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Identification of eicosanoids in the red algae, *Gracilaria asiatica*, using high-performance liquid chromatography and electrospray ionization mass spectrometry

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Abstract

Identification of eicosanoids which are metabolites of arachidonic acid in red algae *Gracilaria asiatica*, one of the popular seaweeds in Japan, was carried out using high-performance liquid chromatography (HPLC) interfaced with electrospray ionization mass spectrometry. Prostaglandin (PG) E_2, 15-keto-PGE_2, and 8-hydroxyeicosatetraenoic acid (HETE) were detected as major eicosanoids and PGA_2, leukotriene B_4 as minor ones in *G. asiatica*. 8- and 12-HETE had the same retention time in HPLC analysis, but using this analytical method, we were able to identify them. © 1998 Elsevier Science B.V.

Keywords: *Gracilaria asiatica*; Eicosanoids; Leukotrienes; Hydroxytetraenoic acids; Prostaglandins

1. Introduction

In the application of liquid chromatography (LC)–mass spectrometry (MS) to biological samples, electrospray ionization (ESI) [1] has been proven to desolvate and ionize fragile chemical species such as protein, peptides, nucleic acids and active compounds [2–4]. The characteristics of this technique are soft ionization and suitability for compounds with various molecular masses (from low to high). However, to our knowledge, few applications to the analyses of eicosanoids have been reported.

A red alga, *Gracilaria asiatica* is one of the popular seaweeds in Japan. In 1994, four cases of human intoxication by eating raw *G. asiatica* including eight victims with three deaths were officially reported in Japan [5]. It is known that this species of red algae contains a high percentage of arachidonic acid (AA) [6], and its metabolite via the cyclooxygenase pathway, prostaglandin (PG) E_2, has been implicated as one of the causative substances [5,7]. It is well known, however, that eicosanoids other than PGE_2 such as leukotrienes (LTs) and hydroxytetraenoic acids (HETEs) via lipoxygenase have more physiologically active characteristics such as chemoattraction for neutrophils, and contraction of muscles, and have relations with various kinds of diseases in mammals [8,9]. Thus, these kinds of eicosanoids would be very important as causative substances of human poisoning if these substances are synthesized in raw red algae. To date, the

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existence of eicosanoids such as LTs and HETEs in red algae has not been known.

The aim of this study was to identify and analyze the eicosanoids in *G. asiatica* using LC–MS equipped with ESI interface system.

2. Experimental

2.1. Materials

Fresh samples of *G. asiatica* collected along the coasts of Chiba Prefecture in Tokyo bay in February, 1996 were used. Authentic standards, PGA_2, PGE_2, PGB_2, PGD_2, 15-keto-PGE_2, LTB_4, LTC_4, 5-HETE, 8-HETE, 12-HETE and 15-HETE were purchased from Cayman (Ann Arbor, MI, USA). All solvents and other reagents were purchased from Wako (Tokyo, Japan).

ODS-silica Sep-pak (Waters Associates, Milford, MA, USA) used for extraction of AA metabolites, was washed with 10 ml of ethanol (EtOH) and 10 ml of water before use.

2.2. Extraction of AA metabolites

Fresh *G. asiatica* was cut finely (2–3 mm) and to 1 g of the sample 2 ml of water was added. Extraction of eicosanoids was carried out according to the method of Powell [10]. An 8 ml volume of EtOH containing 0.002% butylated hydroxytoluene was added to the sample, and homogenized for 60 s. The homogenate was placed in a cold room (5°C), shaken for 1 h and centrifuged at 1000 *g* for 10 min. A 3 ml volume of EtOH soluble fat was diluted with the same volume of water and the pH was adjusted to 3 with 1 *M* HCl. A 2 ml volume of the acidic solution was applied to the ODS-silica Sep-Pak cartridge, and polar and nonpolar lipids were removed from the column with 10 ml 15% EtOH and

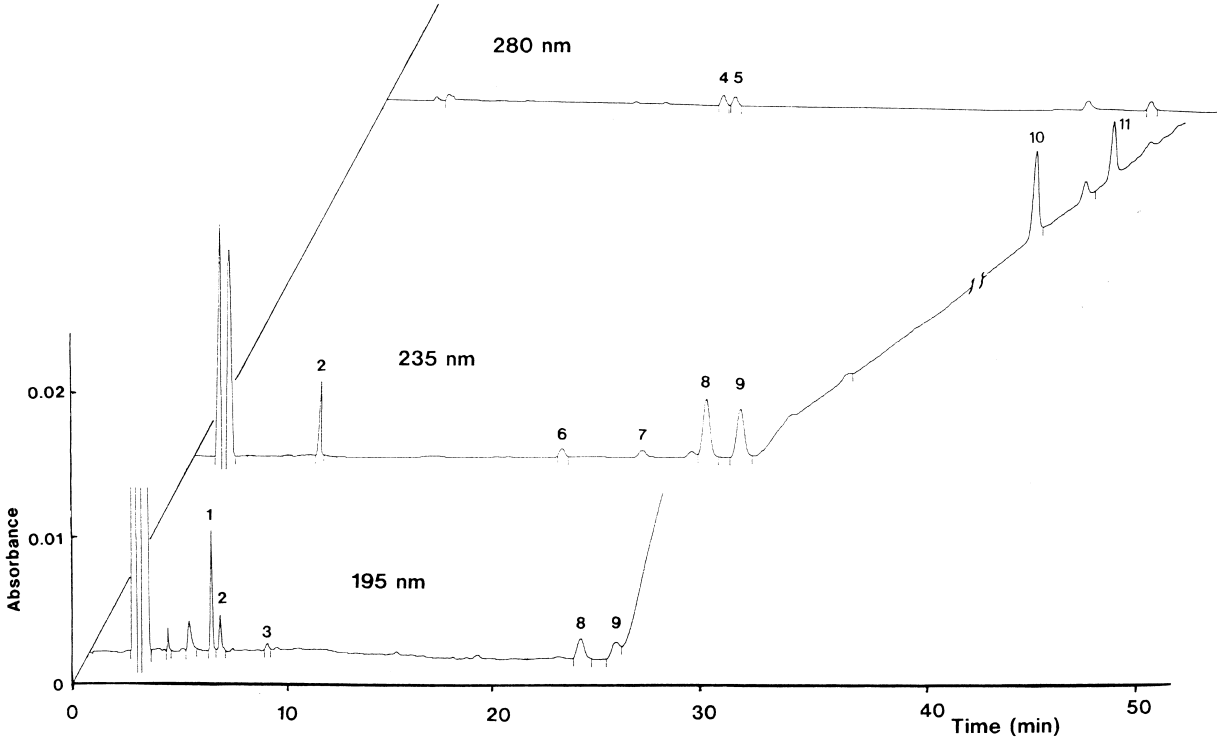

Fig. 1. HPLC chromatograms of eicosanoids in *G. asiatica* at 195, 235 and 280 nm. Mobile phase (I) acetonitrile–MeOH–water–formic acid (40:20:40:0.1, v/v), (II) acetonitrile–MeOH–water–formic acid (40:20:20:0.08, v/v), gradient 0–25 min (I:100%–100%), 25–50 min (I:100%–0%). A 10 μl volume of MeOH sample corresponding to 0.4 mg of *G. asiatica* was injected. For peak nos. see Table 1.

10 ml light petroleum. Eicosanoids were eluted with 6 ml ethyl acetate. The solvent was evaporated under N_2. The residue was dissolved in 0.1 ml methanol (MeOH). It was further diluted to 21-fold with MeOH for HPLC analysis.

2.3. HPLC and MS conditions

A HPLC system, Model PU-980 with CrestPack column, C18T-5 (4.6×250 mm) and a UV-970 detector (UV–vis) equipped with on-flow spectrum system from 200 nm to 350 nm (JASCO, Tokyo Japan), and a Platform mass spectrometer (Micromass, UK) equipped with an ESI interface system were used as analytical instruments. In this study, a gradient solvent system with the following conditions was used. This system was preferred due to its shorter analytical time compared with other solvent systems. Mixing solvents, (I) acetonitrile–MeOH–water–formic acid (40:20:40:0.1, v/v) and (II) acetonitrile–MeOH–water–formic acid (40:20:20:0.08, v/v) were used as a gradient solvent system: 0–25 min (I:100%–100%), 25–50 min (I:100%–0%). For confirmation of PGs, an isocratic solvent system, 0.017 *M* phosphoric acid–acetonitrile (100:50) was also used [11]. Flow-rate and column temperature were 1.0 ml/min and 40°C, respectively. A 10 μl volume of the MeOH sample corresponding to 0.4 mg wet mass of *G. asiatica* was injected into the HPLC. Analytical conditions in the negative ion scanning modes of ESI were as follows: capillary voltage, 2.8 kV; cone voltage, 40 V and 60 V; ion source temperature, 70°C.

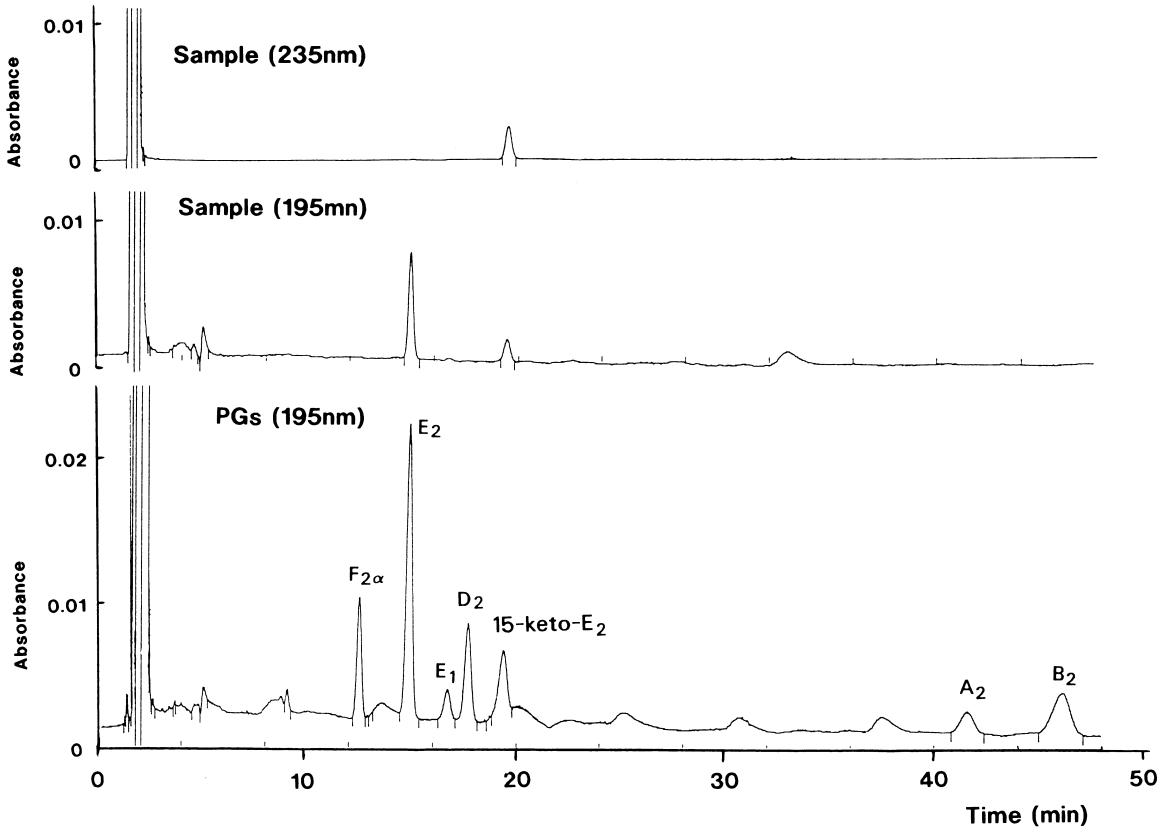

Fig. 2. HPLC chromatograms of PGs in *G. asiatica* at 195, and 235 nm and authentic PGs at 195 nm. Mobile phase, 0.017 *M* phosphoric acid–acetonitrile (100:50). A 10 μl volume of MeOH sample corresponding to 0.4 mg of *G. asiatica* was injected.

2.4. Identification and determination of eicosanoids

Identification of the compounds was made by comparing the HPLC peak retention times and their mass spectra with those of authentic standards. For complete identification of the eicosanoids, co-chromatography using authentic compounds was employed. A multifunction data processor (C-R4A Chromatopac, Shimadzu, Kyoto, Japan) connected to the HPLC system was used for a relative calculation of eicosanoids.

3. Results

HPLC chromatograms of eicosanoids in the *G.*

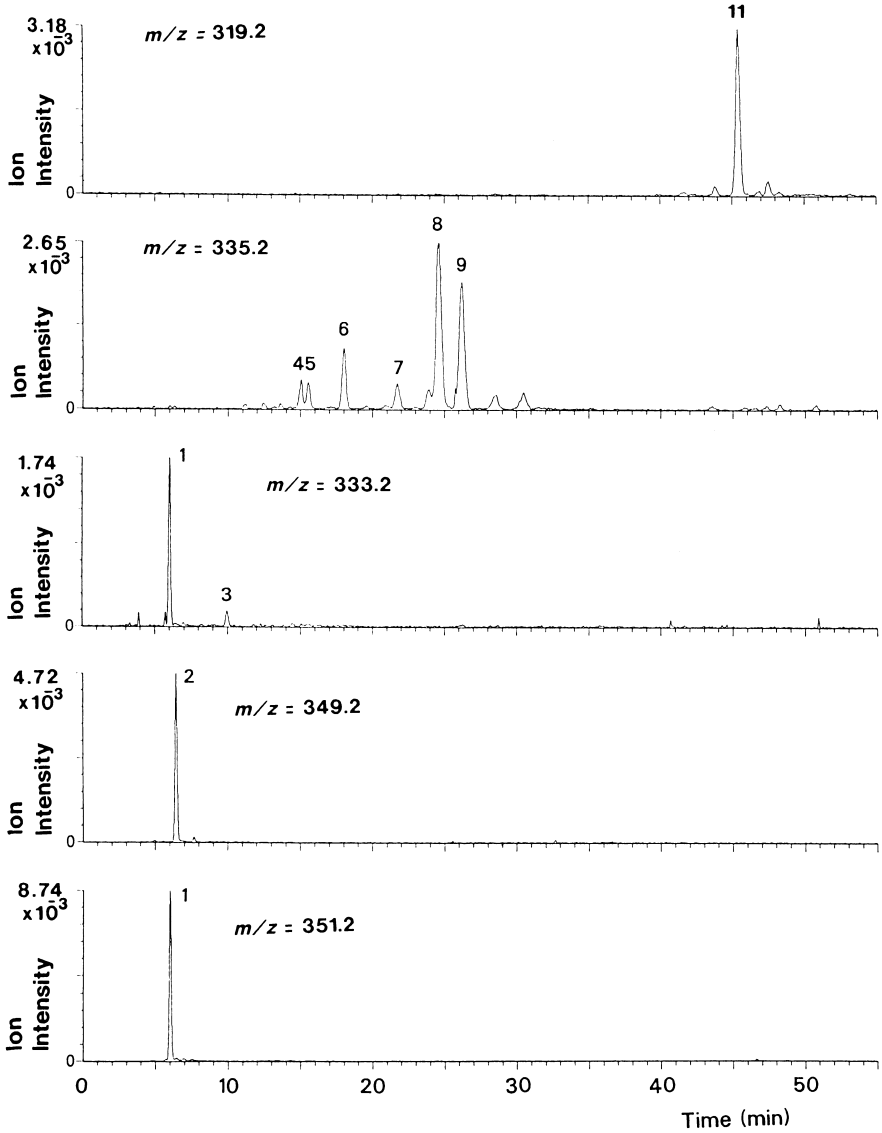

Fig. 3. Ion intensity of eicosanoids in *G. asiatica* detected by LC–MS using ESI. Mobile phase (I) acetonitrile–MeOH–water–formic acid (40:20:40:0.1, v/v), (II) acetonitrile–MeOH–water–formic acid (40:20:20:0.08, v/v), gradient 0–25 min (I:100%–100%), 25–50 min (I:100%–0%) A 10 μl volume of MeOH sample corresponding to 0.4 mg of *G. asiatica* was injected. For peak nos. see Table 1.

asiatica are shown in Fig. 1. PGs, conjugated diene compounds and conjugated triene compounds were monitored at 195 nm, 235 nm and 280 nm, respectively. Eleven peaks were detected on the chromatograms. According to the UV spectra acquired from 200 nm to 350 nm, the UV absorbance maxima of peaks 2, 8, 9, 10 and 11 were found to be at 234 nm, peaks 4 and 5 were at 278 nm, and those of peaks 1 and 3 were below 200 nm. These results suggested that peaks 2, 8, 9, 10 and 11 were substances possessing conjugated diene, peaks 4 and 5 were substances possessing conjugated triene and peaks 1 and 3 were PGs.

To examine the existence of PGs in the same sample, another different solvent system consisting of 0.017 *M* phosphoric acid–acetonitrile (100:50) devised for the isolation of PGs was used. HPLC chromatograms of the samples and authentic PGs are shown in Fig. 2. The retention time of peak 1 corresponded to that of the authentic PGE_2, and that of peak 2 to 15-keto-PGE_2. Peak 2 has UV absorbance maxima at 234 nm. As authentic 15-keto-PGE_2 has also UV maxima at 234 nm, peak 2 was considered as 15-keto-PGE_2. Using the co-chromatography method, peaks 1, 2, 3, 5 and 11 were confirmed to be PGE_2, 15-keto-PGE_2, PGA_2, LTB_4 and 8 or 12-HETE. In this HPLC system, it was impossible to separate 8-HETE from 12-HETE. Further confirmation was carried out using LC–MS. The negative ion mass chromatograms are shown in Fig. 3. The ions associated with peak 1 were *m*/*z* 351 and 333, the latter presumably being a fragment ion due to loss of water. The value for peak 2 was *m*/*z* 349, peak 3 was *m*/*z* 333, peaks 4–9 were *m*/*z* 335 and peak 11 was *m*/*z* 319, respectively. The ion intensities of substances were 1>2>11>8>9>6.

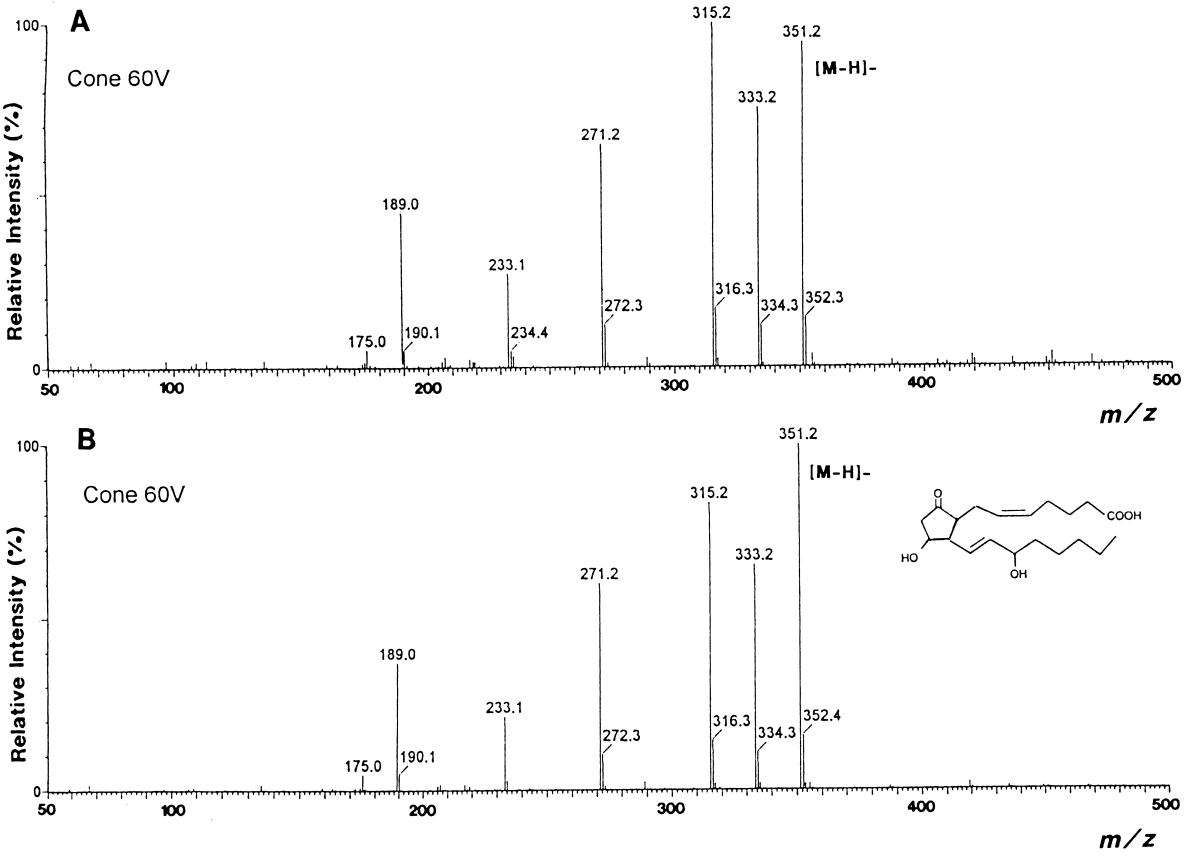

Fig. 4. Mass spectra of HPLC peak 1 (A) and PGE_2 (B) with proposed fragmentation of PGE_2 (B). For peak nos. see Table 1.

Substances 3, 4, 5 and 7 have low ion intensities. Although peak 10 appeared in the UV chromatogram of a compound possessing a conjugated diene, it was impossible to identify because it did not appear in the LC–MS analysis.

Figs. 4–8 show the mass spectra of the authentic eicosanoids and HPLC peaks (1, 2, 3, 4, 5 and 11). It seems that using a cone voltage of 60 V on the interface was more appropriate for mass analysis. In the analysis at 40 V, a few cleavage ions appeared, thus it was considered inefficient. Fig. 4A is the mass spectrum of HPLC peak 2 and Fig. 4B is that of authentic PGE_2. The $[M-H]^-$ ion of PGE_2 was observed at m/z 351. The molecule underwent ESI cleavage resulting in ions at m/z 333 (M−19), 315 (M−37), 271 (M−81), 233 (M−119) and 189 (M−163). Ions at m/z 333 and 315 indicated the loss of water with proton $[M-H-H_2O]^-$ and two moles of water with proton $[M-H-2H_2O]^-$, respectively. The ion at m/z 315 was more abundant than that at m/z 333. Ions at m/z 271 would indicate the loss of a carboxylic group from ion $[M-H-2H_2O]^-$. The mass spectrum pattern of peak 1 (Fig. 4A) agreed well with that of PGE_2 (Fig. 4B). Fig. 5A is the mass spectrum of HPLC peak 2 and Fig. 5B is that of authentic 15-keto-PGE_2. The $[M-H]^-$ ion of 15-keto-PGE_2 was detected at m/z 349. The molecule underwent ESI cleavage resulting in ions at m/z 331 (M−19), 287 (M−63) and 235 (M−115). Ions were considered as $[M-H-H_2O]^-$ at m/z 331 and $[M-H-H_2O-COOH]$ at m/z 287. Though the relative intensity of the ion at m/z 235 was significantly high, the cleavage position is not clear. The mass spectral pattern of peak 2 (Fig. 5A) agreed

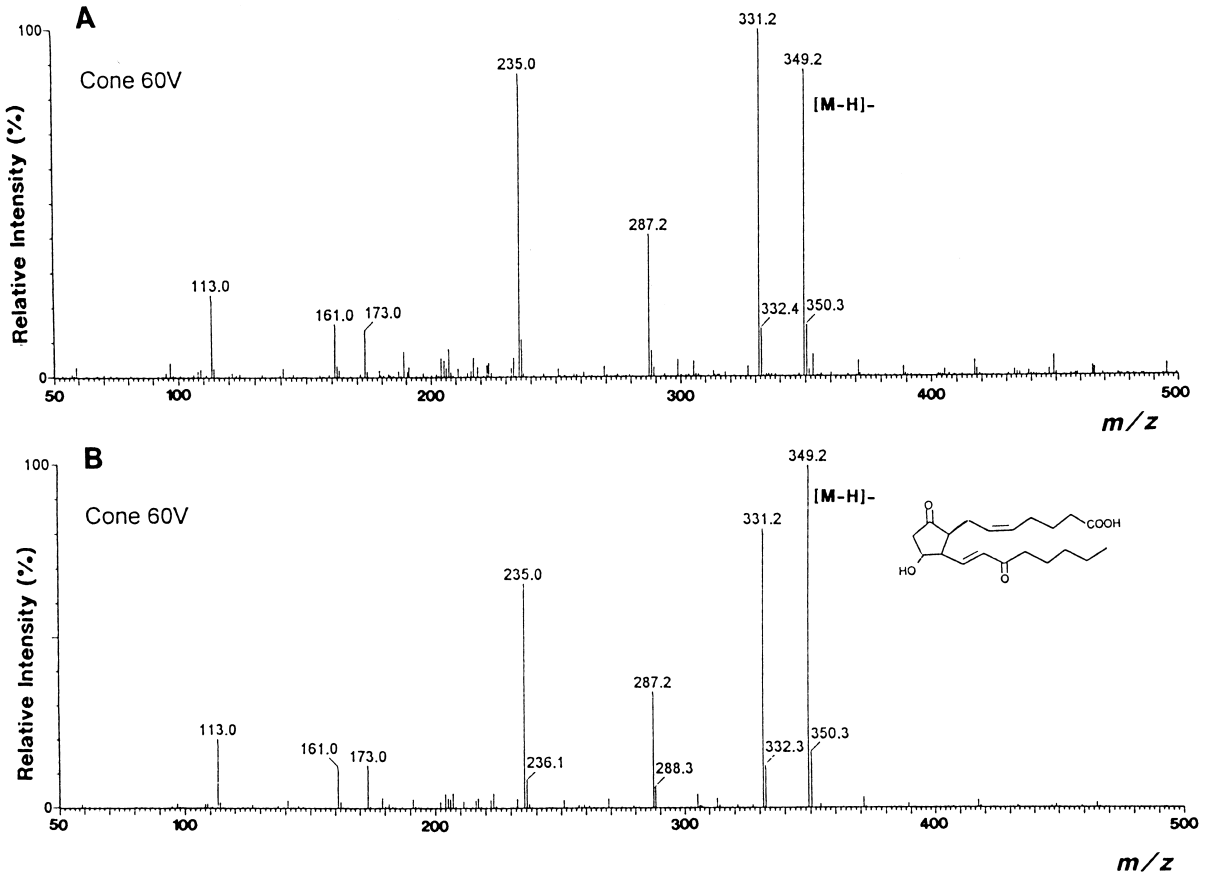

Fig. 5. Mass spectra of HPLC peak 2 (A) and 15-keto-PGE_2 (B) with proposed fragmentation of 15-keto-PGE_2 (B). For peak nos. see Table 1.

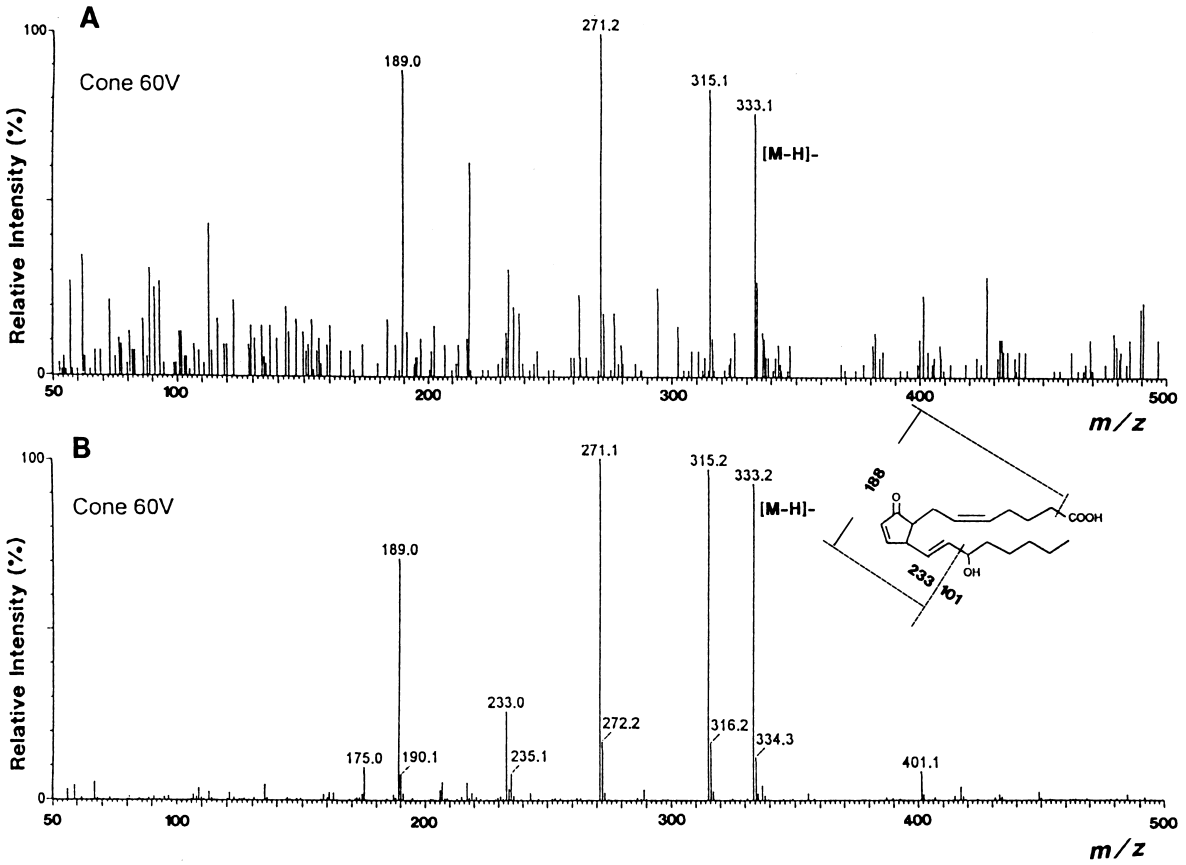

Fig. 6. Mass spectra of HPLC peak 3 (A) and PGA_2 (B) with proposed fragmentation of PGA_2 (B). For peak nos. see Table 1.

well with that of 15-keto-PGE_2 (Fig. 5B). Fig. 6A is the mass spectrum of HPLC peak 3 and Fig. 6B is that of authentic PGA_2. The molecular ion $[M-H]^-$ of PGA_2 was observed at m/z 333. The ESI cleavage ions were observed at m/z 315 (M−19), 271 (M−63), 233 (M−101) and 189 (M−145). Ions at m/z 351 and 271 were considered as $[M-H-H_2O]^-$ and $[M-H-H_2O-COOH]^-$, respectively. The relative intensity of these two ions was higher than that of ions $[M-H]^-$ at m/z 333, indicating that this compound loses water and carboxylic groups quite easily. Ions at m/z 233 and 189 were characteristic fragments of PGA_2. The proposed fragmentation is shown in Fig. 6B. As the HPLC peak 3 was very small, its mass spectrum had a lot of noise peaks. Nevertheless, major ions at 333, 315, 271, 233 and 189 were observed in the mass spectrum, which indicated peak 3 to be PGA_2. Fig. 7(A, B and C) show the mass spectra of HPLC peaks 4, 5 and LTB_4, respectively. The molecular ion $[M-H]^-$ of LTB_4 was observed at m/z 335. Only a few fragments were obtained even at a cone voltage of 60 V, which suggested that this compound seems difficult to fragment. The molecule underwent ESI cleavage resulting in ions at m/z 317 (M−19) and 195 (M−141). The ion at m/z 317 was considered as $[M-H-H_2O]^-$. The ion at m/z 195 was a characteristic fragment of LTB_4 where the proposed fragmentation is shown in Fig. 7C. This mass spectrum agreed with the HPLC peaks 4 (Fig. 7A) and 5 (Fig. 7B) suggesting that compounds 4 and 5 might be isomers of LTB_4. The mass spectra of 8-HETE and 12-HETE are shown in Fig. 7(B and C). The ions $[M-H]^-$, $[M-H-H_2O]^-$ and $[M-H-H_2O-COOH]^-$ were observed at m/z 319 (M−1), 301 (M−19) and 257 (M−63), respectively in both compounds. However,

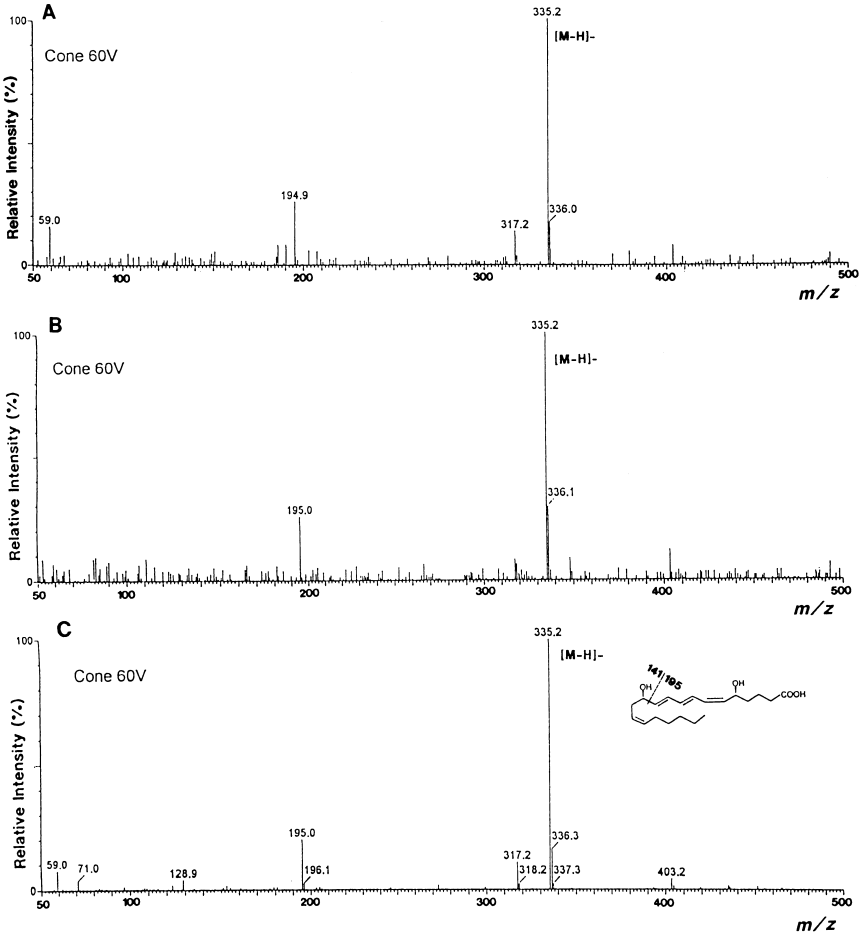

Fig. 7. Mass spectra of HPLC peak 4 (A) and 5 (B), and LTB_4 (C) with proposed fragmentation of LTB_4. For peak nos. see Table 1.

other ions such as those at *m/z* 163 (M−157), 155 (M−165) and 127 (M−193) for 8-HETE were different from those at *m/z* 208 (M−112) and 179 (M−141) for 12-HETE. From the fragment ions, the proposed fragmentations of these compounds are shown in Fig. 8(B and C). The mass spectrum of HPLC peak 11 coincided with 8-HETE.

HPLC profiles and the concentrations of eicosanoids are summarized in Table 1. The concentration of PGE_2 was similar to the value found using the radioimmunoassay method previously reported [6]. The concentrations of eicosanoids identified were high in order, 8-HETE>15-keto-PGE_2>PGE_2>PGA_2>LTB_4 in *G. asiatica*.

4. Discussion

There are many reports [5,12,13] concerning the PGs existence in red algae such as PGE_2, PGE_1 and PGA_2 using HPLC, gas chromatography–MS and ^1C and ^{13}C NMR techniques. An application of LC–MS using ESI as interface to the sea product, red algae, for analyzing eicosanoids is considered to be a first trial. A number of eicosanoids were identified in this study.

It was difficult to identify peak 11 by HPLC alone because it was impossible to separate authentic 8-HETE from 12-HETE using a gradient solvent system. It was also impossible even when a more

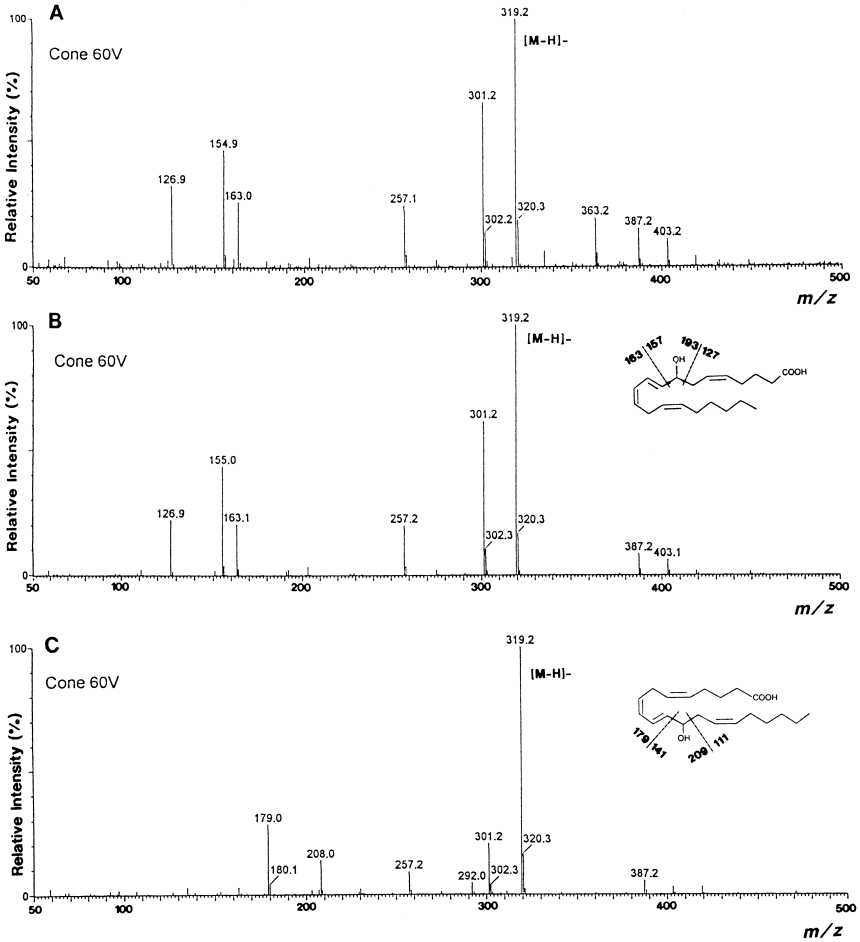

Fig. 8. Mass spectra of HPLC peak 11 (A), 8-HETE (B) and 12-HETE (C) with propeosed fragmentation of 8-HETE and 12-HETE. For peak nos. see Table 1.

hydrophilic solvent system (acetonitrile–MeOH–water–formic acid, 100:50:100:0.2, v/v) was used. Using LC–MS, however, enabled a complete identification. The mass spectrum suggested that this peak was 8-HETE. Thus, ion fragmentation is effective in identifying eicosanoids.

Although identification of the eicosanoids was partially achieved, some problems remained. For example, out of the eleven major compounds detected by HPLC, one compound (peak 10) was not detected in LC–MS suggesting that ionization of this compound did not occur. Moreover, the order in the ion intensity of eicosanoids did not agree with the order of the signals of eicosanoids measured by HPLC. The ion intensity of 8-HETE was lower than that of PGE_2, but their concentrations were in the reverse. This situation might be attributed to the polarity of the compounds. Banks [14] reported that the inclusion of 0.1% trifluoroacetic acid in the LC mobile phase inhibited the electrospray process, resulting in complete loss of ion signal in analysis of protein and peptides. An acidic mobile phase was used in this study, since acidic mobile phase is commonly used [11,15–17] due to its efficient separation in most analyses of eicosanoids by HPLC. The problems in ionization using an on-line

Table 1
Summary of the HPLC profiles and concentrations of eicosanoids in *Gracilaria asiatica*

Peak No.[a]	Compounds	Retention time[b] (min)	λ_{max} (nm)	$[M-H]^-$ (m/z)	Ion intensity[c] ($\cdot 10^{-3}$)	Concentrations[d] ($\mu g/g$ wet mass)	Method of identification
1	PGE_2	6.0	<200	351	8.7	150±20	HPLC, MS
2	15-Keto-PGE_2	6.4	234	349	4.7	120±20	HPLC, MS
3	PGA_2	9.9	<200	333	0.2	24±6	HPLC, MS
4	Isomer of LTB_4	15.0	278	335	0.2	–	HPLC, MS
5	LTB_4	15.4	278	335	0.2	18±2	HPLC, MS
6	Unknown	18.0	234	335	0.9	–	–
7	Unknown	21.7	234	335	0.2	–	–
8	Unknown	24.6	234	335	2.7	–	–
9	Unknown	26.2	234	335	2.0	–	–
10	Unknown	41.1	234	–	–	–	–
11	8-HETE	45.4	234	319	3.2	230±30	HPLC, MS

[a] Peaks No. in HPLC in Fig. 1.
[b] Mobile solvent system with I (acetonitrile–methanol–water–formic acid, 40:20:40:0.1) and II (acetonitrile–methanol–water–formic acid, 40:20:20:0.08) was used as gradient of 0–25 min (I:100%–100%) and 25–50 min (II:100%–0%).
[c] Means of duplicates.
[d] Means of triplicates.

eicosanoids LC separation coupled with ESI-MS might also be due to the characteristics of mobile phase.

The existence of LTB_4 and 8-HETE in *Gracilaria* has not been reported. The existence of 8-HETE and LTB_4 suggested that *G. asiatica* has a metabolic pathway from AA and these eicosanoids might play an important role physically. In addition, a novel substance, 15-keto-PGE_2, was identified in *G. asiatica*. This compound is an oxidative metabolite of PGE_2 and is contained in amounts next highest to PGE_2.

It is well known that LTB_4 and HETEs are potent agents which have chemotactic and chemokinetic properties, aggregation of leukocytes or degranulation and leak of lysosomes from mammalian cells [18–20]. Among various HETEs, 8-HETE has been reported as having a potent migration effect on neutrophils next to 5-HETE [21]. Thus, these substances could not be ruled out as a possible causative substance of poisoning due to eating red algae [1,22].

5. Conclusion

Analysis of eicosanoids in red algae, *G. asiatica* was carried out using LC–MS with ESI for interface. PGE_2, 15-keto-PGE_2, PGA_2, LTB_4 and 8-HETE were identified as major eicosanoids in *G. asiatica*. Although there was a problem regarding the sensitivity for some compounds, this instrument was beneficial for identification of eicosanoids in red algae. This information may give greater insight for researchers in the same field.

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