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The effect of re-dissolution solvents and HPLC columns on the analysis of mycosporine-like amino acids in the eulittoral macroalgae *Prasiola crispa* and *Porphyra umbilicalis*

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Abstract Many macroalgal species that are regularly exposed to high solar radiation such as the eulittoral green alga *Prasiola crispa* and the red alga *Porphyra umbilicalis* synthesize and accumulate high concentrations of mycosporine-like amino acids (MAAs) as UV-sunscreen compounds. These substances are typically extracted with a widely used standard protocol following quantification by various high performance liquid chromatography (HPLC) techniques. However, further preparation steps prior to HPLC analysis as well as different HPLC column types have not been systematically checked regarding separation quality and reproducibility. Therefore pure methanol, distilled water and HPLC eluent were evaluated as re-dissolution solvent for dried *Prasiola* and *Porphyra* extracts, which were subsequently analyzed on three reversed-phase C8 and C18 HPLC columns. The data indicate that distilled water and the HPLC eluent gave almost identical peak patterns and MAA contents on the C8 and C18 columns. In contrast, the application of the widely used methanol led to double peaks or even the loss of specific peaks as well as to a strong decline in total MAA amounts ranging from about 35% of the maximum in *P. crispa* to 80% of the maximum in *P. umbilicalis*. Consequently, methanol should be avoided as re-dissolution solvent for the HPLC sample preparation. An improved protocol for the MAA analysis in

macroalgae in combination with a reliable C18 column is suggested.

Keywords Photoprotection · Reversed-phase column · UV-sunscreen

Introduction

For algae exposed to enhanced ultraviolet radiation (UVR) for substantial parts of their life-cycles, strategies that passively screen this waveband will contribute to prevent UV-induced damage to essential biomolecules such as DNA and proteins (Bischof et al. 2006). In addition, UV-screening may also save metabolic energy by reducing the need for constantly active avoidance and repair processes.

The most common photoprotective sunscreens in many, but not all algal taxa and cyanobacteria are the mycosporine-like amino acids (MAAs), a suite of chemically closely related, colorless, water-soluble, polar and at cellular pH uncharged or zwitterionic amino acid derivatives. MAAs are related to fungal mycosporines which were first isolated from sporulating mycelia (Leach 1965; Favre-Bonvin et al. 1976), and consist of aminocyclohexenone or aminocyclohexenimine ring systems. The various MAA structures result from N-substitutions of different amino acid moieties to the cyclohexenone or cyclohexenimine chromophore. At present, there are only few known aminocyclohexenone-derived MAAs such as mycosporine-glycine, which typically exhibit their absorption maximum in the UVB (280–315 nm, C.I.E. definition) range. All others, about 20 described MAAs are derivatives of the aminocyclohexenimine structure which maximally absorbs at UVA (315–400 nm) wavelengths (Cockell and Knowland 1999; Karentz 2001; Shick and Dunlap 2002). Many algae and cyanobacteria

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contain multiple MAAs, of which some have been identified. However, particularly for cyanobacteria most chemical structures are still to be elucidated (Garcia-Pichel and Castenholz 1993). MAAs were invoked to function as passive shielding solutes by dissipating the absorbed short wavelength radiation energy in the form of harmless heat without generating photochemical reactions (Bandaranayake 1998). These biomolecules exhibit extremely high molar absorptivity for UVA and UVB (molar extinction coefficients between 28.000 and 50.000), and have been reported as photochemically stable structures, both of which are prerequisites for their sunscreen function (Conde et al. 2000).

For qualitative and quantitative analysis of MAAs different HPLC (high performance liquid chromatography) techniques have been applied (Tartarotti and Sommaruga 2002; Caretto et al. 2005 and references therein) with a preference for the widely used method of Dunlap and Chalker (1986). Except the more recent technique of Caretto et al. (2005) all other HPLC methods fail to separate a complex mixture of MAAs from different marine organisms because of the specific physico-chemical properties of individual MAAs. Caretto et al. (2005) coupled two reverse-phase C18-columns with different selectivity for polar compounds and introduced a complex gradient eluent system at low pH. Although this approach is highly suitable for screening of new and unknown MAAs in marine organisms, preparation and analysis are time-consuming and hence are not adequate for routine measurements in organisms with known MAA composition.

The protocols to extract MAAs from biological samples largely differ in the organic solvents used, in the extraction times and temperatures as well as in concomitant cell rupture approaches leading to significant different extraction efficiencies (Tartarotti and Sommaruga 2002). Consequently, these authors compared different protocols and recommended a simple but highly accurate methodological approach for standard MAA extraction. After extraction of MAAs, the extracts are typically evaporated to dryness and, depending on the authors, re-dissolved in different solvents such as aqueous to pure methanol, distilled water or the respective HPLC eluent (Gleason 1993; Dionisio-Sese et al. 1997; Bracher and Wiencke 2000; Caretto et al. 2001; Figueroa et al. 2003). However, to our knowledge the effect of these solvents on the MAA analysis has never been systematically evaluated, particularly not in combination with the widely used reversed-phase C8 and C18 HPLC columns. The motivation of the present study was related to various analytical problems on C8 columns such as suddenly appearing double peaks of standard MAAs, as well as decreasing resolution between the major red algal MAAs shinorine and porphyra-334. Since the standard extraction protocol of Tartarotti and Sommaruga (2002) has always

been successfully used with our macroalgal samples (Karsten et al. 1998) the major goal was to evaluate the re-dissolution step and different column types. As model organisms, the green macroalga *Prasiola crispa* and the red macroalga *Porphyra umbilicalis* were chosen because as eulittoral organisms they are regularly exposed to high solar radiation and hence synthesize and accumulate MAAs.

Methods

Macroalgal samples

Specimens of the green macroalga *Prasiola crispa* (Light-foot) Kützinger were collected during the summer season 2006 from a subaerial rocky cliff. The collection site was located in a distance of 100 m from the shore and about 2–3 m above NN underneath a seagull colony in the inner part of the Kongsfjorden (Ny-Ålesund, Spitsbergen, Norway, 78°55.5'N, 11°56.0'E). The red macroalga *Porphyra umbilicalis* (L.) J. Agardh was collected in 2003 from the rocky upper littoral zone at the island Helgoland, North Sea, Germany, (54.2°N, 7.9°E). All algal samples were collected under air-dried conditions with tweezers and put in black bags to avoid exposure to high irradiance during transportation, followed by short rinsing with tap water in the laboratory to remove sediment and fecal particles. Afterwards, algal thalli were oven-dried at 40°C overnight, and stored in sealed plastic bags under dark, dry and cool conditions (4–10°C) prior analysis. This treatment has no effect on the MAA concentrations, and such samples could be stored for many years without any degradation (unpublished results).

MAA extraction and analysis

Three replicate macroalgal samples each of about 10 mg dry weight (DW) were extracted for 1.5–2 h in screw-capped centrifuge vials filled with 1 mL 25% aqueous methanol (v/v) and incubated in a waterbath at 45°C (Tartarotti and Sommaruga 2002). After centrifugation at 5,000g for 5 min, 700 µL of the supernatants were evaporated to dryness under vacuum (Speed Vac Concentrator SVC 100H). Figure 1 shows a schematic diagram of the further methodological strategy. Dried extracts were re-dissolved in either 700 µL 100% methanol, distilled water or the HPLC eluent [2.5% aqueous methanol (v/v) plus 0.1% acetic acid (v/v) in water], and all samples were vortexed for 30 s. After centrifugation at 5,000g for 5 min the extracts were passed through a 0.2 µm membrane filter, and afterwards analyzed with an Agilent HPLC system equipped with an autoinjector according to the method of Karsten et al. (1998), modified as follows (Fig. 1). MAAs

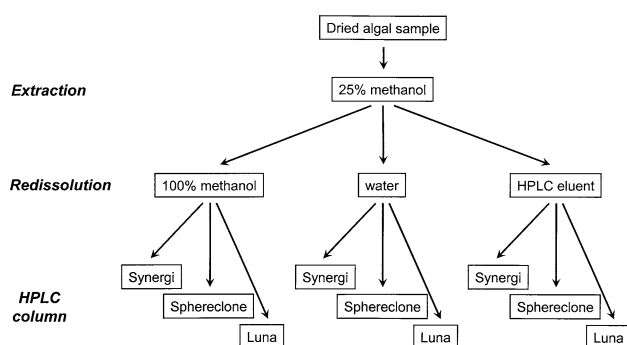


Fig. 1 Methodological protocol with the different re-dissolution solvents and HPLC columns used

in all extracts were one after another separated on three different stainless-steel HPLC columns: (1) Phenomenex Synergi Fusion RP-18 column (4 μm , 250 \times 3.0 mm I.D.) protected with a RP-18 guard cartridge (20 \times 4 mm I.D.) of the same material, (2) Phenomenex Sphereclone ODS (RP-8) column (5 μm , 250 \times 4.6 mm I.D.) protected with a RP-8 guard cartridge (20 \times 4 mm I.D.) of the same material, and (3) Phenomenex Luna C8(2) column (5 μm , 250 \times 4.6 mm I.D.) protected with a RP-8 guard cartridge (20 \times 4 mm I.D.) of the same material. The mobile phase was always 2.5% aqueous methanol (v/v) plus 0.1% acetic acid (v/v) in water, run isocratically at a flow rate of 0.7 ml min⁻¹ with the Sphereclone column, while both Synergi and Luna columns were run at 0.5 ml min⁻¹. MAAs were detected online with a photodiode array detector at 330 nm, and absorption spectra (290–400 nm) were recorded each second directly on the HPLC-separated peaks. Identification was done by spectra, retention time and by co-chromatography with pure standards of palythine, porphyra-334 and shinorine kindly sent by Prof. Yabe, Japan. Quantification was made using the molar extinction coefficients given in Karsten et al. (1998).

Statistics

Mean values of three replicates and standard deviation per treatment were calculated. Statistical significance of differences in the concentrations of total MAA amounts was tested by one-way analysis of variance (ANOVA) followed by a multi-range test using Fisher's protected least significant difference (LSD) according to Sokal and Rohlf (1995). Calculations were done using the program InStat (GraphPad, San Diego, USA).

Results

Prasiola crispa contained two MAAs with identical absorption spectra and a maximum at 324 nm. Therefore

these compounds were described as so-called 324 nm MAAs (Hoyer et al. 2001) (Table 1). Re-dissolution of dried *P. crispa* extracts in methanol led to much lower signals compared to distilled water or HPLC eluent (Fig. 2). In addition, the latter samples exhibited much sharper peaks while utilization of methanol resulted in double peaks, all with the same absorption maximum. Distilled water and the HPLC eluent gave almost identical chromatograms with all columns. The retention times for both MAAs on the Synergi column with distilled water and HPLC eluent samples were 7.6 and 17.5 min, respectively (Fig. 2, Table 1). The resolution of these MAAs on the C8 Sphereclone column was not as good as on the C18 Synergi column, but with retention times of 3.5 and 4.6 min still acceptable (Fig. 2). The methanol extract gave three instead of two peaks on the Sphereclone column. In contrast to the Synergi and Sphereclone columns, the *Prasiola* extracts could not be separated on the C8 Luna column (Fig. 2). Although some small peaks appeared in the chromatograms, the respective absorbance spectra indicated for each peak a mix of unidentifiable compounds. A calculation of the total MAA concentration in *P. crispa* showed strong differences for the re-dissolution solvent and column used. All distilled water and HPLC eluent samples gave with the Synergi and Sphereclone columns similar MAA contents between 5.6 and 6.1 mg g⁻¹ dry weight (Table 1). In contrast, in the methanol samples significantly less MAAs (2.0–2.1 mg MAAs g⁻¹ dry weight; $P < 0.001$) were measured, indicating that utilization of methanol as solvent may lead to a strong underestimation of these green algal MAAs.

Porphyra umbilicalis extracts exhibited three main MAAs which could be identified as palythine, shinorine and porphyra-334, as well as some minor unidentifiable compounds (Fig. 3, Table 2). Quantitatively dominating in all samples was always porphyra-334. Re-dissolution of dried *P. umbilicalis* extracts in distilled water or HPLC eluent led on all columns to much higher signals compared to methanol (Fig. 3). In addition, the methanol samples showed broader or even double peaks while utilization of distilled water and HPLC eluent resulted in sharp and symmetric peaks. The retention times for palythine, shinorine and porphyra-334 on the Synergi column with extracts re-dissolved in distilled water and HPLC eluent were 2.9, 5.4 and 7.7 min, respectively (Fig. 3, Table 2). In contrast with the Sphereclone column, the elution order of these MAAs and extracts changed: shinorine (3.1 min), porphyra-334 (3.8 min) and palythine (5.0 min). Compared to the *Prasiola* extracts the *Porphyra* samples could be separated on the Luna column (Fig. 3, Table 2). However, while shinorine and porphyra-334 could be easily quantified, palythine did not appear on the chromatograms. In addition, the porphyra-334 peak showed shoulders or some tailing on the Luna column. The calculated total MAA concentrations of

Table 1 MAA separation and concentrations in extracts of the green alga *Prasiola crispa* after re-dissolution in different solvents and after using different HPLC columns

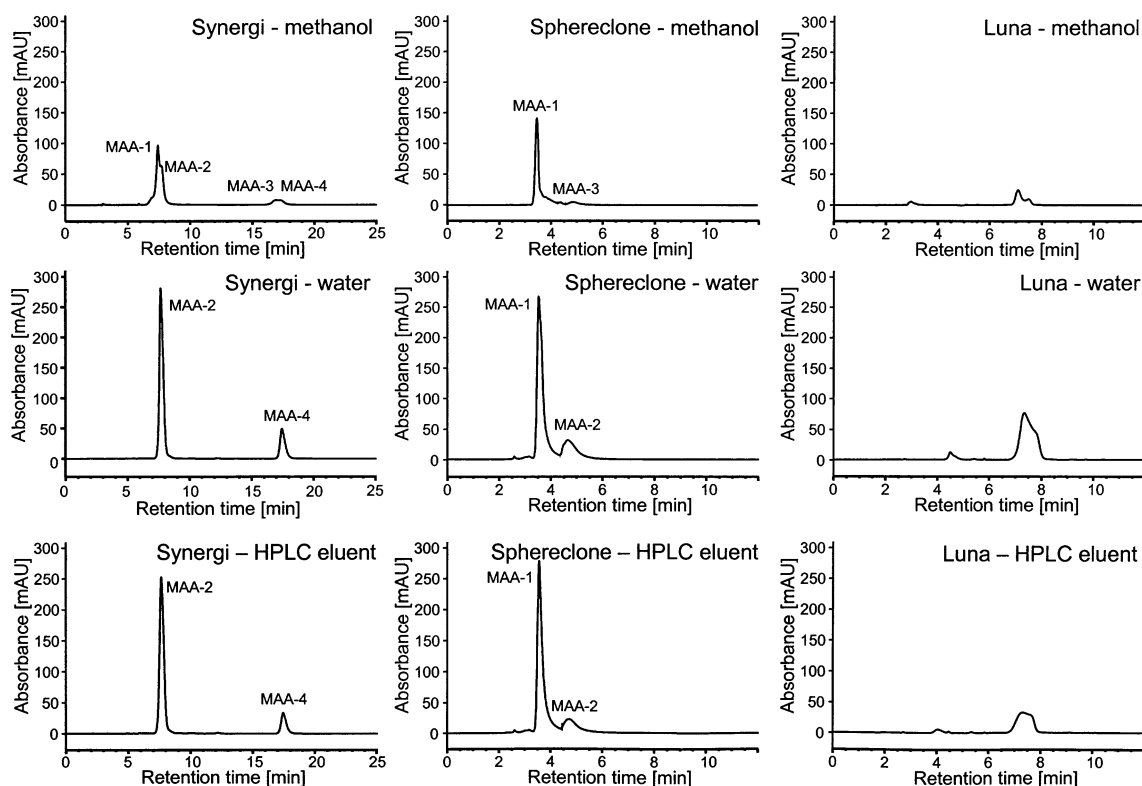
Column	Solvent	Concentration (mg g ⁻¹ DW)				Total
		MAA-1 (324 nm)	MAA-2 (324 nm)	MAA-3 (324 nm)	MAA-4 (324 nm)	
Synergi C18	Methanol	1.22 ± 0.1 (7.7 min)	0.64 ± 0.07 (17.2 min)	0.14 ± 0.01 (17.5 min)	0.12 ± 0.02	2.12 ± 0.19
Synergi C18	Water	ND	4.91 ± 0.12 (7.6 min)	ND	0.77 ± 0.02 (17.5 min)	5.68 ± 0.14
Synergi C18	HPLC eluent	ND	4.87 ± 0.12 (7.6 min)	ND	1.05 ± 0.25 (17.5 min)	5.92 ± 0.21
Sphereclone C8	Methanol	1.84 ± 0.16 (3.4 min)	0.06 ± 0.01 (4.4 min)	0.14 ± 0.01 (4.9 min)	ND	2.04 ± 0.18
Sphereclone C8	Water	4.70 ± 0.14 (3.5 min)	0.90 ± 0.04 (4.6 min)	ND	ND	5.6 ± 0.21
Sphereclone C8	HPLC eluent	4.85 ± 0.32 (3.5 min)	1.27 ± 0.26 (4.6 min)	ND	ND	6.12 ± 0.17
Luna C8	Methanol	^a				<0.5
Luna C8	Water	^a				<0.5
Luna C8	HPLC eluent	^a				<0.5

The absorbance maxima of the different MAAs and the retention time in minutes of the respective peaks is given in brackets. Data given represent mean value ± SD (*n* = 3). ND not detected

^a Mixed unidentifiable peaks

the methanol extracts were significantly 18–37% lower for all columns compared to the distilled water extracts (Table 2; *P* < 0.0001). While use of Synergi and Sphereclone columns led to identical total MAA concentrations in *Porphyra* ranging from 7.88 to 8.01 mg g⁻¹ dry weight in

extracts re-dissolved in distilled water and HPLC eluent, the water extract on the Luna column gave c. 40% higher MAA amounts than the HPLC eluent extract (Table 2). As already mentioned with the *Prasiola* extracts, the methanol samples of *Porphyra* gave also much lower total MAA

**Fig. 2** Representative HPLC chromatograms of MAA extracts from the green macroalgal species *Prasiola crispa*. The evaporated extracts were re-dissolved in either pure methanol, distilled water or the HPLC

eluent, analyzed on different reversed-phase columns and individual peaks detected at 330 nm. Retention time, maximum absorbance and identification of individual peaks are summarized in Table 1

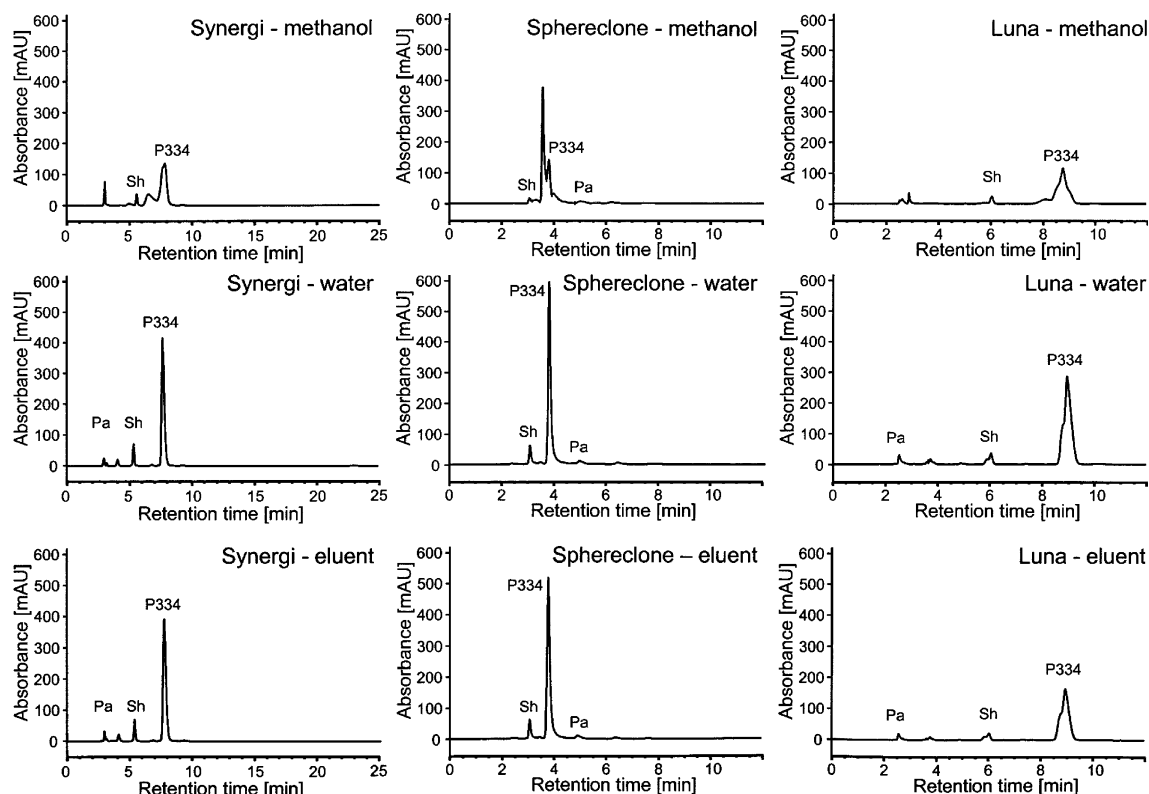


Fig. 3 Representative HPLC chromatograms of MAA extracts from the red macroalgal species *Porphyra umbilicalis*. The evaporated extracts were re-dissolved in either pure methanol, distilled water or HPLC eluent, analyzed on different reversed-phase columns and

individual peaks detected at 330 nm. Retention time, maximum absorbance and identification of individual peaks are summarized in Table 2. *Pa* palythine, *Sh* shinorine, *P334* porphyra-334

concentrations supporting the fact that utilization of this solvent for re-dissolution will lead to an underestimation of the values.

Discussion

The data presented clearly indicate that the selection of the re-dissolution solvent after the extraction and evaporation protocol (Tartarotti and Sommaruga 2002) has a significant effect on the qualitative and quantitative MAA analysis in the macroalgal species *Prasiola crispa* and *Porphyra umbilicalis*. While distilled water and the HPLC eluent with small percentages of methanol and acetic acid led to almost identical peak patterns and MAA concentrations on the C18 Synergi and C8 Sphercclone columns, application of pure methanol resulted in different patterns and much lower MAA contents. These results can be confirmed by further investigations on the red algae *Corallina elongata* and *Asparagopsis armata* from the Mediterranean Sea where re-dissolution of extracts in methanol, distilled water or HPLC eluent led to lower MAA concentrations in methanol and much higher variance between replicate injections

(data not shown). In addition, utilization of methanol was accompanied by the formation of double peaks such as for porphyra-334 on the Sphercclone column, the loss of specific MAAs such as palythine on the Synergi column and a decrease in total MAA amounts ranging from about 35% of the maximum in *P. crispa* to 80% of the maximum in *P. umbilicalis*. The variability in peak areas and/or peak tailing when extracts were re-dissolved in methanol may result from an incompatibility between sample liquid phase and column properties as well as from the much lower solvent concentration of the HPLC eluent. If the chromatographic elution strength of the solvent is much higher compared to the mobile phase this may result in lower plate numbers and hence lower separation efficiency (Sadek 2002). Consequently, methanol should be avoided as re-dissolution solvent during the sample preparation protocol prior HPLC analysis. Since many scientists used methanol as re-dissolution solvent the respective MAA concentrations published should be taken with great caution because they seem to be underestimated (Table 3).

The results indicate also that MAAs exhibit different physico-chemical properties, which may strongly influence their chromatographic behavior on various reversed-phase

Table 2 MAA separation and concentrations in extracts of the red alga *Porphyra umbilicalis* after re-dissolution in different solvents and after using different HPLC columns

Column	Concentration (mg g ⁻¹ DW)									
	Solvent	Palythine (320 nm)	MAA-5 (324 nm)	Shinorine (333 nm)	MAA-6 (334 nm)	MAA-7 (334 nm)	Porphyra-334 (334 nm)	MAA-8 (334 nm)	MAA-9 (322 nm)	Total
Synergi C18	Methanol	^a	ND	0.30 ± 0.01 (5.5 min)	1.36 ± 0.27 (6.5 min)	ND	4.26 ± 0.08 (7.8 min)	ND	ND	6.05 ± 0.34
Synergi C18	Water	0.21 ± 0.01 (2.9 min)	0.22 ± 0.02 (4.1 min)	0.63 ± 0.04 (5.4 min)	ND	ND	6.90 ± 0.04 (7.7 min)	ND	ND	8.01 ± 0.05
Synergi C18	HPLC eluent	0.21 ± 0.01 (2.9 min)	0.22 ± 0.02 (4.1 min)	0.64 ± 0.03 (5.4 min)	ND	ND	6.83 ± 0.05 (7.7 min)	ND	ND	7.88 ± 0.06
Sphereclone C8	Methanol	0.18 ± 0.02 (5.0 min)	ND	0.15 ± 0.01 (3.1 min)	ND	3.49 ± 0.11 (3.6 min)	1.59 ± 0.10 (3.8 min)	0.78 ± 0.04 (4.0 min)	0.11 ± 0.02 (6.2 min)	6.54 ± 0.26
Sphereclone C8	Water	0.22 ± 0.01 (5.0 min)	ND	0.62 ± 0.03 (3.1 min)	ND	ND	6.95 ± 0.11 (3.8 min)	ND	0.11 ± 0.01 (6.4 min)	8.00 ± 0.06
Sphereclone C8	HPLC eluent	0.23 ± 0.04 (5.0 min)	ND	0.61 ± 0.02 (3.1 min)	ND	ND	6.87 ± 0.07 (3.8 min)	ND	0.12 ± 0.01 (6.4 min)	7.90 ± 0.10
Luna C8	Methanol	^a	ND	0.40 ± 0.02 (6.0 min)	ND	ND	5.40 ± 0.20 (8.8 min)	ND	ND	5.97 ± 0.24
Luna C8	Water	0.29 ± 0.02 (2.5 min)	0.16 ± 0.01 (3.7 min)	0.62 ± 0.05 (6.0 min)	ND	ND	8.52 ± 0.66 (8.9 min)	ND	ND	9.59 ± 0.73
Luna C8	HPLC eluent	0.21 ± 0.01 (2.5 min)	0.12 ± 0.01 (3.7 min)	0.31 ± 0.01 (6.0 min)	ND	ND	6.08 ± 0.16 (8.9 min)	ND	ND	6.72 ± 0.19

The absorbance maxima of the different MAAs and the retention time in minutes of the respective peaks is given in brackets. Data given represent mean value ± SD (*n* = 3). ND not detected

^a Mixed unidentifiable peaks

Table 3 The analytical HPLC set-ups available from the literature for the separation of MAAs with an emphasis on the re-dissolution solvents used

Re-dissolution solvent	Elution/column	Mobile phase	Reference
100% methanol	Isocratic/C8	0.1% acetic acid; 25% aqueous methanol	Dunlap and Chalker (1986), Banaszak et al. (2006)
100% methanol	Isocratic/C8	0.1% acetic acid; 25% aqueous methanol	Neale et al. (1988), Karsten et al. (1998)
100% methanol	Isocratic/C8	0.1% acetic acid; 80% aqueous methanol	Moisan and Mitchell (2001)
100% methanol	Isocratic/C8	0.1% acetic acid; 5% aqueous methanol	Figueroa et al. (2003)
100% methanol	Isocratic/C8	0.1% acetic acid; 2.5% aqueous methanol	Korbee et al. (2005), Karsten et al. (2005), de la Coba et al. (2008)
80% methanol	Isocratic/C8	0.1% acetic acid; 55% aqueous methanol	Carroll and Shick (1996)
25% methanol	Isocratic/C8	0.1% acetic acid; 25% aqueous methanol	Tartarotti and Sommaruga (2002)
Deionized water	Isocratic/C8	0.1% acetic acid; 25% aqueous methanol	Shick et al. (1999)
80:20 methanol:THF	Gradient/C18	From 0.2% acetic acid to 0.1% acetic acid; 25% aqueous methanol	Dionisio-Sese et al. (1997)
100% water	Gradient/C18	From 0.2% acetic acid to 0.1% acetic acid; 25% methanol; 25% acetonitrile	Caretto et al. (2001)
0.2% aqueous trifluoroacetic acid/ammonium acetate	Gradient/two coupled C18 columns	From 0.2% trifluoroacetic acid/ammonium hydroxide in water to 0.2% trifluoro-acetic acid/ammonium hydroxide in water:methanol; acetonitrile (80:10:10)	Caretto et al. (2005)

columns as extensively discussed in Caretto et al. (2005). While the typical 324 nm-MAAs in *P. crispa* did not give any clear peak on the C8 Luna column independent on the re-dissolution solvent used, the MAAs shinorine and porphyra-334 from *P. umbilicalis* were easy to identify with all 3 solvents applied on this column. The chemical structure and features of palythine, shinorine and porphyra-334 are known for many decades (Bandaranayake 1998, and references therein). Although the 324 nm-MAA has been described from various taxa of the subaerial green macroalgal genus *Prasiola* (Hoyer et al. 2001; Gröniger and Häder 2002), as well as from other closely related subaerial green microalgae of the Trebouxiophyceae colonising building facades (Karsten et al. 2005), the chemical structure is still to be elucidated. However, various preliminary experiments with partially purified extracts and the application of mass spectroscopy indicate the typical aminocyclohexenimine ring system linked to an unknown sugar moiety (data not shown). To our knowledge only one scientific publication on the occurrence of a MAA with a covalently linked oligosaccharide exists which is located in the extracellular glycan of the terrestrial cyanobacterium *Nostoc commune* (Böhm et al. 1995). It is reasonable to assume that such a chemical modification will strongly influence the chromatographic behavior of the 324 nm-MAAs on the Luna column.

The first author was using for more than a decade reversed-phase C8 columns of different manufactures including the Spherclone for MAA analysis (e.g. Karsten

et al. 1998), and the general experience is a gradual loss in resolution after several hundreds of injections. Although according to the manufacturer's recommendation various regeneration and cleaning procedures for these columns such as rinsing with different solvents at low and high temperatures had been tested, none of the protocols was successful. It seems that unknown contaminations irreversibly bind to the stationary phase which reduce the separation capacity. Consequently, the rate of column replacement and hence concomitant cost was very high for routine MAA analysis.

In contrast, the experience with the C18 Synergi column indicate long-term reproducibility in the separation of MAAs also after more than 2.000 analysis. Besides the separation of palythine (2.9 min), shinorine (5.4 min) and porphyra-334 (7.7 min), also standards of asterina-330 (3.3 min) and mycosporine-glycine (4.6 min) can be well separated on this column (data not shown). These five MAAs represent the most abundant UV-absorbing compounds in red algae (Karsten et al. 1998).

While a general methodological approach for the re-dissolution of dried macroalgal MAA extracts and specific reversed-phase HPLC columns does not exist, the data presented clearly indicate that the widely used pure methanol as well as C8 columns may lead to analytical problems. Therefore, re-dissolution in distilled water or in HPLC eluent is recommended in combination with the C18 Synergi column. Following this technique guarantees separation and routine analysis of the most abundant MAAs in macroalgae.

Whether this method is also applicable for specific MAAs from marine animals has to be tested in further studies. For analyzing complex MAA mixtures the HPLC gradient method of Caretto et al. (2005) should be used.

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