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RESEARCH ARTICLE

J. I. Carreto · M. O. Carignan · N. G. Montoya

A high-resolution reverse-phase liquid chromatography method for the analysis of mycosporine-like amino acids (MAAs) in marine organisms

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Abstract Mycosporine-like amino acids (MAAs) are a group of about 20 structurally related water-soluble compounds, widely distributed among freshwater and marine organisms. To provide a better assessment of the diversity and concentration of MAAs in aquatic environments a high-performance liquid chromatography (HPLC) method of analysis based on reverse-phase C_{18} column and trifluoroacetic acid and an ammonium-containing mobile phase was developed. The improvements with respect to previous methods and the extraction and clean-up procedures are described here. With this method the clean-up recovery of MAAs of high polarity (shinorine), medium polarity (palythinol), and low polarity (palythene) is greater than 99% ($\pm 1\%$). The method is selective enough to resolve in a single run most of the characterized MAAs found in marine organisms, including the critical and highly polar compounds shinorine, mycosporine-2-glycine, and palythine-serine, the medium polarity pair palythenic acid and shinorine methyl ester (M-333), and the low polarity isomeric pair usujirene and palythene. A chromatogram of a mixture of over 20 MAAs such as might be found in complex samples of marine organisms is given. Good precision was obtained in the separations. The relative standard deviation for retention times was below 1% and the mean relative standard deviation for integrated area estimations was below 2%. A mean column recovery of standards was 99% ($\pm 1\%$) whereas limits of detection (signal-to-noise, $S/N = 2$) for different MAAs varied between 0.08 and 0.47 pmol injected. The applicability of the method was tested using extracts of three microalgae cultures, three natural phytoplankton populations, two scleractinian corals, and one species of sea anemone. Results reveal the occurrence of several unknown MAAs not previously reported in the literature. The selectivity of the method toward some recently discovered MAAs makes it especially suitable not only for studying new field samples, but also for re-examining the MAA composition of previously studied organisms.

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J. I. Carreto (✉) · M. O. Carignan · N. G. Montoya
Instituto Nacional de Investigación y
Desarrollo Pesquero (INIDEP),
Paseo Victoria Ocampo N°1,
B7602HSA, 7600 Mar del Plata,
Argentina
E-mail: jcarreto@inidep.edu.ar

Introduction

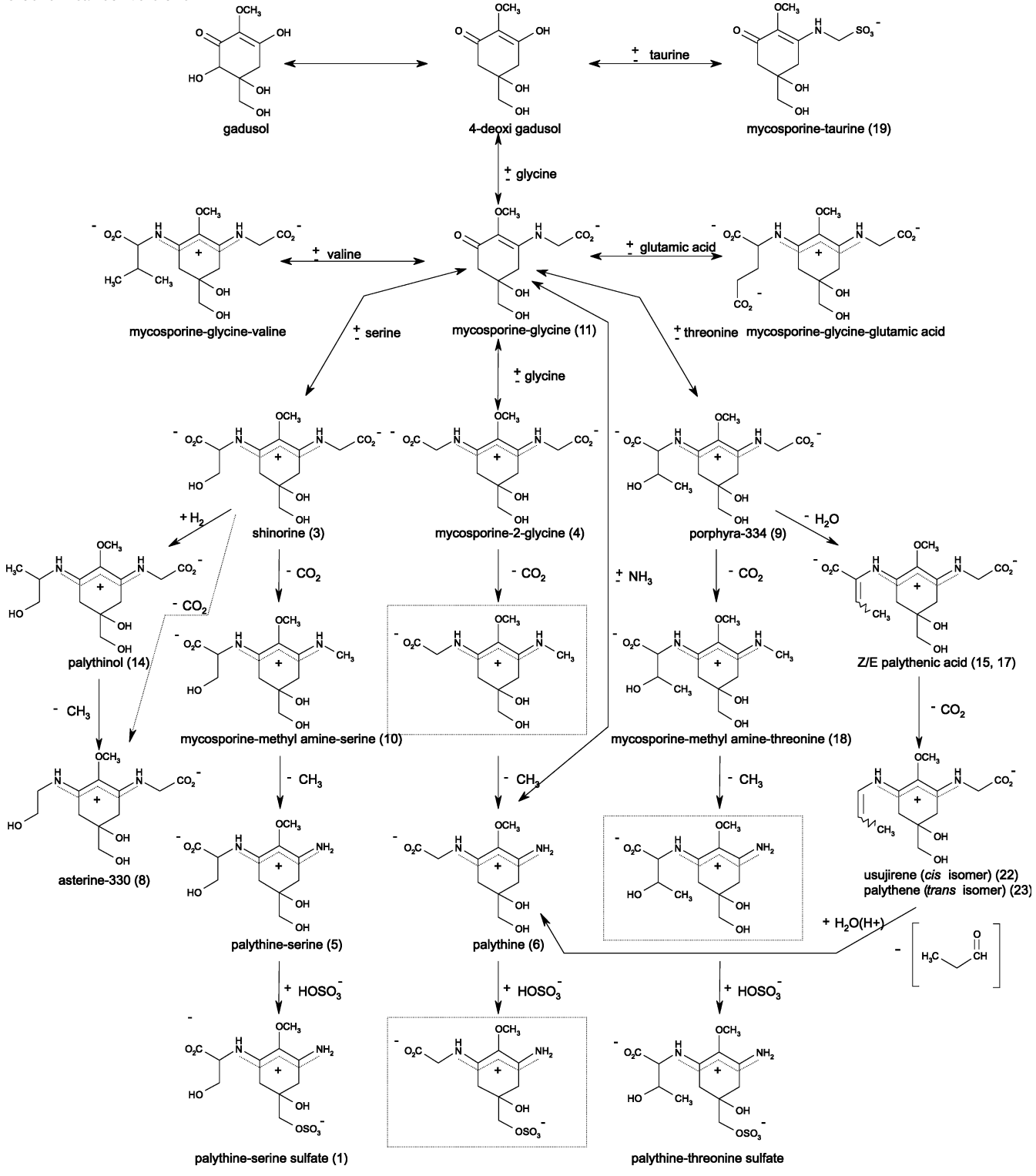
Considerable interest has been centered on the mycosporine-like amino acids (MAAs) because of their ultraviolet (UV)-photoprotective role. This has been inferred from their efficient UV absorption (Takano et al. 1978) and from their light-dependent induction of synthesis (Carreto et al. 1989), which has been recently verified experimentally (Adams and Shick 1996; Neale et al. 1998; Karsten et al. 1999). In addition some MAAs may act as antioxidants to prevent cellular damage resulting from UV-induced production of toxic oxygen species (Dunlap et al. 2000).

These compounds, which are widely distributed among freshwater and marine organisms (Bandaranayake 1998; Sinha et al. 1998; Shick and Dunlap 2002), are composed of a cyclohexenone or cyclohexenimine chromophore conjugated with the nitrogen substituent of an amino acid, amino alcohol, or amino group. There are now more than 20 fully characterized MAAs with maximum absorption ranging from 309 to 360 nm. In addition, several partially characterized and other unknown MAAs have been recently detected as a consequence of the increase in the number of studied organisms and the development of more efficient high-performance liquid chromatography (HPLC) separation techniques (Jeffrey et al. 1999; Subramaniam et al. 1999; Carreto et al. 2001). Generally a glycine sub-unit is present on the C3 of the cyclohexenimine ring. Some MAAs also contain sulfate esters (Wu Won et al. 1997).

Other MAAs are covalently linked to oligosaccharides (Böhm et al. 1995) or among themselves (Carreto et al. 2001). The structural relationship among several MAAs commonly detected in the marine environment is illustrated in Fig. 1.

Until now, only a few HPLC methods for MAA separation in marine organisms have been reported. No method has been able to separate a mixture of over 20 MAAs such as might be found in the most complex samples from marine organisms. Techniques for the separation of MAAs include those of Nakamura et al. (1982), Dunlap and Chalker (1986) and their modifications (Stochaj et al. 1994; Shick et al. 1999), and Carreto

Fig. 1 Structural relationships between the different mycosporine-like amino acids (MAAs) and their feasible chemical and/or biochemical conversions



et al. (2001). The classical HPLC method was based on reverse-phase low silanol-free group octadecylsilica (C_{18}) columns and isocratic elution with 0.02% acetic acid as mobile phase at 15°C (Nakamura et al. 1982). Under these conditions 7 of the 9 MAAs known at the time were separated according to hydrophobic properties. This method had the disadvantage that it did not elute the more hydrophobic compounds such as usujirene and palythene and several of the new complexes of MAAs recently described (Carreto et al. 2001). In addition its utility for the separation of the new highly polar compounds recently discovered in corals (Teai et al. 1997, 1998; Wu Won et al. 1997; Shick et al. 1999) was not proved.

Dunlap and Chalker (1986) first introduced the use of monomeric octylsilica (C_8) columns. The original method was based on reverse-phase non-endcapped C_8 column and isocratic elution with 0.1% acetic acid and 10% methanol. Under these conditions the more weakly acidic compounds (mycosporine-glycine, palythine, asterina-330, palythinol, and palythene) were separately eluted. However, it was unable to separate clearly the strongly acidic shinorine from porphyra-334. After the discovery of mycosporine-2-glycine and mycosporine-taurine in the sea anemone *Anthopleura elegantissima* (Stochaj et al. 1994) a new drawback was added as the method was not able to separate mycosporine-2-glycine from porphyra-334 and mycosporine-glycine from mycosporine-taurine. Stochaj et al. (1994) showed that the chromatographic separation of the acidic compounds could be improved if the methanol content of the mobile phase was increased up to 75%. In this condition, the highly polar compounds interacted with the weak anion exchange properties of the silanol groups to give an improved chromatographic separation of these compounds. Although these methods and their further modifications using the same stationary phase achieved good separation of several compounds (Shick et al. 1999), none was able to separate in a single run the strongly acidic MAAs and the more weakly acidic compounds. Separation of these mixtures was only achieved by isocratic elution with two different mobile phases (Helbling et al. 1996; Adams and Shick 2001; Shick et al. 2002). However, resolution for some critical pairs, such as mycosporine-glycine and mycosporine-taurine, the MAA-sulfated esters and the isomeric mixture of usujirene and palythene, was insufficient. Even the most recent modifications of these methods do not completely resolve these MAA mixtures. The resolution of mycosporine-glycine and the mixture of MAA-sulfated esters was recently improved with the use of ion-exchange chromatography on a bonded-phase amino column (Wu Won et al. 1997).

Dionisio-Sese et al. (1997) were the first to use reverse-phase C_{18} columns and gradient elution with aqueous methanol, but their application was only successful for the separation of four MAAs. In a recent paper Carreto et al. (2001) showed that using acetonitrile-based eluents and polymeric double-endcapped C_{18} columns, a mixture of strongly acidic and neutral MAAs could be separated in a single run. This technique also resolved the critical isomeric pair usujirene and palythene and its application revealed the occurrence of several new low-polarity atypical MAAs not previously reported in the literature. Nevertheless, this method and its further modifications (Conde et al. 2003) failed in the separation of certain highly polar MAAs (shinorine from mycosporine-2-glycine and palythine-serine), characteristics of some scleractinian corals (Teai et al. 1997, 1998; Wu Won et al. 1997; Shick et al. 1999). Recently Whitehead et al. (2001) and Whitehead and Hedges (2002) developed a mass spectral approach to MAA characterization using liquid chromatography coupled with electrospray ionization mass spectrometry (LC/MS). With the exceptions of the isomeric pair E/Z palythenic acid and usujirene/palythene, this approach allows the identification of individual MAAs without the need for a high chromatographic resolution. However the high cost of LC/MS systems and the level of expertise required for their operation limit their use in many laboratories.

In the present work we describe an improved HPLC separation method for the analysis of complex samples of MAAs from marine organisms. This method combines a C_{18} column system with an optimized aqueous mobile phase including trifluoroacetic acid (TFA) and ammonium as ion-suppression/ion-pairing agents. The results obtained from the analysis of algal cultures, natural phytoplankton populations, red macroalgae, and symbiotic organisms show that the new method is able to separate in a single run complex mixtures of over 20 MAAs, such as might be found in the most complex samples from marine organisms.

Materials and methods

Samples

Four algal cultures, three natural phytoplankton populations, four macroalgae, and three symbiotic organisms were selected for this study to include most of the MAAs found in marine organisms. Extracts from these samples were used for method development and evaluation.

Algal cultures

The four species used were *Alexandrium tamarense* (Lebour) Balech clone MDQ 1096 (Dinophyceae) isolated from the Mar del Plata coast (Argentina), *A. catenella* (Weedon and Kofoid) Balech clone CC08 (Dinophyceae) isolated from the XI region of Chile, *Emiliania huxleyi* clone CCMP 370 (Prymnesiophyceae) from the Provasoli-Guillard Centre for Culture of marine phytoplankton (CCMP), and *Pseudonitzschia multiseries* (Bacillariophyceae) isolated from the Mar del Plata coast (Argentina).

Phytoplankton assemblages

Two phytoplankton samples were collected in the coastal waters off Mar del Plata, one during a bloom of the heterotrophic dinoflagellate *Noctiluca scintillans* and the other during a bloom of the dinoflagellate *Gymnodinium catenatum*. Another sample was collected in waters from the Brazil Current. Samples were filtered onto GF/F filters and maintained in liquid nitrogen until analysis.

Macroalgae

The macroalgae *Palmaria decipiens* and *Iridea* sp. were collected at Potter Cove (South Shetland Islands, Antarctica) and were kindly donated by Dr. G. Ferreyra. *Porphyra columbina* were collected at Chubut coast (Argentina) and were kindly donated by Dr. A. Boraso. Lyophilized *Porphyra* sp. (Nori) was kindly donated by Dr. P. Zimba. All of these organisms were stored at −20°C until analysis.

Symbiotic organisms

Lyophilized purified (C_{18} column) extracts of the coral *Pocillopora eydouxi* collected at Tahiti island (French Polynesia) were kindly donated by Dr. T. Teai. Lyophilized purified (C_{18} column) extracts of the coral *Stylophora pistillata* were kindly donated by Dr. M. Shick. Specimens of the sea anemone *Anthopleura elegantissima* were collected in California (USA) and were kindly donated by Dr. M. Shick. All of these materials were immediately stored at −20°C.

Chemicals

All solvents used were HPLC grade from Riedel-de Haën. Distilled water was further purified to HPLC grade by passage through a Barnstead water purification system (Nanopure UV) equipped with ion exchange, carbon cartridges, and UV radiation. TFA (99.9% purity) was purchased from J. Baker and the ammonia solution (37%) from Merck.

The MAAs shinorine, phorphyra-334, palythine, asterine, palythinol, mycosporine-glycine, palythenic acid, usujirene, palythene, M-320, M-333, and M-335/360 were obtained as previously described (Carreto et al. 2001). Palythine-serine, mycosporine-methylamine-serine, and mycosporine-methylamine threonine standards purified from the coral *P. eydouxi* were kindly donated by Dr. T. Teai. Secondary standards of mycosporine-2-glycine and palythine-serine sulfate from the coral *S. pistillata* and mycosporine-taurine from the sea anemone *A. elegantissima* were kindly donated by Dr. M. Shick. Mycosporine-2-glycine and mycosporine-taurine were isolated and purified from these sources.

Preparation of samples for HPLC analysis

Sample extraction

Algal cultures and natural phytoplankton samples were concentrated on 25-mm Whatman GF/F filters and when necessary stored at −20°C. Three different protocols were used to extract MAAs with HPLC grade methanol: (1) lyophilized or filtered phytoplankton samples were cut into small pieces and sonicated 1 min in a pulse mode at 0°C in centrifuge tubes, using 2 ml 100% methanol. A Vibra Cell sonicator (Sonic and Materials, Inc.) equipped with a 4-mm-diameter probe was operated at 40 W. The decanted extract was collected and the filter debris was re-extracted twice with 100% methanol. The combined extracts were filtered (Whatman GF/F) to remove debris and their absorption spectra was recorded. (2) Samples were soaked with small volumes of water (water:sample mass ratio approximately 9:1) overnight in the dark at 4°C before applying the extraction procedure described in protocol 1. (3) For comparison we also investigated a complementary extraction method using more aggressive conditions: samples were extracted with 25% aqueous methanol (v:v) for 2 h in a water bath at 45°C (Sommaruga and Garcia-Pichel 1999; Tartarotti and Sommaruga 2002). The decanted extract was collected and the filter debris was re-extracted twice in the same conditions with 25% aqueous methanol. Extraction efficiencies for these three extraction methods were determined after exhaustive serial extraction of the remaining pellet using protocol 3. For these extraction methods, the recovery and stability of the main individual MAAs found in *Alexandrium catenella* were also determined.

The obtained extracts were evaporated to dryness using a centrifugal vacuum evaporator (Centrivap, Labconco, Co.) and the residue re-dissolved in 500 µl of a pH 3.15 solution of aqueous trifluoroacetic acid 0.2% and ammonium hydroxide (mobile phase A). Lyophilized purified extracts of *P. eydouxi* and *S. pistillata* were dissolved in methanol, evaporated to dryness, and redissolved in mobile phase A prior to injection. To test the stability of MAAs in 100% methanol and in mobile phase A, three replicates of a solution of a mixture of standards were re-injected into the HPLC after having been kept at ambient temperature for 24 h and at −2 °C in the freezer for 7 days. The recovery and stability of more labile MAAs were compared with those of the fresh extracts.

Clean-up procedure

The aqueous MAA extracts were passed through a 100-kDa ultrafilter (Ultra spin, Alltech) to remove water-insoluble materials and large molecules. The recovery after the clean-up procedure was determined using three replicate extracts spiked with shinorine, palithynol, and palythene standard solutions.

Chromatographic apparatus and conditions

HPLC

Method development was performed using a high-pressure gradient system HPLC Shimadzu LC 10 A consisting of a continuous degassing system DGU-14 A, two pumps model LC-10 AT, an auto-sampler injector SIL-10Axl, and a column oven CTO-10 AC. An SPD-M10Avp diode array detector connected via an interface module to a computer running CLASS-LC10 software was used for detection and quantification. The prepackaged columns used were (1) a polymeric double-endcapped C_{18} column (5 μm, 4.6 mm i.d. × 150 mm length; Alltima, Alltech) and (2) a polymer-coated silica reversed-phase C_{18} column (5 μm, 4.6 mm i.d. × 250 mm length; CapCell Pak UG, Shiseido) protected with a guard column cartridge (4.6 mm i.d. × 20 mm length; Alltima, Alltech). The final high-resolution separations were performed with the two columns connected in series and thermostated at 35°C.

Mobile phase and elution gradient

Eluent A was a pH 3.15 solution of aqueous trifluoroacetic acid 0.2% and ammonium hydroxide (see below) while eluent B was a solution containing aqueous trifluoroacetic acid 0.2% and ammonium hydroxide at pH 2.20:methanol:acetonitrile (80:10:10,v:v:v). The aqueous 0.2% TFA solutions were prepared as follows: 2.0 ml of TFA were added to 900 ml of water in a 1-l flask and mixed using a magnetic stirrer. Ammonia solution was then added dropwise until the desired pH was obtained. The mixture was diluted to 1,000 ml with water and the pH rechecked. The optimum gradient employed after all other chromatographic conditions were fixed is shown in Table 1.

MAA identification, resolution, and quantification

Detection was made by monitoring absorption at 360, 330, 310, and also at 270 nm to ensure that the samples were devoid of contaminants with absorption at lower wavelengths. Individual peaks were identified by online absorption spectra, retention time, and when possible by co-chromatography with standards. Resolution (Rs) between a peak and the preceding one was calculated using the following equation: $Rs = 2(Rt_2 - Rt_1)/Wt$, where Rt_2 and Rt_1 are the retention times of two adjacent peaks, and Wt is the sum of peak widths at baseline. Quantification was accomplished by comparing the areas of peaks from unknowns with those from standard solutions calibrated using the molar extinction coefficients (ϵ) at the wavelengths of maximum absorption reported by Bandaranayake (1998). For MAAs whose molecular structure has not yet been completely elucidated or whose extinction coefficients have not been reported we use the calibration coefficients of closely related compounds.

Table 1 Final analytical high-performance liquid chromatography (HPLC) gradient protocol for mycosporine-like amino acid (MAA) separation. Eluent A: 0.2% TFA + ammonium hydroxide (pH 3.15); eluent B: 0.2% TFA + ammonium hydroxide (pH 2.20):methanol:acetonitrile; 80:10:10 (v:v:v). Temperature 35°C

Time (min)	Percent eluent A	Percent eluent B
0	100	0
2	100	0
15	80	20
30	50	50
50	50	50

MAA recovery during chromatography

Recovery of MAAs during chromatography was calculated by comparing peak areas for shinorine, palythinol, and palythene, using the gradient and column described above, with those obtained using isocratic phase A and stainless steel tube instead of the columns (three replicates).

Results and discussion

Extraction

Extraction with methanol or aqueous methanol was used for most MAA analysis (Nakamura et al. 1982; Dunlap and Chalker 1986; Carreto et al. 1990; Karentz et al. 1991; Karsten and García Pichel 1996; Shick et al. 1999; Tartarotti and Sommaruga 2002). The literature has reported several extraction techniques involving soaking, grinding, or ultrasonic disruption in various solvent combinations, which have had varying degree of success. In our results (Table 2) sonication in 100% methanol followed by filtration to remove debris appears to be a practical and efficient (>95%) extraction technique for algal cultures. It would be advantageous if the extract could be split and used for both pigment (Jeffrey et al. 1997) and MAA analysis. However, the efficiency of the extraction appears to be dependent on several factors, especially on the matrix nature. Tartarotti and Sommaruga (2002) reported that in lyophilized red macroalgae and freshwater phytoplankton assemblages, the mean total concentration of MAAs obtained in 25% aqueous MeOH at 45°C was, respectively, ~13 and ~3 times higher than in extractions made with 100% MeOH at 4°C. In contrast, Whitehead et al. (2001) reported that in lyophilized samples of the pteropod *Clione antartica*, extraction efficiencies, after exhaustive serial extractions with 20% aqueous MeOH at −14°C for 12–16 h, were very low (32%). Our results with lyophilized organisms also showed low extraction efficiency after three serial extractions with 100% methanol (Table 2). In this matrix the MAAs appear to be sequestered in a bound form not accessible to the

Table 2 Efficiencies (percent) for different MAA extraction methodologies applied on the studied organisms. Data given as means ± SD ($n = 3$)

Protocol	Organisms			
	Alexandrium catenella	*Porphyra columbina*	*Porphyra* sp. (Nori)	*Anthopleura elegantissima*
1. Methanol 100% (3 times)	97.7 ± 5.2	34.5 ± 6.0	36.1 ± 10.4	28.1 ± 14.0
2. Rehydration + methanol 100% (3 times)	–	98.9 ± 5.9	130.2 ± 15.8	88.1 ± 12.7
3. Aqueous methanol 25% 45°C, 2 h (3 times)	100.0 ± 0.0	97.8 ± 1.4	97.6 ± 0.7	99.9 ± 0.2
4. Exhaustive extraction using protocol 3	100.0	100.0	100.0	100.0

methanol. However, high extraction efficiency (>90%) was obtained if lyophilized samples were previously soaked with water in the dark at 4°C overnight (Table 2). We suspect that hydration of polar groups of proteins and other macromolecules could be the MAAs' liberating mechanisms. Extraction in 25% aqueous MeOH at 45°C for 2 h also increases the extraction efficiency from lyophilized samples, but in *Porphyra* sp. the mean total concentration obtained with this method was about 30% lower than in extractions made after hydration of the sample (protocol 2).

Another question is that extraction made in the most aggressive conditions of the protocol 3 could produce the transformation or degradation of some labile MAAs. In our results it was evident that using protocol 3, hydrolysis of shinorine methyl ester and of the MAA complex M335/360 occurred, with the concomitant increase of their constitutive MAAs (Carreto et al. 2001). Accordingly, the amount of shinorine was 3.5 times higher using protocol 3 than using protocol 1, while M335/360 practically disappears in the extract obtained with protocol 3 (Fig. 2). The increased amount of palythine observed during the experiment is related to the hydrolysis of palythene and the complex M-335/360, which probably occurred in the aggressive conditions used in protocol 3.

Similar hydrolytic cleavage of an unknown MAA—probably shinorine methyl ester—with the corresponding increase in the proportion of shinorine was observed by Tartarotti and Sommaruga (2002) in freshwater phytoplankton samples extracted with 25% aqueous MeOH. We therefore would recommend that routine fresh samples should be directly sonicated in methanol whereas for lyophilized samples a previous soaking with water is required. Nevertheless, extraction efficiencies of the methods and stability of the more labile MAAs should be determined for the type of organism to be analyzed.

The proposed method required evaporation of the extract under reduced pressure prior to HPLC injection and re-dissolution of the residue with mobile phase A. This time-consuming step is also necessary to remove low polarity substances such as lipids and lipoproteins that present problems for HPLC analysis (Shick et al. 1999). On the other hand, these methods do give the best overall enrichment for trace determinations since the extract can be ultimately concentrated in a small volume. Because some MAAs such as usujirene and palythene are unstable in acidic medium, they yielded palythine by treatment with dilute hydrochloric acid (Takano et al. 1978; Carreto et al. 1990). The stability of this and other MAAs in mobile phase A was also investigated. After standing in mobile phase A at ambient temperature for 24 h, a significant decrease in concentration of usujirene (9.3 ± 1.3%) and palythene (12.2 ± 1.7%), accompanied by a significant increase in the amounts of palythine (15.7 ± 1.0%), was observed (Table 3). The yield of palythine calculated from the amounts of usujirene and palythene lost was estimated at about 77.2%. Changes in the concentration of other MAAs were small. Similar decreases in concentration of usujirene (14.2 ± 2.5%) and palythene (26.5 ± 3.4%) were obtained after standing in mobile phase A at −20°C in the freezer for 7 days (Table 3). However, the yield of palythine was very low, indicating the existence of another alternative degradation pathway. Standard MAA solutions made with methanol were found to be very stable at ambient temperature for 24 h (Table 3),

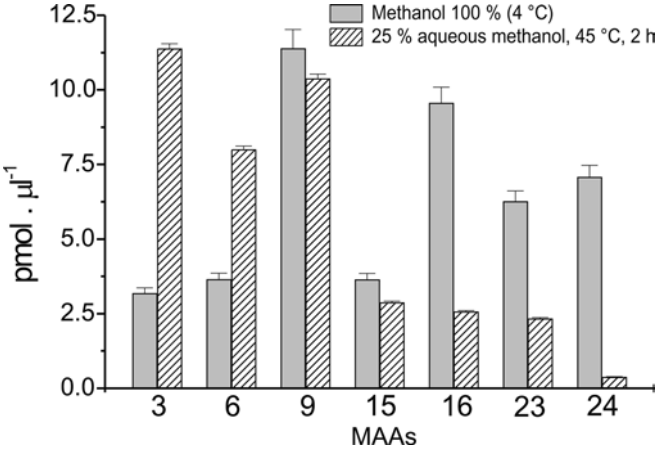


Fig. 2 Concentration of main individual MAAs resulting from the extraction protocols 1 (methanol 100%, 4°C) and 3 (25% aqueous methanol, 45°C, 2 h) made on an *Alexandrium catenella* culture. MAA number identification as in Table 4. Values are means ± standard error (SE), where $n = 3$ in each MAA

but some decrease (7–10%) in concentration of all MAAs tested was observed after 7 days in the freezer (Table 3).

Interferences and clean-up

Solid phase extraction (SPE) on C-18 cartridges gave good results and reproducibility (Teai et al. 1997; Shick et al. 1999) and specifically removed substances such as pigments and lipids that would have eluted at long retention times from the HPLC. However, care should be taken to minimize loss of the less-polar MAAs. Ultrafiltration of the recomposed aqueous extract is another alternative that, in addition to the water-insoluble material, removes molecules larger than the membrane molecular weight cut-off rating. Applying this procedure to three replicates of control extracts spiked with shinorine, palythinol, and usujirene, mean recoveries of $100.4 \pm 0.3\%$, $100.0 \pm 0.6\%$, and $98.2 \pm 0.8\%$, respectively, were obtained.

Nevertheless, highly polar, low molecular weight substances such as 4-deoxygadusol—the postulated precursor of mycosporines—and structurally related compounds such a gadusol (Chioccara et al. 1980; Bandaranayake et al. 1997) and other UV-absorbing substances unrelated to MAAs were also present in extracts of marine organisms (Bandaranayake et al. 1997). As these interferences with absorption maxima typically ranging between 264 and 270 nm were not removed by solid phase extraction (Newman et al. 2000) or ultrafiltration, care should be taken to ensure that peak absorptions are devoid of contaminants with absorption overlapping detection wavelength. In our chromatographic conditions these interfering substances eluted together in a sharp peak without severe overlapping with mycosporine-2-glycine and palythine-serine.

HPLC-method development

Initially we tested the method used by Carreto et al. (2001) as it gave a good separation of most dinoflagellate MAAs. However, this method failed in the separation of certain highly polar MAAs [shinorine (3) from mycosporine-2-glycine (4) and palythine-serine (5)], characteristic of some scleractinian corals (Teai et al. 1997; Wu Won et al. 1997; Shick et al. 1999). To overcome such a problem we evaluated the selectivity of a polymer-coated silica reversed-phase C_{18} column (250 mm × 4.6 mm i.d.; CapCell Pak UG, Shiseido) as this novel bonding reduces residual silanol groups that caused peak tailing and increased the selectivity for polar compounds. The initial studies were focused on ten MAAs that eluted in the early part of the chromatogram where the elution conditions are essentially isocratic and the separation is difficult. Extracts of the coral *Stylophora pistillata* containing a complex mixture of highly polar MAAs (Shick et al. 1999) were used as resolution probes for optimizing the mobile phase A. The mobile phase B employed in the initial experiments was fixed as in the Carreto et al. (2001) method, a composition previously found to be optimal for separating low-polarity MAAs.

Measurements using isocratic elution with acetic acid 0.2% have shown that the retention time of shinorine (3) on CapCell Pak UG was much larger than those obtained on the Alltima column, and that in relation to shinorine (3), the elution order of palythine (6) and palythine-serine (5) was reversed. However, in these conditions a serious band broadening, especially of shinorine (3), occurs. MAAs are polyfunctional compounds with several ionizable groups (carboxyl, sulfate, imine, secondary amine), and the retention time and elution sequence of individual MAAs are very sensitive to pH changes (Nakamura et al. 1982). On this base we explored the use of trifluoroacetic acid (TFA) and ammonia solutions instead of acetic acid 0.2%, as mobile phase A. For any combination, the mobile phase A containing TFA 0.2% plus ammonium hydroxide solution at pH 2.9 provided the best separation between shinorine (3), mycosporine-2-glycine (4), and palytine-serine (5) but low efficiency was found. Discouraged by our unsuccessful efforts to combine high peak separation and high efficiency, we coupled the Alltima C_{18} ODS and the CapCell Pak C_{18} columns. We were fortunate, since the separation properties of the combined columns increased peak resolution. To study in detail the effect of pH in these new conditions, the pH of the mobile phase A was varied from 2.7 to 3.2 using mixtures of ammonium solution and TFA acid (final concentration 0.2%),

Table 3 MAA recoveries (percent) from mobile phase A and methanol solutions stored at ambient temperature for 1 day and −20°C for 7 days. Data given as means ± SD ($n = 3$)

MAA	Mean recovery (%)			
	Eluant A		Methanol 100%	
	25°C, 1 day	−20°C, 7 days	25°C, 1 day	−20°C, 7 days
Shinorine	96.6 ± 1.9	97.0 ± 2.3	96.8 ± 0.6	93.4 ± 0.4
Palythine	115.7 ± 1.0	103.6 ± 0.5	101.2 ± 0.1	92.7 ± 0.5
Palythinol	96.6 ± 2.4	98.1 ± 1.7	97.5 ± 1.4	89.4 ± 0.0
Usujirene	90.7 ± 1.7	85.8 ± 2.5	95.5 ± 0.7	93.0 ± 1.1
Palythene	87.8 ± 1.7	73.5 ± 3.4	97.6 ± 0.6	89.2 ± 0.8

and the resolution of polar MAAs measured. The major difficulty encountered was the separation of mycosporine-2-glycine (4) from palythine-serine (5) without affecting the separation of the other MAAs. Lowering the pH of the mobile phase A, an expected increase in retention time for the most acidic MAAs was observed. The best separation of mycosporine-sulfates (1 and 2) was obtained at pH 2.7, but the pair mycosporine-2-glycine (4) and palythine-serine (5) remained unresolved (Fig. 3a).

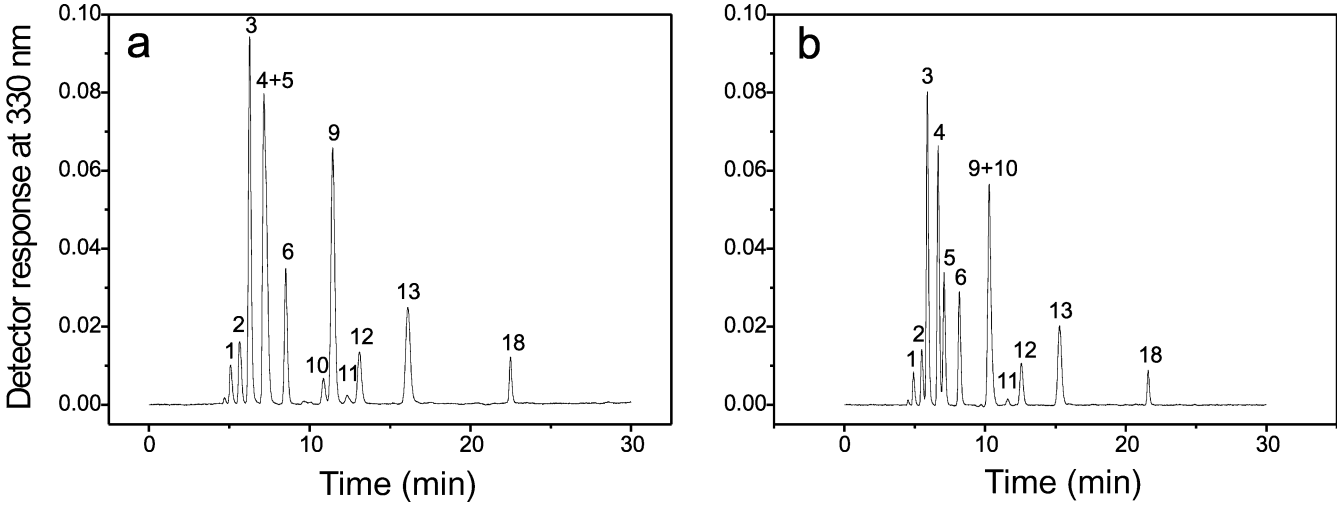
Good resolution of mycosporine-2-glycine (4) and palythine-serine (5) was obtained at pH 2.9 (Fig. 3b). In these conditions the unresolved peaks were reduced to the pair porphyra-334 (9)/mycosporine-NMA-serine (10). Finally, at pH 3.15 the elution sequence of 9 and 10 was reversed and a better resolution of this pair of MAAs was obtained (Fig. 4). In these last conditions all compounds were baseline separated. Only shinorine (3) and the less-polar sulfate ester (2) were partially resolved (Rs = 0.98; Table 4).

The performance of the method in the central region and the less-polar end region of the resulting chromatogram was also examined in detail. Extracts of the coral *S. pistillata* enriched by addition of available MAA standards were used as resolution probes for optimizing the mobile phase B. Using the original mobile phase B (aqueous acetic acid 0.2%:methanol:acetonitrile, 50:25:25) the central region of the chromatogram showed the co-elution of palythenic acid (15) with shinorine-methyl ester (16). Although different slopes in the rate of change from mobile phase A to mobile phase B were applied these compounds remained unresolved. Knowing about the differential selectivity of pH for acidic MAAs we evaluated the effect of lowering the pH of the mobile phase B using TFA 0.2%. For any combination of organic solvents the mobile phase including TFA 0.2% plus ammonium hydroxide (pH 2.2) provided better results than those containing acetic acid. The organic strength of the mobile phase B was also reduced to obtain a significant decreasing pH gradient between the beginning of separation and the elution time of the unresolved peaks. The best results were obtained when mobile phase B was TFA 0.2% plus ammonium hydroxide (pH 2.2):methanol:acetonitrile (80:10:10, v:v:v).This reduction in the organic strength produced an expected increase in retention time of the less-polar compounds that was partially compensated by modifying the gradient profile. As the columns used were sensitive to temperature changes we tested the temperature at 20°C, 30°C, and 35°C. The highest efficiency was obtained at 35°C, improving the separation between shinorine-methyl ester (16) with palythenic acid (15) to near baseline resolution (Rs = 0.71) and shortening the analysis time. The final optimum temperature, gradient, and mobile phase composition are shown in Table 1. Under these conditions, substances 1–24 were separately eluted according to their hydrophobic properties (Fig. 4, Table 4) from 1, the lowest hydrophobicity, to 24, the highest, within 40 min.

MAA detection and identification

A diode array detector (DAD) allowed the acquisition of UV absorption spectra. Due to the lack of fine spectral absorption, the only spectral characteristics available for MAA identification were the positions of the absorption maximum (λ_{max}). However wavelength absorption maximums for some specific MAAs are identical or are only 2 nm apart, which makes it difficult to distinguish these compounds based on absorption spectra only. Confirmation of peak identity with a high degree of confidence is possible by matching the retention time and UV spectrum with that of authentic MAAs. While this methodology has proven useful, the lack of commercial standards makes identification and quantification of individual MAAs difficult. In addition, these two

Fig. 3a, b Effect of pH of mobile phase A on MAA resolution. **a** Mobile phase A at pH 2.7. **b** Mobile phase A at pH 2.9. Sample was the coral *Stylophora pistillata*. Detection by absorbance at 330 nm. Peak identification as in Table 4



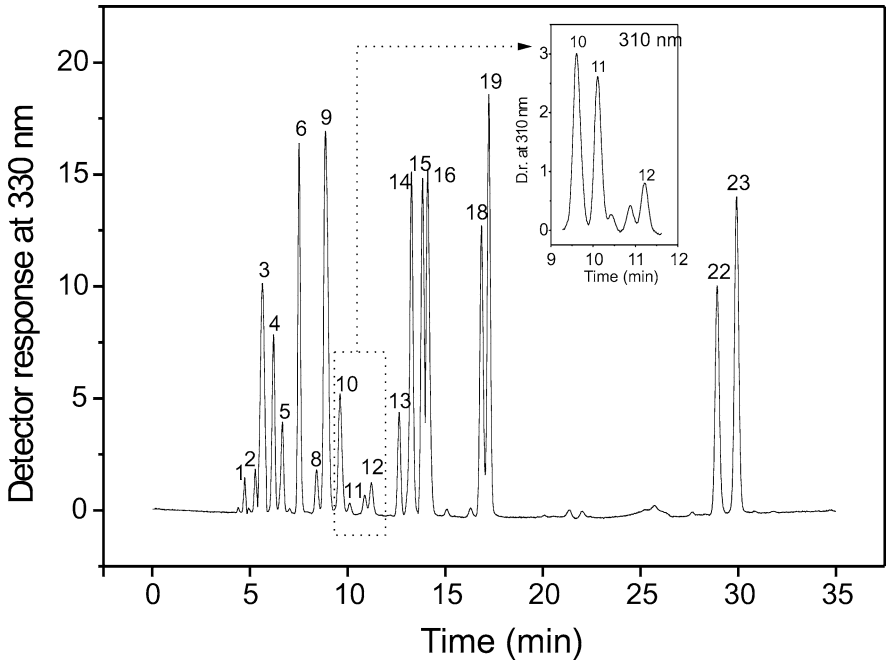


Fig. 4 Chromatogram covering three polarity ranges. Sample was an MAA extract from the coral *S. pistillata* mixed with several MAA standard solutions. Detection at 330 nm (the *insert* shows the detection of mycosporine-glycine at 310 nm). Peak identification as in Table 4

properties alone are generally not considered sufficient for a secure identification of organic compounds. In natural product chemistry, co-chromatography in at least two chromatographic systems as well as mass spectrometry is required. Ion spray LC/MS is just as sensitive and serves as an excellent method of confirmation. This approach also allows the quantification of individual MAAs without the need for complete resolution. However, one limitation was the inability to distinguish geometric isomers such as usujirene/palythene (22/23) and E/Z palythenic acid (15/17). To achieve more structural information, tandem mass spectrometry (MS/MS) may be used to generate fragment ion spectra (Whitehead et al. 2001). It is interesting to note that recent studies on marine organisms (Carreto et al. 2001; Whitehead et al. 2001; Whitehead and Hedges 2002) using mass spectrometry detection and identification (LC/MS and LC/MS MS) showed a wide distribution of

Table 4 Peak identification table. Resolution factor (*Rs*) between MAA pairs is indicated when Rs Wavelengths given in parenthesis denote shoulders

Peak no.	MAA	Retention time (min)	Rs (between peaks)	λ_{max} (nm) in eluant A
1	Palythine-serine sulfate	4.76		320
2	Mycosporine sulfate ester	5.25		318
3	Shinorine	5.65	0.98 (2/3)	333
4	Mycosporine-2-glycine	6.19		332
5	Palythine-serine	6.68		321
6	Palythine	7.52		320
7	Unknown from *Noctiluca* sp.	8.18		310
8	Asterine	8.43		329
9	Porphyra-334	8.87		333
10	Mycosporine-methylamine-serine	9.60		327
11	Mycosporine-glycine	10.11		310
12	Unknown from *Stillopora pistillata*	11.24		327
13	Unknown from *Pocillopora eydouxi*	12.62		320
14	Palythinol	13.26		330
15	Z-palythenic acid	13.83		335
16	Shinorine methyl ester	14.10	0.71 (15/16)	332
17	E-palythenic acid (?) from *Noctiluca* sp.	15.97		337
18	Mycosporine-methylamine-threonine	16.88		327
19	Mycosporine-taurine	17.23	0.95 (18/19)	309
20	M-320 from *A. tamarense*	19.54		320
21	Unknown from *G. catenatum*	21.21		370
22	Usujirene	28.95		357
23	Palythene	29.94		360
24	M-335/360 from *A. tamarense*	35.71		335 (360)

palythenic acid (15). However using the commonly adopted methods, this MAA has only been previously identified in a small number of marine organisms (Nakamura et al. 1982; Carreto et al. 1990). These contrasting results are probably related to the fact that based on absorption spectra only, the Z-palythenic acid (15) could be misidentified as mycosporine-glycine-valine (Whitehead et al. 2001). On the other hand, the high cost of LC/MS systems limits their wide use. Therefore a highly selective separation method would represent a good alternative approach for studying complex samples. Our proposed HPLC method is also compatible with ion-spray LC/MS and application of this approach only requires slight methodological changes.

Precision and recovery

Variability in retention time between injections was evaluated using five MAAs eluting in different regions of the chromatogram. Although total column length was now 40 cm, retention times were remarkably constant (Table 5). In addition, the relative standard deviation of the integrated peak area for repeated injections of calibrated MAAs solutions made over several days was on the order of 1–2% (Table 5).

Instrument response was established to be sufficiently linear ($r > 0.999$) over the range up to 80 ng injected, so that a single-point porphyra-334 external calibration was routinely used. The detection limit for the different MAAs was estimated to be between 0.08 and 0.47 pmol injected (signal-to-noise ratio, S/N = 2) with a 10-µl injection volume and the regular extraction and clean-up presented in the experimental section. The mean recovery of shinorine (3), palythinol (14), and usujirene (22), using the gradient and column described above, was 99.2 ± 0.8, 98.8 ± 1.3, and 99.2 ± 0.8%, respectively, of that obtained using isocratic phase A and stainless steel tube instead of the column (three replicates).

Method applicability

HPLC analyses of MAAs in several marine organisms were carried out under the conditions described above and good separations were obtained in all cases.

Table 5 Means and standard deviations ($n = 8$) of the retention times for five typical MAAs and relative standard deviation (*RSD*) associated with the integrated area determination at ~50-pmol level

MAA	Retention time $\bar{x} \pm \sigma$ (min)	RSD of integrated area $\pm$ $(\sigma/\bar{x}) \times 100$
Shinorine	5.65 ± 0.1	± 1.9%
Palythine	7.52 ± 0.1	± 0.9%
Palythinol	13.26 ± 0.1	± 1.3%
Usujirene	28.95 ± 0.2	± 1.9%
Palythene	29.94 ± 0.2	± 1.9%

Phytoplankton cultures

The chromatogram of the toxic dinoflagellate *Alexandrium tamarense* showed the most complex MAA profile among the microalgal cultures analyzed (Fig. 5a). Nine MAAs (3, 6, 9, 11, 15, 16, 20, 23, and 24) previously observed in this species (Carreto et al. 1990, 2001; Whitehead and Hedges 2002; Laurion et al. 2003), including some recently partially characterized complex compounds, such as shinorine-methyl ester (16), M-320 (20), and M-335/360 (24), were baseline separated. Compared with the previous method (Carreto et al. 2001), an improvement in the resolution of the critical MAA pair palythenic acid (15)/shinorine-methyl ester (16) was observed (Table 4).

The chromatogram of the domoic acid producer diatom *Pseudo-nitzschia multiseries* shows (Fig. 5b) the net dominance of shinorine (3) with minor amounts of mycosporine-2 glycine (4), palythine (6), porphyra-334 (9), mycosporine-taurine (19), and one unknown UV-absorbing compound with absorption maximum at 331 nm. The presence of mycosporine-taurine is the most noteworthy feature in this profile, as this rare MAA has only been previously detected in some species of sea anemones of the genus *Anthopleura* (Stochaj et al. 1994; Shick et al. 2002). It is interesting to note that similar to the sea anemones, *Pseudo-nitzschia* species can accumulate large pools of taurine, a free amino acid not detected in other diatom species (Smith et al. 2001).

In the bloom-forming coccolithophorid *Emiliania huxleyi* only shinorine (3) was present in quantifiable amounts, whereas palythine (6) and porphyra-334 (9) were present at trace level (Fig. 5c). The inability of *E. huxleyi* to accumulate MAAs could possibly be related to this species' poor tolerance of ultraviolet radiation (Buma et al. 2000). By contrast, natural blooms of this species appear to be promoted by high light irradiances (Nanninga and Tyrrell 1996).

Natural phytoplankton samples

The chromatogram of a field sample collected during a red tide in the coastal waters off Mar del Plata showed (Fig. 6a) the net dominance of shinorine (3), and minor amounts of palythine-serine (5), porphyra-334 (9), mycosporine-glycine (11), palythinol (14), palythene (23), and two unknown UV-absorbing compounds (U and 21). Compound 21 shared a typical MAA absorption spectra but its absorption maximum was centered at 370 nm, which is an unusually large absorption signal for an MAA (Fig. 6a). Microscopic observations of this sample indicated the dominance of the toxic dinoflagellate *Gymnodinium catenatum* (R. Akselman, personal communication) but pigment analysis showed the net predominance of the unequivocal Cryptophyceae carotenoid marker alloxanthin (results not shown). It is interesting to note that Jeffrey et al. (1999) observed a

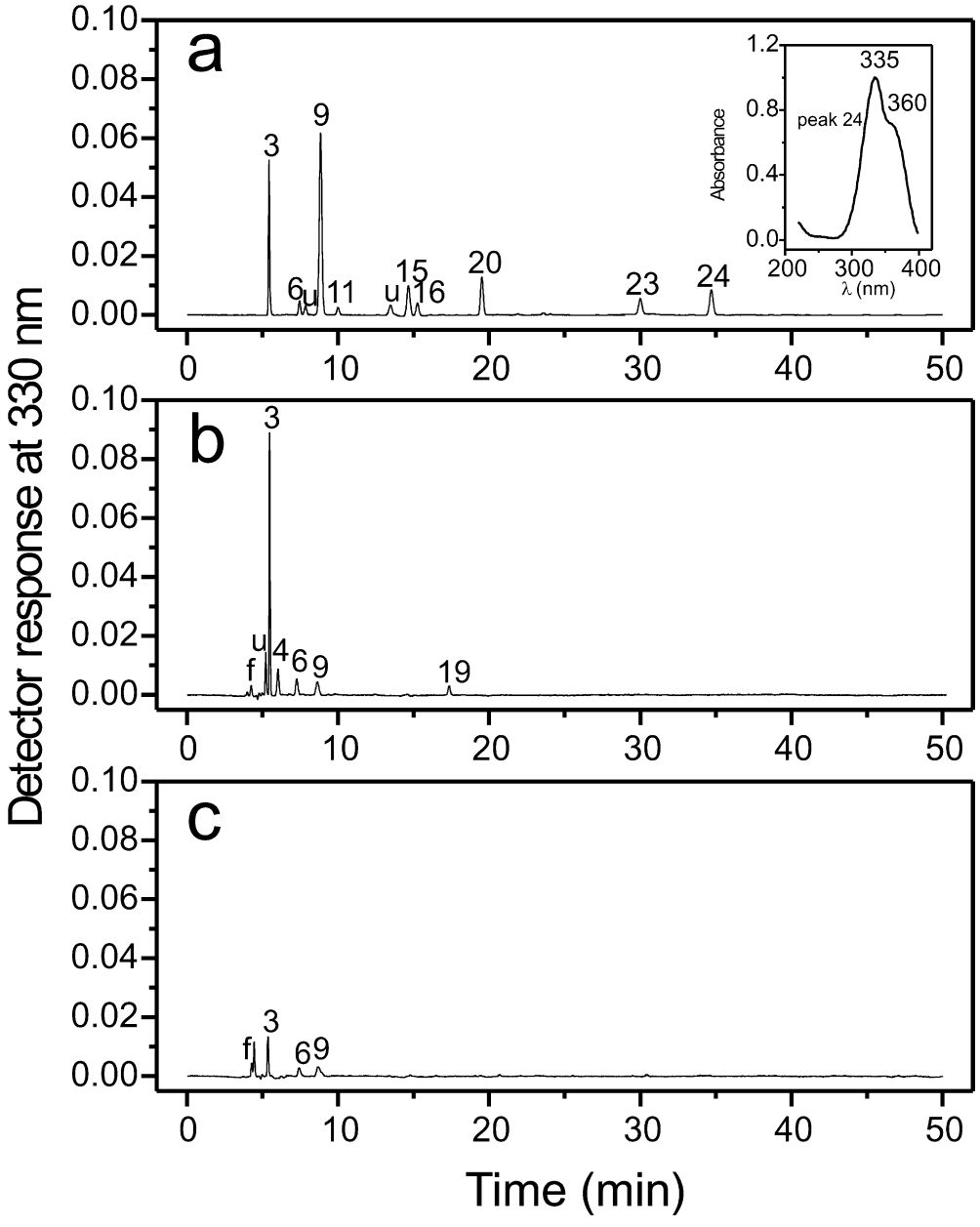


Fig. 5 MAA composition from the algal cultures (**a**) *Alexandrium tamarense* (the *insert* shows the absorbance spectra of peak 24), (**b**) *Pseudo-nitzschia multiseries*, and (**c**) *Emiliania huxleyi*. Detection at 330 nm. Peak identification as in Table 4. *f* Solvent front, *u* unidentified compound

large peak at 370 nm in the UV-visible spectrum of extracts from cultures and natural populations of *G. catenatum*. Although in this species, in addition to shinorine (3), porphyra-334 (9), and mycosporine-glycine (11), several unknown compounds were detected by Jeffrey et al. (1999), the major UV-absorbing component responsible for the large absorption peak at 370 nm was not detected by the HPLC method they used.

The chromatogram of another field sample in which *Noctiluca* sp. was the dominant species is show in Fig. 6b. The most noteworthy feature is the dominance of porphyra-334 (9), which occurred together with several previously known MAAs, shinorine (3), palythine (6), mycosporine-glycine (11), and palythenic acid (15), and two unidentified UV-absorbing compounds. One of them (17) showed the characteristic palythenic acid absorption maximum (337 nm), but differed from our palythenic acid standard isolated from *A. tamarense* (15) in its polarity. These findings and the isolation in these species of both (Z/E) palythenic acid isomers (Okaichi and Tokomura 1980, cited in Kobayashi et al. 1981) suggest that we were dealing with the less-polar trans-isomer of palythenic acid (Nakamura et al. 1982).

The chromatogram from a sample collected at the surface from oligotrophic waters from the Brazil Current (Fig. 6c) showed a complex MAA composition including shinorine (3), palythine (6), porphyra-334 (9), mycosporine-glycine (11), palythenic acid (15), shinorine-methyl ester (16), and minor amounts of three unidentified compounds. The HPLC pigment analysis indicated the presence of *Synechococcus* spp. (44.2% of total chl *a*) and *Prochlorococcus* spp. (36.8% of total chl

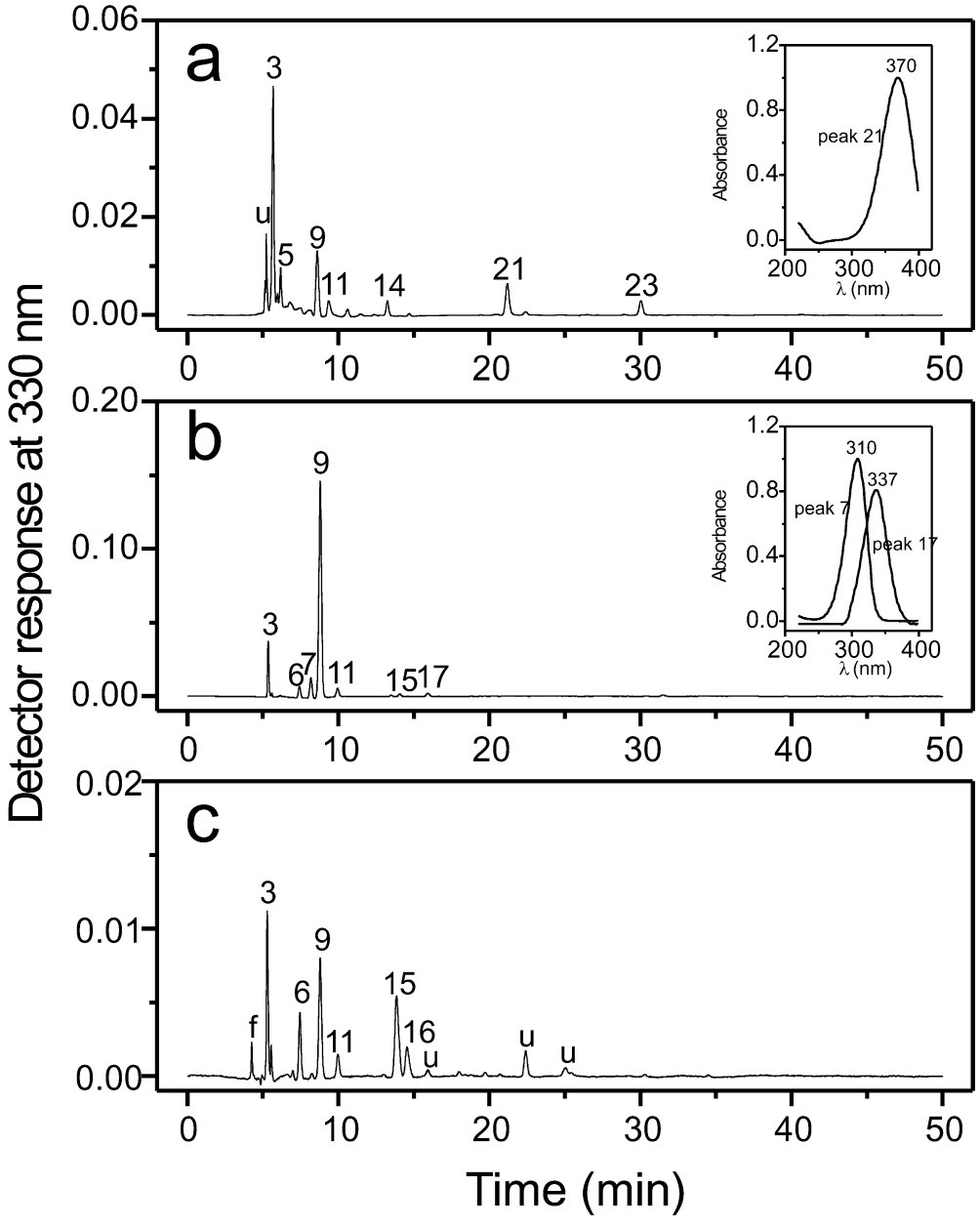


Fig. 6 Chromatograms of phytoplankton MAAs from seawater samples collected from (**a**) a red tide event that occurred in the coastal waters off Mar del Plata (the *insert* shows the absorbance spectra of peak 21), (**b**) a *Noctiluca* sp. red tide event that occurred in the coastal waters off Mar del Plata (the *insert* shows the absorbance spectra of peaks 7 and 17), and (**c**) surface oligotrophic waters from the Brazil Current. Detection at 330 nm. Peak identification as in Table 4. *f* Solvent front, *u* unidentified compound

a) as major components of the phytoplankton assemblage (J.I. Carreto et al., unpublished).

Macroalgae

The seaweeds *Iridea* sp., *Porphyra* sp. (Nori), and *Palmaria decipiens* all showed the net dominance of porphyra-334 (9) together with minor amounts of shinorine (3) and palythine (6) (Fig. 7 a, b, c). However, the *Iridea* sp. and *P. decipiens* MAA compositions were more complex. In addition to shinorine (3), palythine (6), and porphyra-334 (9), the chromatogram from *Iridea* sp. showed (Fig. 7a) minor amounts of usujirene (22), palythene (23), and one unidentified compound (u). The chromatogram from *P. decipiens* also showed (Fig. 7c) the presence of usujirene (22) and palythene (23), besides palythinol (14) and the recently partially characterized complex M-335/360 (24) from *A. tamarense*. This is the first time that this compound was detected in red algae.

Symbiotic organisms

The HPLC chromatograms of MAA extracts from three symbiotic organisms also showed interesting results (Fig. 8). In the sea anemone *Anthopleura elegantissima* (Fig. 8a) four MAAs were present in quantifiable amounts: shinorine (3), mycosporine-2-glycine (4), palythine (6), and mycosporine-taurine (19), with mycosporine-2-glycine (4) being the most concentrated. Porphyra-334 was only detected at trace levels. This profile was similar to that reported for this and other species of *Anthopleura* that contained four constitutive

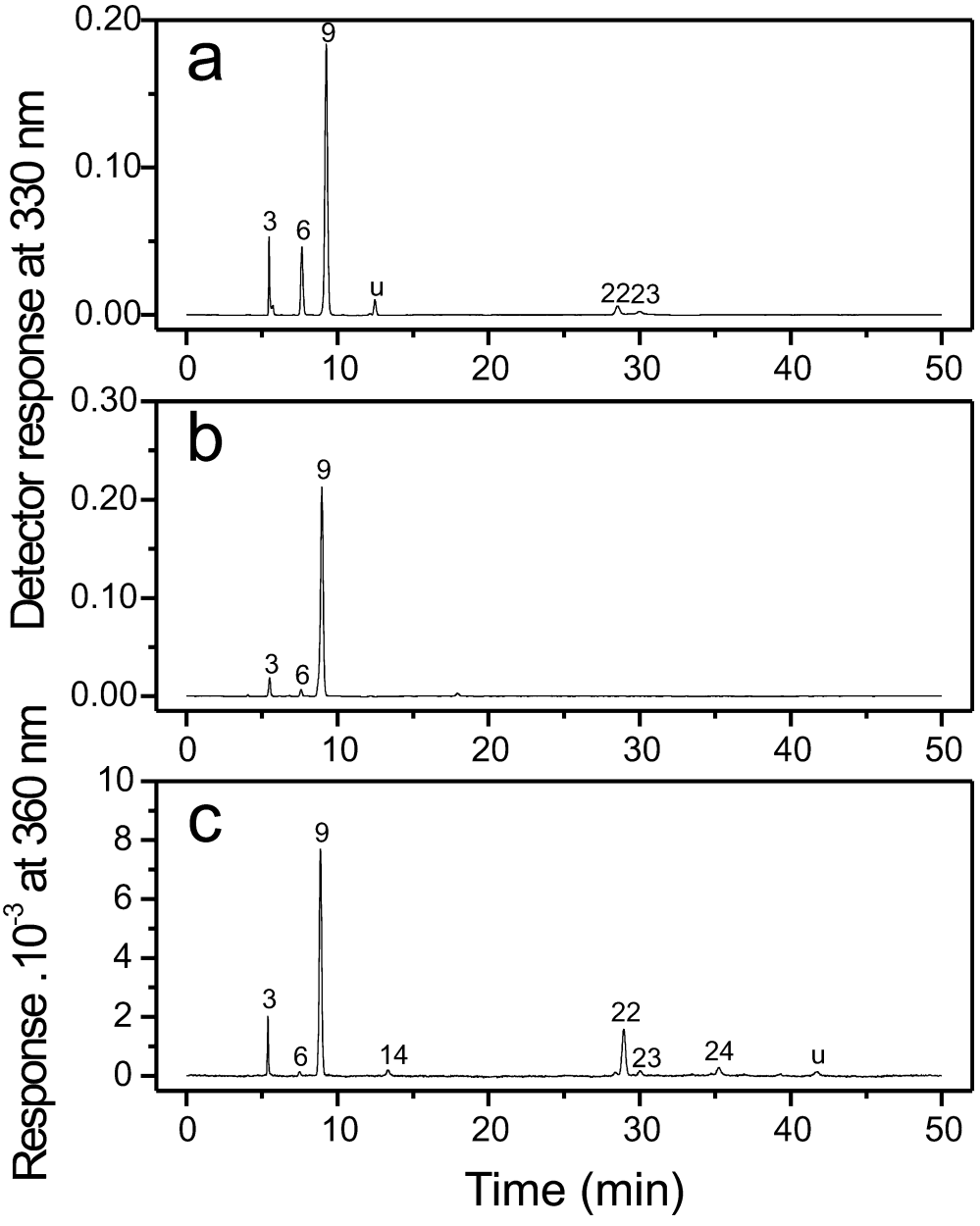


Fig. 7 MAA compositions from the red algae (**a**) *Iridea* sp., (**b**) *Porphyra* sp. (Nori), and (**c**) *Palmaria decipiens*. Detection at 330 nm (**a** and **b**) and 360 nm (**c**). Peak identification as in Table 4. *u* Unidentified compound

major MAAs (3, 4, 9, 19), palythine being the only MAA component of occasional occurrence (Stochaj et al. 1994; Banaszak and Trench 1995; Shick et al. 2002). In our chromatographic conditions, mycosporine-taurine (19)—a rare MAA containing the sulfonic amino acid taurine as unique amino acid (Stochaj et al. 1994)—was highly retained (Table 4) and its separation from the highly polar MAAs was optimal. The chromatogram of the scleractinian coral *Pocillopora eydouxi* showed (Fig. 8b) the resolution of seven MAAs (3, 5, 6, 9, 10, 11, and 18) previously identified in this species (Teai et al. 1997, 1998) and of two additional compounds (1 and 13). One of them (1) was identified as palythine-serine-sulfate (Wu Won et al. 1997). The other unknown compound (13) also showed a maximum absorption at 320 nm, indicative of a chromophore with unsubstituted imine functionality but having a much lower polarity than the latter (1).

The chromatogram of the scleractinian coral *Stylophora pistillata* showed (Fig. 8c) the resolution of nine MAAs (1, 3, 4, 5, 6, 9, 10, 11, 18) previously identified in this species (Shick et al. 1999; Wu Won et al. 1997) and of three additional unknown compounds (2, 12, 13). Palythinol was also present at trace level. The main unknown compound (13) shared the same spectral properties ($\lambda_{max} = 320$ nm) and retention time as those of the compound detected in extracts of the coral *Pocillopora eydouxi* (Fig. 8b). From biochemical considerations (Fig. 1) it seems probable that this low polarity compound previously undetected in corals could be palythine-threonine, a precursor or hydrolytic product of palythine-threonine-sulfate, a highly polar compound

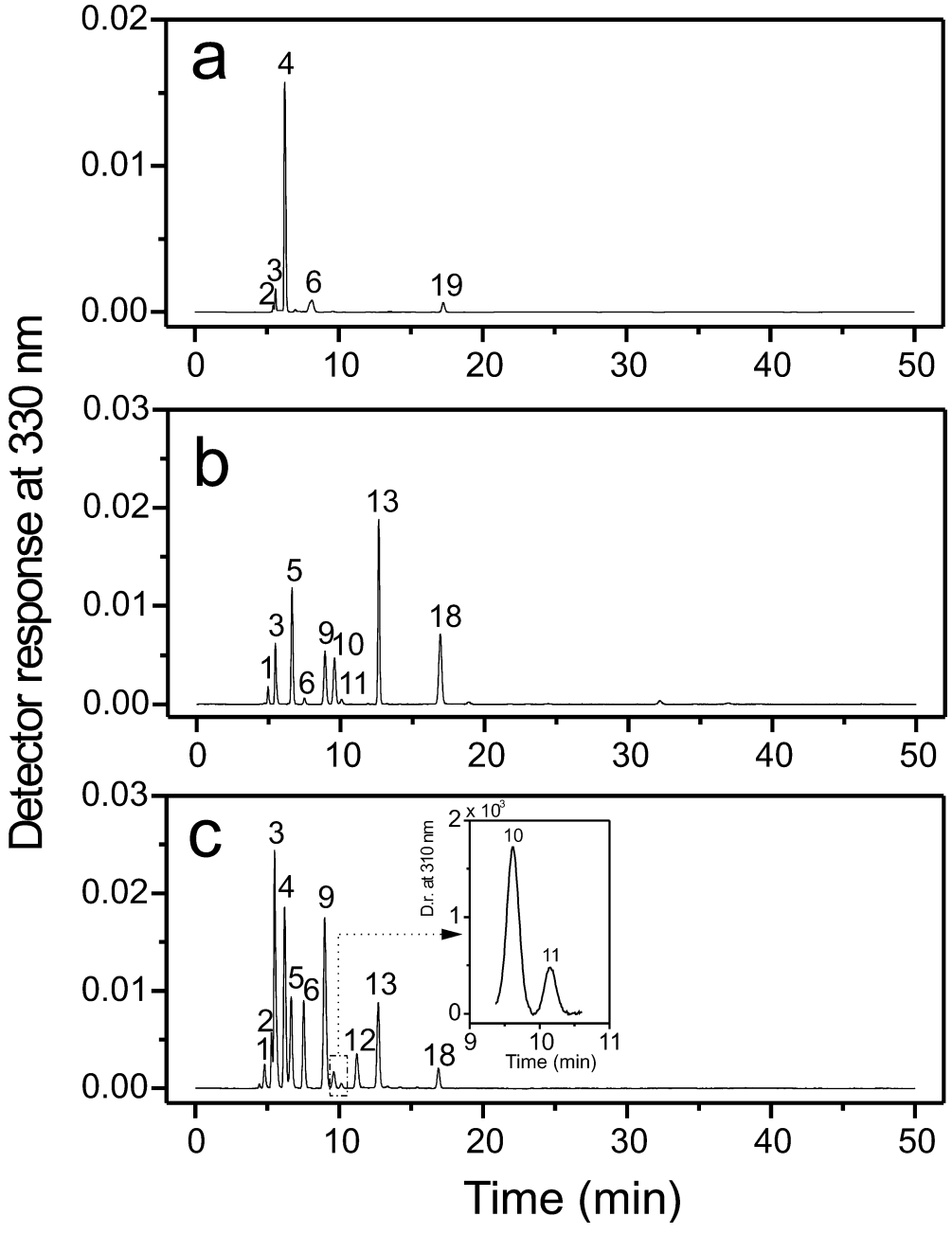


Fig. 8 MAA compositions from (**a**) the sea anemone *Anthopleura elegantissima* and the corals (**b**) *Pocillopora eydouxi* and (**c**) *Stylophora pistillata* (the *insert* shows the detection of mycosporine-glycine at 310 nm). Peak identification as in Table 4. Detection at 330 nm

isolated from *S. pistillata* (Wu Won et al. 1997). Chromatography also revealed the presence of two high-polarity compounds (1 and 2) that eluted before shinorine. One of them (1), which absorbed maximally at 320–321 nm and was also found in the coral *P. eydouxi* (Fig. 8b), was identified as palythine-serine-sulfate (Wu Won et al. 1997). The presence of the other compound (2) is in agreement with recent results obtained by Wu Won (in Shick et al. 1999, J.M. Shick personal communication). This author explained that although original nuclear magnetic resonance spectra indicated the presence of only one compound in the palythine-serine-sulfate isolate from *S. pistillata* (Wu Won et al. 1997), newer chromatography revealed a second peak in that isolate. Thus, compound 2 may be a new mycosporine sulfate ester or decomposition product of this MAA (Shick et al. 1999, 2002). The other low-polarity compound in our chromatograms (12) that could not be identified had a maximum absorption at 327 nm, indicative of a chromophore with an N-methylimine functionality, which was not 10 or 18.

Conclusions

Separation of MAAs in complex samples, such as those found in some marine organisms, requires high resolution because of the large number of components present

and the wide range of polarities encountered among these compounds. Good recovery, precision, and resolution in the separation of more than 20 compounds were obtained with the reverse-phase HPLC gradient elution method here described. In comparison with the commonly adopted isocratic elution method, the proposed method is more selective and resolves in a single run most of the more complex mixtures found in marine organisms. In comparison with other HPLC gradient elution methods the proposed method gives a higher resolution of the more polar MAAs, while retaining or improving the resolution of later-eluting MAAs.

Several partially characterized and other unknown MAAs have been recently discovered and our results suggest that there are still many unknown MAAs. A diode array detector is essential to track spectroscopic resolution, but reproducible retention times and selective co-chromatography with authentic standards should be included to confirm identifications, whenever possible. For better MAA identification and characterization of novel compounds, ion-spray LC/MS is a robust technique than can be adapted to our chromatographic conditions after slight methodological changes.

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