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Short Communication

CE–MS fingerprinting of *Laurencia* complex algae (Rhodophyta)

The use of CE–ESI-MS has been considered as a new chemical strategy for the possible discernment of genera and species of the *Laurencia* complex. After the selection of the CE–MS and the extraction conditions, a total of 28 specimens of the complex, including different species of four genera (*Laurencia*, *Laurenciella*, *Palisada*, and *Osmundea*) collected from five intertidal locations on the Island of Tenerife (Canary Islands, Spain) were analyzed. CE–MS fingerprints revealed that CE–MS can be used as a useful tool for these studies in order to assess similarities and differences between them and that it constitutes an important starting point for further studies in the field.

Keywords: Algae / CE / *Laurencia* complex / MS / Rhodophyta
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1 Introduction

Red algae of the *Laurencia* complex are cosmopolitan species with a wide distribution around the world. Nowadays, six genera are recognized in the *Laurencia* complex: *Laurencia* J.V. Lamouroux, *Osmundea* Stackhouse, *Chondrophycus* (Tokida *et* Saito) Garbary *et* J.T. Harper, *Palisada* (Yamada) K.W. Nam, *Yuzurua* (K.W. Nam) Martin-Lescanne and *Laurenciella* Cassano, Gil-Rodríguez, Sentíes, Díaz-Larrea, M.C. Oliveira, and M.T. Fujii. The complex has been and is currently being studied in different areas of biological and biomedical sciences since it is of interest for the prospecting of bioactive substances due to their potential antibacterial, antifungal, anticholinesterasic, antileishmanial, cytotoxic, and antioxidant activities. From a chemical point of view, species of *Laurencia* and *Laurenciella* genera have been of special concern due to the diversity of secondary metabolites isolated from them [1, 2]. Most of these metabolites, according to their biogenic origin, are terpenes [3–5] and C_{15} acetogenins [6].

The identification of the members of the *Laurencia* complex based on anatomical and morphological characters is extremely difficult due to the high phenotypic plasticity of most species, and to the overlap of many of the morphological characters. In this context, molecular markers appear as a proficient complement to morphological features for species delimitation and to infer their phylogenetic relationships. In fact, several molecular markers have already proven to be useful in delimiting the taxa of this complex and in inferring their phylogenetic relationships [7–12]. At the same time, insight on the metabolite chemistry of different *Laurencia* complex species may prove to be a valuable chemotaxonomic tool at the generic or species level. To the best of our knowledge, most of these approaches have been carried out using different chromatographic techniques coupled to MS detectors [13–16], but they are still few and none of them have used CE.

On the other hand, CE has been demonstrated to be a powerful and complimentary technique for algae characterization. For instance, it has been employed for the determination of amino acids in *Sargassum fusiforme* [17], dimethylsulfoniopropionate in *Ulva pertusa* [18], polyphenols in *Fucus vesiculosus* [19] or positional isomers of sulfated oligosaccharide alditols in *Kappaphycus alvarezii*, *Gigartina skottsbergii*, *Gracilaria domingensis*, and *Acanthophora spicifera* [20]. Specifically, the coupling of CE with MS, which constitutes a powerful analytical technique, has been applied for algae characterization only in very few cases [21–25].

The main objective of this work is to explore the application of CE–MS to the characterization of algae from the *Laurencia* complex as well as to develop a preliminary study in which the chemical fingerprints of each species may help to reveal differences or similarities between them. To the best of our knowledge, this is the first time that the *Laurencia* complex has been studied using CE–MS and also the first data concerning its chemical characterization.

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Abbreviations: **ACN**, acetonitrile; **MeOH**, methanol

2 Materials and methods

2.1 Chemicals and samples

All chemicals were of analytical reagent grade and used as received. Acetic and formic acid were supplied by Riedel-de

Haën, Sigma–Aldrich Química (Madrid, Spain). Methanol (MeOH), 2-propanol, acetonitrile (ACN), and hydrochloric acid (HCl) were from Merck (Darmstadt, Germany). Sodium hydroxide (NaOH) was obtained from Panreac Química S.A. (Barcelona, Spain). Milli-Q water was obtained using a Milli-Q gradient system A10 (Millipore, Bedford, MA, USA).

Specimens of *Laurencia* algae were collected from five intertidal locations on the Island of Tenerife (Canary Islands, Spain), in the period from May to June 2011 (see Fig. S1 of the Supporting Information for sampling locations), as follows: (1) El Pris; (2) Puerto de la Cruz; (3) Punta del Hidalgo; (4) Playa de La Tejita; (5) Playa Paraíso. See Supporting Information Fig. S2 for photographs of each algae specimen collected. Tissues for chemical analysis were frozen with liquid nitrogen and then conserved at –80°C until their analysis.

2.2 CE–MS analysis

CE–MS analyses were performed in a P/ACE MDQ CE system (Beckman Instruments, Fullerton, CA, USA) coupled to an Amazon SL mass spectrometer from Bruker (Bremen, Germany) equipped with an ESI interface and an ion trap analyzer. Karat Software (Beckman Instruments) was used for CE instrument control, while the mass spectrometer was controlled by the Esquire NT software from Bruker.

Separation was carried out using bare fused-silica capillaries of 75 μm id, which are 110 cm total length to the MS detector. Before its first use, each capillary was consecutively rinsed with HCl 1 M for 3 min, Milli-Q water for 3 min, NaOH 0.1 M for 6.5 min, Milli-Q water for 3 min and finally the BGE (1 M acetic acid, previously filtered through 0.45 μm filters, Chromafil PET-45/25 from Macherey-Nagel, Düren, Germany, and degassed) for 5 min, applying in all cases a pressure of 20 psi. At the beginning of the day, the capillaries were conditioned with BGE for 5 min and between injections the capillary was rinsed for 3 min with the BGE. At the end of the day, it was rinsed with Milli-Q water for 5 min and its ends immersed in Milli-Q water until the next day. Hydrodynamic injection was carried out at 0.5 psi for 12 s and the electrophoretic separation was achieved using a voltage of +25 kV. The temperature of the capillary was kept constant at 25°C and the sample vial was maintained at 15°C.

To couple CE to MS, the capillary was fixed into a coaxial sheath-liquid interface so that its outlet protruded from the coaxial steel needle by 0.1 mm. Positive ESI conditions were as follows: capillary voltage of –4500 V, gas nebulizer (N_2) pressure of 6.0 psi; dry gas (nitrogen) flow of 8.0 L/min, and dry gas temperature of 200°C. The mass spectrometer operated in the positive ion mode and scanned between 100 and 700 m/z (target mass = 300 m/z, trap drive level 100%) and the ion trap parameters were selected with the ion charge control activated using a target of 100.000, a maximum accumulation time of 100 ms with 10 averages per experiment. A sheath liquid consisting of a mixture of 1:60:39 v/v/v formic acid/2-propanol/water was used to assist the ionization at a flow rate of 4 μL/min.

2.3 Sample extraction procedure

The algal thallus was introduced in a stainless-steel cup and was treated with liquid nitrogen (–176°C) and ground with a blender. Afterwards, 1 g of the homogenized powder was weighed in a 50 mL glass centrifuge tube and 20 mL of MeOH was added. The extraction was assisted by a T10 basic Ultra-Turrax laboratory disperser (Ika, Germany) at high speed for 10 min and then centrifuged at 4000 rpm (2.5 × *g*) for a further 10 min in a 5702 centrifuge from Eppendorf (Hamburg, Germany). The supernatant was filtered through a 0.45 μm Chromafil® Xtra PET-45/25 filter into an Erlenmeyer flask and evaporated to dryness at 40°C and 230 mbar using a R-200 rotavapor equipped with a V-800 vacuum controller and a V-500 vacuum pump, all of them from Büchi Labortechnik (Flawil, Switzerland). The obtained dry extract was reconstituted with 1 mL of the BGE (1 M acetic acid) and again filtered through a 0.20 μm Chromafil® Xtra PET-20/25 into a 1.5 mL tube. Then, 60 μL of this solution was introduced in a CE vial for its further hydrodinamic injection in the CE system as indicated in Section 2.2.

3 Results and discussion

3.1 Selection of extraction, separation, and detection conditions

In order to develop a simple but, at the same time, effective extraction procedure, liquid nitrogen was initially used to ultra-freeze the samples. The extreme low temperatures provided by liquid nitrogen (–176°C) helps to break the cells, to liberate their content and, as a result, to facilitate extraction [26,27]. After this prior treatment, samples could be well homogenized with a laboratory mixer.

With the aim of developing a suitable comparison among the different species of algae, efforts were made at the very beginning to obtain clean and repeatable extracts with localized peaks. For this reason, 1 g of the algae power previously obtained was extracted with 20 mL of different organic solvents, i.e. MeOH, ACN, and acetone. These solvents were selected in order to extract possible ionizable compounds that may be separated and detectable by CE. Assistance of the extraction was studied both by ultrasound and by agitation using an Ultra-Turrax homogenizer for 10 min. Afterwards, samples were centrifuged and the supernatant evaporated to dryness in a rotavapor and reconstituted in 1 mL of the BGE, which was later injected into the CE–MS system. For this purpose, and based on our own previous experience [28], a general BGE composed of 1 M acetic acid, which is also MS compatible, was used as well as general CE conditions (separation at +25 kV and 25°C). Among the extracts obtained with the selected solvents, methanolic ones were those that

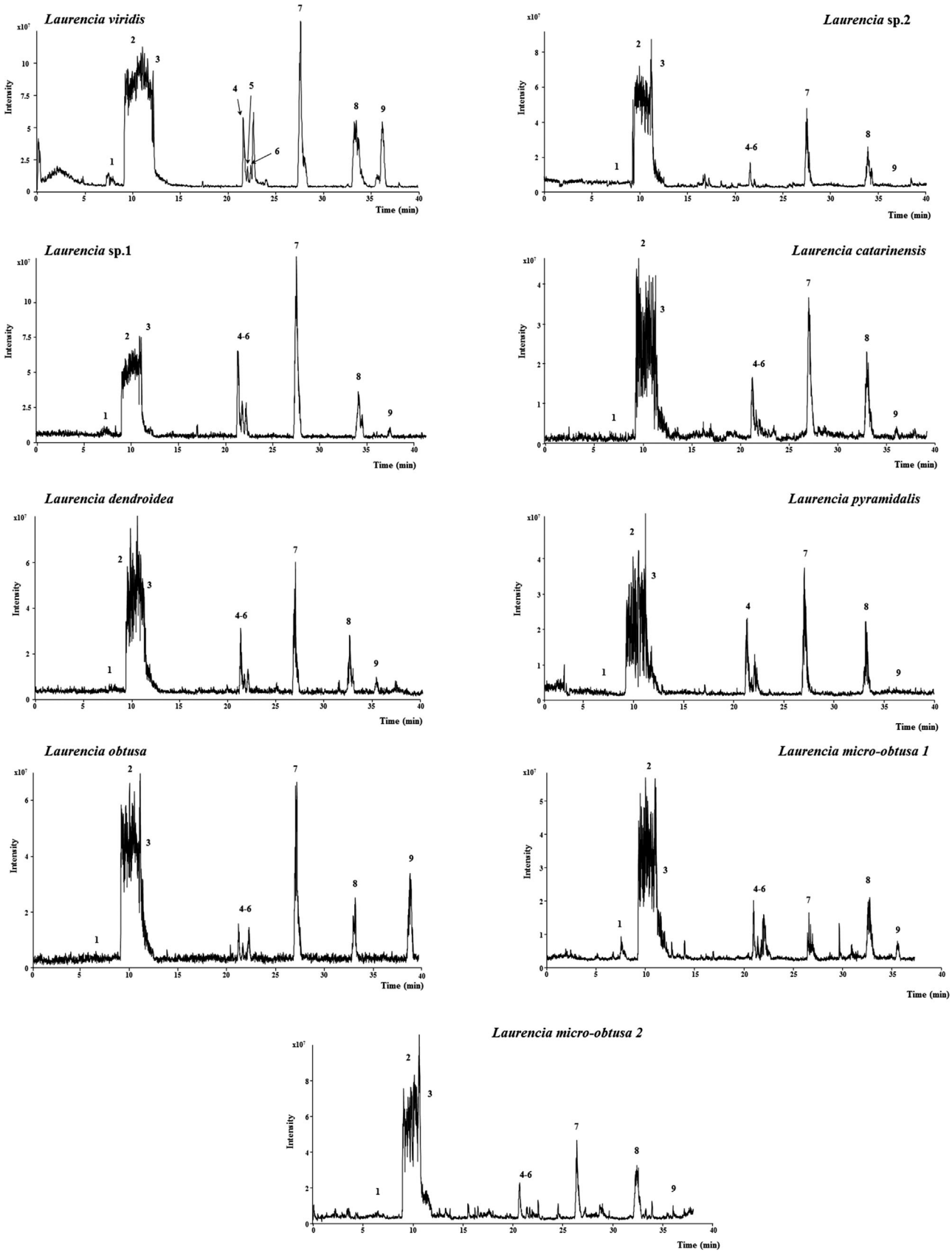

Figure 1. CE–MS base peak electropherograms of the extracts of the nine species of *Laurencia* selected for this study. The most intense peaks are numbered. For details about the extraction and CE–MS conditions see Section 2.

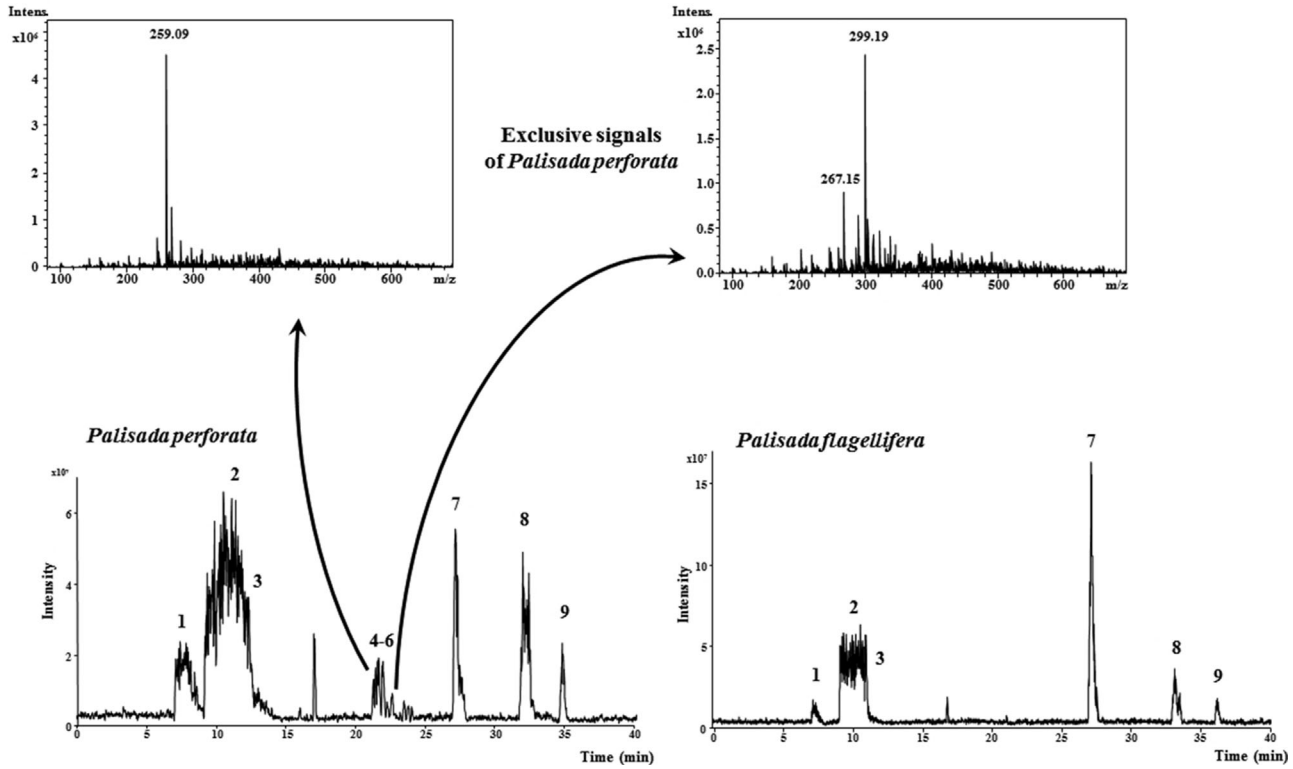

Figure 2. CE–MS base peak electropherograms of the extracts of the two species of the *Palisada* genus. MS spectra of two exclusive signals of *Palisada perforata* are also shown.

provided the highest extraction of compounds and therefore, MeOH was selected for subsequent analysis. As an example, Supporting Information Fig. S3 shows the base peak electropherograms of the different solvent extracts of *Laurenciella marilzae* (Gil-Rodríguez, Sentíes, Díaz-Larrea, Cassano, and M.T. Fujii). As can be seen, a clean profile with an analysis time of less than 40 min was observed for the methanolic extract.

Afterwards, the optimization of the parameters that affect MS ionization was undertaken. In this respect, the composition and flow rate of the sheath liquid was studied in order to effectively assist the ionization in positive mode (ESI+). For this purpose, direct infusion of the methanolic extract was developed. Mass spectra were acquired in the positive ion mode scanning from 100–700 m/z. Since mixtures of 2-propanol/water containing small percentages of formic or acetic acid are usually employed, different amounts of 2-propanol were assayed (20–80% v/v) as sheath liquid, containing acid in different proportions (0–3% v/v) flushing at diverse flow rates (2–10 μL/min). It was observed that 1% v/v of formic acid in 60:40 v/v 2-propanol/water at 4 μL/min provided the best results in terms of S/N for all the obtained signals and the more stable current. Furthermore, the rest of ESI parameters were also studied, e.g. temperature of the dry gas (100–300°C), dry gas flow rate (2–10 L/min), and nebulizer gas pressure (4–16 psi). The conditions that provided the highest signal intensities were 200°C, 8 L/min, and 6 psi, respectively.

3.2 Analysis of samples of the same species collected at different sampling points

CE–MS analysis of all algae revealed that there were no differences between the CE–MS fingerprinting profiles (not even between samples collected in the North and South of the island) and, as a result, apparently, and under the selected extraction, separation, and detection conditions, there does not exist an environmental factor that may suggest that the same algae collected in different parts of the island should be separately studied. As an example, Supporting Information Fig. S4 shows the base peak electropherogram obtained for *Laurenciella marilzae* collected at three different locations (Playa Paraíso, Punta del Hidalgo, and Playa de La Tejita). In all cases, CE–MS electropherograms, of which the spectra at each point were also carefully analyzed, were very similar, independent of the collection location.

3.3 Analysis of different specimens of each genus of the Laurencia complex

In order to establish conclusions about the differences or similarities between the different species of each genus, the most widely represented species in the Canary coast have been studied.

Regarding the analysis of *Laurencia* species, which were all analyzed in duplicate, Fig. 1 shows the base peak

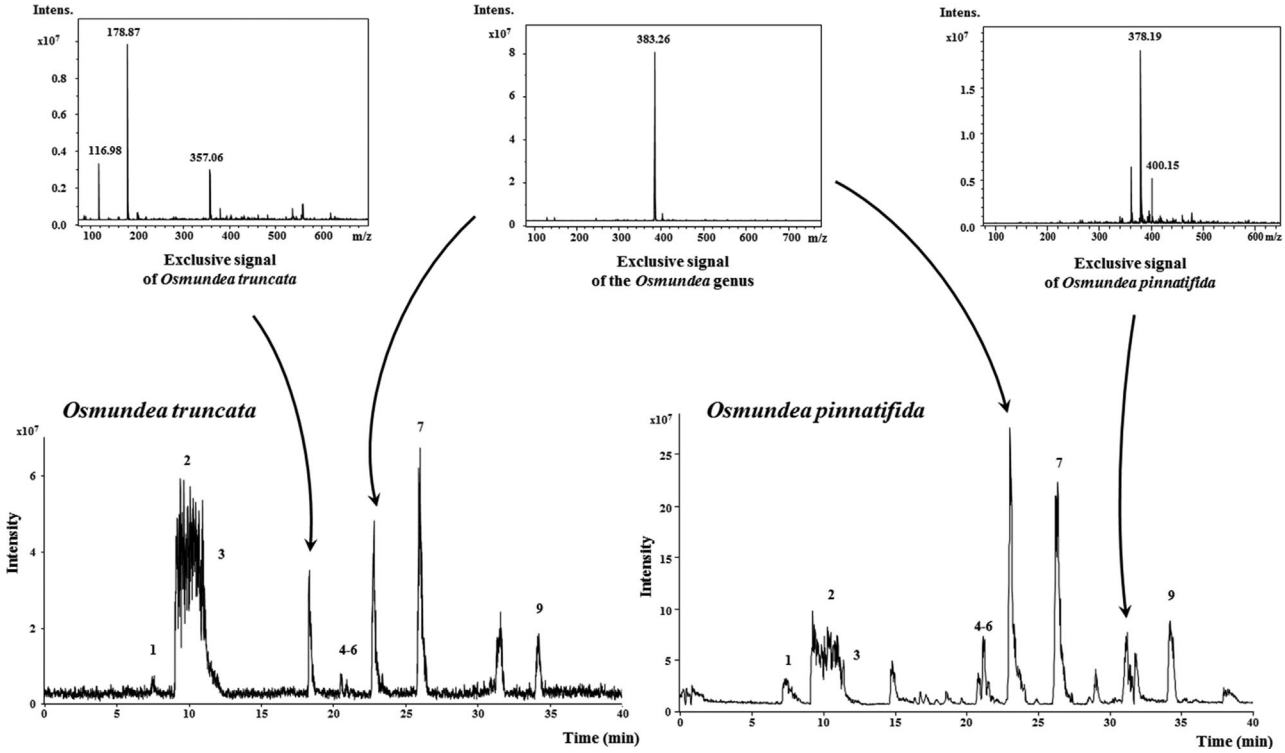

Figure 3. CE–MS base peak electropherograms of the extracts of the two species of the *Osmundea* genus. The MS spectrum of an exclusive signal of the genus is shown, as well as spectra of exclusive signals of both *Osmundea truncata* and *O. pinnatifida*.

electropherograms of the extracts of the nine selected species of the genus in which the most intense peaks have been numbered and indicated in order to clearly appreciate differences and similarities between them. Supporting Information Fig. S5 shows the MS spectra of the nine peaks. As can clearly be observed, they all show very similar CE–MS profiles. A deep analysis of the CE–MS spectra also revealed that they all had in common the same compounds, except *Laurencia pyramidalis* Bory de Saint Vincent ex Kützing that did not show peaks 5 and 6 corresponding to m/z ratios of 289.05 and 303.08.

Concerning *Laurenciella marilzae*, as can be seen in its base peak electropherogram (Supporting Information Fig. S6), it does not also show peak 6 (which seems to be a typical compound of most of the species of the *Laurencia* complex, as it will be later seen) but a new exclusive signal, which spectra has been properly indicated in the figure. Since *Laurenciella* is a monospecific genus, it can be concluded that the genus and the species can be characterized by this exclusive signal, since it is clear that it does not appear in the rest of the species of the *Laurencia* complex analyzed.

In the case of the *Palisada* genus [*Palisada perforata* (Bory de Saint-Vincent) K.W. Nam and *P. flagellifera* (J. Agardh) K.W. Nam], shown in Fig. 2, it is clear that they both show a characteristic CE–MS profile. *P. perforata* lacks a signal that appears in the rest of the studied algae (m/z 245.10), while *P. flagellifera* shows two particular and exclusive signals corresponding to m/z ratios of 259.09 and 299.19.

The two species of *Osmundea* [*Osmundea truncata* (Kützing) K.W. Nam and Maggs and *O. pinnatifida* (Hudson) Stackhouse] showed the most complex of the CE–MS profiles, in particular *O. pinnatifida*. This genus can be characterized by an exclusive signal (m/z 383.26) that does not appear in the rest of the genera, as well as by particular signals of each species, for example those corresponding to m/z 178.87 for *O. truncata* and 378.19 for *O. pinnatifida*, as the most intense peaks (see Fig. 3).

4 Concluding remarks

In this work, CE–MS is proposed for the first time for the characterization/differentiation of algae from the *Laurencia* complex. CE–MS profiles of the same algae collected in different points of the island of Tenerife revealed that, apparently, there were no differences between them. The analysis of *Osmundea pinnatifida* extracts revealed that this species has the most complex CE–MS profile. In general, several exclusive signals of each species or genera were identified. Although these results are of interest and promising, they still represent a starting point in the characterization of these species and a suitable identification of the compounds should be developed by means of complimentary analytical techniques or the use of a more appropriate MS analyzer.

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The authors have declared no conflict of interest.

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