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Chemical profiles of less-volatile organic compounds from the Adriatic Sea macroalgae obtained by supercritical CO_2 extraction

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ABSTRACT

Due to the lack of less-volatile compounds composition data of macroalgal supercritical CO_2 (SC-CO_2) extracts, the main goal of this study was to investigate their chemical profiles. SC-CO_2 extraction (40 °C and 300 bar) was performed on seven macroalgal species including five brown (*Halopteris filicina*, *Fucus virsoides*, *Dictyopteris polypodioides*, *Gongolaria barbata* and *Ericaria amentacea*), one green (*Codium bursa*) and one red (*Amphiroa rigida*), that were collected from the Adriatic Sea. After the analysis by gas chromatography and mass spectrometry, the results revealed that fatty acids were the main components of the extracts. Hexadecanoic acid was found as dominant fatty acid in most of the species, while 3-hexyl-4,5-dithiacycloheptanone was dominant in *D. polypodioides*. Performed phytochemical study contributes to the knowledge of less-volatile composition of analyzed species indicating that the "species biodiversity" factor was the most influent regardless of classification to brown, green or red macroalgae.

Introduction

Macroalgae synthesize various volatile organic compounds (VOCs) during their growth and adaptation to abiotic stress. VOCs are usually serving not only as communication agents in the interaction with the environment, but also for the protection against predators (Santos Leite Neta and Narain, 2018). Higher concentrations of volatile short-chain fatty acids (up to 10 C atoms) were found in different types of macroalgae when treated with the marine pathogen Vibrio haveyi (Pham et al., 2008). Cook et al. (1948) determined that VOCs can act as pheromones. The environmental conditions, macroalgal maturity, geographical origin of the macroalgae, as well as the drying process and methods used for VOCs extraction, have significant influence on their concentration and composition (Loper-Perez et al., 2016). VOCs are present during photosynthesis and respiration so the presence or absence of light does not affect the concentration of VOCs. On the other hand, oxidative stress occurs when macroalgae are exposed to either air or drying. Consequently, the concentration of VOCs increases (Bravo-Linares et al., 2010).

The use of supercritical fluids to extract volatile compounds eliminates the disadvantages of other methods, such as heating of the sample during hydrodistillation, which can cause degradation of some thermolabile compounds (Pourmortazavi and Hajimirsadeghi, 2007). Another advantage is the production of solvent-free extract due to the usage of non-toxic and volatile fluid, such as supercritical CO_2 (SC-CO_2). During supercritical extraction, when high pressures are applied, it is possible to obtain the compounds of higher molecular weight such as fatty acids and less-volatile compounds (Hattab et al., 2007). The studies reporting the usage of SC-CO_2 extraction for the isolation of volatile compounds from macroalgae are not well represented. Its application is

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mainly related to the extraction of pigments, lipids, polyphenols and vitamins (Michalak et al., 2015). Hattab et al. (2007) analyzed volatile compounds obtained with SC-CO_2 from brown macroalga *Dicytopteris membranacea* (now accepted as *Dictyopteris polypodioides* (Guiry and Guiry, 2022)) and noted the presence of sesquiterpenes, C_{11}-hydrocarbons and sulfur compounds with 3-hexyl-4,5-dithiacycloheptanone as the dominant. Our previous study on SC-CO_2 extract of *Codium bursa* revealed the dominance of (*E*)-phytol followed by hexadecanoic acid (Jerković et al., 2019). Other reports of the VOCs from species that were also investigated in this study included hydrodistillation (HD) and headspace solid-phase microextraction (HS-SPME). Some of these reports include VOCs composition of *Gongolaria barbata* (Radman et al., 2022; Ozdemir et al., 2006), *Fucus virsoides* (Jerković et al., 2021), *Amphiroa rigida* (Cikoš et al., 2021) and *Halopteris filicina* (Jerković et al., 2018), while Riad et al. (2019) and Ozdemir et al. (2006) used HD for the isolation of VOCs from *D. polypodioides*. Hattab et al. (2007) used three different methods including HD, focused microwave-assisted hydrodistillation (FMAHD) and SC-CO_2 extraction for the comparison of VOCs isolated from *D. polypodioides* (ex. *D. membranacea*). There are no reports on VOCs from *Ericaria amentacea*.

Therefore, main goals of the present research were to: a) determine less-volatile organic compounds isolated with SC-CO_2 extraction from selected macroalgae species from the Adriatic Sea (*H. filicina*, *G. barbata*, *E. amentacea*, *F. virsoides*, *D. polypodioides*, *A. rigida* and *C. bursa*) as the first time report on SC-CO_2 extracts from Adriatic Sea algae (except for *C. bursa*); b) indicate the potential of SC-CO_2 for the extraction of less-volatile compounds; c) determine and compare the chemical biodiversity of found VOCs among investigated species; d) determine the impact of "species biodiversity" factorand to classify macroalgae as brown, green or red according to the obtained VOCs profile; e) compare the obtained results with previously published data on VOCs obtained with different methods; d) continue the investigation on chemical characterization of the algae from the Adriatic Sea within the project "Bioprospecting of the Adriatic Sea" (BioProCro).

Materials and methods

Collection and preparation of macroalgae samples

Macroalgal samples were collected at various locations in the Adriatic Sea:

- *Codium bursa* (Olivi) C. Agardh 1817 was collected at Rtina peninsula(44°19'14''N; 15°55'42''E) in February 2020;
- *Gongolaria barbata* (Stackhouse) Kuntze 1891 was collected at Šepurine near Zadar (44°12'42''N; 15°09'23''E) in February 2020;
- *Amphiroa rigida* J.V. Lamouroux was collected close to Šepurine (Zadar) (44°12′42″ N; 15°09′23″ E) in September 2020;
- *Fucus virsoides* J. Agardh 1868 was collected at Novigrad sea area (44°12'02''N; 15°28'51''E) in February 2021;
- *Ericaria amentacea* (C. Agardh) Molinari & Guiry 2020 was collected in April 2021 offshore Dugi otok island, 1 km nortwest of Brbišćica Bay (43°03'16''N; 14°59'14''E);
- *Halopteris filicina* (Grateloup) Kützing 1843 and Dictyopteris polypodioides (A.P.De Candolle) J.V.Lamouroux 1809 were collected close to Okrug Donji, Čiovo Island (43°29'55"N; 16°13'24"E) in September 2020.

Single point sample collection provided representative sample for all the species investigated. The species were separately placed in air tight plastic bags together with surrounding seawater and immediately transported to the laboratory. *C. bursa*, *G. barbata*, *A. rigida*, *F. virsoides* and *E. amentacea* were freeze-dried before the extractions. The samples were washed 5 times with water and 2 times with deionized water before being cut into 5-10 mm slices. Cut samples were frozen at -60 °C for 24 h in ultra-low freezer and then subjected to freeze-drying in a laboratory freeze dryer (CoolSafe PRO, Labogene, Denmark). The freeze-drying process was performed for 24 h with -30 °C and 20 °C as the primary and secondary drying temperatures under high vacuum from 0.13 to 0.55 hPa.

Supercritical CO_2 (SC-CO_2) extraction

SC-CO_2 extractions were performed in a system for supercritical fluid extraction previously explained in detail (Jokić et al., 2015). Before the extraction, the samples were milled in laboratory mill (MRC Sample Mill C-SM/450-C, Holon, Israel) and 100 g of each sample was used for the process. The extraction was performed for 60 min at the temperature of 40 °C and pressure of 300 bar. The SC-CO_2 passed through the sample matrix with mass flow of 2 kg/h. The obtained extracts were subjected to gas

chromatography and mass spectrometry (GC-MS) analysis.

Gas chromatography and mass spectrometry (GC-MS)

Gas chromatography and mass specrometry (GC-MS) analyses were performed on the Agilent Technologies (Palo Alto, CA, USA) gas chromatograph model 7890A equipped with a mass selective detector (MSD) model 5977E (Agilent Technologies) and HP-5MS capillary column (30 m × 0.25 mm, 0.25 μm film thickeness, Agilent Technologies, Palo Alto, CA, USA). The GC–MS conditions were the same as described previously (Jerković et al., 2018). The compounds percentage compositions were calculated from the GC peak areas using the normalization method (without correction factors) and were calculated as mean values from triplicate GC–MS analyses of all extracts. The structural identification was based on the comparison of GC retention indices (RI) determined relative to the retention times of C_9–C_{25} n-alkanes and compared with those available in the literature (National Institute of Standards and Technology) and their mass spectra with the mass spectra libraries Wiley 9 library (Wiley, New York, NY, USA) and NIST14 (National Institute of Standards and Technology, Gaithersburg, MD, USA) (NIST, 2021).

Results and discussion

For the first time the SC-CO_2 extraction was successfully applied for the extraction of less-volatile compounds from seven macroalgal species collected at various locations in the Adriatic Sea. It is evident from the results (Table 1) that there are significant differences among investigated species and their chemical profiles, as was expected. The dominant VOCs found in the supercritical extracts were fatty acids with the exception of *D. polypodioides*,where 3-hexyl-4,5-dithiacycloheptanone was dominant. One of the advantages of using SC-CO_2 is the protection of thermolabile compounds as high temperatures are avoided during the extraction. It was shown that the pressure has the highest influence on the extraction of fatty acids, as well as on volatile metabolites. Different studies used the pressure of around 300 bars for the extraction of both groups of compounds from various macroalgae (Crampon et al., 2011; Machmudah et al., 2018; Choi et al., 1987). According to preliminary results and our previous study (Jerković et al., 2019), where less-volatile compounds were successfully extracted at 300 bars and 40 °C, the same conditions were chosen for the extractions during this study for all macroalgal samples.

Volatile composition of Halopteris filicina (Grateloup) Kützing 1843

It was established (Table 1) that the fatty acids were the most dominant in SC-CO_2 extract with hexadecanoic acid (palmitic acid) as the most represented (44.88%), followed by oleic acid (10.57%). Except the fatty acids, diterpene alcohol (*E*)-phytol was also detected as abundant (5.00%) and it is the product of chlorophyll *a* degradation (Rontani and Volkman, 2003). Methylated long chain fatty acid ketone - hexahydrofarnesyl acetone (phytone), known as the degradation product of (*E*)-phytol, was also detected with low abundance (0.53%). C_8-compounds that were found as abundant in the headspace of *H. filicina* (Jerković et al., 2018) are not found in the SC-CO_2 extract. Alkanes, such as heptadecane (1.19%) and pentadecane (0.34%) were identified. Hydrocarbons are characteristic volatile compounds found in brown macroalgae, with pentadecane as the most abundant compound and alkanes represent the fatty acid degradation products (Youngblood et al., 1971; Youngblood and Blumer, 1973). The degradation products of carotenoids, including C_{13}-norisoprenoids such as β-ionone were also detected in SC-CO_2 extract of *H. filicina* (0.13%) indicating that the carotenoid degradation did not occur on a large scale during the extraction.

It is evident from the results that the profile of volatile compounds in SC-CO_2 extract of *H. filicina* differ significantly when compared to the headspace profile (Jerković et al., 2018). The composition of VOCs isolated by HS-SPME revealed the dominance of dimethyl sulfide (DMS; 12.8%). The aliphatic compounds, particularly C_8-compounds ((3*Z*,5*E*)-octat-1,3,5-triene, octan-1-ol, octanal, octan-3-one, (3*E*)-octa-1,3-diene and oct-1-en-3-ol) were the most represented in the headspace profile. The presence of halogenated compounds, such as tribromomethane and 1-iodopentane, was also established. These differences were expected since HS-SPME is not able to detect less volatile compounds, but only high-

volatile compounds that are in equilibrium with the sample at given temperature.

Volatile composition of Fucus virsoides J. Agardh 1868

In this study, the extraction of *F. virsoides* with SC-CO_2 was used in order to obtain less volatile chemical profile. The volatile composition of *F. virsoides* was analyzed in our previous study (Jerković et al., 2021) by HS-SPME and HD, but there is no report on the volatile compounds obtained by SC-CO_2. The results (Table 1) showed that SC-CO_2 extract of *F. virsoides* was rich in fatty acids. Oleic acid was found as dominant (31.36%), followed by tetradecanoic acid (22.38%), hexadecanoic acid (18.24%), octadecanoic acid (6.87%), palmitoleic acid (1.10%) and other acids that were present less than 1% (i.e. pentadecanoic acid, hexanoic acid, nonanoic acid, octanoic acid, heptanoic acid, pentanoic acid). In comparison to HS-SPME and HD (Jerković et al., 2021), fatty acids were found only in small amounts, while pentadecane was the main compound of headspace (60.27-71.55%) and hydrodistillate (3.28-5.87%). Pentadecane in *F. virsoides* SC-CO_2 extract was significantly lower (1.56%). The common marine terpene, dihydroactinidiolide, was found in low amounts (0.13%) in the present study. Previously, it was reported that it made an important contribution to the volatile profile of *Ulva prolifera* where it was present in great abundance (8.4%) (Yamamoto et al., 2014). Monoterpenoid hydroxylactone loliloide was also detected (0.34%) and it was present in the greatest t abundance when compared to other analyzed species (Table 1). Loliolide was not detected in headspace and volatile oil of *F. virsoides* that were analyzed in our previous report (Jerković et al., 2021). Even though it posseses simple structure, there are some reports implicating its biological activity, including antioxidant activity (Yang et al., 2011), germination inhibitory activity, anti-repellent activity (Okunade and Wiemer, 1985), immunosuppressive activity (Okada et al., 1994), as well as activity of growth inhibition of murine lymphocytic leukemia and human nasopharynx carcinoma (Pettit et al., 1980).

Volatile composition of Dictyopteris polypodioides (A.P.De Candolle) J.V.Lamouroux 1809

GC-MS analysis of SC-CO_2 extract (Table 1) of *D. polypodioides* revealed that 3-hexyl-4,5-dithia-cycloheptanone (27.79%) was the dominant compound in the extract, followed by hexadecanoic acid (16.51%) and oleic acid (13.36%). 3-Hexyl-4,5-dithiacycloheptanone was also detected as dominant in SC-CO_2 extract of *D. membranacea* (known as *D. polypodioides*) that was analyzed by Hattab et al. (2007). Sulfur compounds are characteristic for *Dictyopteris* species and are known for their biological activities, such as antibiotic (Wratten and Faulkner, 1976) and anti-inflammatory (Dimou et al., 2016). It was reported that they act as chemical defenses against herbivores and some *Dictyopteris* species produce high concentrations of these valuable compounds (Hay et al., 1988). They can originate from oxidative degradation of highly unsaturated fatty acids and they are biosynthetically related to C_{11}-hydrocarbons (Roller et al., 1971). Hattab et al. (2007) used two additional methods for the isolation of volatile compounds, including HD and FMAHD. C_{11}-hydrocarbons were dominant in the hydrostillate, sesquiterpenes were the main compounds when FMAHD was applied, while sulfur compounds were found as dominant in SC-CO_2 extract. In this study, C_{11}-hydrocarbons, including dictyopterene A (8.00%), dictyopterene C (0.87%) and dictyopterene D (0.08%), were found in the extract of *D. polypodioides*. They are structurally similar to sexual attractants and act as odoriferous compounds (Zatelli et al., 2018). Dictyopterens are usually included in the preparation of cosmetic or pharmaceutical products. They can also be found in the composition of fragrances and deodorants (Kajiwara et al., 2003; Gedouin et al., 2007). C_{11}-hydrocarbons are produced by various rearrangements from arachidonic acid into divinylpropane, which decomposes into various C_{11}-hydrocarbons via Cope rearrangement (Stratmann et al., 1993). Riad et al. (2019) investigated volatile composition of volatile fraction and essential oil of *D. polypodioides*. Their results revealed the predominance of the sulfur C_{11}-compound 3-hexyl-4,5-dithiacycloheptanone and dictyopterene A in both volatile fraction and essential oil, which was similar

to our finding. Futhermore, fatty acids that were found in this study, namely, hexadecanoic and oleic acids, are known as the most dominant in *Dictyopteris* (Karaki et al., 2013).

Volatile composition of Gongolaria barbata (Stackhouse) Kuntze 1891

The most dominant compound in SC-CO_2 extract of *G. barbata* was hexadecanoic acid (29.02%), while the presence of other fatty acids such as palmitoleic acid (11.14%), octadecanoic acid (7.22%), tetradecanoic acid (6.40%), heptadecanoic acid (2.40%) and pentadecanoic acid (0.73%) was also revealed. The palmitic acid (hexadecanoic acid) is known as the dominant fatty acid in *G. barbata* which was established when the profile of fatty acids was investigated by Vizetto-Duarte et al. (2015). Some compounds that were previously reported (Ozdemir et al., 2006; Milkova et al., 1997) as abundant in *G. barbata* were not found in this study. Milkova et al. (1997) reported that the main volatile compounds obtained with HD were halogenated hydrocarbons and it represented the first study where these compounds were reported for Phaeophyta. Ozdemir et al. (2006) found mostly hydrocarbons in the volatile oil of *G. barbata* with docosane (7.61%) and tetratriacontane (7.47%) as the most abundant, followed by eicosane (5.05%), tricosane (4.43%), hexadecane (4.16%) and heptadecane (1.35%). Pentadecane (0.15%) and heptadecane (0.83%) (Table 1) were found in SC-CO_2 extract of *G. barbata*, but in very low abundance when compared to the headspace (Radman et al., 2022). Our previous study on *G. barbata* was the first reporting the headspace composition of fresh and dried samples of this alga (Radman et al., 2022). The dominance of sesquiterpenes, particularly cadinane-type sesquiterpenes (τ-cadinol (41.25%; 35.65%) and δ-cadinene (6.16%; 3.86%)), was found in headspace and volatile oil of fresh sample, while drying process caused significant changes in profiles. The amount of sesquiterpenes in headspace was significantly reduced, while the amounts of alkanes (pentadecane (10.62%; 7.72%), heptadecane (4.12%; 3.11%)) increased due to more intense fatty acid degradation. In the volatile oil of *G. barbata* fresh sample (Radman et al., 2022) aliphatic compounds were found as one of the abundant groups with octadecan-1-ol (6.81%) as dominant. In this study, octadecan-1-ol was not found in the SC-CO_2 extract of *G. barbata* indicating that SC-CO_2 extraction performed at 300 bars and 40 °C is not adequate method for the isolation of aliphatic compounds and alcohols. On the other hand, volatile oil of dried sample of *G. barbata* revealed the presence of linoleic acid (5.02%), oleic acid (2.32%) and (*E*)-phytol (1.47%) (Radman et al., 2022). These compounds were also found in this study, but in higher amounts with oleic acid as the most abundant (12.01%), followed by linoleic acid (9.80%) and (*E*)-phytol (3.48%) (Table 1).

Volatile composition of Ericaria amentacea (C. Agardh) Molinari & Guiry 2020

The main components of SC-CO_2 extract of *E. amentacea* were fatty acids (Table 1) including hexadecanoic acid as dominant (32.16%), followed by palmitoleic acid (19.66%), oleic acid (16.17%), tetradecanoic acid (11.69%), linoleic acid (2.81%), octadecanoic acid (1.43%) and other that were present in very low abundance (i.e. pentadecanoic, heptadecanoic, hexanoic, dodecanoic, heptanoic, octanoic and pentanoic acids). The study on fatty acid profile of *E. amentacea* (Bouafif et al., 2018) revealed that palmitic acid was dominant, as was found in this study. Furthermore, the lipid extract that was analyzed and showed a high amounts of saturated fatty acids, around 40.51% (Bouafif et al., 2018). There is no literature that reports the volatile composition of *E. amentacea*, so this study is the first to give an insight into the less-volatile compounds. Available literature reported meroditerpenes including 4'-methoxy-(2*E*)-bifurcarenone and its chromene derivative from *Cystoseira amentacea* var. *stricta* collected at the French Riviera coast, as well as 2,12-diepineobalearone from species collected at Galite islands coast (Mesguiche et al., 1997). Demethoxy cystoketal chromane and cystoketal quinone are reported for *C. amentacea* var. *stricta* also collected at the French Riviera coast in another study by Valls et al (1996). Different *Cystoseira* species collected from the Sicilian coast have been studied more and for them the presence of tetraprenyltoluquinols with regular diterpenoid moiety (balearone, amentol, cystoketal, cystoketal

chromane, strictaepoxide, strictaketal, isocystoketal, isostrictaketal, isobalearone, (2*E*)-bifurcarenone, amentaepoxide and amentadione), as well as rearranged dieterpenoid moiety (neobalearone, 2-epineobalearone), is reported in different studies (Amico et al., 1984a; Amico et al., 1984b; Amico et al., 1987). Furthermore, the volatile fraction of *C. stricta* var. *amentacea* revealed the presence of 97 compounds with sesquiterpene alcohols as predominant chemical group with cubenol as the major constituent (30.82%). C_{11} unsaturated hydrocarbons were also found, but in traces (Gally et al., 1993). Only three common compounds were found in *C. stricta* var. *amentacea* (Gally et al., 1993) and *E. amentacea* examined in this study. Naimely, *p*-anethole and methyl palmitate were present in higher amounts in this study (0.35% and 0.16%, respectively), while β-ionone was found in similar percentages (0.11% for *E. amentacea*; 0.10% for *C. stricta* var. *amentacea*). Dibutyl phtalate (1.58%) was found in SC-CO_2 extract of *E. amentacea* during this study. Its presence can be explained by the fact that brown algae are natural producers of this compound which has the application as plasticizer or solvent in a wide range of industrial products (Namikoshi et al., 2006). Furthermore, the presence of hydrocarbons, such as nonadecane (0.59%) and heptadecane (0.23%), was also detected, as well the presence of terpenes including dihydroactinidiolide (0.13%) and hexahydrofarnesyl acetone (0.20%).

Volatile composition of Codium bursa (Olivi) C. Agardh 1817

During this study, the dominance of hexadecanoic acid (40.63%), followed by oleic acid (13.30%), tetradecanoic acid (8.33%), palmitoleic acid (5.09%), linoleic acid (1.39%) and pentadecanoic acid (1.03%) was noticed. Even though we have analyzed the composition of SC-CO_2 extract of *C. bursa* (Jerković et al., 2019), we wanted to re-examine the content of VOCs, because it can be influenced by different collection time of the samples. There are many reports on changes in the chemical profiles of algae that are collected in different period of the year (Paiva et al., 2014; Generalić Mekinić et al., 2021; Kim et al., 1996). *C. bursa* used in this study was collected in February 2020, while in the previous study (Jerković et al., 2019) the samples were collected in May 2018. The dominant compound in *C. bursa* collected in May 2018 (Jerković et al., 2019) was (*E*)-phytol (42.30%), while its amount in *C. bursa* investigated during this study was 4.20%. Probably more intense chlorophyll degradation occurred in the sample collected in May 2018 that lead to higher (*E*)-phytol concentrations. Neophytadiene (3.20%), structurally related to (*E*)-phytol, was found in previous study (Jerković et al., 2019), but it was not present in the extract obtained from *C. bursa* collected in February 2020. The same trend was observed also for loliolide (3.51%) (Jerković et al., 2019). Hexadecanoic acid, that was dominant in this study, was found in lower abundance in our previous study (17.51%). These differences are pointing out that the collection time has a significant role in the chemical composition of VOCs. Mirzayeva et al. (2021) studied how collection time, species, geographical origin and pretreatment procedures influenced the volatile profile of different macroalgae from Spanish coasts. The results revealed that collection time had the highest influence on volatile content. In their research only dimethyl sulfide, 2-butylfuran, 6-methylheptan-2-one, hexyl acetate, 2-phenylethyl acetate and isopropyl palmitate were not affected by the collection time. Generally, species collected in autumn contained higher values of volatile compounds when compared to the spring samples. Furthermore, the ‘’species’’ factor was the most influent one after collection time, since the big differences were noticed with respect to total volatile profiles of different macroalgal groups. According to their study, green macroalgae contained richer volatile profiles, while red macroalgae were shown as the poorest ones. Geographical origin was the next one to have an influence on volatile composition, while the pretreatment procedures were revealed as the factor with the lowest influence with regards to volatile compounds profiles including raw, salted and dehydrated samples. Mirzayeva et al. (2021) detected dimethyl sulfide, ethyl acetate, heptadecane, α-ionone, 2-phenylethyl acetate and dimethyl sulfoxide as the main compounds in *Codium* sp. In this study only heptadecane (8.05%) and α-ionone (0.19%) were found, but in lower amount.

Volatile composition of Amphiroa rigida J.V. Lamouroux

According to the results of GC-MS analysis of SC-CO_2 extract obtained from *A. rigida*, the dominance of fatty acids in volatile profile was determined (Table 1). Hexadecanoic acid was found as dominant (40.63%), followed by oleic acid (13.30 %), (*E*)-phytol (9.14%), tetradecanoic acid (8.33%), palmitoleic acid (5.09%), heptadecane (3.00%) and linoleic acid (1.39%). Heptadecane (3.00%) and pentadecane (0.11%) were found in lower amounts when compared to our previous study (Cikoš et al., 2021), where headspace and hydrodistillate of fresh and air-dried samples of *A. rigida* were dominant compounds. Furthermore, (*E*)-phytol was also present in lower amount (9.14%) when compared to hydrodistillate (41.75%) where it was the dominant compound (Cikoš et al., 2021). Even though it was present in lower amount, its presence can also indicate chlorophyll *a* degradation during the CO_2 extraction and freeze-drying process. Since the amount of β-ionone was low (0.19%), it can be suggested that the degradation of carotenoids did not occur intensively as it occurred during air-drying process and hydrodistillation considering that β-ionone content (2.55%) was higher in that case (Cikoš et al., 2021). Generally, the volatile profile of red macroalgae is more complex than the brown and green macroalgae. Previously (Kamernarska et al., 2006), it was determined that the fatty acids esters and hydrocarbons are the main groups in volatile profile of red macroalgae, as was found also in this study. The content of fatty acid esters can vary between algae belonging to the same genus which was found for red macroalgae belonging to the genus *Corallina* and *Polysiphonia* (Kamenarska et al., 2000; Riguera et al., 1984). Available literature shows that fatty acid esters play very important role in algae, as they do in terrestrial plants, where they are responsible for insect-plant communication and are thought to have a similar mechanism in algae (Kamenarska et al., 2006). Kanias et al. (1992) found that the antibiotic activity of some algae can be attributed to stearic, linoleic, palmitic, myristic, lauric and capric acid, and all these acids were also found in the SC-CO_2 extract of *A. rigida* (Table 1). Kamenarska et al. (2006) observed that the red macroalga *Callithamnion granulatum* contains only short-chain fatty acids (4-6 C atoms) and concluded that fatty acids may be taxonomic markers of certain species. Furthermore, terpenes are very important volatile compounds, but in red macroalgae they are found in low concentrations. Dihydroactinidiolide and hexahydrofarnesil acetone are known to be present in most red macroalgae and they represent degradation products of β-carotene (Kamenarska et al., 2006). Mentioned compounds were found in this study but in low amounts with 0.36% and 0.33%, respectively. Sakan et al. (1967) found that these compounds posses various bioactivities and act as pheromones for the recognition of *Solenopsis invicta* (red fire ant) and as a protective barrier against fungal pathogens.

Table 1. VOCs composition determined by GC-MS analysis of SC-CO_2 extracts of different macroalgae

No.	Compound	RI[a]	Area percentage (%)						
			HAFI[b]	FUVI[c]	DIPO[d]	GOBA[e]	ERAM[f]	COBU[g]	AMRI[h]
1.	Pentanoic acid	< 900	0.03	0.01	0.01	0.02	0.01	0.05	0.01
2.	Hexanoic acid	< 900	0.62	0.75	0.15	0.20	0.50	1.58	0.14
3.	Octanal	997	-	0.04	-	-	-	-	-
4.	*cis*-3-Butyl-4-vinyl-cyclopentene	1065	-	-	0.16	-	-	-	-
5.	Heptanoic acid	1084	0.18	0.03	0.05	-	0.11	0.15	-
6.	Nonanal	1104	-	0.11	-	-	-	-	-
7.	Cycloocta-1,3-diene	1118	0.05	-	-	-	-	-	-
8.	Dictyopterene A	1118	-	-	8.00	-	-	-	-
9.	1-[(1*E*)-Hex-1-en-1-yl]-2-vinylcyclopropan	1120	-	-	1.09	-	-	-	-
10.	Dictyopterene D'	1155	-	-	0.08	-	-	-	-
11.	Dictyopterene C'	1172	-	-	0.87	-	-	-	-
12.	Benzoic acid	1175	0.23	-	-	-	-	-	-
13.	Octanoic acid	1181	0.10	0.03	0.03	-	0.01	0.20	0.03
14.	Decanal	1206	-	0.01	-	-	-	-	-
15.	3-Ethyl-4-methyl- 1H-Pyrrole-2,5-dione	1237	0.09	0.07	-	-	-	-	-

No.	Compound	RI[a]	Area percentage (%)						
			HAFI[b]	FUVI[c]	DIPO[d]	GOBA[e]	ERAM[f]	COBU[g]	AMRI[h]
16.	Phenylacetic acid	1260	0.16	-	0.13	-	-	-	-
17.	(*E*)-Dec-2-enal	1264	-	0.03	-	-	-	-	-
18.	Nonanoic acid	1278	0.08	0.05	0.05	-	-	0.28	0.04
19.	*p*-Anethole	1287	0.05	0.04	0.10	-	0.35	0.15	0.09
20.	(*E*,*E*)-Deca-2,4-dienal	1294	-	0.07	-	-	0.17	-	-
21.	(*Z*,*Z*)-Deca-2,4-dienal	1317	-	0.05	-	-	-	-	-
22.	Decanoic acid	1375	0.05	-	-	-	-	0.21	0.03
23.	*trans*-β-Caryophyllene	1417	0.36	0.07	-	-	0.07	-	0.04
24.	α-Ionone	1428	-	-	-	-	-	0.19	-
25.	α-Humulene	1452	0.11	0.01	-	-	-	-	-
26.	2,6-Di(t-butyl)-4-hydroxy-4-methyl-cyclohexa-2,5-dien-1-one	1492	-	-	-	-	-	0.12	0.09
27.	β-Ionone	1485	0.13	0.08	-	-	0.11	0.16	0.19
28.	Pentadec-1-ene	1492	0.24	0.01	-	0.13	-	-	0.05
29.	Bicyclogermacrene	1494	-	-	0.30	-	-	-	-
30.	Pentadecane	1500	0.34	1.56	0.13	0.15	0.06	-	0.11
31.	Dihydroactinidiolide	1526	0.13	0.13	0.24	0.15	0.13	0.28	0.36
32.	Dodecanoic acid	1570	0.20	0.09	0.18	0.20	0.13	1.52	0.11
33.	Caryophyllene oxide	1580	0.20	0.07	-	-	-	-	0.08
34.	Hexadecane	1600	-	-	-	-	-	-	0.03
35.	Diethyl phtalate	1603	-	-	-	-	-	-	0.05
36.	Tridecanoic acid	1667	-	0.04	-	-	-	-	-
37.	(*E*)-Heptadec-8-ene	1678	-	-	0.18	-	0.43	0.66	0.47
38.	Heptadec-1-ene	1694	0.56	0.07	-	0.10	0.09	-	0.17
39.	Heptadecane	1700	1.19	0.07	0.09	0.83	0.23	8.05	3.00
40.	Methyl myristate	1727	-	0.32	-	-	-	-	-
41.	Loliolide	1762	0.24	0.34	0.13	-	0.11	-	0.14
42.	Tetradecanoic acid	1771	7.10	22.38	5.31	6.40	11.69	3.27	8.33
43.	Hexahydrofarnesyl acetone	1846	0.53	0.13	0.28	0.25	0.20	0.97	0.33
44.	Pentadecanoic acid	1866	0.77	0.71	0.19	0.73	0.82	0.20	1.03
45.	Diisobutyl phthalate	1867	-	-	-	0.39	-	0.69	-
46.	3-Hexyl-4,5-dithia-cycloheptanone	1874	-	-	27.79	-	-	-	-
47.	Hexadecan-1-ol	1880	0.36	0.31	-	0.40	0.48	0.38	0.75
48.	Nonadec-1-ene	1892	-	-	-	-	0.24	-	0.09
49.	Nonadecane	1900	-	-	-	-	0.59	-	0.08
50.	Methyl palmitate	1927	-	0.20	-	0.56	0.16	-	-
51.	*cis*-Hexadec-9-enoic acid (Palmitoleic acid)	1949	8.48	1.10	2.58	11.14	19.66	12.13	5.09
52.	Dibutyl phtalate	1961	-	-	-	-	1.58	-	0.58
53.	Hexadecanoic acid	1976	44.88	18.24	16.51	29.02	32.16	31.05	40.63
54.	Octadecan-1-ol	2056	0.80	-	-	-	0.33	-	0.83
55.	Heptadecanoic acid	2063	2.14	-	1.30	2.40	0.70	-	0.71
56.	(*E*)-Phytol	2108	5.00	-	1.66	3.48	0.98	4.20	9.14
57.	(*Z*,*Z*)-Octadeca-9,12-dienoic acid (Linoleic acid)	2129	2.32	-	2.39	9.80	2.81	5.71	1.39
58.	Oleic acid	2140	10.57	31.36	13.36	12.01	16.17	12.27	13.30
59.	Octadecanoic acid	2159	3.51	6.87	1.82	7.22	1.43	2.24	0.28
60.	*cis*-Octadec-13-enoic acid	2185	0.11	1.44	0.54	2.62	-	-	0.04

[a]RI – retention indices relative to C_9-C_{25} alkanes; [b]HAFI – *Halopteris filicina;* [c]FUVI – *Fucus virsoides*; [d]DIPO – *Dictyopteris polypodioides*; [e]GOBA – *Gongolaria barbata*; [f]ERAM – *Ericaria amentacea*; [g]COBU – *Codium bursa*; [h]AMRI – *Amphiroa rigida*

Conclusions

In the conclusion, SC-CO_2 was shown as a successful extractant of less-volatile compounds, especially of fatty acids that were found in all of the analyzed extracts. Hexadecanoic acid was the most represented fatty acid in most of the extracts, with *Halopteris filicina* containing the highest amount. *Fucus virsoides* showed the dominance of oleic acid, while in *Dictyopteris polypodioides* 3-hexyl-4,5-dithiacycloheptanone was dominant and it was not detected in other extracts. The chlorophyll degradation products, such as (*E*)-phytol, were also detected in all of the anayzed extracts, except in *F. virsoides*. C_{13}-norisoprenoids, dihydroactinidiolide and hexahydrofarnesil acetone, that represent carotenoid degradation products, were also detected. The presence of some biologically active compounds, such as loliolide, dihydroactinidiolide, hexahydrofarnesyl acetone and different fatty acids that were found during the study, can suggest that CO_2 extracts can be used for different applications. Performed phytochemical study contributes to the knowledge of less-volatile composition of analyzed species indicating that the "species biodiversity" factor was the most influent, regardless of the classification to brown, green or red macroalgae. Further studies must be conducted to determine biological activity, as well as the potential of these extracts for the implementation in different products.

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