Unveiling the Metabolomic, Phytochemical and Bioactive Profile of Twelve Macroalgae From the Adriatic Sea: A Comprehensive Analysis Using MSPD-UHPLC-QTOF

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Abstract

The present study provides an exhaustive exploration of twelve macroalgal species from the Adriatic Sea, including seven brown algae (Ericaria amentacea, Fucus virsoides, Cutleria multifida, Cystoseira compressa, Cystoseira corniculata, Gongolaria barbata and Padina pavonica), three green algae (Codium adhaerens, Codium vermilara and Ulva lactuca), and two red algae (Scinaia furcellata and Asparagopsis taxiformis). Matrix solid-phase dispersion (MSPD) was applied as the extraction technique, using solvents recognised as safe (GRAS). The bioactive profile of the extracts was assessed through the quantification of total phenolic content (TPC) and antioxidant activity. Among the three phyla, U. lactuca, F. virsoides and S. furcellata exhibited the highest TPC (0.8, 26 and 3.0 mg GAE·g DW^{-1}) and antioxidant activity (1.9, 38 and 7.5 mg TE·g DW^{-1}), respectively. Targeted HPLC-MS/MS analysis enabled the identification of nineteen phenolic compounds across all taxa. Chlorophyta showed a characteristic profile enriched in coumarins, benzaldehydes and flavanones, including the selective detection of 7-hydroxycoumarin in species with higher antioxidant potential. Additionally, compounds such as chlorogenic, rosmarinic and caffeic acids exhibited taxon-specific distributions that may explain differences in antioxidant activity. Complementary untargeted UHPLC-QToF metabolomics analysis provided broader coverage, revealing eighty metabolites spanning phenolics, sugars, organic acids, lipids, amino acids and their derivatives. Notably, the detection of FAHFA lipids represents the first report of these compounds in macroalgae, alongside a pronounced presence of sulphated phenolics. Overall, these findings provide a robust baseline on the bioactivity and chemical composition of Adriatic macroalgae, highlighting their value as a natural source of functional compounds.

INTRODUCTION

The Adriatic Sea, named by the ancient Greeks in reference to the city of Adria, is the northernmost basin of the Mediterranean Sea, a condition that significantly influences its physical characteristics, even in its southernmost regions (Gačić et al. 2001). Although it is part of the Mediterranean Sea, the Adriatic Sea is considered a separate biogeographical and ecological subunit, which is reflected in the composition and particular properties of its biological communities (Lipej and Dulčić 2004).

The Adriatic Sea is characterised by its shallow depth and a climate that combines hot, dry summers with mild, wet winters. This region has one of the highest levels of solar radiation in the Mediterranean area and is recognised as a critical point for climate threats (Tojčić et al. 2024). In addition, the Adriatic Sea exhibits a weak tidal regime and a marked salinity gradient associated with the exchange of saltier water masses with the Ionian Sea (Wang et al. 2007). These environmental conditions place considerable stress on benthic organisms, particularly macroalgae inhabiting intertidal zones. These algae face partial desiccation and greater solar radiation compared to those that remain constantly submerged.

A significant relationship has been established between abiotic stress and the accumulation of bioactive compounds in seaweed (Lomartire et al. 2021). Adverse factors in aquatic ecosystems induce the

release of phytochemicals by these macroorganisms, acting as defence and adaptation mechanisms. In turn, there is a known positive correlation between increased solar radiation and increased concentrations of bioactive compounds in macroalgae, particularly those of a phenolic nature (Polo and Chow 2020). Hence the growing interest, over the last decade, in extracting bioactive compounds from species characteristic and endemic to the Adriatic**.**

Various extraction techniques have been used to obtain these phytochemicals from macroalgae. However, conventional methods often involve long extraction times and the use of high temperatures, which can negatively affect the stability and yield of bioactive compounds (Santos et al. 2019). This limitation has driven a growing trend towards the development of innovative and sustainable extraction technologies aimed at obtaining extracts of high biological value (Mekinić et al. 2021). One of the most recent applications in this field is solid-phase matrix dispersion (MSPD), a technique that has proven effective in extracting bioactive compounds from different species of macroalgae in the Adriatic Sea (Castillo et al. 2023). The results obtained are comparable to those achieved by ultrasound-assisted extraction, with the added advantage of requiring lower volumes of solvent, and operating at room temperature and atmospheric pressure, making it a more sustainable alternative.

Despite the development of more efficient extraction techniques, the analytical characterisation of bioactive compounds in macroalgae is mostly limited to the determination of global spectrophotometric parameters. In the case of total phenolic content, the most widely used methodology, the Folin-Ciocalteu method, presents high interference with compounds such as carotenoids and chlorophylls in macroalgae, which makes it difficult to accurately interpret the results (Andriopoulos and Kornaros 2024). Similarly, antioxidant activity has been evaluated using methods that show clear interference when applied to the phytochemicals of these species, as well as significant differences in units or modes of expression, which complicates comparison between studies and the establishment of a consensus (Castillo et al. 2023). On the other hand, studies addressing the individual identification of phenolic compounds tend to focus on previously described or target analytes, such as florotannins, bromophenols, and specific flavonoids (Steevensz et al. 2012; Hofer et al. 2019).

In this context, new strategies have been developed that go beyond traditional spectrophotometric analyses through the application of metabolomic approaches based on high-resolution techniques designed to detect and identify new metabolites present in macroalgae (Ebrahimi et al. 2024). However, for many resilient algae species from the Adriatic Sea, the available information on their chemical composition remains scarce, especially regarding their polyphenolic content (Jerković et al. 2018).

In this context, the present study provides a comprehensive assessment of the bioactive composition of twelve macroalgal species from the Adriatic Sea, conducted within the framework of the BioProCro marine bioprospecting project. For the first time, the MSPD extraction technique was applied to this group using GRAS solvents, covering seven brown algae (*E. amentacea, F. virsoides, C. multifida, C. compressa, C. corniculata, G. barbata* and *P. pavonica*), three green algae (*C. adhaerens, C. vermilara* and *U. lactuca*) and two red algae (*A. taxiformis* and *S. furcellata*).

The global bioactive potential of the extracts was evaluated through TPC, antioxidant capacity and mean inhibitory index (IC_{50}). Phenolic constituents were further characterised using a targeted LC–MS/MS strategy. Additionally, an untargeted UHPLC–QTOF metabolomic workflow enabled the annotation of eighty metabolites, offering deeper insight into the chemical diversity and interspecific relationships within this macroalgal assemblage.

MATERIALS AND METHODS

Seaweed collection and sample handling

Ericaria amentacea (C. Agardh) Molinari & Guiry, 2020 (ex *Cystoseira amentacea*) was collected in April 2021 at Dugi Otok, Adriatic Sea, Croatia (44°03'16" N; 14°59'14" E) from a depth of 0.5 m. *Fucus virsoides* was collected in March 2022 on the southwestern coast of the Novigrad Sea, Croatia (44°12'02" N; 15°28'51" E) at 0.5 m depth. *Culteria multifida* was sampled in March 2022 in Bregdetti Bay, Zadar, Croatia (44°05'51" N; 15°15'00" E) from 2 m depth. *Cystoseira compressa* was collected in March 2021 at Zadar Kolovare II, Adriatic Sea, Croatia (44°07'00" N; 15°14'00" E) from 0.5 m depth, while *Cystoseira corniculata* was gathered in April 2021 at Dugi Otok, Adriatic Sea, Croatia (44°03'16" N; 14°59'14" E) from 0.5 m depth. *Gongolaria barbata* (Stackhouse) Kuntze (ex *Cystoseira barbata*) was collected in May 2021 near Zadar, Adriatic Sea, Croatia (44°12'42" N; 15°09'23" E) at depths of 4–6 m. *Padina pavonica* (Linnaeus) Thivy, 1960 was sampled in July 2020 at Zadar Kolovare II, Adriatic Sea, Croatia (44°07'00" N; 15°14'00" E) from 1 m depth. *Codium adhaerens* was collected in March 2021 at Zadar Punta Bajlo, Croatia (44°05'50" N; 15°14'43" E) from 4 m depth, whereas *Codium vermilara* was gathered in July 2021 at Firule Bay, Split, Adriatic Sea, Croatia (43°29'58" N; 16°27'10" E) from 2 m depth. *Ulva lactuca* was collected in May 2020 at Zadar Kolovare II, Adriatic Sea, Croatia (44°07'00" N; 15°14'00" E) from 1 m depth. *Asparagopsis taxiformis* and *Scinaia furcellata* was sampled in July 2020 in Split, Adriatic Sea, Croatia (43°30'28" N; 16°23'21" E) at depths of 1–2 m.

Fresh macroalgal material was washed separately five times with seawater and twice with deionized water. Samples were cut into small pieces, frozen at −60 °C for 24 h in an ultra-low-temperature freezer and subsequently freeze-dried using a laboratory freeze dryer (CoolSafe PRO, Labogene, Denmark). Lyophilization was performed for 24 h under high vacuum (0.13–0.55 hPa), with primary and secondary drying temperatures of −30 °C and 20 °C, respectively. The resulting freeze-dried material was used for extraction.

Chemicals and reagents

For 60 target polyphenols, their CAS numbers, molecular mass, retention time and MS/MS transitions are shown in Supplementary Table S1. Water and methanol (MS grade), and ethanol were supplied by Scharlab (Barcelona, Spain). The Folin-Ciocalteu's phenol reagent (2M), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox®), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), formic acid, sodium carbonate and sand (200–300 µm mesh) were supplied by Sigma-Aldrich (Darmstadt, Germany).

MSPD

1 g of freeze-dried macroalgal material was gently homogenised with 4 g of SiO_2 dispersing sorbent in a porcelain mortar using a pestle until a uniform mixture was obtained. The blend was then transferred into a polypropylene cartridge fitted with a PTFE–cellulose frit at the bottom and layered with an additional 1 g of SiO_2 to enhance fractionation and sample clean-up. To prevent the formation of preferential solvent channels, an extra layer of SiO_2 was added on top to function as a flow distributor. Extraction was performed by gravity using ethanol/water (50:50, v/v), controlling the elution rate with a regulating valve, and collecting 5 mL of extract in a volumetric flask. The final extracts were stored in amber vials at −20 °C for subsequent analyses.

Total polyphenolic content (TPC)

TPC was determined using the Folin–Ciocalteu (FC) colorimetric method following a modified version of Zhang's microtitre protocol for 96-well plates (Zhang et al. 2006). Briefly, 20 µL of each diluted extract was mixed with 100 µL of FC reagent (1:10, v/v) and 80 µL of sodium carbonate solution (7.5 $g \cdot L^{-1}$). The mixture was shaken and incubated in the dark for 30 min. The absorbance was then measured at 760 nm using a microplate reader (BMG LABTECH, Ortenberg, Germany). TPC was quantified using a gallic acid calibration curve covering 20–160 $mg \cdot L^{-1}$ (absorbance range: 0.200–0.800). Results were expressed as milligrams of gallic acid equivalents per litre of extract (mg $GAE \cdot L^{-1}$) and per gram of sample (dry weight) (mg $GAE \cdot g^{-1}$).

Antioxidant activity (AA)

AA was determined using the 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) assay following the method described by (Gonzalez-Iglesias et al. 2025) with minor modifications. Briefly, a 7 mM ABTS stock solution was prepared in water and reacted in equal volumes with a 2.45 mM potassium persulfate solution. The mixture was kept in the dark at 25 °C for 16 h. The resulting solution was then diluted with water to obtain an absorbance of 0.700 (±0.004) at 748 nm. By performing successive dilutions, 50 µL of each extract were transferred into a 96-well plate and mixed with 200 µL of the working ABTS solution. The mixture was incubated in the dark for 7 min, after which absorbance was measured at 748 nm. All analyses were performed in triplicate. For quantification, an AA calibration curve was prepared using Trolox, covering a concentration range of 3–31 $mg \cdot L^{-1}$ (absorbance: 0.200–0.800). AA was expressed as milligram of Trolox equivalents per litre of extract (mg$TE \cdot L^{-1}$) and per gram of dry algae (mg$TE \cdot g^{-1}$). The IC_{50} of the samples was also calculated, expressed as the milligrams of dry algae per litre of extract ($mg \cdot L^{-1}$) required to inhibit 50% of the $ABTS^{+}$ radicals.

Liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis

For the quantification of targeted polyphenols in the macroalgal extracts, analyses were carried out by LC–MS/MS using a Thermo Scientific system (San José, CA, USA) consisting of a TSQ Quantum Ultra™ triple-quadrupole mass spectrometer equipped with a HESI-II (heated electrospray ionization) source,

coupled to an Accela Open autosampler fitted with a 20 µL injection loop. Chromatographic separation was achieved on a Kinetex C18 column (100 × 2.1 mm, 2.6 µm) protected with a SecurityGuard™ ULTRA pre-column (Phenomenex, Torrance, CA, USA). The column was maintained at 50 °C, and 10 µL of extract were injected for each run. The mobile phase consisted of water (A) and methanol (B), both acidified with 0.1% formic acid. The gradient began at 5% B (kept for 5 min), increased linearly to 90% B over 11 min (held for 3 min), and then returned to initial conditions within 5 min. The flow rate was maintained at 200 $\mu L \cdot min^{-1}$, resulting in a total run time of 20 min per sample. The mass spectrometer operated in both positive and negative ionization modes, and data acquisition was performed in Selected Reaction Monitoring (SRM), monitoring two to three transitions per analyte for robust identification and quantification (Table A.1). Instrument control and data processing were carried out using Xcalibur 2.2 and TraceFinder™ 3.2 software.

UHPLC-QTOF Analysis

The macroalgal extracts were filtered through 0.22 µm PTFE syringe filters prior to analysis. Untargeted metabolomic profiling was performed using an ultra-high-performance liquid chromatography system (Elute UHPLC 1300) coupled to a compact quadrupole time-of-flight (QTOF) mass spectrometer (Bruker Daltonics, Fremont, CA, USA). Chromatographic separation was achieved on a Bruker Intensity Solo 2 C18 column (100 × 2.1 mm, 2.0 µm) maintained at 40 °C. The mobile phases consisted of water with 0.1% formic acid (A) and methanol with 0.1% formic acid (B). Prior to analysis, mass accuracy was calibrated using a sodium hydroxide solution (1×10^{-3} M) prepared in an isovolumetric mixture of 2-propanol and water containing 0.2% formic acid. The chromatographic gradient began at 95:5 (A/B) for 0.4 min, followed by a stepwise decrease of phase A to 70% at 4.5 min, 63% at 8 min, 50% at 9 min, and 10% at 11 min, which was maintained for 4 min. Initial conditions were restored at 16 min and held for an additional 4 min, resulting in a total run time of 20 min. Mass spectrometric acquisition was performed in AutoMS/MS mode under negative electrospray ionization (ESI), primarily detecting $[M-H]^-$ ions. Data were collected at 8 Hz with 1 s cycles, applying collision energies between 10 and 105 eV and scanning ions in the m/z 20–1000 range. Instrument control and data acquisition were performed using Compass HyStar 5.0 (Bruker, USA). Each macroalgal species was extracted in triplicate, and every extract was injected three times into the UHPLC-QTOF system, resulting in nine analytical injections per species. This design ensured high reproducibility and robustness of the untargeted metabolomic dataset.

Data acquisition and initial processing were performed using Compass HyStar together with Bruker Compass DataAnalysis (Version 6.1, Build 331.2.0). The untargeted annotation workflow was subsequently carried out in Bruker Compass MetaboScape 2024b (Version 13.0.3, Build 13497), which includes updated algorithms for feature detection, isotopic pattern extraction and component grouping. Compound identities were proposed using the SmartFormula module, integrating signal intensity, isotopic pattern fit and high-resolution mass accuracy. For molecular formula generation, a maximum mass deviation of 5 mDa and an mSigma threshold < 50 were applied. Putative identifications were further evaluated via comparison with major online chemical repositories, including the National Center for Biotechnology Information (NCBI) (National Center for Biotechnology Information 2024) and

Chemical Entities of Biological Interest (ChEBI) (Hastings et al. 2015) databases. Fragment-ion interpretation was complemented with *in silico* fragmentation tools such as Compound Crawler and MetFrag. In addition, experimental MS/MS spectra were matched against reference spectral libraries, including MassBank of North America (MoNA) (MassBank of North America 2026) and the MassBank European Database (Horai et al. 2010).

Statistical Analysis

Using MetaboScape® 2024b (version 13.0.3, build 13497) and its T-REX 3D feature-extraction algorithm, the raw UHPLC-QToF-MS/MS data (Supplementary File S1), comprising more than 2500 detections, were processed and organised according to the predefined experimental groups. For subsequent statistical exploration, all pre-processed datasets were exported to the MetaboAnalyst 6.0 platform (Pang et al. 2024). The analytical settings applied in this environment are described in Supplementary Section S1.

Data visualisation included principal component analysis (PCA), overlapping response area plots, the supervised Partial Least Squares Discriminant Analysis (PLS-DA) model, and Variable Importance in Projection (VIP) values, represented as a heatmap of key discriminant metabolites. To validate the robustness of the multivariate statistical models and to exclude potential random separation ($p < 0.05$), permutation tests with 2000 iterations were performed. The set of identified phytochemicals met all established identification criteria ($\Delta m/z < 5$ mDa and mSigma < 50).

RESULTS

Bioactive Analysis

The total phenolic content, determined using the FC method, is the most used parameter in the analysis of bioactive content from macroalgae (Torres et al. 2024). However, in many cases it is interpreted more as an indicator of the overall content of bioactive compounds with reducing capacity than as an absolute measure of phenolic content, given the possible interference of pigments such as chlorophylls and carotenoids in the reaction (Andriopoulos and Kornaros 2024). Similarly, the evaluation of antioxidant activity using the DPPH assay, the most widely reported in macroalgae, can be affected by pigments such as phycoerythrin, chlorophyll and carotenoids, whose absorption at around 515 nm interferes with the measurement (Castillo et al. 2022). Furthermore, the lack of standardisation in the presentation of results, expressed as dry extract, wet extract or fresh seaweed, and the frequent absence of precise numerical values, replaced by graphical representations, make it difficult to compare studies (Castillo et al. 2023).

In this context, the objective of this section is to report the quantified bioactivities of macroalgae that have been limited in scope or insufficiently described, in order to generate a reference database for these species. The results are presented mainly in relation to dry weight, which allows for direct comparison between species, as well as in terms of extract volume. This approach seeks to integrate the different criteria and units of measurement reported in the literature, with the aim of facilitating more consistent

comparisons and a more accurate assessment of their bioactive potential. Accordingly, Table 1 presents a comparative analysis of the twelve macroalgal species examined, highlighting their TPC, antioxidant capacity, and IC_{50}. In addition, the activities of the extracts expressed per extract volume are shown in Supplementary Fig. S1.

Target Analysis

In order to obtain a more detailed chemical profile of the reported bioactive content, a targeted analysis of phenolic compounds in the twelve species of macroalgae was performed using high-performance liquid chromatography coupled with triple quadrupole mass spectrometry (HPLC–MS/MS). This approach allowed the detection and identification of a total of 19 phenolic compounds distributed among the different species analysed (Fig. 1).

Table 1 Comparative evaluation of total phenolic content (TPC), antioxidant capacity (ABTS assay), and inhibitory concentration (IC_{50}) in macroalgae extracts obtained by MSPD with ethanol/water (50:50, v/v).

Family	Macroalga	TPC (FC)		AA (ABTS)		IC50	
		mgGAE/g	ΔmgGAE·g^{-1}	mgTRE·g^{-1}	ΔmgTRE·g^{-1}	g·L^{-1}	Δg·L^{-1}
Chlorophyta	*C. vermilara*	0.65	0.069	0.08	0.011	N/D	N/D
	C. adhaerens	0.59	0.060	1.41	0.094	4.9	0.38
	U. Lactuca	0.8	0.12	1.9	0.12	2.7	0.24
Phaeophyceae	*G.a barbata*	3.2	0.42	6.7	0.18	0.76	0.026
	E. amentacea	1.3	0.19	3.5	0.76	1.6	0.43
	C. corniculata	0.38	0.074	0.6	0.14	9	1.9
	C. multifida	0.57	0.069	0.72	0.079	8	1.0
	C. compressa	1.5	0.33	3.8	0.51	1.6	0.45
	P. pavonica	0.148	0.0066	0.42	0.019	12.8	0.66
	F. virsoides	26	2.8	38	3.8	0.14	0.021
Rhodophyta	*S. furcellata*	3.0	0.20	7.5	0.34	0.73	0.046
	A. taxiformis	0.22	0.014	0.22	0.049	23	3.0

The metabolites identified belong mainly to the families of coumarins, flavonoids, hydroxycinnamic acids and benzaldehyde derivatives, showing characteristic profiles according to the taxonomic group. In particular, brown algae presented the greatest diversity of phenolic compounds, while green algae exhibited a profile dominated by 7-hydroxycoumarin and naringenin.

In-Depth Metabolomic Profiling of Twelve Macroalgal Species from the Adriatic Sea

To thoroughly examine the bioactive profile of twelve species of macroalgae from the Adriatic Sea, a metabolomic analysis was performed using ultra-high-performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry (UHPLC-QTOF). The annotation of the eighty analytes listed in Table 2 was performed by considering both the fragmentation spectra in contrast to the spectral

databases cited and the exact mass values, following a previously described algorithm based on a mass deviation >5 ppm and an isotopic pattern >50 mSigma (Castillo et al. 2020).

Fig. 2A shows an unsupervised principal component analysis (PCA) performed on the metabolomic data acquired by UHPLC-QTOF for the twelve species. This analysis aimed to identify global patterns of variation in the detected metabolites and to assess potential natural clustering among the samples. The model enabled a clear differentiation among species, with well-defined groups in the plane formed by the first two principal components (PC1 and PC2), which explained the largest proportion of total variance. The overlaid area-intensity plot further supports this intragroup separation, revealing distinctly delineated regions.

Complementarily, a supervised PLS-DA analysis is presented (Fig. 2B), accompanied by a VIP score plot highlighting the 30 analytes (Fig. 3C) with the greatest discriminant power.

The most distinct groups corresponded to *F. virsoides* and *U. lactuca*. In *F. virsoides*, this separation aligns with the high bioactivity observed in its antioxidant profile and TPC, as well as with the presence of characteristic phenolic compounds. In *U. lactuca*, the separation relative to other green algae was less pronounced, positioning it in a PC1–PC2 space adjacent to the rest of the green species.

DISCUSSION

Bioactive Properties of the Studied Macroalgae

To contextualise the quantitative patterns shown in Table 1, the following sections examine the bioactive profiles of the studied macroalgae by major taxonomic group. This organisation allows the characteristic responses of green, brown and red species to be evaluated in a comparative framework and highlights group-specific trends in phenolic content and antioxidant capacity.

Green Algae

Among green macroalgae, the total bioactive content followed the order *U. Lactuca* > *C. adherens* > *C. vermilara*. *U. lactuca* is the most extensively studied green alga among the species analysed in this work. However, as with most macroalgae, its bioactivities are commonly expressed per gram of dry extract rather than per algal dry weight, which limits direct comparison due to the lack of information on extraction yields (García et al. 2020; Pangestuti et al. 2021). Reported TPC values range from 0.954 (Pappou et al. 2022) to 2 mgGAE·g^{-1} (García et al. 2020), with the present study yielding 0.8 mgGAE·g^{-1}, the highest value among the analysed green species. The comparison of antioxidant activity is also challenging, as the value obtained here (1.9 mg TE·g^{-1}, IC_{50}= 2.7 g·L^{-1}) is expressed relative to algal biomass, while most literature reports results based on extract dry weight.

C. adherens and *C. vermilara* pose even greater challenges for comparison, as their bioactivities have been only rarely investigated and are consistently expressed relative to extract mass rather than the original algal material (Augusto et al. 2016; Pinteus et al. 2017; Radman et al. 2021). Interestingly, *C.*

adherens exhibited a lower phenolic content (0.59 mg GAE·g⁻¹) than *C. vermilara* (0.65 mg GAE·g⁻¹); however, *C. vermilara* displayed markedly low antioxidant capacity compared with all other analysed macroalgae, failing to reach a measurable half-inhibitory concentration. In contrast, *C. adherens* showed a moderate antioxidant activity of 1.41 mgTE·g⁻¹ with an IC_{50} of 4.9 g·L⁻¹, whereas *C. vermilara* presented an activity of only 0.08 mgTE·g⁻¹ and no detectable IC_{50} value.

Brown Algae

Among brown macroalgae, species belonging to the *Sargassaceae* family showed the following descending order in total bioactive content: *G. barbata* > *C. compressa* > *E. amentacea* > *C. corniculata*. *G. barbata* is one of the most extensively studied macroalgae within the *Sargassaceae* family; however, data on its TPC remain limited. Reported concentrations range from 2.08 (Ak et al. 2022) to 4.95 (Pica et al. 2024) mgGAE·g⁻¹, with an average value of approximately 3.35 mgGAE·g⁻¹ (Cadar et al.; Ak et al. 2022; Pica et al. 2024). The value obtained in the present study (3.2 mgGAE·g⁻¹) falls within this range. Regarding its antioxidant activity, determined by the ABTS assay, a capacity of 6.7 mgTE·g⁻¹ was observed, comparable to that reported by (Castillo et al. 2023) (4.43 mgTE·g⁻¹) when using ultrasound and an ethanol/water solvent mixture. Other studies have reported lower values, ranging from 1.31 to 2.30 mgTE·g⁻¹, when ethanol or acetone were used as solvents under ultrasonic conditions (Manev and Petkova 2021).

In the case of *C. compressa*, reported values of total TPC range from 0.161 mgGAE·g⁻¹ (Güner et al. 2015) (using methanol and UAE) to 2.6 mgGAE·g⁻¹ [19] (following extraction by freezing at −80 °C, grinding, simple dilution in methanol and dichloromethane, evaporation at 40 °C, and redilution in DMSO) (De La Fuente et al. 2022). In our study, the obtained value of 1.5 mgGAE·g⁻¹ was comparable to that reported by (Güner et al. 2015), who used ultrasound-assisted extraction with a solvent mixture of hexane, chloroform, and methanol, yielding a total content of 1.541 mg GAE·g⁻¹.

Among the few studies reporting the antioxidant activity of *C. compressa* expressed per gram of dry algae, ABTS values of 9.76 mg TE·g⁻¹ have been described for purified fucoidan from *C. compressa*, with an IC_{50} of 478.6 mg·L⁻¹ (Rashed et al. 2021). For the DPPH assay, IC_{50} values of 12 mg·L⁻¹ and activities expressed as 7.5 mg TE·g⁻¹ (Mhadhebi et al. 2014) and up to 24.86 mg TE·g⁻¹ (Bouafif et al. 2019) have been reported. In our study, a lower activity of 3.8 mg TE·g⁻¹ was obtained; however, this value maintains the proportional relationship observed by (Rashed et al. 2021), representing approximately one third of the reported activity and requiring nearly three times the concentration (1.6 g·L⁻¹) to achieve 50% inhibition.

Regarding *E. amentacea*, a wide variability in TPC has been reported, with values ranging from 0.65 to 279 mgGAE·g⁻¹ (Alghazeer et al. 2022; Radman et al. 2022b). In the present study, the obtained value (1.3 mgGAE·g⁻¹) falls within this broad range. *E. amentacea* exhibited an antioxidant activity like that of *C. compressa*, with 3.52mgTE·g⁻¹ and an IC_{50} of 1.6 g·L⁻¹, consistent with the values reported in previous studies (Castillo et al. 2023).

E. corniculata, this species remains poorly studied. Under its current taxonomic designation, no specific studies have been identified in the databases consulted. Approximately a dozen works refer to its former classification (*Cystoseira corniculata*), mainly focusing on the characterisation of its volatile compounds and fatty acid profile (Polat and Ozogul 2009; Radman and Jerković 2022). In the present study, *E. corniculata* exhibited a TPC of 0.38 mg GAE·g^{-1} and an antioxidant activity of 0.60 mg TE·g^{-1}, with an IC_{50} value of 9 g·L^{-1}.

Within the *Fucaceae* family, *F. virsoides*, an endemic species of the Adriatic Sea, exhibited the highest TPC among all analysed samples, accounting for approximately 3% of its dry weight in polyphenols. The species recorded a TPC of 26 mgGAE·g^{-1} and an antioxidant activity of 38 mgTE·g^{-1}, with an IC_{50} value of 0.14 g·L^{-1}. Previous research on *F. virsoides* has primarily focused on fucoxanthin extraction (Cikoš et al. 2023), whereas its overall bioactive profile remains poorly characterised. Reported TPC values in the literature range between 2 and 60 mgGAE·g^{-1}, with DPPH IC_{50} values varying widely from 2 to 90 g·L^{-1}, depending on the extraction method and solvent system used (e.g., methanol, petroleum ether, ethyl acetate, butanol) (Grozdanić et al. 2019). Fractionation studies targeting extracts of different polarities have yielded up to 75.47 mgGAE·g^{-1} in specific fractions, complicating comparisons normalised to algal dry weight (Jerković et al. 2021). In addition, non-phenolic compounds have been reported to interfere with the FC response, and variations in environmental and geographical conditions may further influence the chemical composition of this species.

When examining the *Dictyotaceae* family, *P. pavonica* exhibited the lowest bioactive content among the analysed species, with a TPC of 0.148 mgGAE·g^{-1} and an antioxidant activity of 0.42 mgTE·g^{-1}, corresponding to an IC_{50} value of 12.8 g·L^{-1}. The available literature on *P. pavonica* remains scarce regarding its overall bioactivity, as most studies express activity relative to extract yield rather than algal dry weight, hindering direct comparison (El Shafay et al. 2022; Čagalj et al. 2022). One study reported a content of 6.42 mg phloroglucinol equivalents per gram of dry alga. A similar situation occurs with antioxidant assays, where reported values are often expressed based on extract concentration rather than algal biomass.

Turning to the *Cutleriaceae* family, *C. multifida* has been very little investigated, and no detailed bioactive profile has been previously described. In the present analysis, this species exhibited a TPC of 0.57 mgGAE·g^{-1} and an antioxidant activity of 0.72 mgTE·g^{-1}, with an IC_{50} value of 8 g·L^{-1}.

Red algae

Within the set of red macroalgae analysed, *S. furcellata* exhibited notably high bioactivity, ranking as the second most active species among the twelve evaluated. However, *S. furcellata* remains a poorly studied macroalga whose metabolomic profile is largely unexplored, which limits the ability to establish robust comparisons with previous reports. In this study, the species displayed a TPC of 3.0 mg GAE·g^{-1} DW and an antioxidant activity of 7.5 mg TE·g^{-1} DW (IC_{50} = 0.73 g·L^{-1}). Although these values are high relative to

the other species assessed, their comparative interpretation is constrained by the scarcity of existing data for this macroalga.

In contrast, *A. taxiformis* has been more extensively characterised in the literature. A study employing deep eutectic solvents coupled with ultrasound-assisted extraction reported maximum TPC ranging from 0.19 to 0.57 mg GAE·g⁻¹ DW (Gao et al. 2022). This range is consistent with the values obtained in the present study using MSPD, which yielded a TPC of 0.22 mg GAE·g⁻¹ DW. These relatively low concentrations are reflected in its antioxidant performance, with an ABTS activity of 0.22 mg TE·g⁻¹ DW and an IC_{50} of 23 mg·L⁻¹, confirming the limited reducing capacity previously described for this species.

Targeted Analysis of Phenolic Compounds

In this study, the identification of 7-hydroxycoumarin in green macroalgae is particularly noteworthy, as this compound appears to occur almost exclusively within this group. It was detected in *U. lactuca* and *C. adherens*, but not in *C. vermilara*, which also exhibited one of the lowest antioxidant activities among the analysed species. This pattern suggests that the higher antioxidant capacity observed in *U. lactuca* and *C. adherens* may be partly influenced by the presence of this compound.

Previous studies have reported coumarin derivatives and their sulphated or esterified forms such as trihydroxycoumarin, 7-hydroxycoumarin-3,6-disulphate, dihydroxycoumarin sulphate, methoxylated dihydroxycoumarin sulphate, and pyrocoumarins in green macroalgae (*Dasycladus vermicularis* (Hartmann et al. 2018; Radman et al. 2022a); *Dasycladales siphonous* (Kurth et al. 2015)) as well as in brown algae (*Dictyota dichotoma* (Dixit et al. 2020); *Padina tetrastromatica* (Maheswari et al. 2018). The predominance of coumarins in green algae has been linked to their photoprotective function, since the conjugated phenolic structure of these compounds efficiently absorbs ultraviolet radiation (Pérez-Rodríguez et al. 2003).

Another relevant compound detected in all green macroalgae analysed was naringenin, a flavonoid belonging to the flavanone subclass, with multiple beneficial biological effects on human health (Shin and Shin 2024). Naringenin has been previously reported in *U. lactuca* (Korkmaz 2025), but there are no previous records of its occurrence in *C. adherens* or *C. vermilara*. Within the genus *Codium*, it has only been described in *C. fragile* (Kim et al. 2023).

Regarding hydroxylated and methoxylated benzaldehydes (4-hydroxybenzaldehyde, 3-hydroxybenzaldehyde, 4-methoxybenzaldehyde, and 3,5-dimethoxybenzaldehyde), these compounds were also detected in this study, showing moderate abundance in green algae, stronger responses in brown species, and lower presence in red algae. These aromatic aldehydes have been previously reported in several Adriatic algae (Castillo et al. 2023) and are associated for a broad spectrum of bioactivities, including antioxidant, antibacterial, anticancer, anti-inflammatory, and immunomodulatory effects (Kang et al. 2022) . They have been detected in numerous marine macroalgae (Gunathilake et al. 2022; Lee et al. 2024) and are recognised as direct precursors of bromophenols, a group of secondary metabolites with a wide range of biologically beneficial activities (Liu et al. 2011).

Within the flavonoid group, catechin and myricetin were also identified in this study, the former in *U. lactuca* and the latter in *C. vermilara*. Both compounds exhibited stronger signals in brown and red algae, particularly in *G. barbata*, *C. compressa*, and *F. virsoides* for catechin, and in *C. corniculata* and *A. taxiformis* for myricetin. Catechin has been previously reported as an abundant flavonoid in brown algae and in *U. lactuca* (Pappou et al. 2022), showing anti-inflammatory and cytoprotective effects (Ouahabi et al. 2024). Although documented in *Cystoseira amentacea* and *C. sedoides* (Ousmer et al. 2024), no prior records exist for the brown species *C. compressa* and *G. barbata* identified in this study.

Within the group of hydroxycinnamic acids and their derivatives, chlorogenic acid was detected exclusively in *G. barbata*, whereas rosmarinic acid was characteristic of the brown alga *F. virsoides*. In contrast, caffeic acid exhibited a more heterogeneous profile, being identified in most species analysed. This behaviour may be explained by the fact that caffeic acid represents the simplest structure within the hydroxycinnamic acids detected, compared with their more complex ester derivatives.

Chlorogenic acid has been previously reported in species such as *Sargassum* (Guerrero-Higareda and Carrillo-Nieves 2025) and *Ulva fasciata* (Ibrahim et al. 2021); however, its presence in *G. barbata* had not been documented. Similarly, although rosmarinic acid has been identified in brown algae such as *Ascophyllum nodosum* (Obluchinskaya et al. 2024) and in the red alga *Gracilariopsis persica* (Pourakbar et al. 2021), its occurrence in *F. virsoides* was previously unreported. High concentrations of rosmarinic acid have been associated with plant responses to desiccation stress, mediated by increased proline levels (Ruiz-Medina et al. 2022). Caffeic acid has been widely reported in green (Ouahabi et al. 2024), brown (Fonseca-Barahona et al. 2025), and red algae (Carpena et al. 2022).

Quercetin was identified exclusively in *P. pavonica* and *A. taxiformis,* although widely cited in the literature (Lourenço-Lopes et al. 2020; Pereira and Cotas 2023) as a common macroalgal phenolic, only a few studies confirm its specific identification. It has been detected in species such as *Caulerpa racemosa* (Tanna et al. 2018) and in various macroalgae from the Red Sea (Al-Saif et al. 2014). Sulfated derivatives have also been reported, as in the red alga *Alsidium corallinum*, where a protective effect against in vivo oxidative stress was evidenced, linked to the quercetin content, affecting lipid membranes and proteins (Ben Saad et al. 2017).

Comprehensive Metabolomic Profiling of Adriatic Macroalgae

In order to obtain a more detailed characterisation of the metabolites identified in the non-targeted analysis, these were grouped into chemical families: amino acids and derivatives, lipids and lipid-related compounds, phenolic compounds, sugars and polyols, and organic acids. This approach allows a systematic comparison of the relative responses of each species within each chemical family, facilitating the identification of discriminant compounds and the detection of metabolomic patterns associated with both taxonomy and bioactivity of the studied macroalgae.

Amino Acids

In the analysis of amino acid derivatives, glutamic acid, aspartic acid, and taurine were detected. Glutamic and aspartic acids exhibited a strong response in *F. virsoides*, and a weaker response in *E. amentacea* and *C. coniculata*. Generally, among the most common amino acids in the genus *Fucus spp.* (Catarino et al. 2018), concentrations have been reported to relate to respiratory processes, increasing through the consumption of mannitol, a compound also identified in this study. Glutamate has been reported at elevated levels in *Fucus* relative to other species, likely associated with the habitats typically inhabited by this species, mainly estuaries or brackish zones (Munda 1977). This generates a higher proportion of available nitrogen, increasing glutamate production for metabolism.

Taurine showed a high response in an *S . furcellata* and a low response in *E. amantacea*, *U. lactuca*, *C. multifida*, *C. corniculata*, *F. virsoides*, and *A. taxiformis* Taurine content varies according to species, with red algae exhibiting high concentrations, whereas it is rare in green and brown algae (Thiviya et al. 2022). However, concentrations in green algae have been reported, particularly in *U. lactuca*, as observed in this study (Terriente-Palacios and Castellari 2022). Taurine possesses multiple pharmacological functionalities, stimulating interest for marine-derived applications, including antihypertensive and hypocholesterolaemic properties, antioxidant activity, and protection against the toxicity of several heavy metals.

Lipids and Derivatives

The lipid family was the most extensive detected in these macroalgae, including fatty acids (FA), fatty acid esters of hydroxy fatty acids (FAHFA), oxylipin, lysophospholipids (LysoP), lysophosphoglycerolipids (LysoPG), sulfoquinovosylmonoacylglycerols (SQMG), and monogalactosyldiacylglycerols (MGDG).

Among lipid derivatives, FAHFA were particularly noteworthy. FAHFA are defined as oligomers of a hydroxy fatty acid and a fatty acid (Riecan et al. 2022). This lipid class has been scarcely explored, with their endogenous presence in humans reported only about a decade ago (Yore et al. 2014). In aquatic photosynthetic organisms, evidence is very limited, reported only in microalgae such as *Haematococcus pluvialis* and *Coccomyxa melkonianii* (Fais et al. 2021; Casula et al. 2024). Although the presence of FAHFA in macroalgae has not been reported, their constituent compounds, namely fatty acids and hydroxy fatty acids (Kendel et al. 2015), have been described in different macroalgal species.

Identification of FAHFA is complex due to the wide diversity of lipid families in macroalgae, which can lead to incorrect assignment as fatty acid dimers. In this study, four principal ions were detected: m/z 563.50, 551.41, 537.49, and 495.34, with proposed molecular formulas $C_{36}H_{68}O_4$, $C_{36}H_{56}O_4$, $C_{34}H_{66}O_4$, and $C_{32}H_{48}O_4$, respectively. The fragment at m/z 537 contained the common ions 281, 255, and 299, corresponding to octadecenoic acid, palmitic acid (PA), and hydroxy stearic acid (HSA), respectively. This pattern has previously been reported for FAHFA (Yore et al. 2014), where the proposed formula $C_{34}H_{56}O_4$ cannot accommodate double bonds, indicating that the identified octadecanoic acid is generated as a rearrangement following MS fragmentation. Accordingly, the most reasonable structure for ion 537 is an ester combining PA and HSA to produce palmitic acid–hydroxy stearic acid (PAHSA). Similarly, based on

this structural model and the detected masses for the other ions, their structures are: oleic acid–hydroxy stearic acid (OAHSA, m/z 563), linolenic acid–hydroxy stearidonic acid (FAHFA 36:7, m/z 551.41), and hexadecatrienoic acid (16:3)–hydroxy hexadecatetraenoic acid (16:4-OH, m/z 495.34).

The highest FAHFA concentrations were detected across the green algae, such as *Codium adherens*, *C. vermilara*, and particularly *U. lactuca*. In general, relative responses followed the order: FAHFA 36:1 > FAHFA 18:4/18:3 > FAHFA 16:4/16:3 > FAHFA 34:0, with the highest concentration corresponding to the hydroxy stearic ester. These findings are consolidated considering that, among the few hydroxy fatty acid esters, hydroxy stearic acid has been detected previously in the *Ulva* genus (Ghallab et al. 2022).

Phenolics

Most phenolic compounds were detected as sulphated derivatives. The sulphated form of vanillic acid (vanillic acid 4-O-sulphate) exhibited the highest response, mainly in *U. lactuca*. The 2,4-dihydroxyacetophenone 5-sulphate, a sulphated acetophenone derivative, was present in high concentrations in *F. virsoides*. This compound has not previously been reported in macroalgae, although non-sulphated derivatives have been detected in higher plants (Supikova et al. 2022).

Salicylic acid was detected in several species, showing the most prominent response in brown algae such as *G. barbata*, *C. compressa*, and *C. corniculata*. Hydroxybenzoates were predominantly detected in *F. virsoides*, also in *U. lactuca*, and at lower intensity in *G. barbata*, *C. compressa*, and *C. corniculata*. The sulphated forms were observed in the same macroalgae, particularly in *E. amentacea*, which lacks the non-sulphated form. Overall, homovanillic acid was present in most species, with higher levels in *F. virsoides* and *E. amentacea*.

Sugars and Polyols

Mannitol was among the compounds with the highest response, especially in *P. pavonica* and *F. virsoides*, the latter also showing elevated tagatose levels. Fructose and glucose were detected in all species, with particularly high intensities in *F. virsoides* and *B. barbata*. Arabinose 5-phosphate showed a notable response in *D. adherens*. Sorbitol exhibited one of the lowest responses, though it was consistently detected in all evaluated species.

Organic Acids

Several organic acids were detected, including malic, succinic, azelaic, citric, fumaric, itaconic, gluconic, aconitic, and tricarballylic acids. Fumaric acid was one of the predominant compounds, showing very high response, especially in *C. adherens*. This species also displayed the highest levels of malonic acid and malic acid.

Succinic acid and derivatives were most abundant in brown algae, including *Fucus spp.*, *E. amentacea*, *C. corniculata*, *C. compressa*, and *Dictyota multifida*, exhibiting higher intensities than in other species. Citric and azelaic acids, with relatively low responses, were mainly detected in *Fucus spp.* and an *S. furcellata*. Itaconic acid was almost exclusively observed in *C. corniculata*, at lower but detectable levels in some other brown algae.

CONCLUSION

This study provided a comprehensive characterisation of the bioactive and metabolomic profiles of twelve macroalgal species from the Adriatic Sea, an environment whose distinctive conditions (high solar irradiance, salinity gradients and intertidal stress) promote the accumulation of functional compounds. The implementation of MSPD using GRAS solvents enabled the efficient and sustainable extraction of metabolites, overcoming limitations associated with conventional methods and offering a robust foundation for comparative bioactivity analyses.

The results obtained for total phenolic content and antioxidant capacity revealed marked differences among the three taxonomic groups examined, with *Fucus virsoides*, *Ulva lactuca* and *Scinaia furcellata* emerging as the species with the highest bioactive potential among brown, green and red algae, respectively. Targeted LC–MS/MS analysis enabled the identification of nineteen phenolic compounds, showing greater diversity in brown algae and distinct profiles in green species, including the selective detection of 7-hydroxycoumarin in those with higher antioxidant capacity. This finding provides valuable insights into potential chemical markers linked to the observed bioactivity.

Untargeted metabolomic analysis using UHPLC-QTOF significantly broadened the understanding of macroalgal metabolomes, allowing the identification of eighty metabolites and revealing highly differentiated chemical patterns across species. The detection of FAHFA families not previously reported in macroalgae and the annotation of novel compounds in *Codium* and *Scinaia* further highlight the importance of these taxa as sources of still underexplored chemical diversity. Overall, this work established a solid reference framework for the bioactive composition of Adriatic macroalgae and demonstrated their potential as natural sources of functional compounds. The findings open new perspectives for marine bioprospecting, identifying priority species for future research and underscoring the value of integrating sustainable extraction techniques with targeted analyses and high-resolution metabolomics.

Declarations

Associated Content

Information as mentioned in the text File S1, Fig. S1, Table S1 and Section S1.

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Conflict of interest

The authors declare no competing interests.

Data availability

All data generated or analysed during this study are included in this published article and its supplementary material files.

Code availability

Not applicable.

Ethics approval

Not applicable.

Consent to participate

Not applicable.

Consent for publication

Not applicable.

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Table 2

Table 2 is available in the Supplementary Files section.

Figures

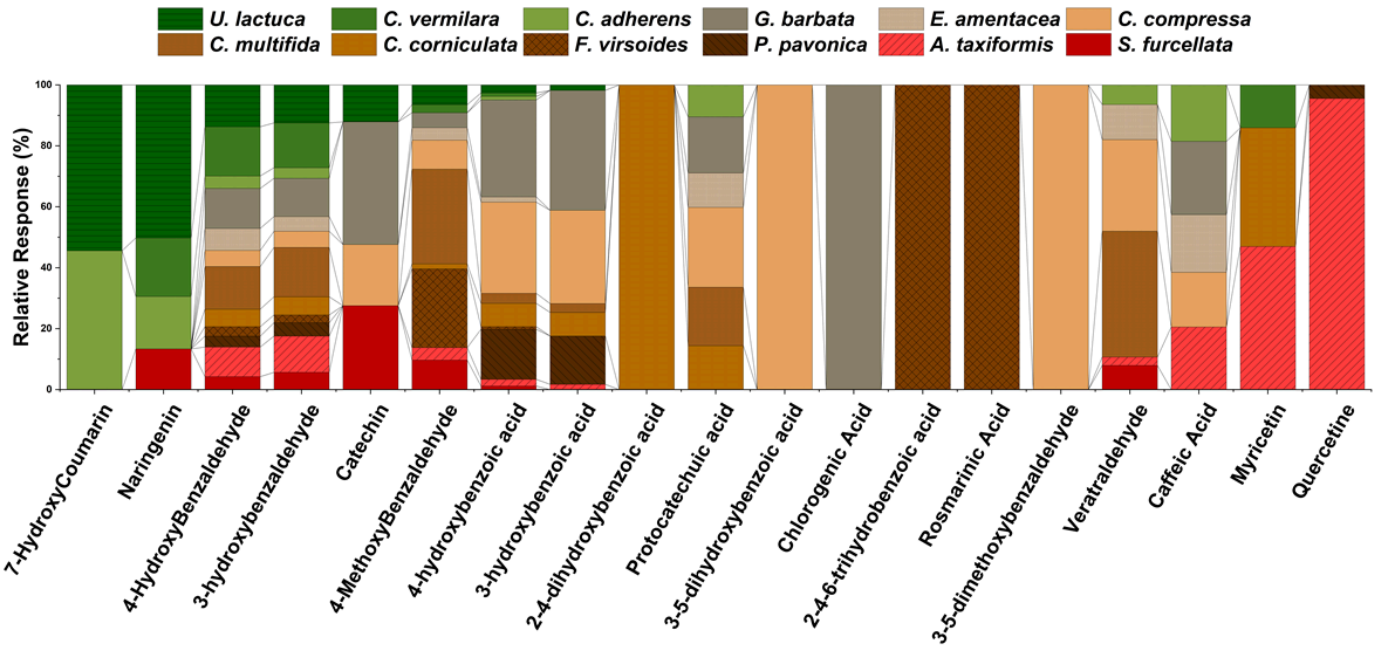

Figure 1

Comparative profile of main phenolic markers identified by targeted HPLC–HESI–MS/MS in twelve macroalgae species, grouped by family and expressed as relative response (%).

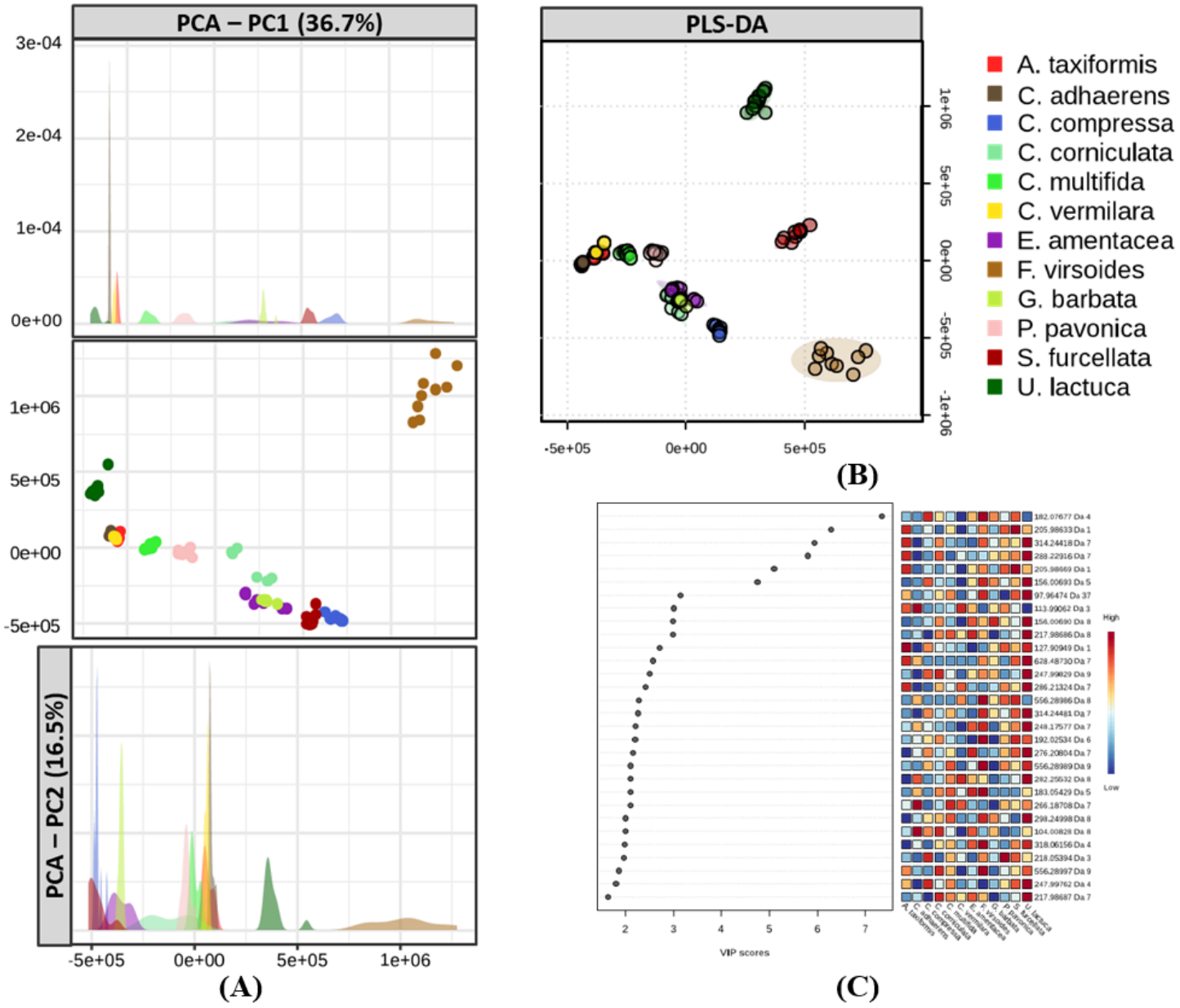

Figure 2

Multivariate metabolomic differentiation of twelve Adriatic macroalgal species based on UHPLC–QTOF data: PCA (A), PLS-DA models (B), and VIP scores with heatmap of key discriminant metabolites (C).

Supplementary Files

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- APPENDIXA.docx
- FILES1.xlsx
- Table2.docx