Title

Analysis of chlorophylls and their derivatives by matrix-assisted laser desorption/ionization-time of flight mass spectrometry

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Abstract

The analysis of chlorophylls and their derivatives by matrix-assisted laser desorption/ionization-time of flight mass spectrometry is described. Four matrixes, sinapinic acid, α-cyano-4-hydroxycinnnamic acid, terthiophene, and 3-aminoquinoline, were examined to determine optimal conditions for analysis of the molecular mass and structure of chlorophyll *a* as a representative chlorophyll. Among them, terthiophene was the most efficient without releasing metal ions, although it caused fragmentation of the phytol-ester linkage. Terthiophene was useful for the analyses of chlorophyll derivatives as well as porphyrin products such as 8-deethyl-8-vinyl-chlorophyll *a*, pheophorbide *a*, pyropheophorbide *a*, bacteriochlorophyll *a* esterified phytol, and protoporphyrin IX. The current method is suitable for rapid and accurate determination of the molecular mass and structure of chlorophylls and porphyrins.

Keywords: MALDI-TOF MS; chlorophylls; photosynthetic pigments; porphyrin.

Introduction

The matrix-assisted laser desorption/ionization (MALDI)-time of flight (TOF) mass spectrometry (MS) method developed by Tanaka et al. [1] and Karas and Hillenkamp [2] has been applied broadly to the analysis of biologically relevant molecules. MALDI is the most general ionization method to detect a substance whose molecular weight exceeds 10,000, and it is not easily affected by the chemical properties of the sample compound. TOF MS is compatible with MALDI. MALDI-TOF MS is able to measure the widest *m/z* range, including high molecular mass areas, and rapidly examine the distribution of the molecular mass and analyze the structure at the same time. Therefore, this method has recently become popular in studies of the biochemistry of proteins and peptides. To date, the analytes have been almost exclusively polymers such as proteins, nucleic acids, or carbohydrates. A few previous studies have shown the possibility of applying MALDI-TOF MS to the analysis of low-molecular-weight compounds [3, 4]. The utility of this method for characterization of secondary metabolites of intact cyanobacteria [5] and lipid compositions in green alga and diatom [6] have been reported. There is growing evidence that MALDI-TOF MS is a powerful tool in such a biochemical research. As a method for measurement of pigments such as chlorophylls (Chls) including the porphyrin ring, fast atomic bombardment combined with tandem MS and liquid chromatography-MS have been used so far [7, 8]. Recently, incorporation of the ^{15}N label into Chls has been measured by MALDI-TOF MS [9]. However, the analysis of pigments using MALDI-TOF MS method has not been basically established yet.

In this study, we examined optimal conditions for the analysis of Chls and their derivatives by MALDI-TOF MS. In addition, we applied this technique to the determination of other pigments such as porphyrin derivatives. The structural formulae of the Chls and their derivatives, including protoporphyrin IX, used in this study are shown in Fig. 1.

Materials and methods

Chemicals

Sinapinic acid (SA), α-cyano-4-hydroxy-cinnamic acid (CCA), and terthiophene (Ter) were purchased from Sigma (Steinheim, Germany). 3-Aminoquinoline (3AQ) was obtained from Alfa Aesar (Lancashire, UK). All other reagents were either from Wako Pure Chemical (Osaka, Japan) or Nacalai Tesque (Kyoto, Japan).

Pigments

Chl *a* was purchased from Wako Pure Chemical and/or extracted from spinach (*Spinacia oleracea* L.) leaves and purified by sugar-column chromatography according to the method of Perkins and Roberts [10]. Bacteriochlorophyll *a* esterified phytol (bacteriochlorophyll a_P) was extracted from the purple photosynthetic bacterium *Rhodobacter sulfidophilus* and purified by diethylaminoethyl-Toyopearl column chromatography according to the method reported by Shioi [11]. 8-Deethyl-8-vinyl-chlorophyll (8-vinyl-Chl) *a* was obtained from DHI Water and Environment (Copenhagen, Denmark). Protoporphyrin IX was purchased from Sigma. Pyropheophorbide (pyropheide) *a* was purchased from Tama Biochemical (Tokyo, Japan). Pheophytin *a* and pheophorbide (pheide) *a* were prepared by acid treatment of the respective Chls as described previously [12]. All samples were subjected to high-performance liquid chromatography (HPLC) analysis to verify the purity.

HPLC Analysis

HPLC analysis was performed according to the methods reported by Shioi et al. [12, 13] and Zapata et al. [14]. The HPLC system employed was a model LC-10A equipped with a degasser and column oven (Shimadzu, Kyoto, Japan). Chls were separated and analyzed using a C_{18} column (4.6 x 25 cm) with methanol at a flow rate of 1.0 ml per min at 40°C [12]. For measurement of pheide and pyropheide, methanol containing 2 M ammonium acetate (95:5, v/v) was used with an isocratic method at 30°C [15]. 8-vinyl-Chl *a* was analyzed by HPLC with a model LC-10AT equipped with a column-temperature controller CTO-10AS (Shimadzu), using Waters Symmetry C_8 column (150 x 4.6 mm) (Milford, MA, USA) [14]. 8-vinyl-Chl *a* was eluted with a programmed binary gradient

elution system at a flow rate of 1.0 ml per min at 25°C. The solvents used were methanol:acetonitrile:water (50:25:25, by volume) containing 62.5 mM pyridine for solvent A and methanol:acetonitrile:acetone (20:60:20, by volume) for B. Separation was performed with a gradient containing the break points of 0 min (100:0, v/v), 22 min (60:40), 28-38 min (5:95), and 41 min (100:0). Separated 8-vinyl-Chl was detected spectrophotometrically with a photodiode array detector SPD-M10A (Shimadzu), measuring from 400 to 700 nm and monitoring mainly at 410 nm.

Mass spectrometry

Mass spectra were obtained on a Bruker Daltonics (Billerica, MA, USA) Autoflex MALDI-TOF mass spectrometer equipped with a pulsed N_2 laser (337 nm). The spectrometer was operated in reflectron mode optimized for positive ions with masses from 0 to 2,000 Da. The nitrogen laser excitation frequency was set at 10 Hz. The laser power was optimized to obtain a good signal-to-noise ratio after averaging 300 single-shot spectra. After mixing analytes with a matrix, 1 µl of the mixture was dried on a stainless target plate for analysis. Ter was dissolved in acetonitrile at a final concentration of 100 mM [16], while 3AQ was dissolved in 95% (v/v) acetone at a final concentration of 200 mM. Saturated CCA and SA in acetonitrile were used.

Results and discussion

Matrices for optimal analysis

To determine the most suitable matrix for analysis of Chl *a* as a representative analyte, we examine four matrices, SA, CCA, Ter, and 3QA. Among them, SA is most frequently used for a variety of different samples from high to low molecular masses from proteins to vitamin B_{12}. In positive mode, Ter is known to form only molecular radical cations [$M^{+\cdot}$] upon laser irradiation [16]. The other two chemicals were selected as additional matrices based on their common use and availability. The positive ion MS spectra of Chl *a* ($C_{55}H_{72}O_5N_4Mg$, molecular weight: 892.54) analyzed using these matrices are shown in Fig. 2 (A-D), and identification of pigments obtained by MS measurement with the known Chl *a* derivatives is summarized in Table 1. When SA was used,

two molecular peaks at *m/z* 871.6 and *m/z* 614.0, corresponding to pheophytin *a* ($C_{55}H_{74}O_5N_4$, molecular weight: 870.57) and chlorophyllide (chlide) *a* ($C_{35}H_{34}O_5N_4Mg$, molecular weight: 614.24), respectively, were observed in addition to a weak Chl *a* signal (Fig. 1A). Two MS peaks of fragments at *m/z* 871.5 and *m/z* 593.3, both of which correspond to Mg-released pigments of Chl *a*, pheophytin *a*, and pheide *a* ($C_{35}H_{36}O_5N_4$, molecular weight: 592.27), were observed with CCA, but almost no Chl *a* signal was detected (Fig. 1B). In this case, a chlide *a* signal was not found, unlike with SA; instead pheide *a* was observed, and the intensity of the pheophytin *a* signal was much higher than that of pheide *a*. When Ter or 3AQ were used, two MS peaks at *m/z* 892.7 and *m/z* 614.0, corresponding to Chl *a* and chlide *a*, were observed (Fig. 2C, D). A high intensity peak of the parent ion, Chl *a*, was obtained when Ter rather than 3AQ was used, though the relative intensity of the peak varied from sample to sample and generally increased with increasing laser intensity. When 3AQ was used, slightly higher molecular masses were observed compared to those of the other matrices. This higher molecular ion is probably due to protonation. As a result, among the matrices used, the molecular weights of Chl *a* and its derivatives were measured most correctly as mostly proton adduct ions [M^+], except for Mg-containing derivatives such as Chl *a* and chlide *a* which do not produce [M^+]. It is quite reasonable to expect detection of ionization of Mg-released derivatives. When measurement was carried out at high sensitivity, small mass peaks that split and spread about 1 Da in each direction were found. The peaks at larger molecular mass are due to the natural abundance of isotopes and those at smaller molecular mass are due to oxidation and losing of proton. The formation of fragment ions, metal-releasing products of Chl *a*, pheophytin *a* and pheide *a*, by SA and CCA were due to Mg^{2+} released from Chl *a* by the mixing of an acid matrix with a pigment during preparation of the sample plates, as confirmed by the results in the absence of a matrix (see section of *Measurement of Chls in absence of matrix*). It is generally well known that Chls easily release their chelating metals under acidic conditions, while chlide *a* is formed by breaking a linkage between the esterifying alcohol phytol and the Chl macrocyclic ring, probably by laser irradiation as shown below, which suggests that the ester linkage is weak. In this fragmentation, CCA appears to act somewhat weaker compared to the other matrices used.

Interstingly, fragmentation ion of methoxycarbonyl group at $C13^2$ was negligible among matrices used. Based on these comparisons, Ter is the most suitable matrix for measurement of Chl *a* among the matrices tested. We therefore selected Ter as a matrix for analyses of Chls in the following studies.

Measurement of Chls in absence of matrix

Chls and their derivatives have a prominent absorption band around 400-460 nm and possibly absorb laser light at 337 nm. It is, therefore, expected that Chls might be ionized by laser absorption in the absence of a matrix and give molecular ions. We analyzed Chl *a* and pheophytin *a* without using a matrix. When Chl *a* was used (Fig. 3A), the Chl *a* peak was not detected, but one main peak at *m/z* 614.2 corresponding to chlide *a* was detected, indicating that the phytol-ester linkage was broken without releasing metal ions. In the case of pheophytin *a* (Fig. 3B), in addition to the peak of pheophytin *a* at *m/z* 870.9, an additional peak at *m/z* 592.0, corresponding to pheide *a*, was found. Similar to Chl *a*, the phytol-ester linkage was also broken. Thus, in the absence of a matrix, only fragmentation of the phytol-ester linkage occurred in both pigments, whereas a parent ion was clearly detected in the presence of a matrix (cf. Fig. 2, C and D). Similar scission at the ester linkage was shown to occur in the analysis of theaflavins and thearubigins, two major groups of pigments in black tea, using MALDI-TOF MS [17]. This indicates that fragmentation at the phytol-ester linkage may occur easily when using MALDI-TOF MS. It is, therefore, likely that the release of Mg is caused by mixing with an acid matrix and the fragmentation by breaking an ester linkage is due to laser light absorption of the pigments. Moreover, the presence of an appropriate matrix such as Ter or 3AQ stabilizes Chl samples and prevents fragmentation.

Measurement of Chl a and 8-vinyl-Chl a using Ter

To examine pigments having similar molecular weights, we analyzed Chl *a* and 8-vinyl-Chl *a* ($C_{55}H_{70}O_5N_4Mg$, molecular weight: 890.52) using Ter as the matrix. The MS spectrum showed that in addition to two small peaks of Chl *a* and 8-vinyl-Chl *a*, two fragmentation products, chlide *a* at

m/z 614.5 and 8-vinyl-chlorophyllide (8-vinyl-chlide) *a* ($C_{35}H_{32}O_5N_4Mg$, molecular weight: 612.22) at *m/z* 612.5, were detected with mass peaks that spread 1 Da at larger molecular mass due to the natural abundance of isotopes (Fig. 4), although peaks of parent ion of Chls were weak with compared to that of Chl *a* (cf. Fig. 2, C) probably due to low laser intensity. In addition, at present we have no idea on peak around 695, which also appears in the analysis of Chl derivatives (see Fig. 5, A and B). This may br derived from contaminated degradation products of the pigments. These Chls and fragmentation products were clearly distinguished by the difference of their molecular weights. This finding indicates that each pigment can be separated by the method used in this study and identified simultaneously despite only a 2 Da difference in molecular mass. In fact, this method was successfully applied for determination of the amount of Chl with all four nitrogen atoms substituted with the ^{15}N isotope [9].

Measurement of Chl derivatives using Ter

Other Chl derivatives, pyropheide *a* ($C_{33}H_{34}O_3N_4$, molecular weight: 534.26), pheide *a*, and bacteriochlorophyll a_P ($C_{55}H_{74}O_6N_4Mg$, molecular weight: 910.55) were analyzed in the same manner using Ter as the matrix. The mass spectra of pyropheide *a* and pheide *a* indicated the correct molecular masses at *m/z* 533.6 and at *m/z* 591.9, respectively, and products of their fragmentation were not observed (Fig. 5A, B). On the other hand, during the analysis of bacteriochlorophyll a_P, not only bacteriochlorophyll a_P at *m/z* 910.5 but also bacteriochlorophyllide *a* ($C_{35}H_{36}O_6N_4Mg$, molecular weight: 632.25) at *m/z* 632.2 was detected (Fig. 5C). However, no other derivatives, such as the Mg^{2+}-released products bacteriopheophytin a_P or bacteriopheophorbide *a*, appeared. The molecular weight of each pigment was measured correctly. These results indicate that MALDI-TOF MS with Ter as the matrix is conveniently applicable for measurement of Chls and their derivatives, although fragmentation of the ester linkage between the esterifying alcohol and Chl macrocyclic ring occurred with Ter. Release of the esterifying alcohol could be a convenient way to obtain information on the Chl macrocyclic ring.

Application to other porphyrin compounds

To determine whether porphyrin compounds other than Chls can be analyzed, protoporphyrin IX ($C_{34}H_{34}O_4N_4$, molecular weight: 562.26) was measured with Ter as the matrix (Fig. 6). The positive ion MS spectrum of protoporphyrin IX showed the main peak at *m/z* 562.5, corresponding to protoporphyrin IX, and two fragment products. A peak at *m/z* 595 was attributed to the oxygenated parent ion [M + O_2], and a peak at *m/z* 518 corresponded to the loss of a carboxyl group from the parent ion [M - COOH]. A similar result was obtained using 3AQ as the matrix.

Conclusion

Several studies of the analysis of pigments using MALDI-TOF MS have already been reported. MALDI-TOF MS has been used for characterization and structural analysis of plant lipids including Chl *a* [6], unknown pigments groups in plants [17] and artificially synthesized pigments [18]. However, there are few studies of analysis of photosynthetic pigments, particularly Chls, by using MALDI-TOF MS [9]. In this study, we established a method for measurement of Chls and their derivatives and porphyrin by MALDI-TOF MS. Additionally, this method allows rapid analysis of a variety of Chls and porphyrin products with ease of handling and high efficiency. Consequently, this technique not only appears to be a reasonable alternative to previously reported methods using fast atomic bombardment ionization MS and electrospray ionization MS, but also a powerful tool for determination, quantification and characterization of Chl and prophyrin research.

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Figure legends

Fig. 1. Structural formulae of chlorophylls (A), bacteriochlorophyll a_P (B), and protoporphyrin IX (C).

Fig. 2. MALDI mass spectrum of Chl *a* using four matrices: SA (A); CCA (B); Ter (C); and 3AQ (D). Observed peaks correspond to Chl *a* (*m/z* = 892.7, 894.0), pheophytin *a* (*m/z* = 871.6, 871.5), chlide *a* (*m/z* = 614.0), and pheide *a* (*m/z* = 593.3). Details of MS measurement are described in the text.

Fig. 3. MALDI mass spectrum of Chl *a* (A) and pheophytin *a* (B) without matrices. Observed peaks correspond to chlide *a* (*m/z* = 614.2), pheophytin *a* (*m/z* = 870.9), and pheide *a* (*m/z* = 592.0).

Fig. 4. MALDI mass spectrum of Chl *a* (A) and 8-vinyl-Chl *a* (B) with Ter as a matrix. Observed peaks correspond to chlide *a* (*m/z* = 614.5) and 8-vinyl-chlide *a* (*m/z* = 612.5). Inset, expanded molecular ion region of peaks.

Fig. 5. MALDI mass spectrum of Chl-like pigments, pyropheide *a* (A), pheide *a* (B), and bacteriochlorophyll a_P (C) with Ter as a matrix. Observed peaks correspond to pyropheide *a* (*m/z* = 533.6), pheide *a* (*m/z* = 591.9), bacteriochlorophyll a_P (*m/z* = 910.5), and bacteriochlorophyllide *a* (*m/z* = 632.2).

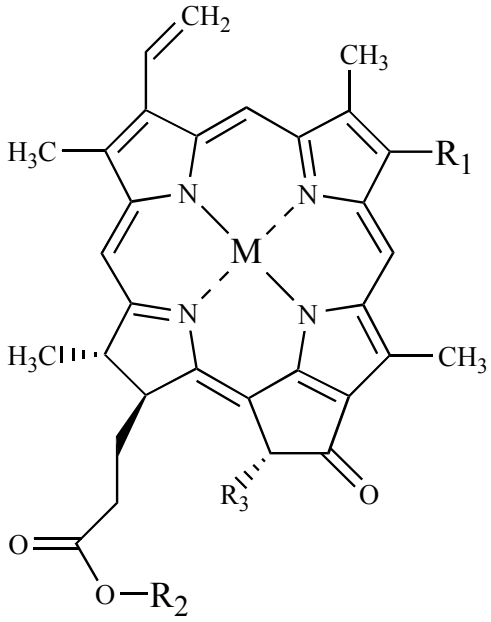
Fig. 6. MALDI mass spectrum of protoporphyrin IX when Ter was used as a matrix. The observed peak corresponds to protoporphyrin IX (*m/z* = 562.5).

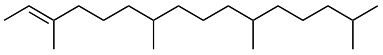
Material	Monoisotopic molecular weight	Matrix	Signal (*m*/*z*)		Identification (Monoisotopic molecular weight)
			Intactness	Fragment	
Chlorophyll *a*	892.54	None	−		
				614.2	Chlide *a* (614.24)
		SA	−		
				871.6	Pheophytin *a* (870.57)
				614.0	Chlide *a* (614.24)
		CCA	−		
				871.5	Pheophytin *a* (870.57)
				593.3	Pheide *a* (592.27)
		3AQ	894.0		
				614.5	Chlide *a* (614.24)
		Ter	892.7		
				614.0	Chlide *a* (614.24)
Pheophytin *a*	870.57	None	870.9		
				592.0	Pheide *a* (592.27)
Divinyl chlorophyll *a*	890.52	Ter	891.0		
				612.5	DV Chlide *a* (612.22)
Pyropheophorbide *a*	534.26	Ter	533.6		
Pheophorbide *a*	592.27	Ter	591.9		
Bacteriochlorophyll *a*	910.55	Ter	910.5		
				632.2	Bacteriochlorophyllide *a* (632.25)
Protoporphyrin IX	562.26	Ter	562.5		

Tabel 1. Summary of measured compounds and detected signals using different matrices.

Fig. 1 (Suzuki et al.)

(A)

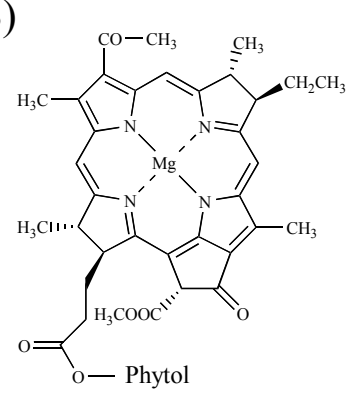




Phytyl ($C_{20}H_{39}$)

Pigment	M	R_1	R_2	R_3
Chlorophyll *a*	Mg	CH_2CH_3	Phytyl	$COOCH_3$
Divinyl chlorophyll *a*	Mg	$CHCH_2$	Phytyl	$COOCH_3$
Chlorophyllide *a*	Mg	CH_2CH_3	H	$COOCH_3$
Pheophorbide *a*	2H	CH_2CH_3	H	$COOCH_3$
Pyropheophorbide *a*	2H	CH_2CH_3	H	H
Pheophytin *a*	2H	CH_2CH_3	Phytyl	$COOCH_3$

(B)



(C)

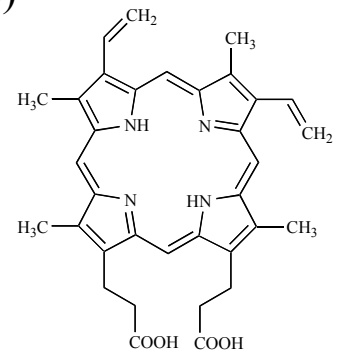
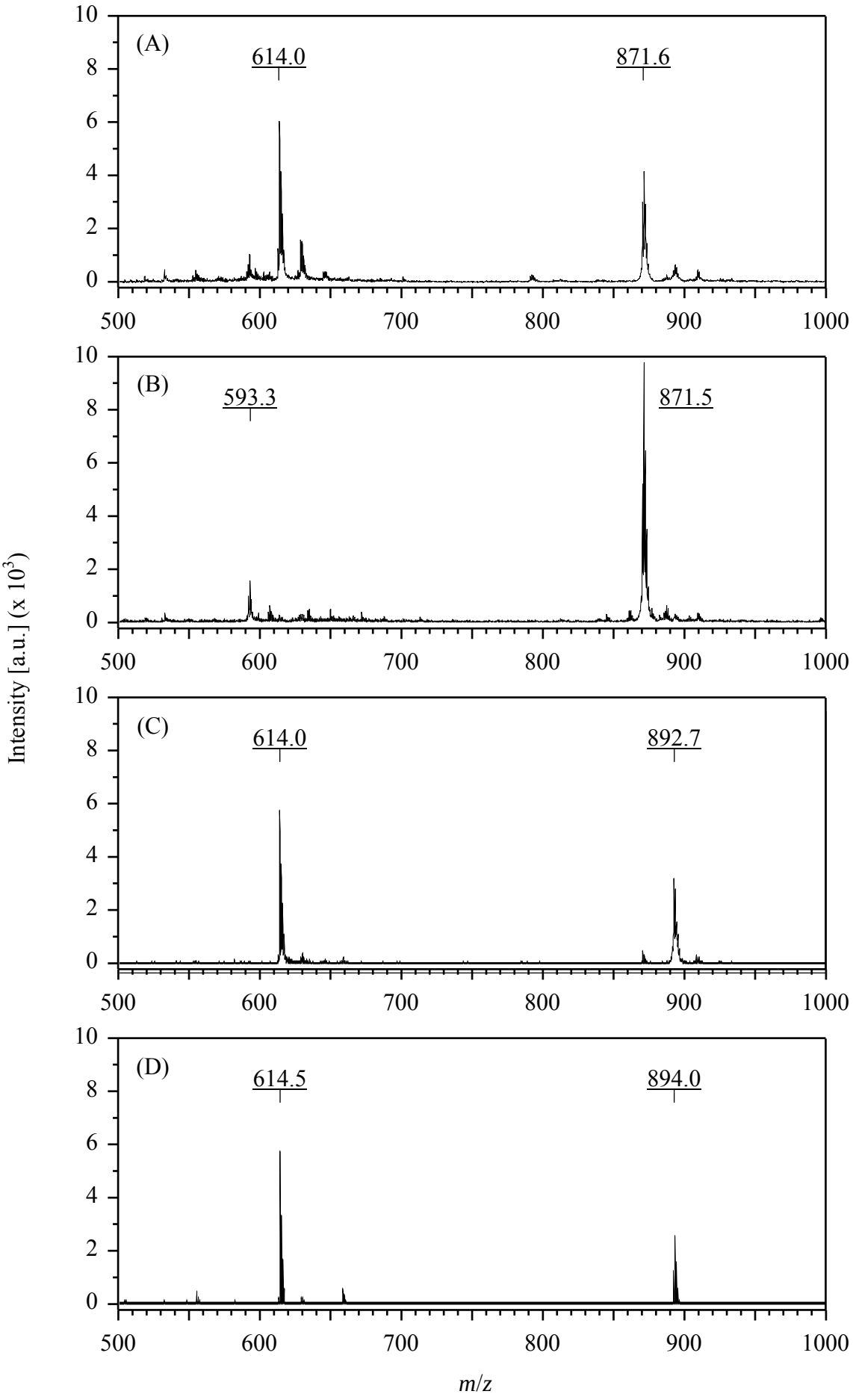


Fig. 2 (Suzuki et al.)



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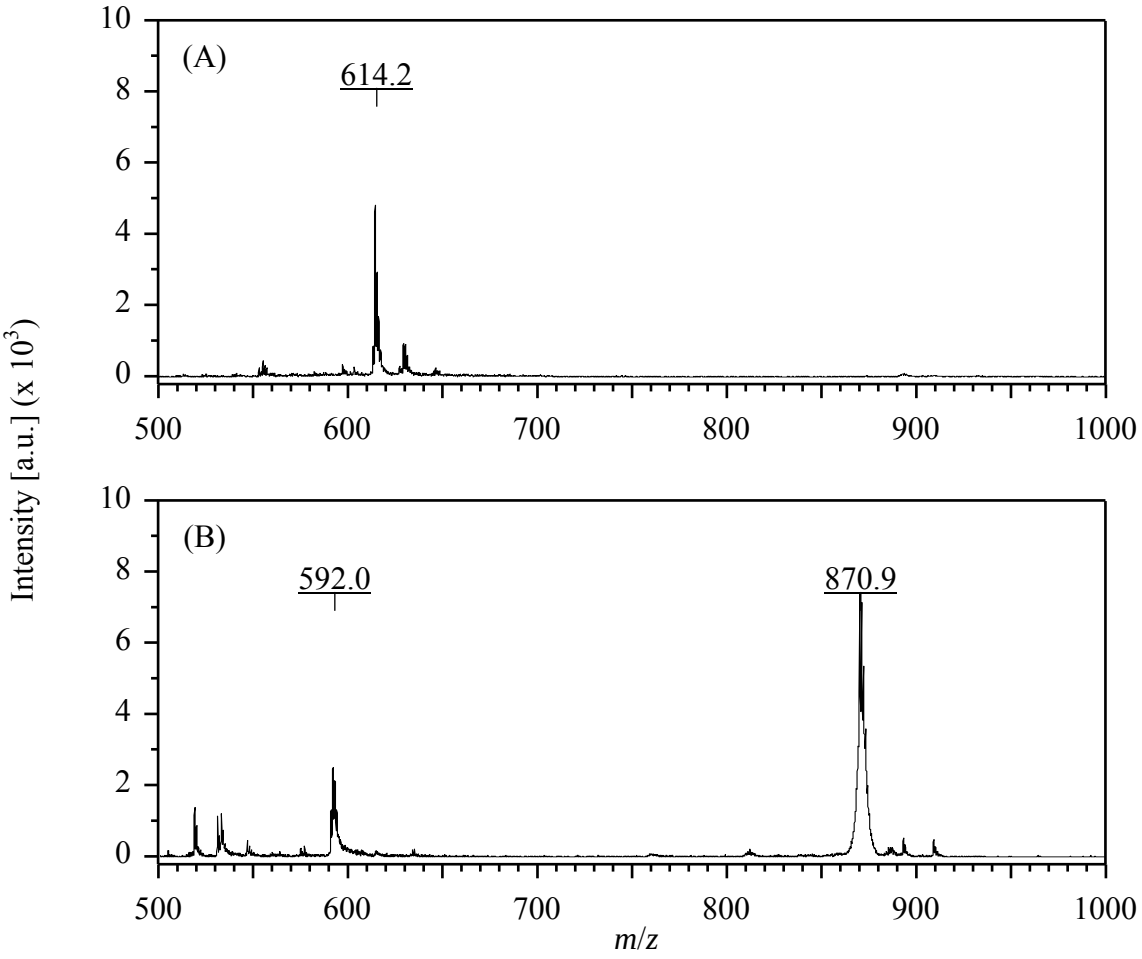
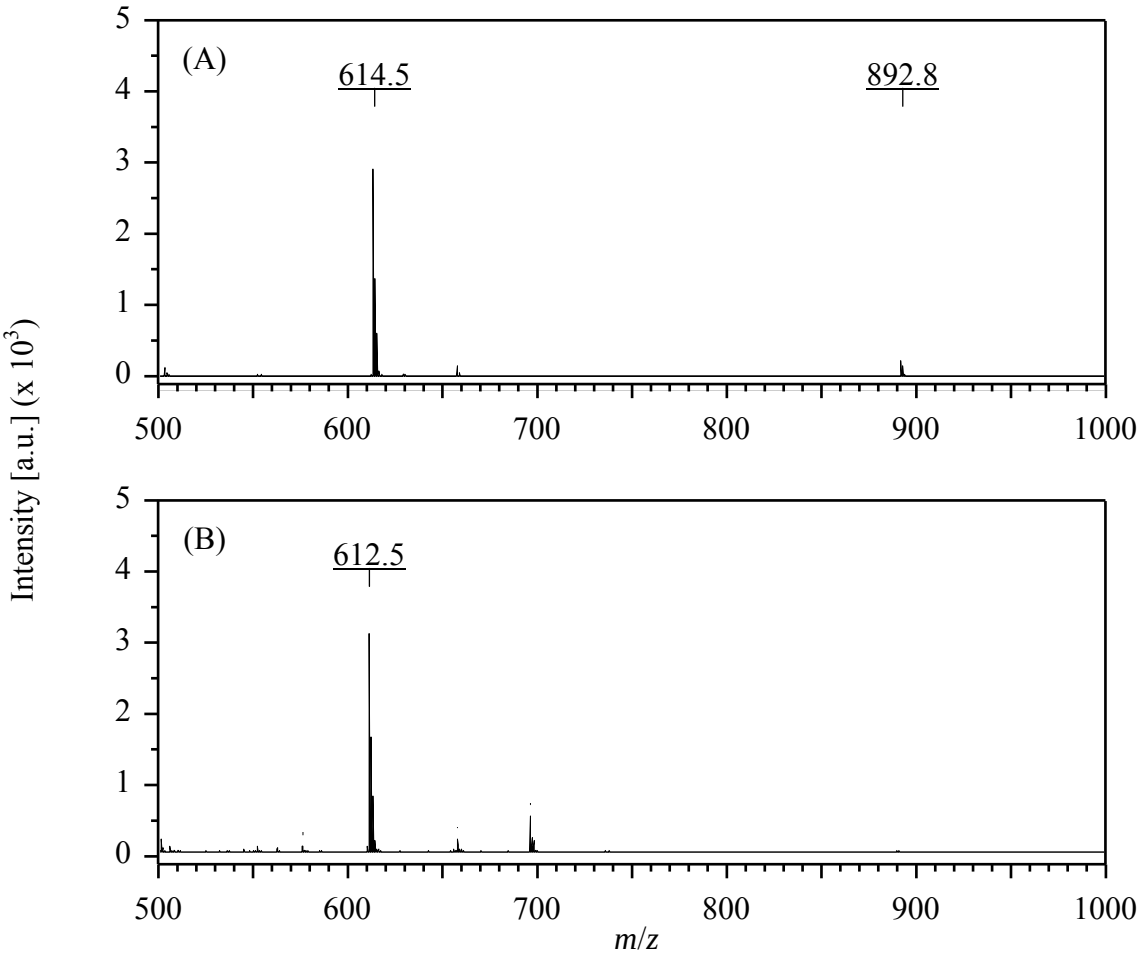


Fig. 4 (Suzuki et al.)



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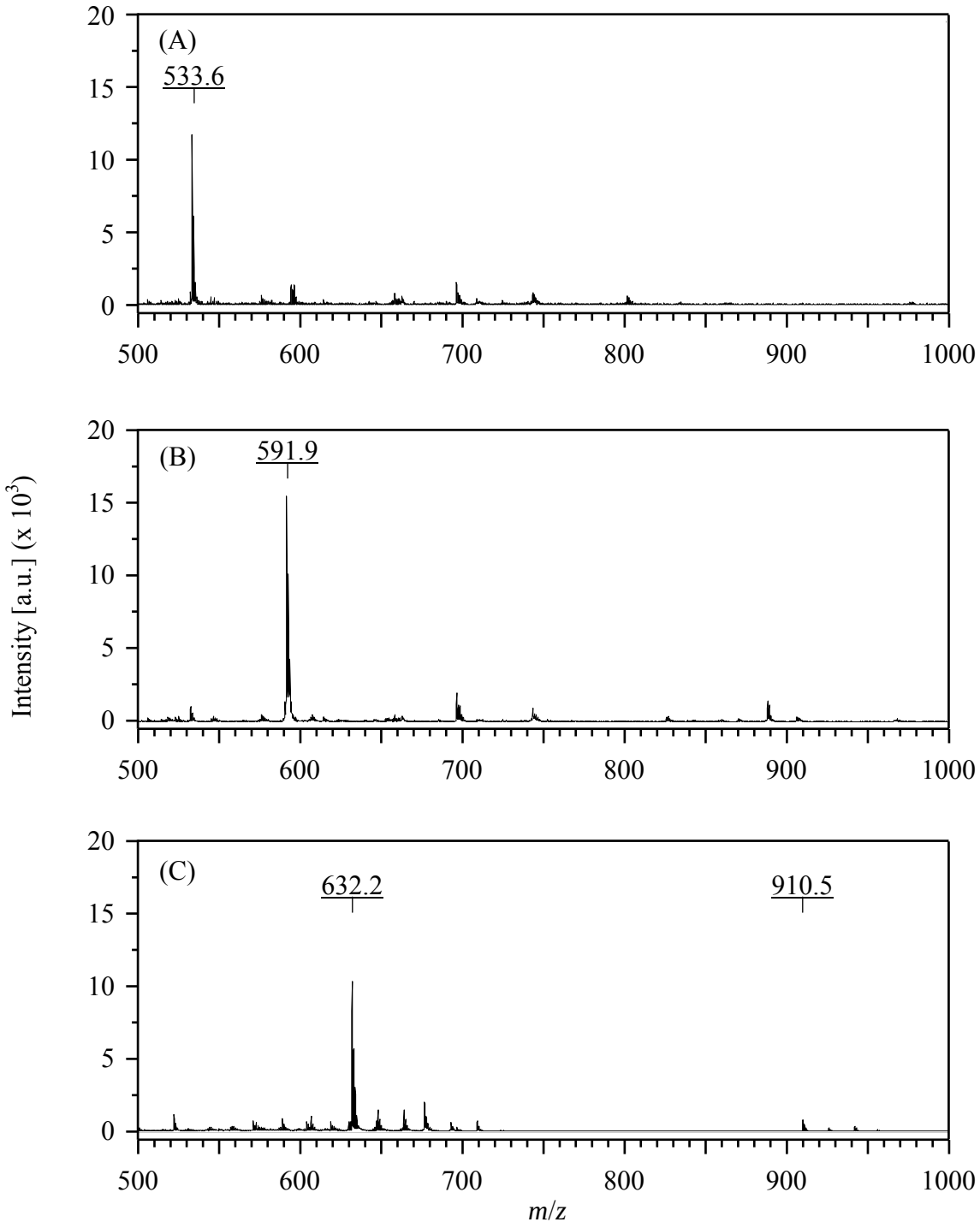

Fig. 6 (Suzuki et al.)