Quantitative and Qualitative HPLC Analysis of Mycosporine-Like Amino Acids Extracted in Distilled Water for Cosmetical Uses in Four Rhodophyta

Patricia Chaves-Peña 1, F. de la Coba 2, Felix L. Figueroa 1 and Nathalie Korbee 1,*

1 Department of Ecology and Geology, Faculty of Sciences, University of Malaga, Institute of Blue Biotechnology and Development (IBYDA), Campus Universitario de Teatinos s/n, E-29071 Malaga, Spain; patricia.ch@uma.es (P.C.-P); Felix_lopez@uma.es (F.L.F.)
2 Photobiology Laboratory, Central Service for Research Support (SCAI), University of Malaga, Campus Universitario de Teatinos, E-29071 Malaga, Spain; pdlacoba@uma.es
* Correspondence: nkorbee@uma.es; Tel.: +34-951953257

Received: 28 November 2019; Accepted: 25 December 2019; Published: 28 December 2019

Abstract: Mycosporine-like amino acids (MAAs) have gained considerable attention as highly active photoprotective candidates for human sunscreens. However, more studies are necessary to evaluate the extraction efficiency of these metabolites in solvents compatible with cosmetics and their subsequent analysis by HPLC. In the present study, MAA extraction using distilled water and 20% methanol in four Rhodophyta was investigated. Different re-dissolution solvents and a C8 and C18 columns were tested for the HPLC analysis. Porphyra-334, shinorine, palythine, palythine-serine, asterina-330, and palythinol were identified by HPLC/ESI-MS. These MAAs were better isolated with the C8 column and using methanol as re-dissolution solvents. Regarding total MAAs concentrations, no differences between the two solvents were found. The highest values were observed injecting them directly in the HPLC. According to these results, distilled water could be an excellent extraction solvent in the production of MAAs extracts and quantification of their total concentrations for different uses in the industry. Nevertheless, the extraction of MAAs using 20% methanol and re-dissolution in pure methanol after dryness is the best option to characterize and identify the most common MAAs in these red algae. Our results entail important implications regarding the use of red macroalgae as promising candidates as environment-friendly sources of natural sunscreens.

Keywords: HPLC column; mycosporine-like amino acids; photoprotection; re-dissolution solvent; Rhodophyta

1. Introduction

Solar radiation exposes the intertidal marine macroalgae to elevated irradiances of UV-B (280–315 nm) and UV-A (315–400 nm). In fact, depletion of the ozone layer in the stratosphere during the past two decades has intensified the levels of solar UV-B radiation, which has reached unexpected levels that can be harmful for many biological processes [1–3]. The UV radiation can produce several detrimental effects on biologically important molecules such as lipids, proteins, or DNA [4–6].

The marine organisms that are exposed to UV radiation can accumulate natural UV-absorbing compounds [7]. Among others, mycosporine-like amino acids (MAAs) [8,9] have drawn special attention as these molecules act as antioxidants and provide photoprotection [10,11]. The main producers of these molecules in the marine coast are the red algae [12–14].

MAAs are a family of intracellular compounds of low molecular weight (<400 Da), they are distributed in a wide range of marine organisms, are water-soluble, and have high molar extinction
coefficients ($\varepsilon = 28,100–50,000 \text{ Lmol}^{-1}\text{cm}^{-1}$). They are secondary metabolites capable of absorbing UV radiation with the maximum absorbance between 310 and 365 nm [15–17]. MAAs have a general structure that consist of cyclohexenone or cyclohexenimine chromophores conjugated with one or two amino acids that are responsible of UV absorption [18]. The high photostability of MAAs over a wide range of temperature and pH, together with their antioxidant properties, make them promising metabolites in the biotechnology industry. These compounds are recognized as strong UV-absorbing molecules that can be used as an active ingredient in sun-care cosmetic products, therefore could be a potential alternative of chemical filters used in sunscreens, also due to the fact that they could satisfy consumers who want the incorporation of natural ingredients. Additionally, MAAs could be effective against actinic erythema, but can also protect the humans against other biological effects such as immune suppression or photo-oxidative damage [11,19–25]. These bioactive functional MAAs are promising functional ingredients for novel cosmeceuticals that are cosmetic products with health benefits. In fact, MAAs have already been commercialized as Helioguard®365. This cosmetic reagent contains the liposomal MAAs, shinorine and porphyra-334, that were originally extracted from the red alga Porphyra umbilicalis and has been successfully commercialized as a natural and safe sunscreen compound. Nowadays, the MAA extraction protocols differ in type of solvents, temperature, and extraction times. Being MAA extraction efficiencies and concentrations affected by these conditions [26–29]. A variety of extraction and separation methods for MAAs have been developed and tested on a broad range of organisms [28]. Ethanol and methanol from 20% to 80% were performed in the extraction of MAAs. However, the potential use of MAAs as sunscreen and antioxidant additives in cosmeceutical products implies an isolation of MAAs using aqueous solution preferably, discarding some organic solvents like methanol due to its toxicity.

On the other hand, different chromatographic techniques have been used for isolation, identification, and quantification of MAAs. Reverse-phase high-performance liquid chromatography (HPLC) employing monomeric octylsilicaC8 column [29] as well as low silanol-free group octadecylsilicaC18 column [16,30] are the most common methods to separate and characterize MAAs. A solvent mixture of distilled water:methanol with a slow percentage of acetic acid run isocratically has most commonly been used as HPLC eluent.

Karsten et al. [28] was the first to evaluate the effect of re-dissolution solvent (100% methanol, distilled water, and HPLC eluent), after dryness, on the extraction efficiency using different columns (Synergi C18, Sphereclone C8, and Luna C8) on a red and a green alga. However, to our knowledge, the extraction efficiency of distilled water as solvent has not been studied. Otherwise, an ideal method for MAA extraction and subsequent characterization is still an unsolved problem. Thus, the aim of the present study was to investigate the effect of different solvents in the extraction of MAAs, as well as the effect of different post-extraction procedures before HPLC analysis in the isolation, identification, and quantification of MAAs presented in four Rhodophyta: Agarophyton vermiculophyllum, Crassiphycus corneus, Gracilaripsis longissima, and Pyropia leucosticta. Thus, two different solvents were used: The most common used in MAA extraction, 20% methanol, and the most appropriated for the cosmetic industry, distilled water. For each, different post-extraction protocols were tested: Direct sample analysis by HPLC or evaporate the sample to dryness and then use different re-dissolving solvents (distilled water, HPLC eluent, and 100% methanol) before injection in HPLC. Additionally, for each protocol, two of the most used HPLC columns (C8 and C18) were tested.

2. Results

In our study, we tested seven different methodological protocols for the extraction of MAAs and subsequent HPLC analysis in four Rhodophytes: Agarophyton vermiculophyllum, Crassiphycus corneus, Gracilaripsis longissima, and Pyropia leucosticta. Figure 1 illustrates those protocols. For each species, we tested two extraction solvents, 20% methanol, and 100% distilled water. Some MAA samples were directly analyzed by HPLC, and others were dried off and subsequently re-dissolved in distilled water, in HPLC eluent (1.5% methanol plus 0.15% of acetic acid), and in 100% methanol before being
injected in HPLC. We did not consider the extraction in distilled water and re-dissolution in distilled water treatment.

Figure 1. Extraction and re-dissolution protocols used to analyze mycosporine-like amino acids (MAAs); 20% methanol as extraction solvent in M1, M2, M3, and M4. Distilled water as extraction solvent in W1, W2, and W3. Number 1 indicates HPLC analysis done without re-dissolution. Number 2: Re-dissolution in distilled water. Number 3: Re-dissolution in HPLC eluent. Number 4: Re-dissolution in 100% methanol.

Chromatograms of *P. leucosticta* have been selected using C8 column (Figure 2) and C18 column (Figure 3). Chromatograms for the other species are shown in Figures S5–S10 (Supplementary Materials). In general, the use of the C18 column resulted in a deficient MAA separation (Figure 3). In three of the seven protocols, we could not identify different MAAs, so we observed a few mixed unidentifiable peaks. We have only been able to identify different MAAs using HPLC eluent or distilled water as a re-dissolution solvent with independence of the solvents of extraction. The most used method for MAA analysis based on extract with 20% methanol and re-dissolve in 100% methanol (M4 protocol) resulted in a mix of MAAs using the C18 column. Regarding the MAA analysis using the C8 column, all protocols led to the separation of different MAAs. In fact, the protocols in which an improved MAA separation were obtained were M1, M4, and W4 for C8 column, while the opposite ones M2, M3, W1, and W3 for C18 column. However, none could separate all MAAs. According to these results, the incoming data presented in this work have been obtained applying the different protocols using the C8 column.

The qualitative distribution of MAAs for each species was determined by ESI-MS and up to five different MAAs were identified in the analyzed samples: *Porphyra*-334, shinorine, palythine, asterina-330, palythinol, and palythine-serine (Table 1). Also, the relative proportions expressed in percentage were calculated in each alga according to the total concentrations obtained applying the protocols M4 and W4 (Table 1). *Porphyra*-334, shinorine, and asterina-330 were common in all studied species. Palythine and *porphyra*-334 were the dominants MAAs in *A. vermiculophyllum*, shinorine in *C. corneus*, palythine in *G. longissima*, and *porphyra*-334 in *P. leucosticta*. Asterina-330 was the minority MAAs in all studied species except *P. leucosticta*, and palythine-serine was only present in *C. corneus*. Palythinol was only identified in three of these species: *G. longissima*, *C. corneus*, and *P. leucosticta*.
Species 1 (palythine), 2 (asterina-330), 3 (palythinol), and B (mixed unidentifiable peaks).

Figure 2. HPLC normalized chromatograms of MAAs identified in Pyropia leucosticta by seven methodological protocols using the Luna-C8 column. The code of protocol is indicated based on extraction and re-dissolution solvents used (see Figure 1 legend). Numbers indicate: 1 (palythine), 2 (asterina-330), 3 (palythinol), 4 (shinorine), 5 (porphyra-334), u (unidentifiable peak), and A (mixture of palythinol and shinorine).

Figure 3. HPLC normalized chromatograms of MAAs identified of Pyropia leucosticta by seven methodological protocols using the Infinity Lab Poroshell 120 C18 column. The code of protocol is indicated based on extraction and re-dissolution solvents used (see Figure 1 legend). Numbers indicate: 1 (palythine), 2 (asterina-330), 3 (palythinol), and B (mixed unidentifiable peaks).
Table 1. Composition of MAAs, relative percentage (%), and characterization of MAAs by mass spectrometry for each analyzed species. AV: *Agarophyton verniculophyllum*. CC: *Crassiphycus corneus*. GC: *Gracilaria longissima*. PL: *Porphyra leucosticta*.

| Species | MAAs | % Relative | Mol. Formula | λ<sub>max</sub> (nm) | Exact (ppm) | Calculated (m/z [M + H]<sup>+</sup>) | Observed (m/z [M + H]<sup>+</sup>) |
|---------|------|------------|--------------|----------------------|------------|-------------------------------------|----------------------------------|
| AV      | Porphyra-334 | 40.19 ± 1.33 | C<sub>34</sub>H<sub>52</sub>N<sub>4</sub>O<sub>6</sub> | 334 | 0.8 | 347.14489 | 347.14365 |
|         | Shinorine    | 10.63 ± 2.71 | C<sub>34</sub>H<sub>52</sub>N<sub>4</sub>O<sub>6</sub> | 334 | 1.4 | 333.12924 | 333.12799 |
|         | Palysthine   | 42.61 ± 3.09 | C<sub>34</sub>H<sub>52</sub>N<sub>4</sub>O<sub>6</sub> | 330 | 2.9 | 245.11320 | 245.11247 |
|         | Asterina-330 | 4.97 ± 0.57  | C<sub>32</sub>H<sub>50</sub>N<sub>4</sub>O<sub>6</sub> | 330 | 1.8 | 289.13941 | 289.13840 |
| CC      | Porphyra-334 | 18.04 ± 3.85 | C<sub>34</sub>H<sub>52</sub>N<sub>4</sub>O<sub>6</sub> | 334 | 0.8 | 347.14489 | 347.14316 |
|         | Shinorine    | 49.20 ± 2.80 | C<sub>34</sub>H<sub>52</sub>N<sub>4</sub>O<sub>6</sub> | 334 | 1.4 | 333.12924 | 333.12781 |
|         | Palysthine-serine | 6.12 ± 1.42 | C<sub>34</sub>H<sub>50</sub>N<sub>4</sub>O<sub>6</sub> | 320 | 3.8 | 275.12376 | 275.12271 |
|         | Asterina-330 | 1.27 ± 0.41  | C<sub>32</sub>H<sub>50</sub>N<sub>4</sub>O<sub>6</sub> | 330 | 1.8 | 289.13941 | 289.13840 |
|         | Palysthine   | 30.28 ± 1.74 | C<sub>32</sub>H<sub>50</sub>N<sub>4</sub>O<sub>6</sub> | 332 | 0.9 | 303.15506 | 303.15399 |
| GL      | Porphyra-334 | 5.36 ± 3.18  | C<sub>34</sub>H<sub>52</sub>N<sub>4</sub>O<sub>6</sub> | 334 | 0.8 | 347.14489 | 347.14371 |
|         | Shinorine    | 37.05 ± 9.79 | C<sub>34</sub>H<sub>52</sub>N<sub>4</sub>O<sub>6</sub> | 334 | 1.4 | 333.12924 | 333.12805 |
|         | Palysthine   | 1.70 ± 0.20  | C<sub>34</sub>H<sub>52</sub>N<sub>4</sub>O<sub>6</sub> | 320 | 1.2 | 245.11320 | 245.11290 |
|         | Asterina-330 | 1.63 ± 0.45  | C<sub>34</sub>H<sub>52</sub>N<sub>4</sub>O<sub>6</sub> | 330 | 1.8 | 289.13941 | 289.13849 |
|         | Palysthine   | 59.28 ± 9.52 | C<sub>32</sub>H<sub>50</sub>N<sub>4</sub>O<sub>6</sub> | 332 | 0.9 | 303.15506 | 303.15421 |
| PL      | Porphyra-334 | 79.83 ± 1.98 | C<sub>34</sub>H<sub>52</sub>N<sub>4</sub>O<sub>6</sub> | 334 | 0.8 | 347.14489 | 347.14343 |
|         | Shinorine    | 3.77 ± 0.99  | C<sub>34</sub>H<sub>52</sub>N<sub>4</sub>O<sub>6</sub> | 334 | 1.4 | 333.12924 | 333.12799 |
|         | Palysthine   | 6.26 ± 0.95  | C<sub>34</sub>H<sub>52</sub>N<sub>4</sub>O<sub>6</sub> | 320 | 1.2 | 245.11320 | 245.11227 |
|         | Asterina-330 | 7.69 ± 0.80  | C<sub>34</sub>H<sub>52</sub>N<sub>4</sub>O<sub>6</sub> | 330 | 1.8 | 289.13941 | 289.13821 |
|         | Palysthine   | 2.44 ± 0.16  | C<sub>32</sub>H<sub>50</sub>N<sub>4</sub>O<sub>6</sub> | 332 | 0.9 | 303.15506 | 303.15411 |

In addition, the flavonoid myricetin was identified by ESI-MS in *A. verniculophyllum*, but no chromatographic peak was found associated to this molecule in any protocols or columns.

Using the C8 column all MAAs described by ESI-MS were detected in *A. verniculophyllum* for all protocols. In *P. leucosticta*, only the M1, M4, and W4 protocols allowed a good identification (Table 2), i.e., extracting MAAs in distilled water or 20% methanol and subsequent re-dissolution in 100% methanol or extraction in 20% methanol and also directly analyzing by HPLC without drying off. In *C. corneus* and *G. longissima*, the protocols M4 and W4 offered similar results, i.e., only extraction in distilled water or 20% methanol and subsequent re-dissolution in 100% methanol must be accepted for the qualitative MAAs analysis. According to the obtained data, M4 and W4 would be the most appropriate protocols for being applied in the qualitative HPLC analysis of the following MAAs: Porphyra-334, shinorine, palysthine, asterina-330, palysthine, and palysthine-serine.

In general, total MAA concentration did not differ between C18 and C8 columns, in some cases being even lower than the concentration using the first column (data not shown for C18). The concentration of MAAs calculated depended on the protocol in the four species (Figure 4). For C8 column, the highest MAA concentrations were observed in M1 and W1 protocols in the four Rhodophytes (*p* < 0.05). No significant differences were found using distilled water or 20% methanol as extraction solvent in any species (Figure 4). The average value of MAA concentration for these two treatments (M1 and W1) in *P. leucosticta* was 9.6 ± 0.04 mg g<sup>-1</sup> DW, followed by *C. corneus* (2.11 ± 0.1 mg g<sup>-1</sup> DW), *G. longissima* (0.69 ± 0.09 mg g<sup>-1</sup> DW), and *A. verniculophyllum* (0.4 ± 0.09 mg g<sup>-1</sup> DW). In general, protocols M4 and W4 offered the lowest MAA concentrations (*p* < 0.05) in the most studied algae, with the results in *P. leucosticta* being 4.3 ± 0.26 mg g<sup>-1</sup> DW, *C. corneus* being 1.19 ± 0.68 mg g<sup>-1</sup> DW, *G. longissima* being 0.26 ± 0.07 mg g<sup>-1</sup> DW, and *A. verniculophyllum* being 0.1 ± 0.04 mg g<sup>-1</sup> DW. We found a tendency of progressive increases in MAA concentration as the percentage of methanol in the re-dissolution solvent decreased (Figure 4).
Table 2. Mycosporine-like amino acids (MAAs) identified in each analyzed species and protocols for their identification by HPLC using the C8-Luna column.

| MAAs              | Agarophyton vermiculophyllum | Protocols |
|-------------------|-----------------------------|-----------|
|                   | M1  | M2  | M3  | M4  | W1  | W3  | W4  |
| Porphyra-334      | ●   | ●   | ●   | ●   | ●   | ●   | ●   |
| Shinorine         | ●   | ●   | ●   | ●   | ●   | ●   | ●   |
| Palythine         | ●   | ●   | ●   | ●   | ●   | ●   | ●   |
| Asterina-330      | ●   | ●   | ●   | ●   | ●   | ●   | ●   |

| MAAs              | Crassiphycus corneus | Protocols |
|-------------------|----------------------|-----------|
|                   | M1  | M2  | M3  | M4  | W1  | W3  | W4  |
| Porphyra-334      | ●   | ●   | ●   | ●   | ●   | ●   | ●   |
| Shinorine         | ●   | ●   | ●   | ●   | ●   | ●   | ●   |
| Palythine-serine  | ●   | ●   | ●   | ●   | ●   | ●   | ●   |
| Asterina-330      | ●   | ●   | ●   | ●   | ●   | ●   | ●   |
| Palythinol        | ●   | ●   | ●   | ●   | ●   | ●   | ●   |

| MAAs              | Gracilariopsis longissima | Protocols |
|-------------------|---------------------------|-----------|
|                   | M1  | M2  | M3  | M4  | W1  | W3  | W4  |
| Porphyra-334      | ●   | ●   | ●   | ●   | ●   | ●   | ●   |
| Shinorine         | ●   | ●   | ●   | ●   | ●   | ●   | ●   |
| Palythine         | ●   | ●   | ●   | ●   | ●   | ●   | ●   |
| Asterina-330      | ●   | ●   | ●   | ●   | ●   | ●   | ●   |
| Palythinol        | ●   | ●   | ●   | ●   | ●   | ●   | ●   |

| MAAs              | Pyropia leucosticta | Protocols |
|-------------------|---------------------|-----------|
|                   | M1  | M2  | M3  | M4  | W1  | W3  | W4  |
| Porphyra-334      | ●   | ●   | ●   | ●   | ●   | ●   | ●   |
| Shinorine         | ●   | ●   | ●   | ●   | ●   | ●   | ●   |
| Palythine         | ●   | ●   | ●   | ●   | ●   | ●   | ●   |
| Asterina-330      | ●   | ●   | ●   | ●   | ●   | ●   | ●   |
| Palythinol        | ●   | ●   | ●   | ●   | ●   | ●   | ●   |

Figure 4. Concentration of total MAAs (mg g⁻¹ DW) of each species obtained by seven different protocols. The code of protocol is indicated based on extraction and re-dissolution solvents used (see Figure 1 legend). (A) Agarophyton vermiculophyllum. (B) Crassiphycus corneus. (C) Gracilariopsis longissima. (D) Pyropia leucosticta. Different letters indicate significant differences among MAA concentrations for each species.
In Table 3, the strengths and weaknesses of each protocol used in this work are summarized.

Table 3. List of protocols indicating extraction and re-dissolution solvents, abbreviation, strengths, and weaknesses of each of them for Luna-C8 column.

| Extraction Solvent | Re-dissolution Solvent | Abbreviation | Strengths | Weaknesses |
|--------------------|------------------------|--------------|-----------|------------|
| 20% Methanol       | Direct HPLC analysis without re-dissolution | M1 | High concentration of extracted MAA | Accumulation of impurities on the column; Toxic for cosmetic use |
|                    | Distilled water        | M2 | High concentration of extracted MAA | Some unidentified MAA; Toxic for cosmetic use |
|                    | HPLC Eluent            | M3 | Medium concentration of extracted MAA | Some unidentified MAA; Toxic for cosmetic use |
| 100% Methanol      | Direct HPLC analysis without re-dissolution | M4 | Improved separation MAA | Low concentration of extracted MAA; Toxic for cosmetic use |
| Distilled water    | Direct HPLC analysis without re-dissolution | W1 | High concentration of extracted MAA; Suitable for cosmetic | Accumulation of impurities on the column |
|                    | HPLC eluent            | W3 | Medium concentration of extracted MAA; Suitable for cosmetic | Some unidentified MAA |
|                    | 100% Methanol          | W4 | Improved MAA separation; Suitable for cosmetic | Low concentration of extracted MAA |

3. Discussion and Conclusions

It is well known that there is high potentiality for including MAAs in cosmetic formulations as they have high bioactive activities [20]. The potential uses of MAAs as sunscreen and antioxidant additives in cosmeceutical products implies the use of extraction solvents with no certified toxic effects and that preserve their physico-chemical and absorption properties. MAAs have high solubility in aqueous solution; therefore, distilled water could be a good solvent in terms of extraction efficiency.

In order to study the influence of distilled water, as a solvent of extraction in MAA analysis, four Rhodophytes were selected based on their medium-high MAA contents and their different MAA profiles [17,31,32]: Agarophyton vermiculophillum, Crassiphycus corneus, Gracilariopsis longissima, and Pyropia leucosticta. We have selected different protocols and chromatographic columns that are commonly used for MAA analysis in order to elucidate which of them would be the best option for the isolation, identification, and quantification of MAAs extracted in distilled water for cosmetic purpose.

Different solvents had been used for MAA extraction, the most common being ethanol and methanol from 20% to 80% [14,17,30,33,34]. However, other solvents such as 2-octyl dodecanol, octyldodecyl ester of L-pyrrolidone carboxylic acid (OEL-PCA) (cosmetic emollient and moisturizer), and ethyl acetate revealed that UV absorption spectra of the extracts, number of elution chromatographic peaks, and their maxima absorption were affected by the nature of the solvents, being the extract in OEL-PCA, which showed the poorest UV absorption, and only palythenic acid could be identified in these extracts [35]. In our study, we compared the extraction in 20% methanol and distilled water and no significant differences between them in terms of total MAA concentrations were found when these extracts were injected directly into HPLC. Furthermore, under this treatment, no hypsochromic shifts in UV-absorption maxima of MAAs identified were observed.

Regarding qualitative and quantitative analysis of MAAs, HPLC-MS is the most used technique nowadays [36]. However, other techniques had been used such as capillary electrophoresis [33]. Reverse-phase high-performance liquid chromatography (HPLC) employing monomeric octylsilicaC8 column [29] as well as low silanol-free group octadecylsilica C18 column [16,30,34] are the most common HPLC columns used to separate and characterize MAAs. The solvent mixture of distilled water:methanol with a slow percentage of acetic acid run isocratically is the most common HPLC eluent. Except for the technique of Carreto et al. [37], there is no isolation and identification MAA protocol able to completely isolate large MAAs mixtures on marine organisms. The method used
by Carreto et al. [37] combines a C18 column with an acid solvent. Although this approach is able to separate high mixtures of MAAs, it is a time-consuming analysis that we recommend only in case of samples with a complex mix of MAAs.

We have analyzed our two MAAs extracts (20% methanol and distilled water) using a Luna-C8 column and a Poroshell-C18 column. The characteristic of both differed; while the C8 had 5 µm particle size and a dimension of 4.06 mm x 250 mm, the C18 column had 4 µm particle size and a dimension of 3 mm x 250 mm. Therefore, besides the different material of each column, we have different particles sizes and thickness. Thus, this is why we had to decrease the flow using C18 from 0.5 mL/min to 0.3 mL/min. Recently, Rosic et al. [34] described a general method for the isolation and characterization of MAA compounds from red algae and symbiotic dinoflagellates using methanolic extracts and HPLC and LC-MS with electrospray ionization source interface. They used a C18 column (1.7 µm particle size) and concluded that porphyra-334, palythine, and mycosporine-glycine could not be separated, as these mixed MAAs appeared only in an elution peak at the beginning of the chromatogram. According to the results obtained in this work, C18 column offers the worst result in terms of MAA separation using the seven treatments evaluated, although the particle sizes of the columns were different. However, Karsten et al. [28], who used three reverse-phase C8 and C18 HPLC columns, obtained better results than us using the Synergi C18 column with the same particle size and dimensions, but other company, Phenomenex versus Agilent. They compared, similarly to this work, pure methanol, distilled water, and HPLC eluent as redissolution solvent for dried Prasiola and Porphyra extracts (Pyropia former Porphyra) [28]. On the contrary, Karsten et al. [28] found analytical problems using the C8 columns, this was not the case for this study; in fact, we used the same C8 Luna column, and we obtained better results than with the C18 column. One explanation to the mixed MAAs could be related to other highly polar, low molecular weight UV-absorbing substances unrelated to MAAs that are generally present in extracts of marine organisms. These molecules may interact with the eluent solvent and MAAs presents in the samples increasing their molecular weights and changing their polarity, and therefore their elution times [30]. Matrix effect is minimized in analysis of purified and partially purified MAA extracts [20,38]. Although, why this matrix effect could occur under some conditions and not others must be investigated.

According to our results, the highest MAA concentration was obtained by injecting the sample directly after its extraction, regardless if it is extracted in distilled water or 20% methanol. The drying and subsequent re-dissolution of the pellets in the different studied solvents declined total MAA concentrations, as occurred in Karsten et al. [28]. With the independence of solvent of extraction, the MAA concentration when the sample was injected directly into the HPLC was approximately double compared to after drying the sample and resuspending it in 20% methanol. Consequently, methanol should be avoided as a re-dissolution solvent for the HPLC analysis in terms of total concentration. Opposite to this, these treatments (M4–W4), together with W1, led to improved peak separations. However, the use of methanol as a re-dissolution solvent provokes double peaks with similar absorption maxima and, therefore, limits its use for qualitative MAA analysis. This result could also be related to matrix effect [39].

Other authors had studied MAA composition and concentration in the same species included in this work. Roleda et al. [40] found, in A. vermiculophyllum (former Gracilaria vermiculophylla), two MAAs but they were not identified. Lalegerie et al. [14] found eight different MAAs in the same species, some of them unidentified, but no total MAA concentration was shown. Barceló-Villalobos et al. [32] identified four MAAs, the same as in this work, porphyra-334, shinorine, palythine, and asterina-330. However, total MAA concentration of samples collected from the field was at least two times higher than our MAA concentration. Regarding Crassiphycus corneus (formerly Hydropuntia or Gracilaria cornea), Sinha et al. [41] identified shinorine and porphyra-334, but they did not show total MAA concentration. Figueroa et al. [42] found shinorine, pophrya-334, and palythine. The concentration in this species was very similar to our work. Álvarez-Gómez et al. [31] found four MAAs, but the concentration was much lower than those presented in this work. In another article, similar concentration and composition
to that in our work were found [43,44], around 1 mg g\(^{-1}\), and porphyra-334, shinorine, palythine, asterina-330, and palythinol. MAAs in \(G.\) \(longissima\) were analyzed firstly by Torres et al. [45], but they identified asterina-330, palythinol, palythene, and usujirene. However, similar concentration and composition (porphyra-334, shinorine, palythine, asterina-330, and palythinol, and a concentration around 1 mg g\(^{-1}\)) have been shown in this species by other authors [31,43,44]. Finally, concentration and composition of \(Porphyr\)a species previously published did not differ from the results presented here. This species has porphyra-334 as the dominant MAA, and in minor concentrations, shinorine, palythine, asterina-330, and palythinol. It belongs to the order Bangiales, which has the highest MAA concentration [17,46,47]. To our knowledge, it is the first time in the literature in which myricetin, a flavonoid found in several foods, has been detected in one species of the Family \(Gracilariaeae\). Other flavonoids have been found in this taxonomic group, such as camptothecin and quercetin [48]. More studies are needed to quantify myricetin in this species and investigate the regulation of its synthesis. This flavonoid has shown anti-photoaging effects through its antioxidant and anti-inflammatory properties [49,50]. The beneficial effects have encouraged researchers to develop photo-protective products from natural sources [51]. This flavonoid is one of the most studied, however it is known that onions, kale, lettuce, tomatoes, apples, grapes, berries, tea, and red wine are rich sources of flavonols [52]. However, it is not deeply studied in algae. A recent study has described the presence of myricetin, among other flavonoids, in \(Caulerpa\) spp. [53]. The last study revealed that \(Caulerpa\) spp. are a promising functional food ingredient and could be explored as daily dietary supplements. Higher content of flavonoids in seaweeds were reported [54]. In contrast, the flavonoid content of some of them, \(Ulva\) \(lactuca\), \(Sargassum\) \(whitii\), and \(Enteromorph\) \(aspirulina\), was reported to be very low [55].

In summary, our data may allow us to obtain a generalization of results for other species with similar MAA profiles: Those containing porphyra-334, shinorine, palythine, asterina-330, and palythinol. In fact, these are the most abundant MAAs in macroalgae. For a routine analysis of those MAAs, particularly in species potentially used for cosmetic purpose, we suggest the following protocol. In order to guarantee the best MAA isolation, the better protocol would be to extract MAAs in distilled water, dry the extracts, and re-dissolute in 100% methanol. However, in order to have a better approximation of the total MAA concentration, the extraction in distilled water and direct injection in HPLC must be used.

Our results entail important implications regarding the use of red macroalgae as promising candidates as environment-friendly sources of industrially important compounds, like MAAs, because of their photosautotrophic properties, which can convert solar energy and carbon dioxide into useful chemicals as it has been reported by Navarro et al. [56]. MAAs, in addition to protecting against erythema, can reduce other negative biological effects as photocarcinogenesis, immunosupression CHYS, photomerization of urocanic acid, or photo-aging [11,25]. The genus \(Gracilaria\) has high potential as a source of high-value compounds and extracts for several applications [57]. However, only agar is commercially exploited. A better use of the biomass from commercial cultivation of species from the family \(Gracilariaeae\) may be an important alternative for exploiting the other bioactive components, such as MAAs and flavonoids. Further studies must be carried out.

4. Materials and Methods

4.1. Biological Material

Specimens of \(Agarophyt\)on \(vermiculophyl\llum\) (Ohmi) Gurgel, J.N.Norris et Fredericq were collected at Ria de Aveiro (40°38′ N, 8°43′ W), Portugal. \(Crassiphycus\) \(corneus\) (J.Agardh) Gurgel, J.N.Norris & Fredericq was collected from cultures in tanks located in a greenhouse of Gran Canaria, Spain (27°59′28″ N; 15°22′8″ W). \(Graciliariopsis\) \(longissima\) (S.G.Gmelin) M.Steentoft, L.M.Irvine & W.F.Farnham were collected on River San Pedro (36°32′52″ N; 6°12′33″ W), Tarifa, Cádiz, Spain. \(Pyropia\) \(leucosticte\) (Thuret) Neefus & J.Brodie were collected on rocky shores from Lagos (36°28′ N, 4°1′ W), Málaga,
Spain. Algae were dried by silica gel and stored under controlled humidity. Dried algal material was crushed to powder prior to extraction to guarantee homogeneity.

4.2. Extraction and Identification of MAAs

Analysis of MAAs was assayed according to Korbee-Peinado et al. [17] with some modifications. The extraction of MAAs was done with two different solvents: 20% aqueous methanol (v/v) and distilled water. Samples of dried algal (20 mg DW) were extracted for 2 h in screw-capped centrifuge vials filled with 1 mL 20% aqueous methanol (v/v) or distilled water in a waterbath at 45 °C. After this, 700 µL of the supernatant was evaporated to dryness under vacuum (Jouan evaporator centrifuge, France), except two samples of each species whose extracts in 20% aqueous methanol or distilled water were passed through a 0.2 µm membrane filter to be analyzed directly by HPLC. All samples were extracted in triplicate. In Figure 1, the seven protocols used in this work are represented. Three replicates were analyzed directly by HPLC (protocols with number 1). The other extracts were dried and re-dissolved in 700 µL 100% aqueous methanol (protocols with number 4), distilled water (number 2), or HPLC eluent (1.5% methanol (v/v) plus 0.15% acetic acid (v/v) in distilled water) (number 3) and mixed for 30 s. After passing through a 0.2 µm membrane filter, samples were analyzed with a distilled waters 600 HPLC system (Distilled waters Cromatografia, Barcelona, Spain). Sample volumes of 20 µL were injected into a reverse-phase Luna C8 column (5 µm particle size; 4.06 mm × 250 mm, Phenomenex, Aschaffenburg, Germany) with a pre-column (C8, Octyl, MOS; Phenomenex) and an Infinity Lab Poroshell 120 reverse-phase C18 column (4 µm particle size; 3 mm × 250 mm, Agilent, Santa Clara, CA, USA). The mobile phase used as eluent was 1.5% methanol (v/v) plus 0.15% acetic acid (v/v) in distilled water, run isocratically at 0.5 mL min⁻¹ with the Luna column and 0.3 mL min⁻¹ with the Agilent column, because the pressure was very high with higher fluxes.

MAAs were detected online with a distilled waters Photodiode Array Detector 996 at 330 nm, and absorption spectra (290–400 nm) were recorded each second directly on the HPLC-separate peaks. Identification of MAAs was performed by comparison of the absorption spectra and retention times and by co-chromatography using high purified grade MAAs extracts (porphyra-334, shinorine, palythine y asterina-330) provided from the Unit of Photobiology of the Central Service for Research Support (SCAI, University of Málaga, Málaga, Spain). Quantification was made by using published extinction coefficients [58,59] and the formula found in Pliego-Cortes et al. [60]. When we obtained a mixed unidentifiable peak, we estimated the total MAA concentration using an average extinction coefficient for those MAAs present in the species [38].

The qualitative analyze was complemented by electrospray ionization mass spectrometry (ESI-MS) with a high-resolution mass spectrometer (model Orbitrap Q-Exact, Thermo Scientific S.L., Bremen, Germany) provided with an electrospray ionization heated probe (HESI-II), in the premises of the Central Service for Research Support (SCAI, University of Málaga, Málaga, Spain). The mass spectrometer indicated 70–700 m/z in positive mode for MAA and negative mode for myricetin with a voltage of 3.5 kV.

4.3. Statistical Analysis

Values of mean concentration and their standard deviations per treatment and column were calculated from three replicates. Total MAA concentration was tested by one-way analysis of variance (ANOVA) for each species. Cochran’s test was used to check the homogeneity of variances. A post-hoc Student Newman–Keuls (SNK) multiple comparison test was applied and significant differences are indicated with different letters in Figure 4. Analyses were done using the program STATISTICA (8.0, Dell and StatSoft, CA, USA).

Supplementary Materials: The following are available online at http://www.mdpi.com/1660-3397/18/1/27/s1.

Figure S1: Mass spectrum of A (porphyra-334), B (shinorine), C (palythine), D (asterina-330), and E (myricetin) in Agarophyton vermiculophyllum. Figure S2: Mass spectrum of A (porphyra-334), B (shinorine), C (palythine-serine), D (asterina-330), and E (palythinol) in Crassiphycus corneus. Figure S3: Mass spectrum of A (porphyra-334),
B (shinorine), C (asterine-330), and D (palythinol) in *Gracilaria longissima*. Figure S4: Mass spectrum of A (porphyra-334), B (shinorine), C (palythine), and D (asterina-330) in *Pyropia leucosticta*. Figure S5: HPLC normalized chromatograms of MAAs identified in *Agarophyton vermiculophyllum* by seven methodological protocols using the Luna-C8 column. Figure S6: HPLC normalized chromatograms of MAAs identified in *Crassiphycus corneus* by seven methodological protocols using the Infinity Lab Poroshell 120 C18 column. Figure S7: HPLC normalized chromatograms of MAAs identified in *Cassidulina conicus* by seven methodological protocols using the Luna-C8 column. Figure S8: HPLC normalized chromatograms of MAAs identified in *Cassidulina conicus* by seven methodological protocols using the Infinity Lab Poroshell 120 C18 column. Figure S9: HPLC normalized chromatograms of MAAs identified in *Gracilaria longissima* by seven methodological protocols using the Infinity Lab Poroshell 120 C18 column. Figure S10: HPLC normalized chromatograms of MAAs identified in *Gracilaria longissima* by seven methodological protocols using the Infinity Lab Poroshell 120 C18 column.

**Author Contributions:** Conceptualization, N.K., F.L.F., and F.d.l.C.; data curation, P.C.-P., N.K., and F.d.l.C.; formal analysis, P.C.-P., N.K., and F.d.l.C.; funding acquisition, F.L.F.; investigation, P.C.-P., N.K., F.L.F., and F.d.l.C.; methodology, P.C.-P., N.K., and F.d.l.C.; project administration, F.L.F.; resources, F.L.F. All authors have read and agreed to the published version of the manuscript.

**Funding:** This research was supported by the Ministry of Education and Science of Spain (ACUDES) and Junta de Andalucía to the research group “Photobiology and Biotechnology of aquatic organisms” (RNM-295), Programa Operativo de Empleo Juvenil code UMATU14 (FEDER).

**Conflicts of Interest:** The authors declare no conflict of interest.

**References**

1. Franklin, L.A.; Forster, R.M. The changing irradiance environment: Consequences for marine macrophyte physiology, productivity and ecology. *Eur. J. Phycol.* 1997, 32, 207–232. [CrossRef]
2. Misonou, T.; Saitoh, J.; Oshima, S.; Tokitomo, Y.; Maegawa, M.; Inoue, Y.; Hori, H.; Sakurai, T. UV-absorbing substance in the red alga *Porphyra Yezoensis* (Bangiales, Rhodophyta) block thymine photodimer production. *Mar. Biotechnol.* 2003, 5, 194–200. [CrossRef] [PubMed]
3. Rastogi, R.P.; Sinha, R.P.; Singh, S.P.; Häder, D.P. Photoprotective compounds from marine organisms. *J. Ind. Microbiol. Biotechnol.* 2010, 37, 537–558. [CrossRef] [PubMed]
4. Buma, A.G.J.; Engelen, A.H.; Gieskes, W.W.C. Wavelength-dependent induction of thymine dimers and growth rate reduction in the marine diatom *Cyclotella* exposed to ultraviolet radiation. *Mar. Ecol. Prog. Ser.* 1997, 153, 91–97. [CrossRef]
5. Helbling, E.W.; Villafañe, V.E.; Buma, A.G.; Andrade, M.; Zaratti, F. DNA damage and photosynthetic inhibition induced by solar UVR in tropical phytoplankton. *Eur. J. Phycol.* 2001, 36, 157–166. [CrossRef]
6. Vicent, W.F.; Roy, S. Solar ultraviolet-B radiation and aquatic primary production: Damage, protection, and recovery. *Environ. Rev.* 1993, 1, 1–12. [CrossRef]
7. Häder, D.P.; Lebert, M.; Sinha, R.O.; Barbieri, E.; Herbling, E.W. Role of protective and repair mechanisms in the inhibition of photosynthesis in marine macroalgae. *Photochem. Photobiol. Sci.* 2002, 1, 809–814. [CrossRef]
8. Karantz, D. Chemical defenses of marine organisms against solar radiation exposure: UV-absorbing mycosporine-like amino acids and scytonemin. In *Marine Chemical Ecology*; McClincky, J.B., Baker, B.J., Eds.; CRC Press: Boca Raton, FL, USA, 2010; pp. 481–520.
9. Cockell, C.S.; Knowland, J. Ultraviolet radiation screening compounds. *Biol. Rev.* 1999, 74, 311–345. [CrossRef]
10. Yoshiki, M.; Tsuge, K.; Tsuruta, Y.; Yoshimura, T.; Koganemaru, K.; Sumi, T.; Matsui, T.; Matsumoto, K. Production of new antioxidant compound from mycosporine-like amino acid, porphyra-334 by heat treatment. *Food Chem.* 2009, 113, 1127–1132. [CrossRef]
11. Dela Coba, F.; Aguilera, J.; Figueroa, F.L.; De Gálvez, M.V.; Herrera, E. Antioxidant activity of mycosporine-like amino acids isolated from three red macroalgae and one marine lichen. *J. Appl. Phycol.* 2009, 21, 161–169. [CrossRef]
12. Karsten, U.; Franklin, L.A.; Lüning, K.; Wiencke, C. Natural ultraviolet and photosynthetic active radiation induce formation of mycosporine-like amino acids in the marine macroalgae *Chondrus crispus* (Rhodophyta). *Planta* 1998, 205, 257–262. [CrossRef]
13. Hoyer, K.; Karsten, U.; Wiencke, C. Induction of sunscreen compounds in Antarctic macroalgae by different radiation conditions. *Mar. Biol.* 2002, 141, 619–627.

Mar. Drugs 2020, 18, 27

11 of 14
14. Lalegerie, F.; Lajili, S.; Bedoux, G.; Taupin, L.; Stiger-Pouvreau, V.; Connan, S. Photo-protective compounds in red macroalgae from Brittany: Considerable diversity in mycosporine-like amino acids (MAAs). Mar. Environ. Res. 2019, 147, 37–48. [CrossRef] [PubMed]
15. Nakamura, H.; Kobayashi, J.; Hirata, Y. Separation of mycosporine-like amino acids in marine organisms using reverse-phase high-performance liquid chromatography. J. Chromatogr. 1982, 250, 113–118. [CrossRef]
16. Shick, J.M.; Dunlap, W.C. Mycosporine-like amino acids related to Gadusols: Biosynthesis, accumulation and UV-protective functions in aquatic organisms. Annu. Rev. Physiol. 2002, 64, 223–262. [CrossRef]
17. Korbee-Peinado, N.; Abdala Diaz, R.T.; Figueroa, F.L.; Helbling, E.W. Ammonium and UV radiation stimulate the accumulation of mycosporine-like amino acids in Porphyra columbina (Rhodophyta) from Patagonia, Argentina. J. Phycol. 2004, 40, 248–259. [CrossRef]
18. Singh, S.P.; Kumari, S.; Rastogi, R.P.; Singh, K.L.; Sinha, R.P. Mycosporine-like amino acids (MAAs): Chemical structure, biosynthesis and significance as UV absorbing/screening compounds. Indian J. Exp. Biol. 2008, 146, 7–17.
19. Conde, F.R.; Churio, M.S.; Previtali, C.M. The photoprotector mechanism of mycosporine-like amino acids. Excited-state properties and photo-stability of porphyra-334 in aqueous solution. J. Photochem. Photobiol. B Biol. 2000, 56, 139–144. [CrossRef]
20. De la Coba, F.; Aguilera, J.; Korbee, N.; De Galvez, M.V.; Herrera, E.; Álvarez-Gómez, F.; Figueroa, F.L. UVA and UVB photoprotective capabilities of topical formulations containing mycosporine-like amino acids (MAAs) through different biological effective protection factors (BEPFs). Mar. Drugs. 2019, 17, 55. [CrossRef]
21. Schmid, D.; Schürch, C.; Zülli, F.; Nissen, H.-P.; Prieur, H. Mycosporine-like amino acids: Natural UV-screening compounds from red algae to protect the skin against photoaging. SOFW J. 2003, 129, 38–42.
22. Ryu, J.; Park, S.-J.; Kim, I.-H.; Choi, Y.; Nam, T.-J. Protective effect of porphyra-334 on UVA-induced photoaging in human skin fibroblasts. Int. J. Mol. Med. 2014, 34, 796–803. [CrossRef] [PubMed]
23. Suh, S.-S.; Hwang, J.; Park, M.; Seo, H.H.; Kim, H.-S.; Lee, J.H.; Moh, S.H.; Lee, T.-K. Anti-inflammation activities of mycosporine-like amino acids (MAAs) in response to UV radiation suggest potential anti-skin aging activity. Mar. Drugs. 2014, 12, 5174–5187. [CrossRef] [PubMed]
24. Morliere, P.; Annie, M.; Isabelle, T. Action spectrum for UV-inducen lipid peroxidation in cultured human skin fibroblasts. Free Radic. Biol. Med. 1995, 19, 365–371. [CrossRef]
25. de la Coba, F.; Aguilera, J.; De Galvez, M.V.; Alvarez, M.; Gallego, E.; Figueroa, F.L.; Herrera, E. Prevention of the ultraviolet effects on clinical and histopathological changes, as well as the heat shock protein-70 expression in mouse skin by topical application of algal UV-absorbing compounds. J. Dermatol. Sci. 2009, 55, 161–169. [CrossRef] [PubMed]
26. Tartarotti, B.; Sommaruga, R. The effect of different methanol concentrations and temperatures on the extraction of mycosporine-like amino acids (MAAs) in algae and zooplankton. Arch. Hydrobiol. 2002, 154, 691–703. [CrossRef]
27. Volkmann, M.; Gorbushina, A.A. A broadly applicable method for extraction and characterization of mycosporines and mycosporine-like amino acids of terrestrial, marine and fresh distilled water origin. FEMS Microbiol. Lett. 2006, 255, 286–295. [CrossRef]
28. Karsten, U.; Escoubeyrou, K.; Charles, F. 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. Helgol. Mar. Res. 2009, 63, 231–238. [CrossRef]
29. Dunlap, W.C.; Chalker, B.E. Identification and quantification of near-UV absorbing compounds (S-320) in a hermatypics cleractinian. Coral Reefs 1986, 5, 155–159. [CrossRef]
30. Carreto, J.J.; Carignan, M.O.; Montoya, N.G. Comparative studies on mycosporine-like amino acids, paralytic shellfish toxins and pigment profiles of the toxic dinoflagellates Alexandrium tamarense, A-catenella and A-minutum. Mar. Ecol. Prog. Ser. 2001, 223, 49–60. [CrossRef]
31. Álvarez-Gómez, F.; Korbee, N.; Figueroa, F.L. Analysis of antioxidant capacity and bioactive compounds in marine macroalgal and lichenic extracts using different solvents and evaluation methods. Cienc. Mar. 2016, 42, 271–288. [CrossRef] [PubMed]
32. Barceló-Villalobos, M.; Figueroa, F.L.; Korbee, N.; Álvarez-Gómez, F.; Abreu, M.H. Production of mycosporine-like amino acids from Gracilaria vermiculophylla (Rhodophyta) cultured through one year in an integrated multi-trophic aquaculture (IMTA) system. Mar. Biotechnol. 2017, 19, 246–254. [CrossRef] [PubMed]
33. Hartmann, A.; Murauer, A.; Ganzer, M. Quantitative analysis of mycosporine-like amino acids in marine algae by capillary electrophoresis with diode-array detection. *J. Pharm. Biomed. Anal.* 2017, 138, 153–157. [CrossRef] [PubMed]

34. Rosic, N.N.; Braun, C.; Kvaskoff, D. Extraction and analysis of mycosporine-like amino acids in marine algae. *Methods Mol. Biol.* 2015, 1308, 119–129. [PubMed]

35. Bedoux, G.; Hardouin, K.; Marty, C.; Taupin, L.; Vandanjon, L.; Bourgougnon, N. Chemical characterization and photoprotective activity measurement of extracts from the red macroalga *Soleria chordalis*. *Bot. Mar.* 2014, 57, 291–301. [CrossRef]

36. Whitchcad, K.; Hedges, J.I. Analysis of mycosporine-like amino acids in plankton by liquid chromatography electrospray ionization mass spectrometry. *Mar. Chem.* 2002, 80, 27–39.

37. Carreto, J.L.; Carignan, M.O.; Montoya, N.G. A high-resolution reverse-phase liquid chromatography method for the analysis of mycosporine-like amino acids (MAAs) in marine organisms. *Mar. Biol.* 2005, 146, 237–252. [CrossRef]

38. Orfanoudaki, M.; Hartmann, A.; Karsten, U.; Ganzer, M. Chemical profiling of mycosporine-like amino acids in twenty-three red algal species. *J. Physiol.* 2019, 55, 393–403. [CrossRef]

39. Carreto, J.L.; Carignan, M.O. Mycosporine-like amino acids: Relevant secondary metabolites. Chemical and ecological aspects. *Mar. Drugs* 2011, 9, 387–446. [CrossRef]

40. Roleda, M.Y.; Nyberg, C.D.; Wulf, A. UVR defense mechanisms in eurytopic and invasive *Gracilaria vermiculophylla* (Gracilariales, Rhodophyta). *Physiol. Plantarum.* 2012, 146, 205–216. [CrossRef]

41. Sinha, R.P.; Klisch, M.; Gröninger, A.; Häder, D.P. Mycosporine-like amino acids in the marine red algae *Gracilaria cornea*—Effects of UV and heat. *Environ. Exp. Bot.* 2000, 43, 33–43. [CrossRef]

42. Figueroa, F.L.; Korbee, N.; Abdala, R.; Jerez, C.G.; López-de la Torre, M.; Güenaga, L.; Gómez-Pinchetii, J.L. Biofiltration of fishpond effluents and accumulation of N-compounds (phycobiliproteins and mycosporine-like amino acids) versus C-compounds (polysaccharides) in *Hydropuntia cornea* (Rhodophyta). *Mar. Pollut. Bull.* 2012, 64, 310–318. [CrossRef] [PubMed]

43. Álvarez-Gómez, F.; Korbee, N.; Casas-Arrojo, V.; Abdala-Diaz, R.T.; Figueroa, F.L. UV photoprotection, cytotoxicity and immunology capacity of red algal extracts. *Molecules* 2019, 24, 341. [CrossRef] [PubMed]

44. Álvarez-Gómez, F.; Korbee, N.; Figueroa, F.L. Effects of UV radiation on photosynthesis, antioxidant capacity and the accumulation of bioactive compounds in *Gracilariapispis longissima*, *Hydropuntia cornea* and *Halopithys incurve* (Rhodophyta). *J. Phycol.* 2019, 55, 1258–1273. [CrossRef]

45. Torres, P.B.; Chow, F.; Ferreira, M.J.P.; dos Santos, D.Y.A.C. Mycosporine-like amino acids from *Gracilariapispis tenuifrons* (Gracilariales, Rhodophyta) and its variation under high light. *J. Appl. Phycol.* 2016, 28, 2035–2040. [CrossRef]

46. Diehl, N.; Michalik, D.; Zuccarello, G.C.; Karsten, U. Stress metabolite pattern in the eulittoral red alga *Pyropiapiicata* (Bangiales) in New Zealand—Mycosporine-like amino acids and heterosides. *J. Exp. Mar. Biol.* 2019, 510, 23–30. [CrossRef]

47. De Ramos, B.; da Costa, G.B.; Ramlov, F.; Maraschin, M.; Horta, P.A.; Figueroa, F.L.; Korbee, N.; Bonomi-Barufi, J. Ecophysiological implications of UV radiation in the interspecific interaction of *Pyropia acanthopora* and *Grateloupiatururatu* (Rhodophyta). *Mar. Environ. Res.* 2019, 144, 36–45. [CrossRef]

48. Ashwini, S.; Manjula, S.; Suresh, B. Comparative HPLC analysis of camptothecin and quercetin contents in ethanolic extracts of *Gracilariapispis corticata* (J. Agardh). *IJPSR.*, 2017, 8, 4710–4715.

49. Jung, S.K.; Lee, K.W.; Kim, H.Y.; Oh, M.H.; Byun, S.; Lim, S.H.; Hae, Y.S.; Kang, N.J.; Bode, A.M.; Dong, Z. Myricetin suppresses UVB-induced wrinkle formation and MMP-9 expression by inhibiting Raf. *Biochem. Pharm.* 2010, 79, 1455–1461. [CrossRef]

50. Sim, G.S.; Lee, B.C.; Cho, H.S.; Lee, J.W.; Kim, J.H.; Lee, D.H.; Kim, J.H.; Pyo, H.B.; Moon, D.C.; Oh, K.W.; et al. Structure activity relationship of antioxidative property of flavonoids and inhibitory effect on matrix metalloproteinase activity in UVA-irradiated human dermal fibroblast. *Arch. Pharm. Res.* 2007, 30, 290–298. [CrossRef]

51. Cavinato, M.; Waltenberger, B.; Baraldo, G.; Grade, C.V.C.; Stuppner, H.; Jansen-Durr, P. Plant extracts and natural compounds used against UVB-induced photoaging. *Biogerontology* 2017, 18, 499–516. [CrossRef] [PubMed]

52. Panche, A.N.; Diwan, A.D.; Chandra, S.R. Flavonoids: An overview. *J. Nutr. Sci.* 2016, 5, e47. [CrossRef] [PubMed]
53. Tanna, B.; Brahmbhatt, H.R.; Mishra, A. Phenolic, flavonoid, and amino acid compositions reveal that selected tropical seaweeds have the potential to be functional food ingredients. *J. Food Process. Preserv.* 2019, 43, e14266. [CrossRef]

54. Lin, M.C.; Tsai, M.J.; Wen, K.C. Supercritical fluid extraction of flavonoids from *Scutellariae Radix*. *J. Chromatogr. A* 1999, 830, 387–395. [CrossRef]

55. Cox, S.; Abu-Ghannam, N.; Gupta, S. An assessment of the antioxidant and antimicrobial activity of six species of edible Irish seaweeds. *IFRJ* 2010, 17, 205–220.

56. Navarro, N.P.; Figueroa, F.L.; Korbee, N.; Bonomi, J.; Álvarez Gómez, F.; de la Coba, F. Mycosporine-like amino acids form red algae to develop natural UV sunscreens. In *Sunscreens: Source, Formulation, Efficacy and Recommendations*; Ragesh, P.R., Ed.; Nova Science Publishers Inc.: Hauppauge, NY, USA, 2018; pp. 99–129. ISBN 978-1-53613-294-6.

57. Torres, P.; Pires Santos, J.; Chow, F.; dos Santos, D. A comprehensive review of traditional uses, bioactivity potential, and chemical diversity of the genus *Gracilaria* (Gracilariaceae, Rhodophyta). *Algal Res.* 2019, 37, 288–306. [CrossRef]

58. Takano, S.; Uemura, D.; Hirata, Y. Isolation and structure of a new amino acid, palythine, from the zoanthid *Palythoa tuberculosa*. *Tetrahedron Lett.* 1978, 26, 2299–2300. [CrossRef]

59. Tsujino, I.; Yabe, K.; Sekekawa, I. Isolation and structure of a new amino acid, shinorine, from the red alga *Chondrus strictus*. *Bot. Mar.* 1980, 23, 65–68.

60. Pliego-Cortés, H.; Bedoux, G.; Boulho, R.; Taupin, L.; Freile-pelegrín, Y.; Bourgougnon, N.; Robledo, D. Stress tolerance and photoadaptation to solar radiation in *Rhodymenia pseudopalmat a* (Rhodophyta) through mycosporine-like amino acids, phenolic compounds and pigments in an Integrated Multi-Trophic Aquaculture system. *Algal Res.* 2019, 41, 101542. [CrossRef]