Field Sample 2	Bueu, Galicia, Spain	MeOH extract	+	-	-	OAs
D. acuminata+D. caudata Field Sample 3	Bueu, Galicia, Spain	MeOH extract	+	881 $[M+Na]^+$, 827 $[M+Na]^+$	-	OAs, PTXs
Gambierdiscus spp (VGO1022)[a]	Gran Canaria Island, Spain	MeOH extract	+	1063 $[M+Na]^+$, 1079 $[M+H]^+$, 1101 $[M+Na]^+$	1079> 132, 490, 948, 1057	CTXs
		Cells	+	1101 $[M+Na]^+$ traces	-	CTXs
Gambierdiscus excentricus (VGO790)[a]	Tenerife Island, Spain	MeOH extract	+	1026?, 1042$[M+H]^+$, 1079$[M+H]^+$, 1101$[M+Na]^+$	1026> 497, 914, 1008	CTXs
		Cells	+	1026?, 1042$[M+H]^+$, 1079$[M+H]^+$	-	CTXs
Pseudo-nitzschia australis (VGO 1045)[a]	Galicia, Spain	MeOH 50% extract	+	312 $[M+H]^+$ traces	-	DA
		Cells	+	312 $[M+H]^+$ traces	-	DA

[a] CCVIEO code for dinoflagellate strains from the phytoplankton cultures, [b] Parent ion> daughter ions. MS values were rounded up.

Figure 1. MALDI-TOFMS of palytoxin standard (a), *Ostreopsis ovata* VGO 1049 strain MeOH extract (b) and whole cells (c) obtained in positive ionization mode with DHB matrix.

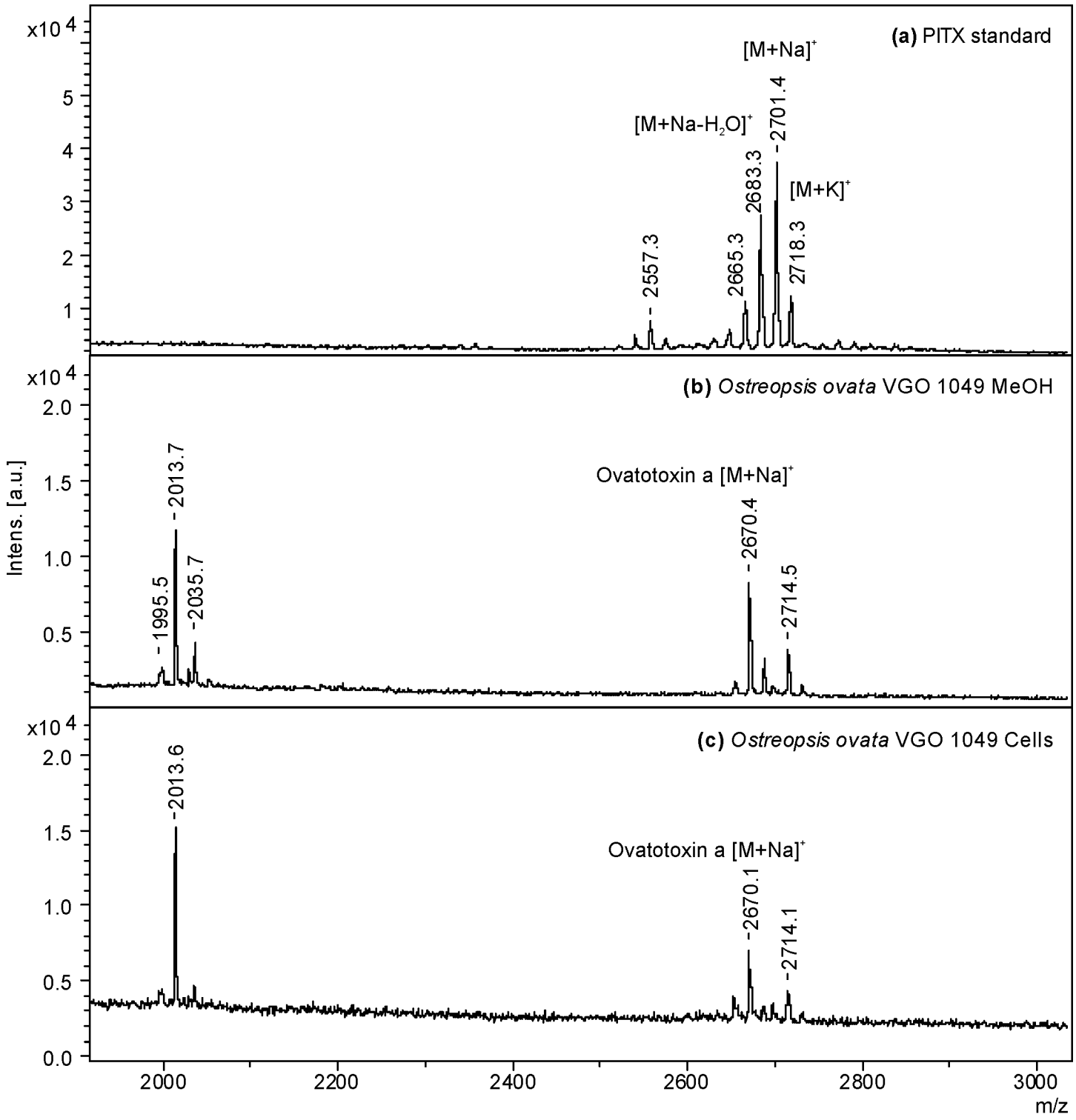


Figure 2. MALDI-TOFMS of YTX standard (a), *Protoceratium reticulatum* strain VGO 905 MeOH extract (b) and cells (c); and strain VGO 764 MeOH extract (d) and whole cells (e). Spectra were acquired in negative ionization mode with DHB matrix.

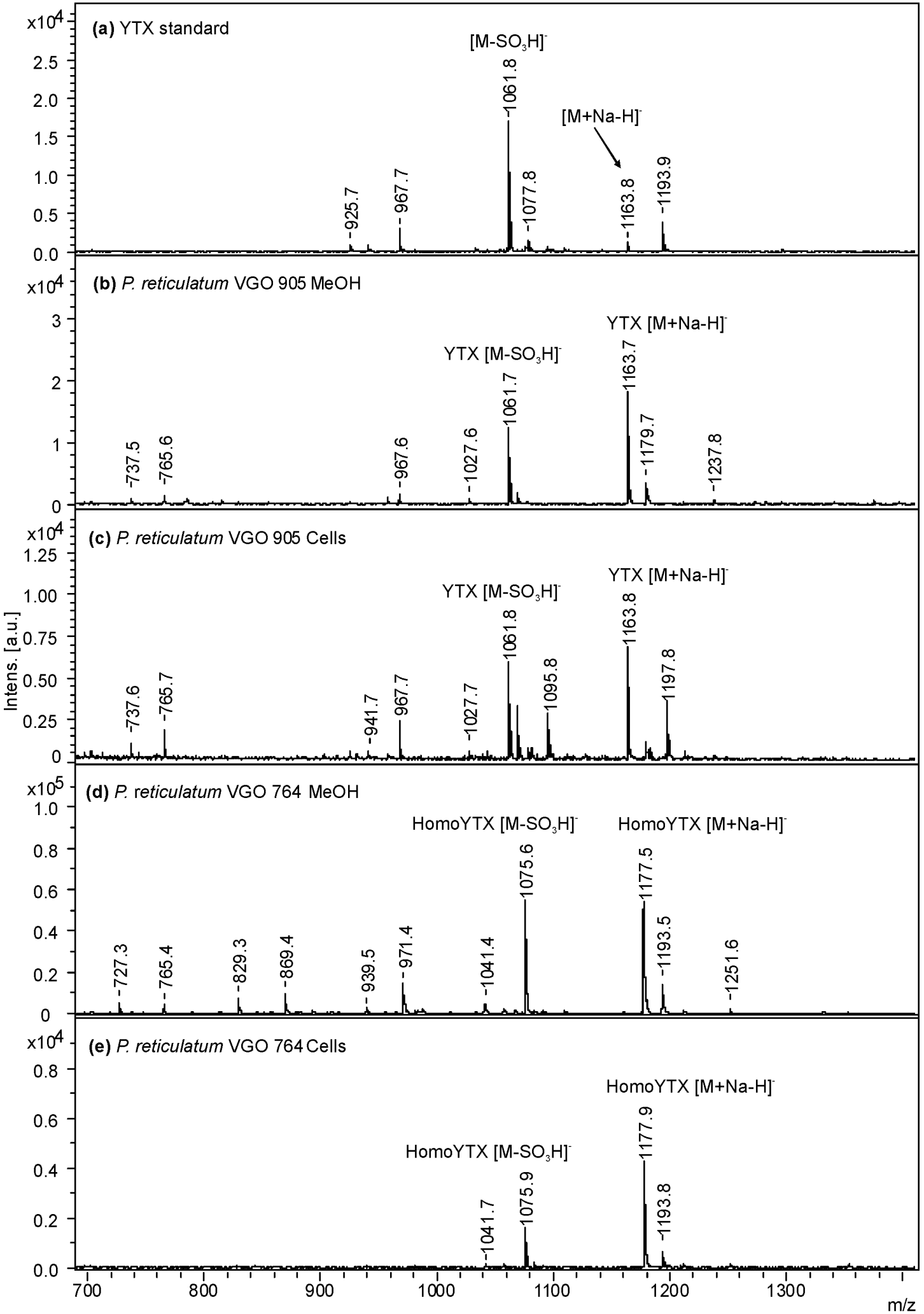


Figure 3. MALDI-TOFMS of OA (a) and PTX2 (b) standards obtained in positive ionization mode with DHB matrix.

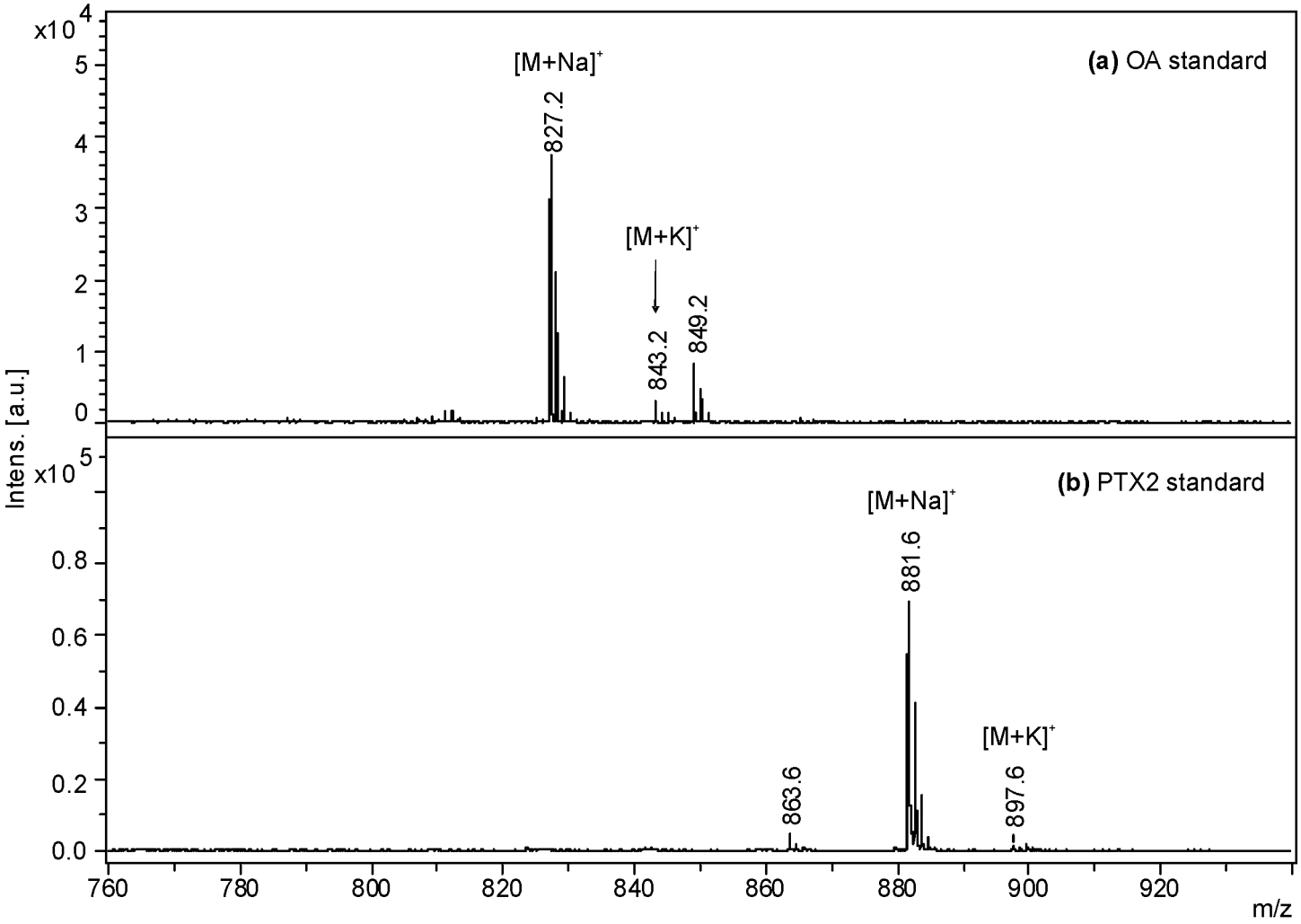


Figure 4. MALDI-TOFMS of *Prorocentrum belizeanum* PBMA01 strain in MeOH extract (a) and whole cells (b); and *P. lima* strain PLRN02 in MeOH (c) and whole cells (d). Spectra were acquired in positive ionization mode with DHB matrix.

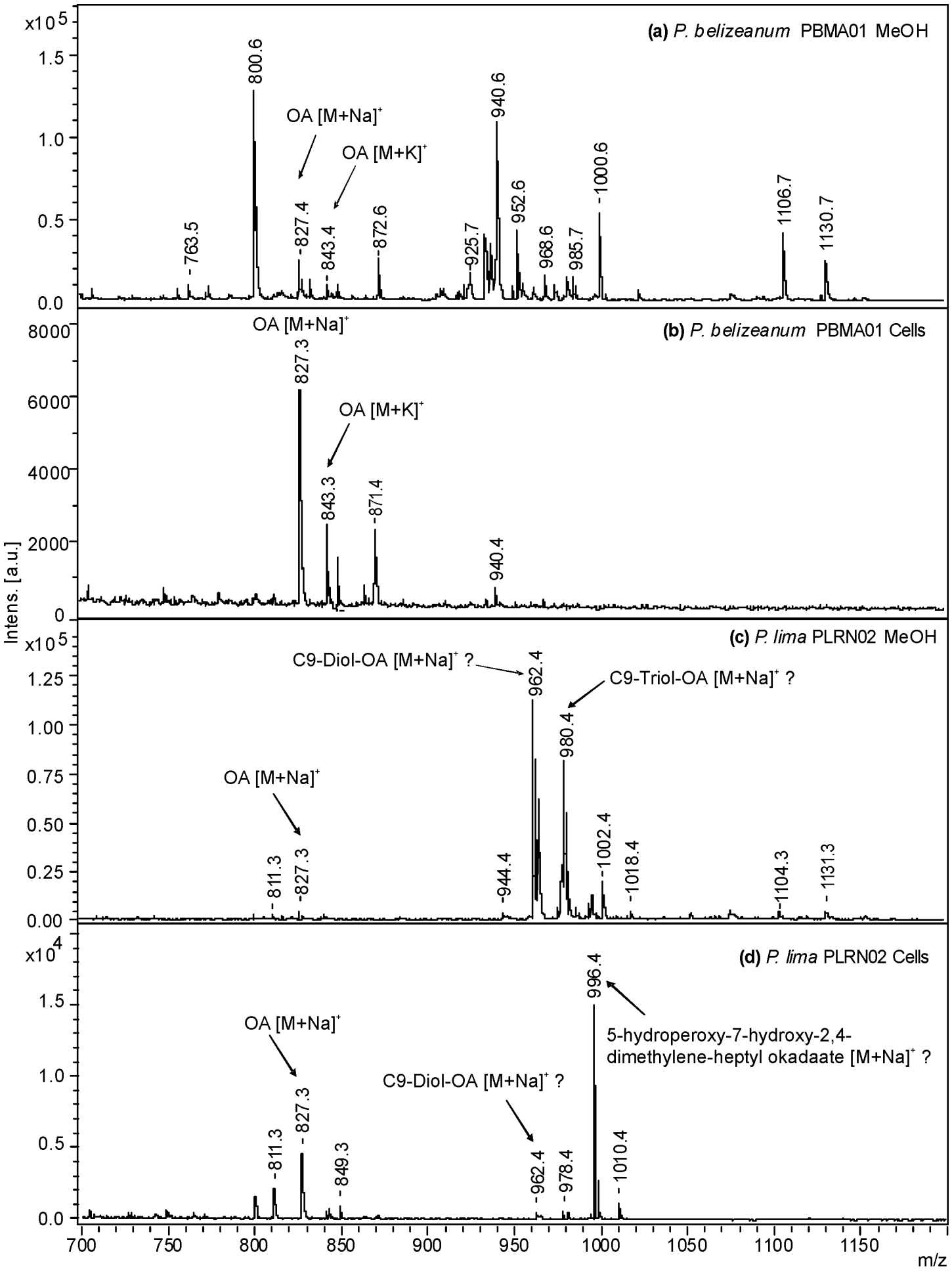


Figure 5. MALDI-TOFMS of *Dinophysis tripos* strain for MeOH extract (a) and whole cells (b) obtained in positive ionization mode with DHB matrix

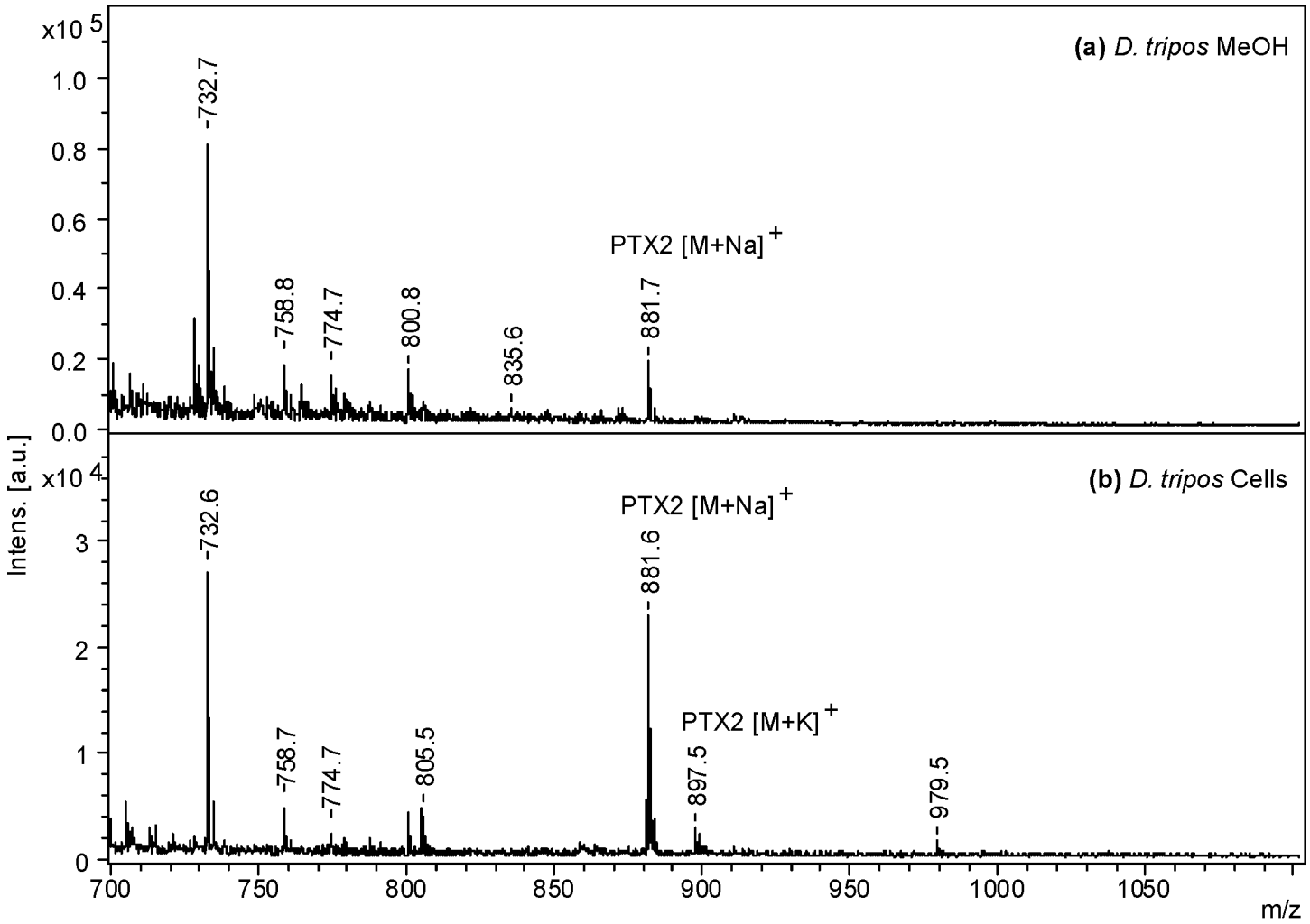


Figure 6. MALDI-TOFMS of a *Dinophysis* filtrate (containing *D. caudata* and *D. acuta*) for MeOH extract (a) and whole cells (b); and MeOH extracts of *Dinophysis* filtrates 1 (c), 2 (d) and 3 (e) (containing *D. acuminata* and *D. caudata*) collected in different dates. Data were acquired in positive ionization mode and DHB matrix.

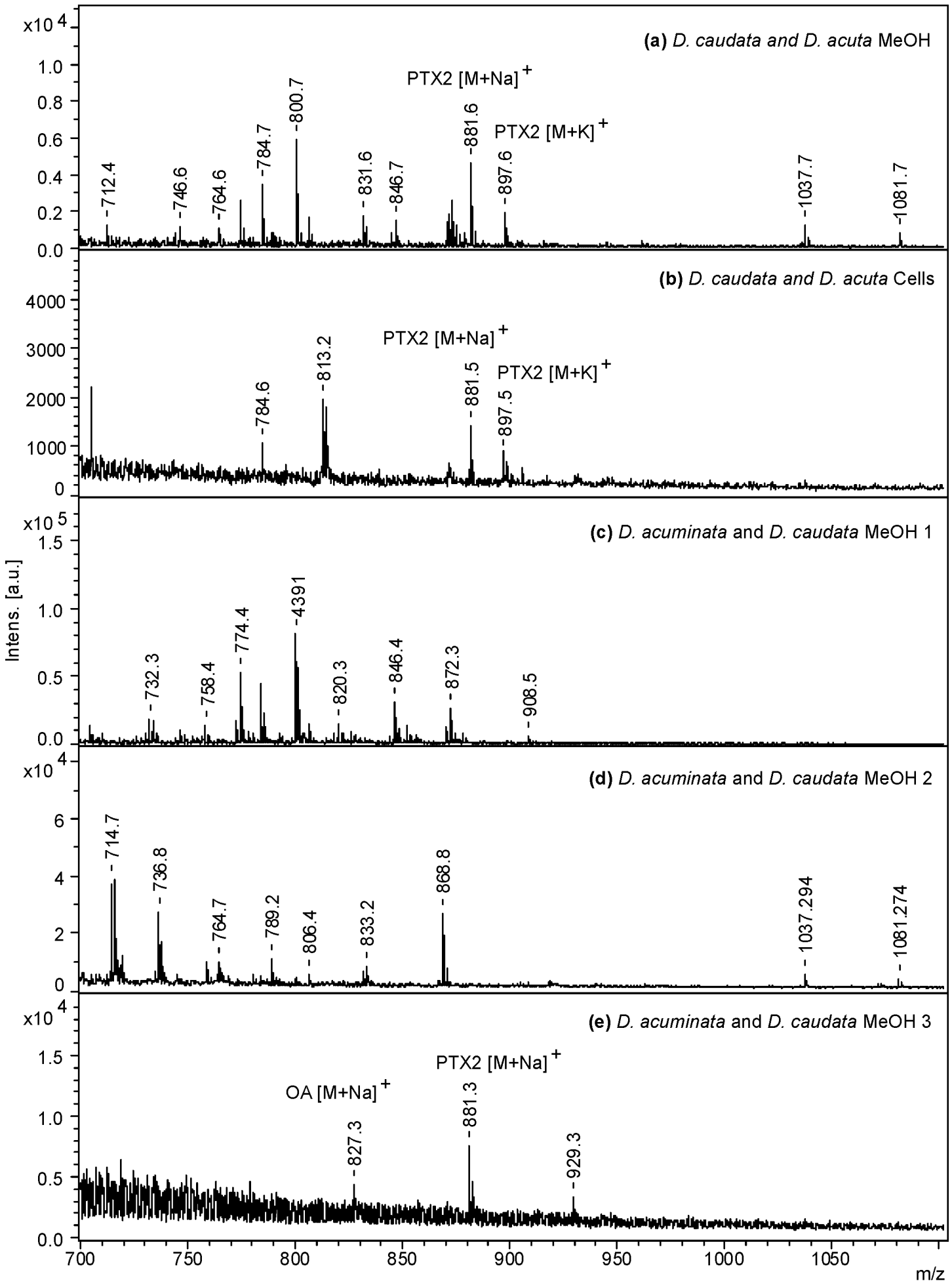


Figure 7. MALDI-TOFMS of *Gambierdiscus* spp. strains VGO 1022 MeOH extract (a) and cells (b); and *G. excentricus* VGO 790 MeOH extract (c) and whole cells (d) obtained in positive ionization mode with DHB matrix.

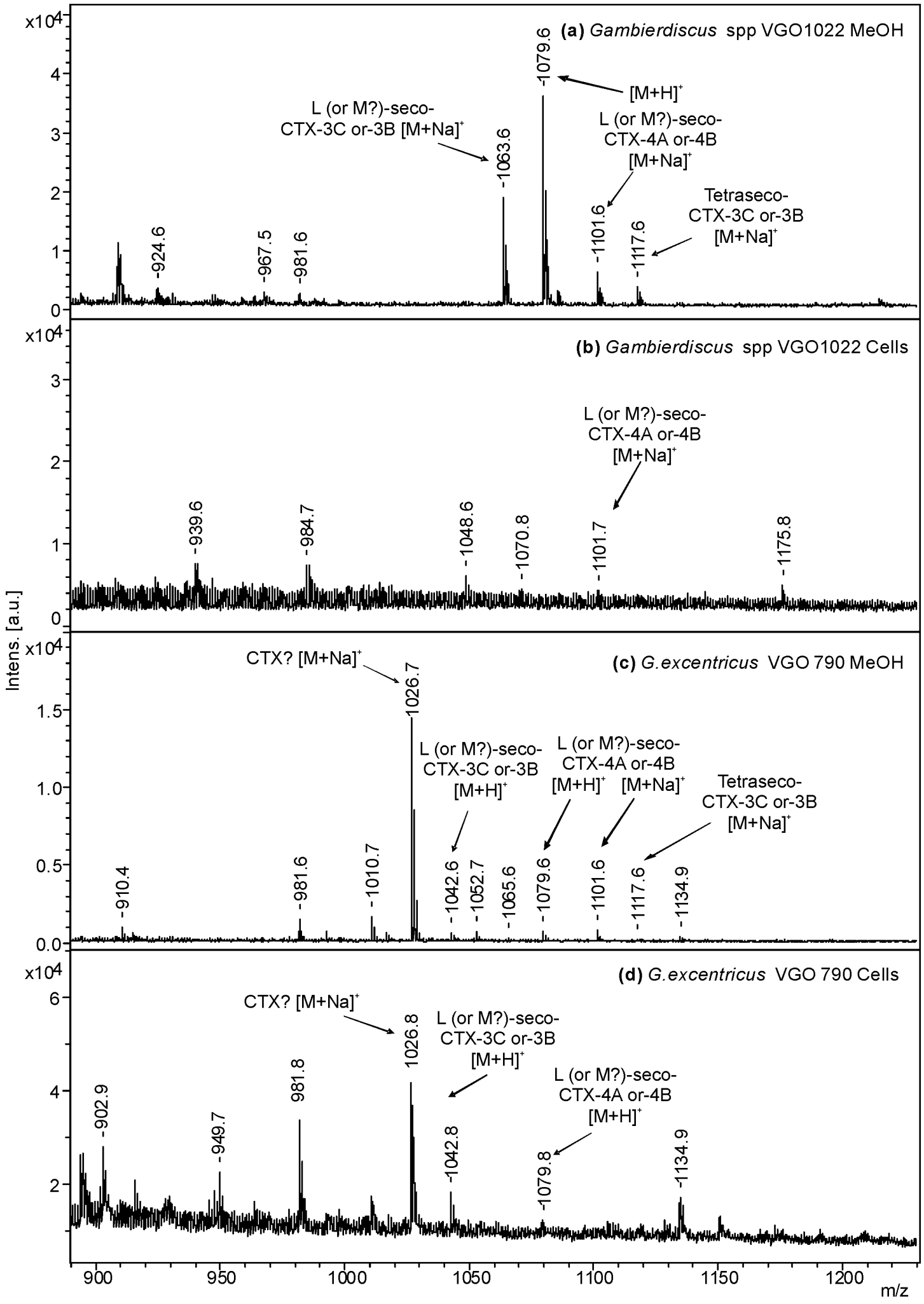
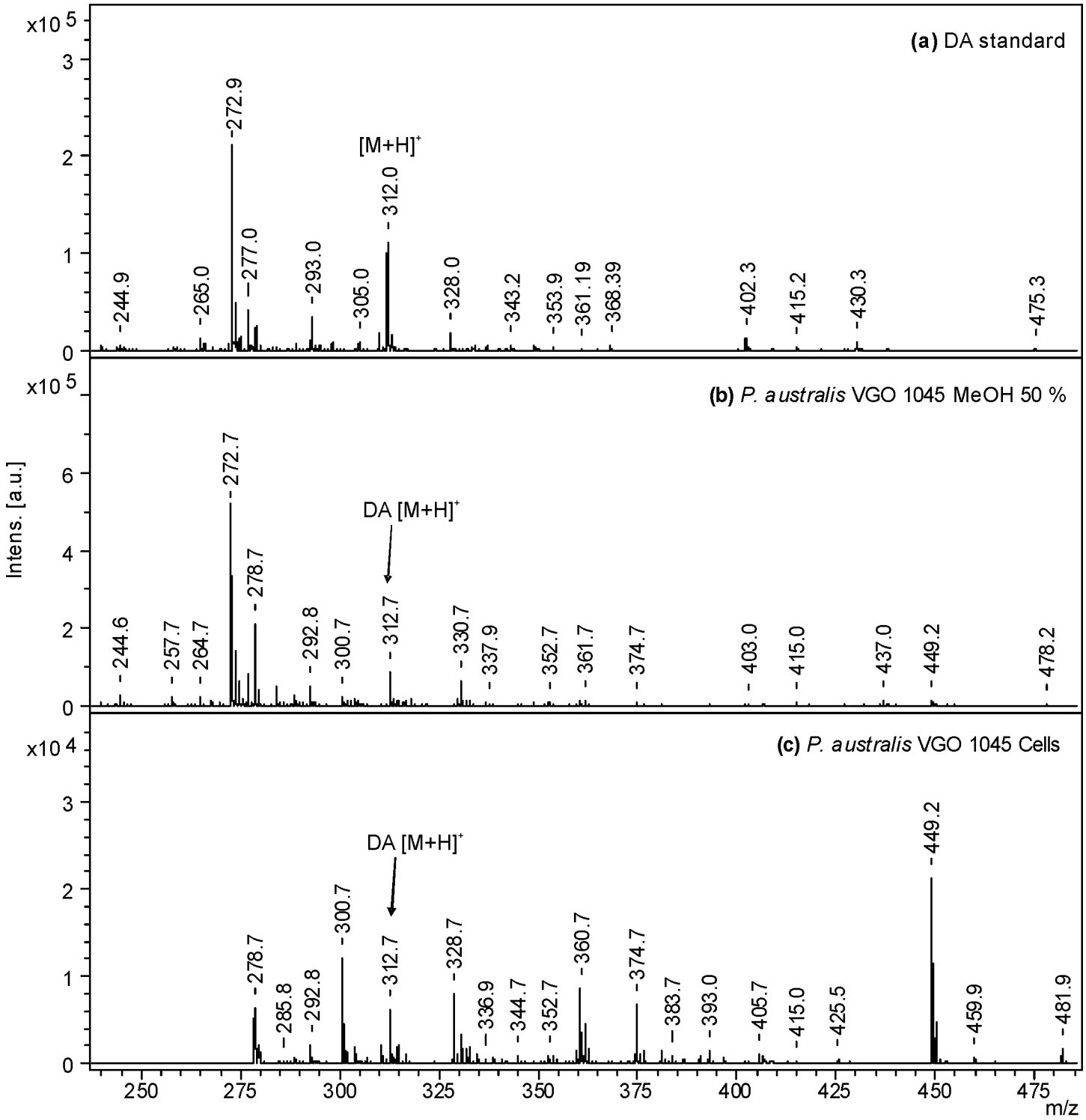


Figure 8. MALDI-TOFMS of DA standard (a), *Pseudo-nitzchia australis* strain VGO 1045 in MeOH extract (b) and whole cells (c) acquired in positive mode with DHB matrix.



Supporting Information

Preliminary study for rapid determination of phycotoxins in microalgae whole cells using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry

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Supplementary Figure Captions

Figure S1. MALDI-TOF/TOFMS of the precursor ion with *m/z* 2701 (a), ion with *m/z* 2683 (b) from the palytoxin standard, and ion with *m/z* 2670 (c) from the MeOH extract of *Ostreopsis ovata* VGO 1049 strain. Spectra were acquired in positive ionization mode with DHB matrix.

Figure S2. MALDI-TOF/TOFMS of the precursor ion with *m/z* 1062 from the YTX standard (a), ion with *m/z* 1062 from whole cells of the *P. reticulatum* strain VGO 905 (b), and ion with *m/z* 1075 from whole cells of the *P. reticulatum* strain VGO 764 (c). Spectra were acquired in negative ionization mode with DHB matrix.

Figure S3. MALDI-TOF/TOFMS of the precursor ions with *m/z* 827 from the OA (a) and with *m/z* 881 from the PTX2 (b) standards obtained in positive ionization mode with DHB matrix.

Figure S4. MALDI-TOF/TOFMS of the precursor ion with *m/z* 881 of *Dinophysis tripos* strain from MeOH extract (a) and whole cells (b). Spectra were obtained in positive ionization mode with DHB matrix.

Figure S5. MALDI-TOF/TOFMS from *Prorocentrum* strains. For *P. belizeanum* PBMA01 of the precursor ion with *m/z* 827 from MeOH extract (a) and whole cells (b). For *P. lima* PLRN02 of the precursor ions with *m/z* 827 (c) and *m/z* 962 (d) from MeOH extract. Results were obtained in positive ionization mode with DHB matrix.

Figure S6. MALDI-TOF/TOFMS of the precursor ion with *m/z* 881 of the *Dinophysis* filtrate (containing *D. caudata* and *D. acuta*) for MeOH extract (a) and whole cells (b). Data were acquired in positive ionization mode and DHB matrix.

Figure S1. MALDI-TOF/TOFMS of the precursor ion with *m/z* 2701 (a), ion with *m/z* 2683 (b) from the palytoxin standard, and ion with *m/z* 2670 (c) from the MeOH extract of *Ostreopsis ovata* VGO 1049 strain. Spectra were acquired in positive ionization mode with DHB matrix.

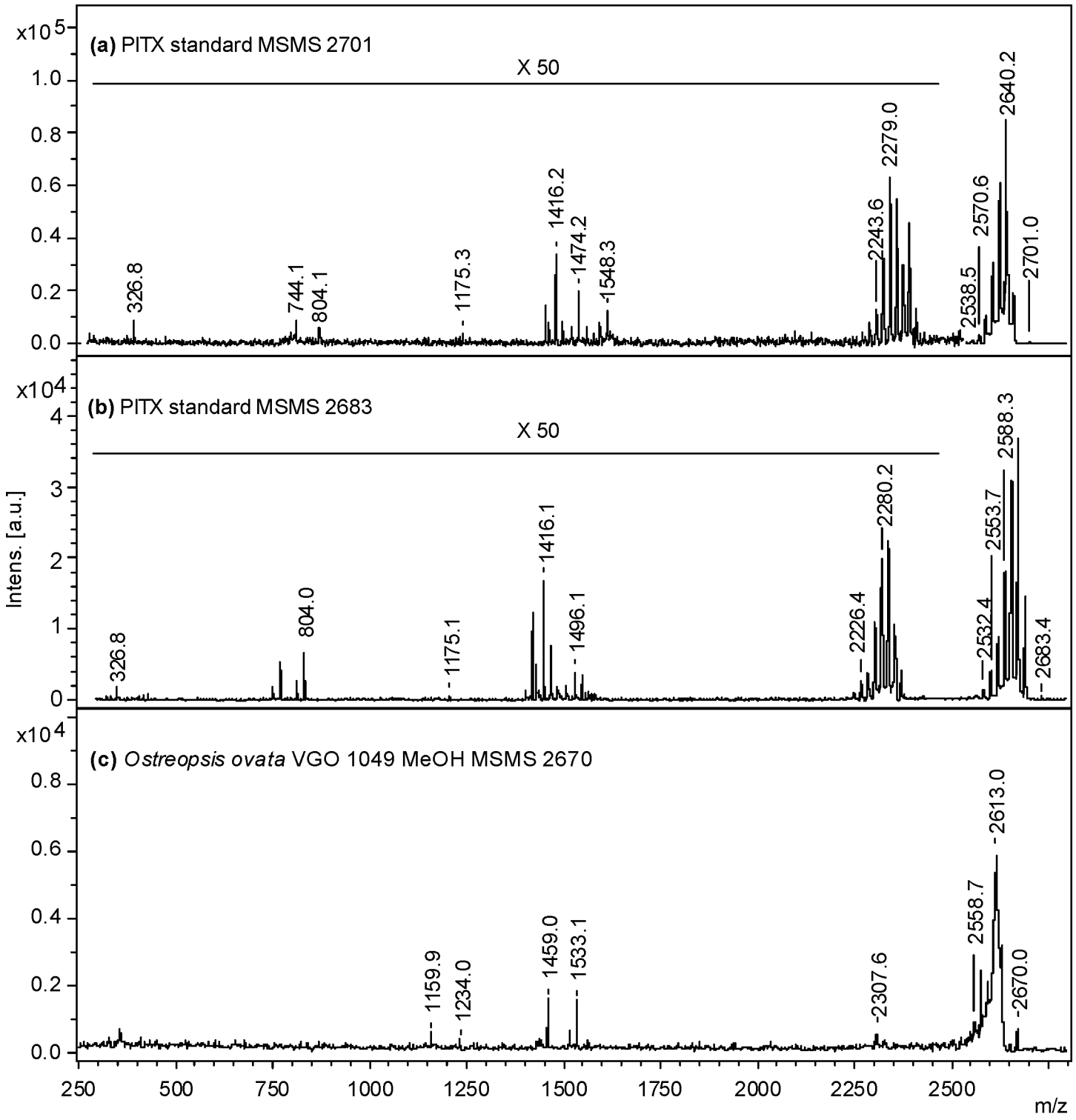

Figure S2. MALDI-TOF/TOFMS of the precursor ion with *m/z* 1062 from the YTX standard (a), ion with *m/z* 1062 from whole cells of the *P. reticulatum* strain VGO 905 (b), and ion with *m/z* 1075 from whole cells of the *P. reticulatum* strain VGO 764 (c). Spectra were acquired in negative ionization mode with DHB matrix.

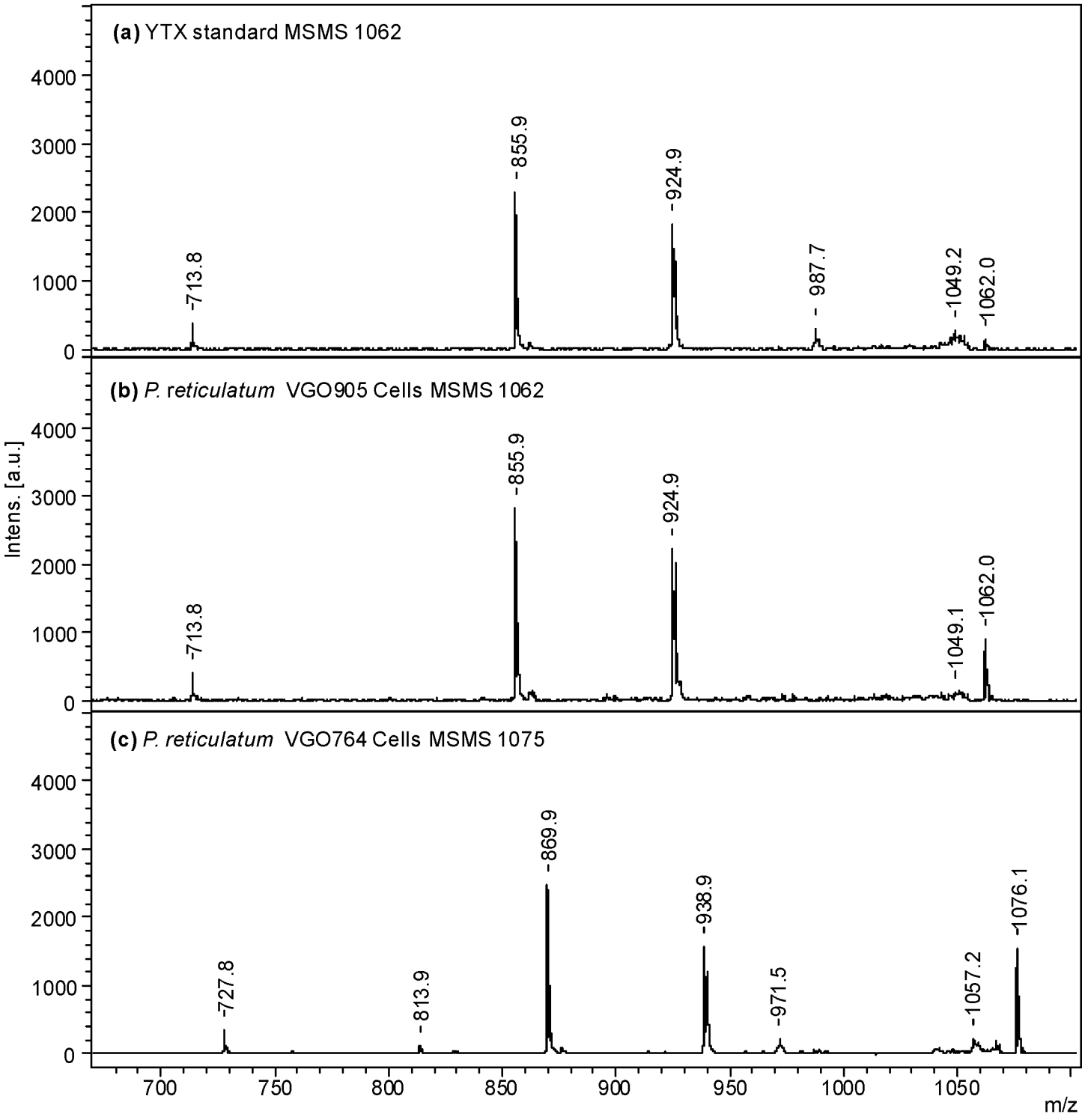

Figure S3. MALDI-TOF/TOFMS of the precursor ions with *m/z* 827 from the OA (a) and with *m/z* 881 from the PTX2 (b) standards obtained in positive ionization mode with DHB matrix.

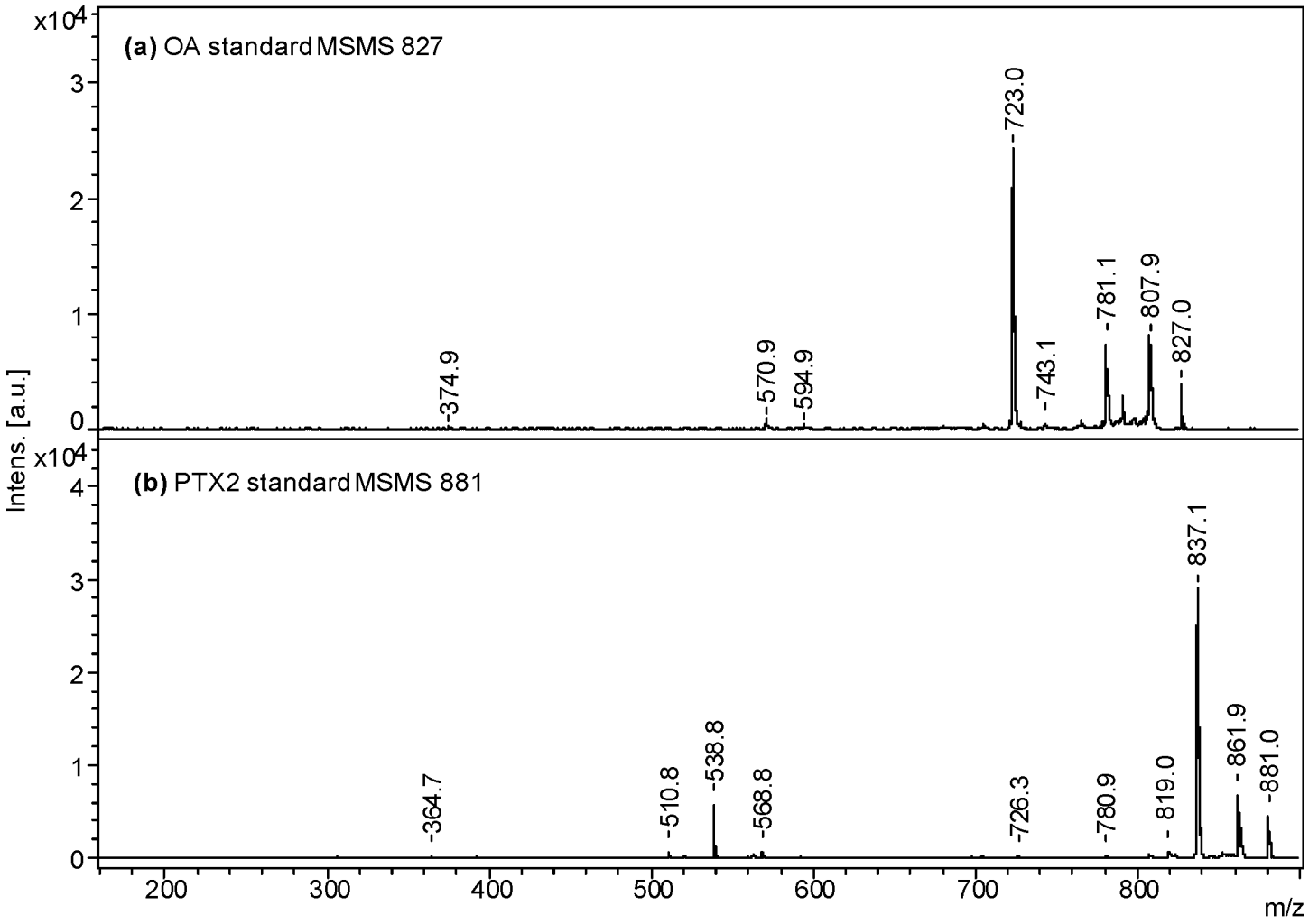

Figure S4. MALDI-TOF/TOFMS of the precursor ion with *m/z* 881 of *Dinophysis tripos* strain from MeOH extract (a) and whole cells (b). Spectra were obtained in positive ionization mode with DHB matrix.

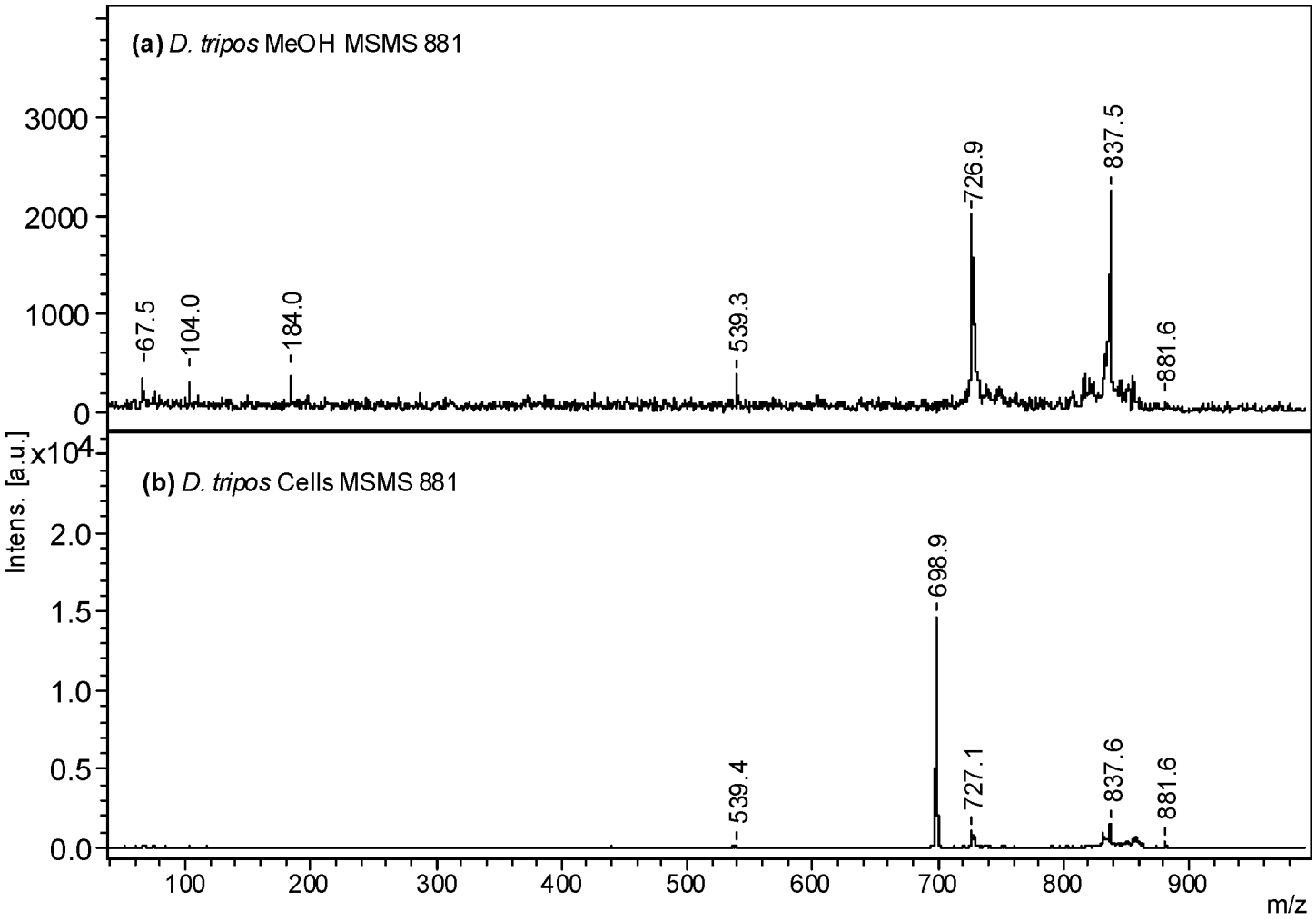

Figure S5. MALDI-TOF/TOFMS from *Prorocentrum* strains. For *P. belizeanum* PBMA01 of the precursor ion with *m/z* 827 from MeOH extract (a) and whole cells (b). For *P.lima*PLRN02 of the precursor ions with *m/z* 827 (c) and *m/z* 962 (d) from MeOH extract. Results were obtained in positive ionization mode with DHB matrix.

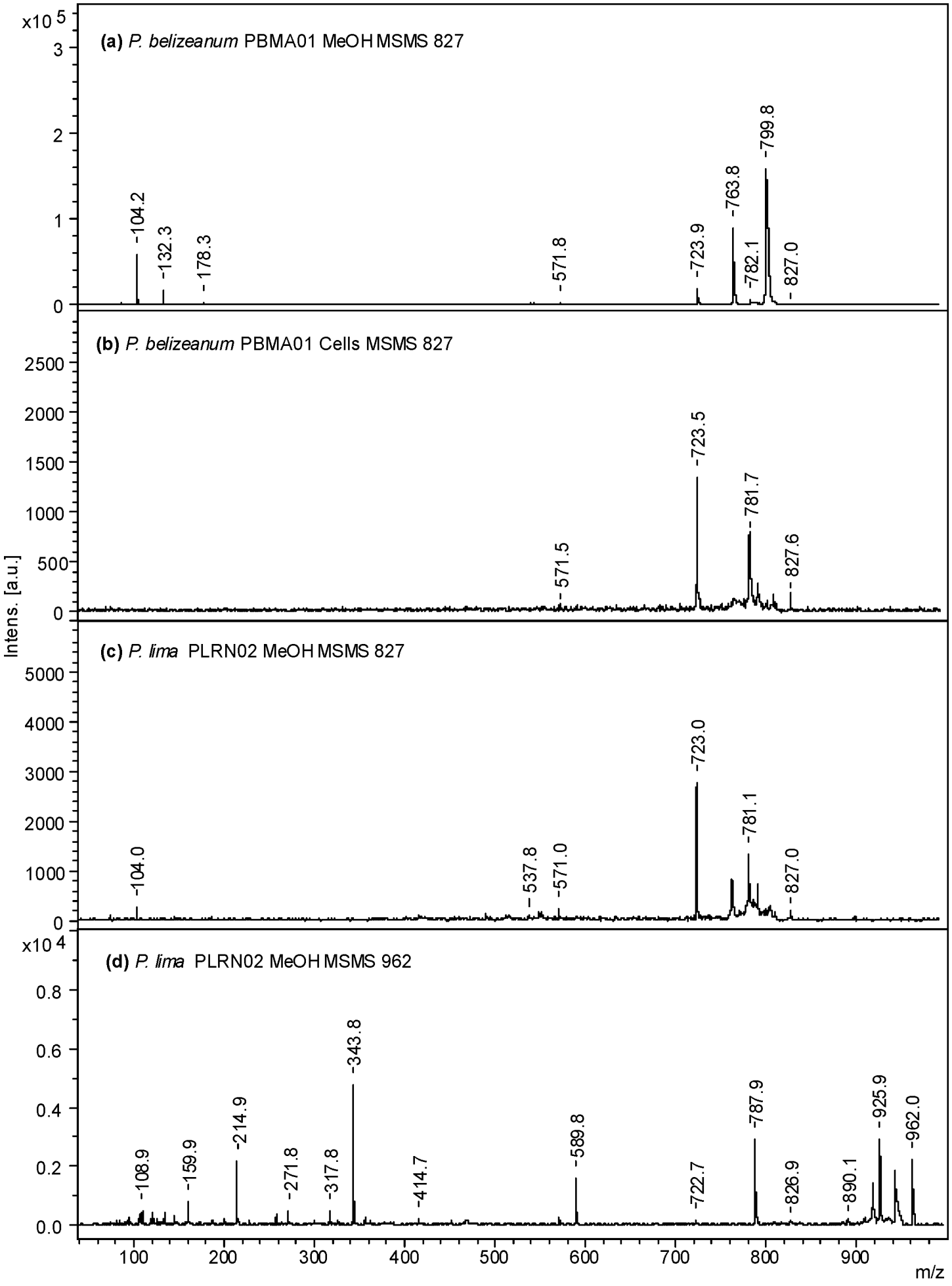

Figure S6. MALDI-TOF/TOFMS of the precursor ion with *m/z* 881 of the *Dinophysis* filtrate (containing *D. caudata* and *D. acuta*) for MeOH extract (a) and whole cells (b). Data were acquired in positive ionization mode and DHB matrix.