UTJECAJ SEZONE RASTA NA KEMIJSKI SASTAV I BIOLOŠKI POTENCIJAL ODABRANIH VRSTA SMEĐIH ALGI IZ JADRANSKOG MORA

Čagalj, Martina

SVEUČILIŠTE U SPLITU, SVEUČILIŠNI ODJEL ZA STUDIJE MORA
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Doktorski studij Primijenjene znanosti o moru

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Ova je disertacija izrađena u laboratorijima Sveučilišnog odjela za studije mora i Zavoda za prehrambenu tehnologiju i biotehnologiju Kemijsko-tehnološkog fakulteta Sveučilišta u Splitu pod vodstvom izv. prof. dr. sc. Vide Šimat i izv. prof. dr. sc. Danijele Skroza u sklopu Doktorskog studija „Primijenjene znanosti o moru" pri Sveučilištu u Splitu i Sveučilištu u Dubrovniku. Istraživanje je podržano od strane programa PRIMA u sklopu projekta „Bio-protective cultures and bioactive extracts as sustainable combined strategies to improve the shelf-life of perishable Mediterranean food" (BioProMedFood, ID: 1467). PRIMA program je pod pokroviteljstvom Europske unije.

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Sažetak

Smeđe alge su rasprostranjen, nedovoljno istražen i iskorišten te ekonomski vrijedan prirodni resurs. Prepoznate su kao izvor različitih skupina spojeva s dokazanim biološkim aktivnostima (antibakterijska, antioksidacijska, antiviralna, antitumorna, citotoksična aktivnost, itd.). Kemijski sastav algi ovisi o brojnim ekološkim čimbenicima koji variraju tijekom sezone rasta, o uvjetima predobrade algalnog materijala kao i različitim parametrima ekstrakcijskog procesa. U ovom istraživanju optimizirane su metode sušenja i odabrani uvjeti zelenih postupaka za ekstrakciju bioaktivnih tvari iz alge *Padina pavonica* s ciljem povećanja prinosa fenolnih spojeva, a time i njihove antioksidacijske aktivnosti. Metoda liofilizacije je odabrana kao najbolja metoda sušenja dok su se ekstrakcije potpomognute mikrovalovima i ultrazvukom pokazale učinkovitijima za dobivanje ekstrakata snažne biološke aktivnosti s višim sadržajem fenolnih spojeva. Po prvi put, izolirani ekstrakt *P. pavonica* korišten je za razvoj bioaktivnog premaza za filmove od polilaktične kiseline čime je potvrđen potencijal primjene ove alge u ambalažnim materijalima koji se koriste u prehrambenoj industriji. Kemijski sastav i biološka aktivnost algi *Cystoseira compressa* i *P. pavonica* pokazali su varijabilnost kroz sezonu rasta. Najviši sadržaj ukupnih fenolnih spojeva i najsnažnija antioksidacijska aktivnost zabilježeni su kod uzoraka branih u lipnju, a najveća antimikrobna aktivnost dokazana je za one brane u lipnju, srpnju i kolovozu, kada su izmjerene najviše temperature mora. Kemijski profil eteričnog ulja alge *C. compressa* mijenjao se sa sezonom rasta. Najviši udio masnih kiselina zabilježen je u svibnju i lipnju, dok je u srpnju i kolovozu zabilježen najviši udio alkohola. Istražen je i utjecaj postupka sušenja i sezone rasta na sastav hlapljivih organskih spojeva alge *Halopteris scoparia*. Uzorci svježih algi su se značajnije razlikovali kroz mjesece uzorkovanja u odnosu na uzorke suhih algi. U uzorcima vršnih para su dominantne skupine spojeva svježih i suhih algi bili alifatski spojevi i derivati benzena dok su u hidrodestilatima svježih algi najzastupljeniji bili alifatski spojevi u svibnju i rujnu te terpeni u lipnju, srpnju i kolovozu. Temeljem navedenog, rezultati ovih istraživanja ukazuju na činjenicu da su smeđe alge Jadranskog mora izvor bioaktivnih spojeva s potencijalnom primjenom u prehrambenoj i farmaceutskoj industriji.

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Ključne riječi: antioksidacijska aktivnost, antimikrobna aktivnost, smeđe alge, razvoj novog pakiranja, zelena ekstrakcija

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THE EFFECT OF THE GROWING SEASON ON CHEMICAL COMPOSITION AND BIOLOGICAL POTENTIAL OF SELECTED BROWN SEAWEEDS FROM THE ADRIATIC SEA

Martina Čagalj

Thesis performed at University of Split, University Department of Marine Studies and Department of Food Technology and Biotechnology, Faculty of Chemistry and Technology.

Abstract

Brown seaweeds are abundant, underexplored, underused and economically valuable natural resource. They are recognized as a source of various compounds with proven biological activity (antibacterial, antioxidant, antiviral, antitumor, cytotoxic activity, etc.). The chemical composition of seaweeds depends on a number of environmental factors that vary during the seasonal growth, on the pre-treatment of algal material and on the extraction parameters. In this study, the drying methods and the selected conditions of the green procedures for the extraction of bioactives from *Padina pavonica* were optimized with the aim of increasing the yield of phenolic compounds and its antioxidant activity. Freeze-drying was chosen as the best drying method, and extractions assisted by microwaves and ultrasound were found to be the most suitable to obtain extracts with strong biological activity and higher total phenolic content. For the first time, *P. pavonica* extract was used to develop a bioactive coating for polylactic acid films, confirming the potential of this seaweed for the use in packaging materials for food industry. The chemical composition and biological activity of *Cystoseira compressa* and *P. pavonica* showed variations throughout the growing season. The highest content of total phenolic compounds and the strongest antioxidant activity were found in June samples, and the highest antimicrobial activity in June, July and August samples, when the highest sea temperatures were recorded. The chemical profile of the *C. compressa* essential oil changed during seasonal growth. The highest percentage of fatty acids was recorded in May and June, while the highest percentage of alcohol was recorded in July and August. The influence of drying procedures and growing season on the composition of *Halopteris scoparia* volatile organic compounds was investigated. Fresh seaweed samples differed more significantly between sampling months than dry seaweed samples. In the headspace samples, the predominant groups of compounds of fresh and dry samples were aliphatic compounds and benzene derivatives, while in the hydrolats of fresh samples, aliphatic compounds were most represented in May and September and terpenes in June, July, and August. Brown seaweeds of the Adriatic Sea are a potential source of bioactive compounds that can be used in the food and pharmaceutical industries.

Thesis deposited in National and University Library in Zagreb and Split University Library.

Keywords: antioxidant activity, antimicrobial activity, brown seaweeds, new packaging development, green extraction

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Sadržaj

1. Pregled objavljenih radova

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2. Uvod

Morske alge su široko rasprostranjena skupina fotosintetskih organizama unutar koje, temeljem pigmenata koji su zastupljeni u njima, razlikujemo smeđe (Phaeophyceae), crvene (Rhodophyceae) i zelene (Chlorophyceae) alge. Smeđim algama pripada preko 3700 vrsta, od kojih preko 95% raste u morima i oceanima. Njihovi glavni fotosintetski pigmenti su klorofil *a*, *c1* i *c2*, međutim posjeduju i druge pigmente, poput fukoksantina koji daje njihovim stanicama smeđu boju te *β*-karotena i drugih karotenoida (Dawes, 2016). Istočnu obalu Jadranskog mora nastanjuje oko 170 vrsta smeđih algi. Dio su bentoske vegetacije stjenovitih podloga do dubine od 20 - 30 m. Vrste roda *Cystoseira* prevladavaju biomasom u Jadranskom moru, a uz njih su brojni i rodovi *Sargassum*, *Dictyota* i *Padina* (Antolić i sur., 2011; Battelli, 2015). Kod vrsta reda Dictyotales postoji izomorfna izmjena uspravnih, spljoštenih, parenhimatoznih talija. Posebnost ovog reda je modifikacija unilokularnih sporangija kako bi se proizvelo četiri do osam velikih aplanospora. Spolno razmnožavanje im je oogamno. Organizmi iz reda Fucales su parenhimatozni i rastu iz apikalne stanice. Haploidna generacija svedena je na jaje i spermij, dok je ostatak životnog ciklusa jedinki diploidan. Gamete se stvaraju u posebnim šupljinama, konceptakulima, a oplodnja je oogamna. Vrste reda Sphacelariales karakterizira apikalna meristematska stanica koja se dijeli poprečno kako bi proizvela stanice kćeri. Nespolno se razmnožavaju pomoću propagula, specijaliziranih grančica karakterističnog oblika, koje se stvaraju kroz vegetativne dijelove biljaka (Lee, 2008).

Morske alge se tradicionalno koriste već tisućama godina kao hrana u mnogim Azijskim zemljama poput Japana, Kine i Koreje, te u Irskoj i Škotskoj. Proizvodnja, odnosno uzgoj algi u svijetu je 2019. godine iznosio 34,7 milijuna tona, od čega je 97% proizvodnje otpadalo na uzgoj u Aziji. U istoj godini prikupljeno je svega 1,08 milijuna tona divljih jedinki (Cai i sur., 2021; McHugh, 2003). Za uzgoj algi nije potrebna složena oprema, kao ni hrana za rast, što je jedna od glavnih prednosti te ima vrlo malen štetni učinak na okoliš, riblje resurse i morsko dno. Alge se mogu integrirati u uzgojni sustav riba unutar kojeg apsorbiraju zaostale otopljene hranjive tvari iz nepojedene riblje hrane. Najčešće se uzgajaju u zoni plime i oseke koja je lako dostupna, početni kapital je nizak te se mogu prikupljati vrlo brzo nakon sadnje, već nakon šest tjedana (FAO, 2018). Osim za prehranu ljudi, alge se koriste i kao hrana za životinje. Suhe, mljevene alge se dodaju u različite pripravke kao hrana za životinje u različitim omjerima ili se svježim algama hrane druge uzgojne morske vrste, poput puzlatki. Također, iz algi se izoliraju mnogi kemijski spojevi koji se potom koriste u prehrambenoj,

kozmetičkoj i farmaceutskoj industriji za proizvodnju različitih proizvoda, poput polisaharida agara, alginata i karagenana (McHugh, 2003; Polat i sur., 2021).

Znanstvenici u budućnosti predviđaju problem nedostatka hrane zbog rasta globalne populacije, a već sada se prehrana velikog dijela populacije suočava s nedovoljnim unosima hranjivih tvari. Uzgoj i branje morskih algi se smatraju održivim iz nekoliko razloga: alge imaju veliku stopu rasta, njihov uzgoj ima neutralnu ili nisku emisiju stakleničkih plinova, a oceani i mora prekrivaju gotovo 70% površine Zemlje pa uzgoj ne zauzima poljoprivredno zemljište. Zbog svega navedenog, morske alge predstavljaju široko dostupan i potencijalno ekonomski vrijedan prirodni resurs (Matos i sur., 2021). Brojne vrste smeđih algi prepoznate su kao izvor spojeva, prvenstveno sekundarnih metabolita, različitih bioloških aktivnosti (antioksidacijske, antimikrobne, antiviralne, antitumorne i citotoksične) koje ih čine iznimno zanimljivima za primjenu u prehrambenoj industriji, proizvodnji dodataka prehrani, terapeutika i farmaceutika. Smeđe alge su bogat izvor fenolnih spojeva, od jednostavnih fenolnih kiselina do složenijih polimera kao što su tanini (uglavnom florotanini). Florotanini prisutni u smeđim algama su polimeri floroglucinola i predstavljaju heterogenu skupinu spojeva visoke molekularne mase (od 126 Da do 650 kDa) čija kemijska struktura nije prisutna u kopnenim biljkama (Generalić Mekinić i sur., 2019; Maggio i sur., 2020). Oni imaju iznimno važnu ulogu u staničnom rastu i zacjeljivanju tkiva algi, ali također ih karakteriziraju i snažna biološka svojstva (Abdelhamid i sur., 2018; Messina i sur., 2019; Šimat i sur., 2020; Stiger-Pouvreau i sur., 2014). Biološka aktivnost fenolnih spojeva iz kopnenih biljaka je dobro proučena, dok je proučavanje fenolnih spojeva koje sadrže alge tek nedavno postalo predmetom ciljanih istraživanja (Generalić Mekinić i sur., 2019). Osim fenolnih spojeva, smeđe alge sadrže i druge visokovrijedne komponente poput polisaharida, lipida, pigmenata i vitamina. Iz skupine lipida osobito se ističu višestruko nezasićene masne kiseline (PUFA), poput eikozapentaenske (EPA), dokozaheksaenske (DHA) i linolenske kiseline. Smatra se da PUFA učinkovito smanjuju rizik od pojave kardiovaskularnih bolesti, raka, osteoporoze i dijabetesa. Također, utječu i na razvoj živčanog sustava te su korisne u liječenju depresije i drugih mentalnih poremećaja (Šimat i sur., 2020). Zabilježeno je *in vitro* antimikrobno djelovanje PUFA protiv bakterija, virusa i plijesni. Smeđe alge imaju relativno nizak udio masnih kiselina, dok im je udio polisaharida poput laminarina, fukoidana i alginata izrazito visok. Ovi polisaharidi imaju široku primjenu u različitim industrijama zbog svojih funkcionalnih, ali i bioloških svojstava (Matos i sur., 2021; Šimat i sur., 2020).

Kemijski sastav algi ovisi o brojnim čimbenicima kao što su temperatura, salinitet, UV zračenje, sezona sakupljanja algi, vegetacijsko razdoblje i razvoj talusa, dubina, zemljopisni položaj, itd. Varijabilnost ovih parametara tijekom sezone rasta algi utječe i na proizvodnju različitih kemijskih spojeva, a osobito sekundarnih metabolita. Individualni i sinergistički učinci navedenih parametara na kemijski profil i biološku aktivnost smeđih algi su još uvijek nedovoljno istraženi. Novije studije su pokazale kako sezonske promjene i vegetativni dijelovi talusa imaju značajan utjecaj na kemijski profil algi (Kumar i sur., 2015). U smeđim fukoidnim algama tijekom toplih i suhih ljetnih razdoblja kada su temperature mora povišene zabilježeni su viši udjeli pojedinih nutrijenata, fenola, PUFA, vitamina i minerala (Gosch i sur., 2015; Mancuso i sur., 2019; Praiboon i sur., 2018).

Posljednjih godina istraživanja su usmjerena na identifikaciju novih spojeva iz algi, dok su priprema standardiziranih protokola za sušenje i optimizacija parametara ekstrakcije algi uglavnom zanemareni, iako je poznato da znatno utječu na količinu, sastav i biološku aktivnost izoliranih spojeva/smjesa spojeva (Generalić Mekinić i sur., 2019). Prije nego što se podvrgnu ekstrakciji, alge se tradicionalno suše na suncu, u hladu ili u sušionicima uz kontrolu temperature. Sušenje bi se trebalo odvijati na niskim temperaturama kako bi se spriječila razgradnja spojeva osjetljivih na toplinu poput PUFA, karotenoida i polifenola, stoga je jedna od predloženih metoda sušenja za njihovo očuvanje liofilizacija. Liofilizacija je metoda sušenja koja se provodi na niskim temperaturama (-50 °C) i u vakuumu kod koje se voda iz materijala uklanja sublimacijom, odnosno direktnim prijelazom iz krute u plinovito stanje. Navedenim postupkom se sprječavaju reakcije oksidacije kao i degradacije termolabilnih spojeva (Amorim i sur., 2020; Ummat i sur., 2021).

Nakon sušenja se obično provodi ekstrakcija ciljanih spojeva algi korištenjem različitih metoda i parametara ekstrakcije (otapalo, temperatura, vrijeme, tlak, pH) koje utječu na sastav i sadržaj bioaktivnih spojeva te njihovu aktivnost. Ekstrakcija je proces prijenosa mase koji uključuje difuziju odabranog otapala u algalni materijal, otapanje bioaktivnih spojeva u otapalu te odvajanje ekstrahiranih spojeva iz otapala. Učinkovitost procesa ekstrakcije ovisi o snazi otapala u razaranju stanica, fizikalno-kemijskim svojstvima ciljanih spojeva i navedenim parametrima ekstrakcije. Otapalo također igra važnu ulogu u lizi stanica pa agresivna otapala mogu povećati prinos ekstrakcije (Henriques i sur., 2007; Ummat i sur., 2021). Odabir otapala temelji se na svojstvima ciljanog spoja i namjeni, odnosno primjeni spoja ili ekstrakta nakon ekstrakcije.

Alge se obično ekstrahiraju konvencionalnim postupcima kao što je maceracija, često popraćena miješanjem (Phasanasophon & Kim, 2018). Problemi konvencionalnih metoda ekstrakcije su korištenje velikih količina organskih otapala, dugo trajanje i u konačnici mali prinos ciljanih spojeva. Kako bi se postigle što sigurnije i ekološki prihvatljive ekstrakcije, upotreba toksičnih organskih otapala se nastoji smanjiti i postupno zamijeniti manje štetnim otapalima poput etanola, etil acetata i acetona (Corrêa i sur., 2021). Osim što je ekološki prihvatljivo otapalo, Uprava za hranu i lijekove Sjedinjenih Američkih Država (engl. *U.S. Food and Drug Administration*, FDA) je označila etanol kao općenito priznat kao siguran (engl. *Generally recognised as safe*, GRAS) spoj za upotrebu u prehrambenoj industriji (FDA, 2022). Stoga, posljednjih godina znanstvenici razvijaju i optimiziraju nove zelene metode ekstrakcije.

Poboljšanje industrijske eksploatacije algi zahtijeva izradu standardiziranih protokola za sušenje algi i ekstrakciju ciljanih spojeva te određivanje utjecaja sezone rasta algi na njihov kemijski sastav i biološke aktivnosti. Morske alge su potencijalno održivi izvor prirodnih antioksidansa i antimikrobnih agensa koji se mogu koristiti u prehrambenoj industriji, bilo za poboljšanje nutritivne vrijednosti i kreiranje funkcionalnih proizvoda, sprječavanje kvarenja proizvoda i produljenja vijeka njegova trajanja ili pak za razvoj novih održivih materijala za pakiranje hrane.

2.1. Ciljevi objavljenih radova

Rad 1:

- Istražiti i usporediti različite metode sušenja alge *Padina pavonica* u cilju povećanja prinosa fenolnih spojeva i antioksidacijske aktivnosti.
- Primijeniti zelene postupke za ekstrakciju spojeva iz algi te ih usporediti s konvencionalnim metodama.
- Optimizirati parametre ekstrakcije (vrijeme, izbor otapala).

Rad 2:

- Istražiti kemijski sastav alge *Cystoseira compressa*.
- Utvrditi postoji li utjecaj sezone rasta (svibanj - rujan) na kemijski sastav, antioksidacijsku i antimikrobnu aktivnost navedene alge.

Rad 3:

- Izolirati i analizirati isparljive organske spojeve alge *Halopteris scoparia*.
- Odrediti utjecaj sušenja i sezone rasta (svibanj - rujan) na sastav isparljivih organskih spojeva

Rad 4:

- Utvrditi postoji li utjecaj sezone rasta (svibanj - rujan) na kemijski sastav, sadržaj ukupnih fenola i antioksidacijsku aktivnost alge *P. pavonica*.
- Razviti premaz od hitozana s inkorporiranim ekstraktom alge *P. pavonica* za filmove od polilaktične kiseline te odrediti antioksidacijsku aktivnost novo razvijenog aktivnog filma.

Rad 5:

- Izolirati i analizirati eterično ulje alge *C. compressa* te utvrditi utjecaj sezone rasta (svibanj - kolovoz) na kemijski profil ulja.

3. Materijal i metode

3.1. Metode sušenja i ekstrakcije algi

Odabir metode sušenja potrebno je prilagoditi samoj vrsti alge, kao i njenoj budućoj primjeni. Parametri sušenja, temperatura i vrijeme, značajno utječu na sastav bioaktivnih spojeva algi. U ovom istraživanju uspoređivane su tradicionalne metode sušenja, sušenje na zraku i u sušioniku s liofilizacijom kao novom metodom koja omogućuje bolje očuvanje termolabilnih spojeva. Nakon sušenja, alge su podvrgnute ekstrakciji. Nadalje, uspoređene su konvencionalne metode ekstrakcije i novi zeleni postupci te istraženi optimalni parametri. Kao novi zeleni postupci ekstrakcije u ovom istraživanju korišteni su ekstrakcija potpomognuta ultrazvukom (engl. *Ultrasound assisted extraction*, UAE) i ekstrakcija potpomognuta mikrovalovima (engl. *Microwave assisted extraction*, MAE) (Gullón i sur., 2020; Šimat i sur., 2021). UAE koristi snagu ultrazvučnih valova koji prolaze kroz tekuće, čvrste i plinovite medije i uzrokuju područja negativnog tlaka. Kada je taj tlak veći od vlačne čvrstoće tekućine, dolazi do kavitacije, stvaraju se mjehurići pare koji implodiraju pod utjecajem jakog ultrazvučnog polja (Kadam i sur., 2015). UAE na ovaj način značajno poboljšava prodiranje otapala u stanice, a time i ekstrakciju ciljanih spojeva, postižući veći prinos ekstrakcije (Mason i sur., 1996; Vernès i sur., 2020). Ova metoda ekstrakcije ne zahtjeva složenu opremu što ju čini prikladnom i za industrijsku primjenu (Gullón i sur., 2020; Kadam i sur., 2015). Elektromagnetski valovi kod MAE uzrokuju promjene u staničnoj strukturi, povećavaju temperaturu i tlak unutar stanice što dovodi do pucanja stanične membrane pa otapalo ubrzano prodire u stanicu i ekstrahira njen sadržaj (Veggi i sur., 2013; Vernès i sur., 2020). Otapalo je jedan od najvažnijih čimbenika MAE. Korištenje otapala s većom dielektričnom konstantom i dielektričnim gubitkom povećava sposobnost otapala da apsorbira mikrovalnu energiju što dovodi do njegovog bržeg zagrijavanja. Osim toga, moguće je kombinirati različita otapala kako bi se povećao prinos (Routray & Orsat, 2012). Prilikom MAE smanjuje se potrošnja otapala i skraćuje vrijeme procesa, ali se troši više energije u usporedbi s konvencionalnim postupcima ekstrakcije (Gullón i sur., 2020). Kako bi se povećao prinos ciljanih spojeva ponekad se koristi i/ili objedinjuje nekoliko različitih postupaka ekstrakcije. Nedavna istraživanja su pokazala kako ekstrakti algi dobiveni novim zelenim postupcima ekstrakcije imaju bolje biološke aktivnosti (npr. antioksidacijsku i antimikrobnu aktivnost) u odnosu na ekstrakte dobivene konvencionalnim metodama (Gullón i sur., 2020; Kadam i sur., 2015; Yuan i sur., 2018).

Eterična ulja, mješavine hlapljivih spojeva, se izoliraju iz algalnog materijala destilacijom. U ovom istraživanju korištena je hidrodestilacija tijekom koje je alga u izravnom kontaktu s vodom tijekom zagrijavanja, a hlapljivi spojevi se izoliraju u vodenoj pari i kapljicama lipida te se kondenziraju u hladilu i skupljaju u trapu u središnjoj cijevi modificirane aparature po Clevengeru. Hidrodestilacijom se troši značajna količina energije te je proces dugotrajan, ali nisu potrebne kemikalije, što čini ovu metodu ekološki prihvatljivom (Politeo i sur., 2023). Hlapljivi spojevi se mogu izolirati iz uzorka algi i tehnikom izolacije vršnih para. Vršne pare su najisparljiviji spojevi u atmosferi iznad ili oko uzoraka koji se izdvajaju uz kontinuirano lagano zagrijavanje. Tijekom mikroekstrakcije vršnih para na čvrstoj fazi se koristi silikonsko vlakno presvučeno polimernim filmom koje se nalazi unutar igle. Isparljivi spojevi se adsorbiraju na vlakno koje se izvlači iz igle i postavlja iznad uzorka tijekom zagrijavanja. Nakon završetka ekstrakcije vlakno se uvlači u iglu, igla se uvodi u injektor kromatografa te se ekstrahirani spojevi desorbiraju s vlakna pomoću topline (Pawliszyn, 2012).

3.2. Metode identifikacije kemijskih spojeva

Karakterizacija i identifikacija spojeva iz algi izrazito je složeno područje jer u algama pronalazimo cijeli spektar različitih biološki aktivnih spojeva. Glavne analitičke tehnike koje se koriste za analizu su: UV/Vis spektrofotometrija te tekućinska kromatografija (engl. *liquid chromatography*, LC) i plinska kromatografija (engl. *gas chromatography*, GC) u kombinaciji s različitim detektorima, prvenstveno spektrofotometrijom masa (engl. *mass spectrometry*, MS) (Dunn i sur., 2017).

3.2.1. Spektrofotometrijske metode

Spektrofotometri koriste izvor svjetlosti za osvjetljavanje uzorka svjetlom preko UV do vidljivog raspona valnih duljina te mjere svjetlost koju uzorak apsorbira, prenosi ili reflektira na određenoj valnoj duljini. Spektrofotometrijske metode su ekonomične, jednostavne, brze i tehnički lako izvedive metode kojima se provodi probir velikog broja uzoraka s ciljem dobivanja kemijskog indeksa i informacije o koncentraciji neke grupe spojeva kao što su na primjer ukupni fenoli, flavonoidi i drugi (Matić i sur., 2017). Koncentracija fenola u ekstraktima algi najčešće se određuje spektrofotometrijski kao sadržaj ukupnih fenolnih spojeva (engl. *total phenolic content*, TPC) pomoću Folin-Ciocalteu testa (Hermund, 2018). Međutim, druge koekstrahirane nefenolne tvari (aminokiseline, proteini, tioli, derivati vitamina, i dr.) mogu imati sposobnost reduciranja reagensa što dovodi do netočnih, najčešće puno viših vrijednosti TPC u uzorcima (Everette i sur., 2010; Singleton i sur., 1999). TPC metoda nije specifična i selektivna pa je dobivene informacije vrlo često potrebno potvrditi kromatografskim tehnikama (Matić i sur., 2017). Ujedno postoji i problem usporedbe rezultata različitih studija dobivenih ovom metodom obzirom da se vrlo često za iskazivanje rezultata te kao standarde za izradu kalibracijskih pravaca koriste različiti spojevi (galna kiselina, floroglucinol). Znanstvenici također izražavaju rezultate na masu suhe alge, svježe alge, suhog ekstrakta ili volumen mokrog ekstrakta što dodatno otežava usporedbu rezultata različitih studija (Generalić Mekinić i sur., 2019). Sadržaji ukupnih flavonoida (TFC) i tanina (TTC) se također određuju neselektivnim spektrofotometrijskim metodama.

3.2.2. Kromatografske metode

Kromatografske metode se koriste za odjeljivanje individualnih fenolnih i/ili drugih spojeva u ekstraktima na temelju različite raspodjele komponenata u uzorku između

nepokretne i pokretne faze (tekućina ili plin). One koriste specifične karakteristike molekula (adsorpcija (nepokretna faza je krutina), raspodjela (nepokretna faza je tekućina vezana na čvrstu površinu), afinitet (ligand vezan na nepokretnu fazu), ionski naboj (suprotno nabijena nepokretna faza) i molekulska masa) za njihovu izolaciju i odvajanje iz smjesa (Corrêa i sur., 2021). Identifikacija i kvantifikacija fenolnih spojeva prilično je kompleksna zbog njihove strukturne sličnosti, reaktivnosti s drugim spojevima i velike molekulske mase (Generalić Mekinić i sur., 2019).

3.2.2.1. Tekućinska kromatografija

Visoko djelotvorna tekućinska kromatografija (HPLC) je izvrsna tehnika zbog toga što postiže brzu i preciznu kvantitativnu analizu, može se automatizirati i ima nisku granicu detekcije. Ipak, nedostatak komercijalno dostupnih standarda ograničava upotrebu tekućinkse kromatografije (LC) za identifikaciju i kvantifikaciju florotanina u ekstraktima algi. Ovaj nedostatak čini spektrofotometriju masa uglavnom kvalitativnim alatom za njihovu analizu i identifikaciju. Za promatranje fragmenata fenolnih spojeva i brzu analizu ovih nehlapljivih, polarnih i termolabilnih spojeva, koristi se obično tandemska spektrometrija masa (MS/MS) povezana s različitim LC sustavima (Agregán i sur., 2017; Ford i sur., 2019). Kvantifikacija se najčešće vrši trostrukim kvadrupolom ili kvadrupol-ionskom zamkom. Identifikacija nepoznatih spojeva najčešće se provodi kvadrupol-analizatorom vremena leta (engl. *quadrupol time of flight*, QTOF). Novije izvedbe QTOF-a imaju djelotvornost visoke rezolucije i, osim velike moći identifikacije, koriste se i za kvantifikaciju spojeva.

3.2.2.2. Plinska kromatografija

Jedna od analitičkih metoda koje se koriste u analizi i karakterizaciji isparljivih spojeva (ugljikovodici, alkoholi, aldehidi i ketoni, derivati masnih kiselina, terpeni, derivati pigmenata, itd.) u algama je plinska kromatografija (GC). Ova metoda osigurava visoku osjetljivost i ponovljivost za odvajanje i detekciju različitih spojeva iz algi (Du i sur., 2018; Vendruscolo i sur., 2018). GC-om se odijele isparljive i poluisparljive komponente iz smjese, ali nije ih moguće identificirati. Zbog toga se na GC sustav veže MS kao detektor koji analizira ione komponenti. Softver za obradu podataka je vezan s bazom podataka te se usporedbom spektra masa pojedine komponente s bazom podataka dobiva informacija o molekulskoj strukturi komponente kao i postotak vjerojatnosti. Na taj način se vrši identifikacija (Al-Rubaye i sur., 2017).

3.3. Metode za određivanje biološke aktivnosti

Fenolni spojevi koje sintetiziraju alge, jedna od najvećih i najraznovrsnijih skupina fitokemikalija, su zbog svoje biološke aktivnosti i dobrobiti za ljudsko zdravlje privukli posebnu pozornost (Cotas i sur., 2020). Uz fenolne spojeve, i drugi spojevi koje nalazimo u ekstraktima algi poput polisaharida i pigmenata su također zanimljivi zbog dokazane pozitivne biološke aktivnosti. Mnoga istraživanja potvrđuju antioksidacijsku i antimikrobnu aktivnost različitih ekstrakata algi (Generalić Mekinić i sur., 2019; Güner i sur., 2015; Hermund, 2018; Jacobsen i sur., 2019; Maggio i sur., 2020).

3.3.1. Antioksidacijska aktivnost

Antioksidansi se prema mehanizmima djelovanja mogu svrstati u primarne ili sekundarne antioksidanse. Primarni antioksidansi izravno reagiraju sa slobodnim radikalima pretvarajući ih u stabilnije, neradikalne produkte. Imaju važnu ulogu u oksidaciji lipida jer nastale lipidne radikale mogu pretvoriti u stabilnije produkte i spriječiti njihovu daljnju razgradnju (Decker, 2002; Hermund, 2018). Fenolni spojevi s više od jedne hidroksilne skupine su učinkoviti primarni antioksidansi zbog svoje sposobnosti doniranja atoma vodika slobodnim radikalima čime nastaju relativno nereaktivni fenoksilni radikali (Hermund, 2018). Sekundarni ili preventivni antioksidansi neizravno ograničavaju oksidaciju lipida putem nekoliko mehanizama: keliranjem iona prijelaznih metala, gašenja singletnog kisika i *hvatanja* kisika. Neki sekundarni antioksidansi mogu regenerirati primarne antioksidanse i time nanovo aktivirati njihovu antioksidacijsku sposobnost (Decker, 2002; Hermund, 2018).

Za ispitivanje antioksidacijske aktivnosti nekog uzorka koristi se obično više metoda s različitim mehanizmima djelovanja. Antioksidacijske metode se, obzirom na različite mehanizme djelovanja antioksidansa, dijele u dvije kategorije: metode temeljene na prijenosu elektrona (engl. *electron transfer*, ET) i metode temeljene na prijenosu atoma vodika (engl. *hydrogen atom transfer*, HAT). Redukcijska sposobnost antioksidansa se mjeri ET metodama, dok se antiradikalna aktivnosti mjeri HAT metodama (Granato i sur., 2018; Prior i sur., 2005). Većina metoda za određivanje antioksidacijske aktivnosti *in vitro* je jednostavna, brza i ekonomična.

Sposobnost ekstrakta da reducira feri ione željeza (Fe^{3+}) u fero oblik (Fe^{2+}), tj. redukcijska snaga ekstrakta se mjeri FRAP (engl. *ferric reducing/antioxidant power*) metodom koja je prema mehanizmu djelovanja ET metoda. Sposobnost antioksidansa u

hvatanju (neutraliziranju) molekula slobodnog 2,2-difenil-1-pikrilhidrazil (DPPH) radikala se mjeri DPPH metodom, koja uz ORAC (engl. *oxygen radical absorbance capacity*) metodu spada u HAT metode. ORAC metodom se mjeri kapacitet antioksidansa za neutralizaciju slobodnog peroksil radikala koji nastaje u reakcijskom sustavu (Prior i sur., 2005).

3.3.2. Antimikrobna aktivnost

Bakterije su najčešći uzročnici bolesti koje se prenose hranom, a u najčešće patogene bakterije ubrajaju se *Escherichia coli, Salmonella enteritidis, Enterococcus faecalis, Listeria monocytogenes, Staphylococcus aureus* i *Bacillus cereus* (Bintsis, 2017). *In vitro* metode za određivanje antimikrobne aktivnosti se provode na mikroorganizmima koji uzrokuju bolest ili kvarenje hrane. Metoda serijskog razrjeđivanja kojom se testira sposobnost mikroorganizama da proizvedu vidljivi rast u bujonu (razrjeđivanje bujona) koji sadrži serijska razrjeđenja uzorka se koristi za određivanje minimalne inhibitorne koncentracije (MIC). Najniža koncentracija uzorka koja u definiranim *in vitro* uvjetima sprječava vidljiv rast mikroorganizma unutar definiranog vremenskog razdoblja definirana je kao MIC vrijednost. Ova metoda namijenjena je testiranju čistih kultura aerobnih bakterija koje se lako uzgajaju inkubacijom preko noći u bujonu i dobro rastu u standardiziranim posudama za razrjeđivanje koje sadrže Mueller-Hinton bujon (International Organization for Standardization, 2019).

Minimalna baktericidna koncentracija (MBC) uzorka određuje se kao najniža koncentracija pri kojoj nije otkriven rast mikroorganizama nakon subkultivacije bakterijske suspenzije pipetirane iz jažica u kojima je određen MIC i iz jažica s višim koncentracijama uzorka (Elez Garofulić i sur., 2021).

3.4. Primjena ekstrakata u aktivnim pakiranjima

Posljednjih godina pojačano se istražuje razvoj novih održivih materijala za pakiranje i njihova integracija s novim rješenjima kako bi se smanjio utjecaj na okoliš (u smislu biorazgradivosti i podrijetla sirovina za proizvodnju ambalaže) u prehrambenoj industriji. Osim ekoloških aspekata, nova rješenja za pakiranje bi također trebala biti funkcionalna i pružiti izravnu korist potrošačima produljenjem roka trajanja hrane, poboljšavanjem sigurnosti i nadzorom kvalitete hrane (Soltani Firouz i sur., 2021). Europska komisija je definirala aktivno pakiranje kao pakiranje koje produljuje rok trajanja ili održava i poboljšava stanje hrane, osmišljeno tako da sadrži komponente koje se otpuštaju ili u hranu ili u okolinu koja okružuje hranu (European Commission, 2009). Implementacija novih koncepata u aktivnom pakiranju se može ostvariti razvijanjem novih bio-premaza. Aktivni premazani filmovi s antioksidacijskim ili antimikrobnim svojstvima stupaju u interakciju sa sastojcima hrane čime utječu na procese kvarenja (Bastarrachea i sur., 2015; Wyrwa & Barska, 2017). Biopolimeri koji se koriste za razvoj novih rješenja pakiranja trebaju biti ekonomični, lako dostupni i obnovljivi.

Polilaktična kiselina (PLA), termoplastični biorazgradivi poliester, dobiva se iz obnovljivih izvora. PLA je jedna od najčešće korištenih bioplastika zbog svojih mehaničkih i fizikalnih svojstava. Za postizanje aktivne funkcionalnosti i biorazgradivosti ambalaže za hranu, najčešće korišteni biopolimeri su polisaharidi, poput hitozana (Deshmukh i sur., 2017; Garcia-Garcia i sur., 2020; García Ibarra i sur., 2016; Kehinde i sur., 2022). Hitozan se dobiva iz nusproizvoda nastalih tijekom prerade morskih rakova, lako je dostupan, prirodan i biorazgradiv (Rathod i sur., 2022). Međutim, hitozan ima neka ograničenja, poput niskog antioksidativnog kapaciteta. Stoga se pokazalo da koloidni kompleksi koji su kombinacija hitozana i drugih tvari, kao što su ekstrakti algi, mogu prevladati navedene nedostatke (Ajdnik i sur., 2021; Potrč i sur., 2020). U ovom istraživanju su se, po prvi puta u ovakvoj formulaciji, razvili PLA filmovi s premazom od hitozana inkorporiranog ekstraktom alge *P. pavonica*.

4. Sažeti pregled rezultata objedinjenih radova

4.1. Rad 1

U ovom radu istražen je učinak tri metode sušenja (liofilizacija, sušenje u sušioniku i sušenje na zraku u sjeni) i tri metode ekstrakcije (konvencionalna ekstrakcija uz miješanje, UAE i MAE) na sadržaj ukupnih fenola, flavonoida i tanina te antioksidacijski potencijal ekstrakata smeđe alge *Padina pavonica*. Kao otapalo su korišteni voda, te 50%-tni i 70%-tni etanol. Parametri ekstrakcija su bili slijedeći: i) miješanje 24 sata pri sobnoj temperaturi u mraku, ii) miješanje pri temperaturi od 60 °C tijekom 1 i 2 sata, iii) UAE pri frekvenciji od 40 kHz i temperaturi od 60 °C tijekom 1 i 2 sata, iv) MAE u uređaju za ekstrakciju pomoću mikrovalova pri snazi od 200W i temperaturi od 60 °C tijekom 5, 10, 15 i 30 minuta. Vrijednosti TPC, TFC i TTC ekstrakata alge *P. pavonica* bile su u rasponu od 0,44 do 4,32 mg ekvivalenata galne kiseline (GAE)/g suhe alge, od 0,31 do 2,87 mg ekvivalenata kvercetina (QE)/g suhe alge, odnosno od 0,32 do 10,41 mg ekvivalenata katehina (CE)/g suhe alge. Obzirom na metodu sušenja, najviše vrijednosti TPC su zabilježene za ekstrakte pripremljene od liofiliziranih uzoraka. U slučaju usporedbe među korištenim ekstrakcijskim otapalima, ekstrakti pripravljeni korištenjem 50%-tnog etanola su imali najviše vrijednosti TPC. Očekivano, korištenje mikrovalova tijekom ekstrakcije je rezultiralo ekstraktima s višim TPC. Najveća vrijednost TPC dobivena je u liofiliziranom uzorku ekstrahiranom pomoću MAE tijekom 5 minuta u 50%-tnom etanolu. Također, liofilizacija se pokazala i kao najbolja metoda sušenja za očuvanje flavonoida i tanina. Najpogodnijim otapalom za TFC se pokazao 70%-tni etanol dok su korištenjem vode dobiveni ekstrakti s najvišim sadržajem TTC. Ekstrakcije potpomognute ultrazvukom i mikrovalovima su se pokazale učinkovitijima za dobivanje ekstrakata s visokim udjelom flavonoida i tanina u usporedbi s konvencionalnim metodama. Antioksidacijski potencijal ekstrakata određen je korištenjem FRAP, DPPH i ORAC metoda. FRAP vrijednosti ekstrakata su bile u rasponu od 98,46 do 537,95 μM ekvivalenata troloksa (TE). Najviše vrijednosti su zabilježene kod ekstrakata pripravljenih od liofiliziranih uzoraka i ekstrahiranih korištenjem 50 i 70%-tnog etanola za sve metode ekstrakcije. Najveća vrijednost je zabilježena u ekstraktu pripremljenom pomoću 70%-tnog etanola koristeći MAE tijekom 30 minuta. Sposobnost ekstrakata za *hvatanje* slobodnog DPPH radikala se kretala od 2,33 do 62,88%. Najviše vrijednosti su zabilježene za ekstrakte liofiliziranih uzoraka pripravljene u 50%-tnom etanolu. Sposobnost ekstrakata za hvatanje peroksil radikala, mjerena ORAC metodom, je bila u rasponu od 8,57 do 71,37 μM TE.

Liofilizirani uzorci su imali dva do tri puta veće ORAC vrijednosti od uzoraka sušenih drugim metodama. Iako je najveća ORAC vrijednost zabilježena za liofilizirani uzorak ekstrahiran pomoću 50%-tnog etanola uz miješanje pri temperaturi od 60 °C tijekom jednog sata, slične vrijednosti su zabilježene za liofilizirani uzorak ekstrahiran pomoću 50% etanola i MAE tijekom 10 minuta. Temeljem navedenog je evidentno da korištenje ovog zelenog postupka ekstrakcije smanjuje vrijeme ekstrakcije čak šest puta bez utjecaja na sposobnost ekstrakta alge *P. pavonica* u *hvatanju* peroksil radikala.

4.2. Rad 2

U ovom radu istražen je učinak sezonskog rasta (od svibnja do rujna) na *in vitro* antioksidacijsku (FRAP, DPPH i ORAC) i antimikrobnu aktivnost (MIC i MBC) ekstrakata smeđe alge *Cystoseira compressa*. Također, analiziran je i kemijski profil ekstrakata pomoću uređaja UPLC-PDA-ESI-QTOF te su određeni TPC i TTC. Uzorci algi su prikupljeni svaki mjesec na istoj lokaciji i dubini te liofilizirani kako bi se suhi materijal mogao koristiti u daljnjem istraživanju. Uzorci su ekstrahirani uz pomoć mikrovalova i 50%-tnog etanola pri snazi od 200W i temperaturi od 60 °C tijekom 15 minuta. Dominantni spojevi u ekstraktima su bile masne kiseline, od kojih je dominirala oleinska kiselina (C18:1*n-9*) u udjelu višem od 15% u svim uzorcima, a najviša vrijednost je zabilježena u uzorku iz svibnja. Od ostalih spojeva u značajnoj količini su bile zastupljene palmitoleinska kiselina (C16:1*n-7*) i palmitinska kiselina (C16:0), također s najvišim udjelima u svibnju. U ekstraktima je pronađena i omega-3 masna kiselina, eikozapentaenska kiselina (EPA), čiji je najviši sadržaj detektiran u uzorku iz srpnja. Vrijednosti TPC ekstrakata su se kretale između 48,2 i 83,4 mg GAE/g suhog ekstrakta dok su rezultati TTC bili u rasponu od 2,0 do 8,8 mg CE/g suhog ekstrakta. Najveće TPC i TTC vrijednosti su zabilježene za ekstrakte uzoraka algi prikupljenih u lipnju. Također, najviša FRAP vrijednost od 2,7 ± 0,1 mM TE je zabilježena za lipanj. TPC i FRAP vrijednosti su bile u korelaciji (0,956; $p < 0,01$). Najviša ORAC vrijednost od 72,1 ± 1,2 μM TE dobivena je za ekstrakte uzoraka iz kolovoza. Općenito, svi uzorci su pokazali iznimno visoku sposobnost *hvatanja* slobodnog DPPH radikala (>80% inhibicije). Antimikrobna aktivnost ekstrakata je određena protiv patogenih bakterija koje se prenose hranom: *E. coli* ATCC 25922, *S. enteritidis* ATCC 13076, *E. faecalis* ATCC 29212, *L. monocytogenes* ATCC 7644, *S. aureus* ATCC 25923 i *B. cereus* ATCC 14579. Gram pozitivne bakterije su bile osjetljivije na ekstrakte alge *C. compressa* od gram negativnih bakterija. Najniže MIC vrijednosti (2,5 mg/mL) su zabilježene protiv bakterije *L. monocytogenes* za ekstrakte uzoraka iz lipnja, srpnja i kolovoza, kada je temperatura mora bila najviša, dok je najniži MBC (2,5 mg/mL) za istu bakteriju zabilježen za ekstrakte uzoraka iz lipnja. Također, iste MIC i MBC vrijednosti su zabilježene protiv bakterije *S. aureus* za ekstrakte uzoraka iz lipnja i srpnja. Rezultati potvrđuju najbolju antibakterijsku aktivnost za uzorke algi brane u najtoplijim mjesecima protiv svih bakterija.

4.3. Rad 3

U ovom istraživanju su prvi put izolirani isparljivi organski spojevi (engl. *volatile organic compounds*, VOC) smeđe alge *Halopteris scoparia* prikupljene od svibnja do rujna na istom lokalitetu. Alga se analizirala svježa i suha. Frakcija VOC je izolirana postupkom mikroekstrakcije vršnih para na čvrstoj fazi (HS-SPME) koristeći dva vlakna i hidrodestilacijom (HD) u modificiranoj aparaturi po Clevengeru. Uzorci su analizirani plinskom kromatografijom-spektrofotometrijom masa (GC–MS). U ovom istraživanju je po prvi put proučavan utjecaj sezone rasta (svibanj - rujan) i sušenja na kemijski sastav VOC-a, a dobiveni podaci su obrađeni analizom glavnih komponenti (PCA). Rezultati uzoraka svježih algi dobiveni pomoću HS-SPME na različitim vlaknima su se značajno razlikovali između mjeseci uzorkovanja, za razliku od uzoraka suhih algi. Identificirani spojevi detektirani u VOC mogu se klasificirati u šest različitih skupina, i to: zasićeni alifatski spojevi, nezasićeni alifatski spojevi, derivati benzena, terpeni, C13-norizoprenoidi (produkti razgradnje karotenoida) i ostali spojevi. Dominantne skupine spojeva u uzorcima vršnih para su bili alifatski spojevi i derivati benzena, osim u uzorku svježe alge iz lipnja i kolovoza kada su dominirali derivati benzena. Najzastupljeniji spojevi u vršnim parama su bili benzaldehid (57,9% u kolovozu), pentadekan (kemijski marker smeđih algi; 29,3% u rujnu) i pentadec-1-en (53,2% u rujnu). Sadržaj benzaldehida se sušenjem smanjio, dok je sadržaj benzilnog alkohola porastao (najviše u svibnju, 6,5%). Udio pentadekana i heptadekana, dva zasićena alifatska spoja, se povećao nakon sušenja, dok se udio pentadeka-1-ena smanjio u svim uzorcima, bez obzira na mjesec branja. Sadržaj oktan-1-ola se smanjio od svibnja do rujna ali je pokazao i najviše varijabilnosti rezultata. Od spojeva iz skupine terpena, seskviterpen germakren D i diterpen fitan su bili dominantni. U hidrodestilatu svježe alge najzastupljeniji su bili terpeni s 32,9, 39,8 i 51,8% u lipnju, srpnju i kolovozu, dok su u svibnju i rujnu dominirali nezasićeni alifatski spojevi s 30,9 i 48,2%. (*E*)-Fitol je bio najzastupljeniji spoj tijekom svih mjeseci osim u uzorku iz rujna, s trendom povećanja sadržaja od svibnja do kolovoza sa 11,4 na 47,0%. U hidrodestilatu suhe alge dominirali su terpeni te nezasićeni i zasićeni alifatski spojevi. Još dva diterpenska alkohola (izopačidiktol A i kembra-4,7,11,15-tetraen-3-ol) i seskviterpenski alkohol gleenol također su detektirani u visokom udjelu. Najveći udio gleenola (6,3%) je zabilježen u rujnu za hidrodestilat svježe alge. Među alifatskim spojevima dominantan je bio pentadek-1-en s najvećom koncentracijom u rujnu za svježu i suhu algu sa 26,5 i 19,0%, dok je pentadekan bio prisutan u nižoj količini. Identificirano je šest karboksilnih kiselina, od čega je kapronska kiselina bila najzastupljenija.

Od tri identificirana C13-norizoprenoida, β-jonon je bio najzastupljeniji i to u uzorcima suhe alge. PCA (na temelju analiza dominantnih spojeva) pokazala je jasno odvajanje svježih i suhih uzoraka. Nije utvrđena korelacija između spojeva i promjene temperature mora tijekom sezonskog rasta. Rezultati ukazuju na veliku varijabilnost tijekom sezone rasta izoliranih VOC-eva, kao i razlike između svježih i osušenih uzoraka što ukazuje na očite promjene u profilu hlapljivih spojeva tijekom ovog postupka predobrade algalnog materijala.

4.4. Rad 4

U ovom radu istražen je učinak sezonskog rasta (od svibnja do rujna) na kemijski sastav i antioksidacijsku aktivnost ekstrakata smeđe alge *Padina pavonica*. Dodatno, razvijene su bioaktivne PLA folije s premazom od hitozana s inkorporiranim ekstraktima alge *P. pavonica*. Alge su po branju liofilizirane i ekstrahirane pomoću 50%-tnog etanola korištenjem mikrovalova snage 200 W u trajanju od 15 minuta pri 60 °C. Ekstraktima je određen TPC, a kemijski profil je analiziran pomoću uređaja UPLC-PDA-ESI-QTOF. Antioksidacijski potencijal ekstrakata određen je korištenjem tri metode s različitim mehanizmima djelovanja (FRAP, DPPH i ORAC). Najviša TPC vrijednost je zabilježena u uzorku iz lipnja, dok je najniža zabilježena za uzorak iz svibnja. Rezultati su se kretali od 11,88 do 26,69 mg GAE/g suhog ekstrakta. Najviša antioksidacijska aktivnost je također zabilježena za uzorak iz lipnja i to s rezultatima za FRAP od 352,82 μmol TE/L, DPPH od 52,51% inhibicije i ORAC 76,45 μmol TE/L. Među identificiranim spojevima u ekstraktima su dominantne bile masne kiseline, od čega je oleinska kiselina imala udio veći od 11% u svim mjesecima. Najveći udio oleinske kiseline je zabilježen u svibnju. Osim oleinske, zabilježen je i visoki udio palmitinske i palmitoleinske kiseline u uzorcima. Identificirane su i dvije omega-3 masne kiseline, stearinska kiselina i EPA. Ekstrakt uzorka iz lipnja koji je imao najvišu TPC vrijednost, kao i najbolju antioksidacijsku aktivnost izabran je za razvoj bioaktivnog PLA dvoslojnog filma s hitozanom. Primarni ili kvaternarni hitozan korišten je kao prvi sloj, dok je za drugi sloj korištena suspenzija čestica hitozana s inkorporiranim ekstraktom alge *P. pavonica*. Prisutnost slojeva na površini PLA filmova potvrđena je spektroskopijom fotoelektrona rendgenskim zrakama. Antioksidacijski potencijal novo razvijenih filmova je mjeren ABTS metodom. Najveća zabilježena sposobnost filmova za hvatanje ABTS radikala je bila 63,82% u uzorcima s kvaternarnim hitozanom. Razvijeni filmovi pokazali su i svojstva protiv magljenja pokazujući izrazit potencijal za primjenu u prehrambenoj industriji.

4.5. Rad 5

U ovom radu prvi put je izolirano eterično ulje smeđe alge *Cystoseira compressa* te je istražen učinak sezonskog rasta (od svibnja do kolovoza) na njegov kemijski sastav. Eterično ulje je iz suhog algalnog materijala izolirano hidrodestilacijom u modificiranoj aparaturi po Clevengeru te analizirano GC-MS-om. Identificirano je ukupno sto četiri spoja što je u konačnici činilo 84 – 89% ukupnih detektiranih spojeva. Identificirani spojevi su podijeljeni u osam skupina: masne kiseline, alkoholi, esteri, aldehidi, ketoni, ugljikovodici, terpeni i terpenoidi te ostali spojevi. Visoki udio masnih kiselina je zabilježen u uzorcima alge iz svibnja (56%), lipnja (69%) i srpnja (34%). U navedenim uzorcima je dominantna bila palmitinska kiselina, s najvišim udjelom od 40,15% u uzorku iz svibnja. Veliki udio zasićenih masnih kiselina zabilježen je u svim uzorcima, a njihov trend opadanja je vidljiv kroz sezonu rasta. U odnosu na uzorke iz svibnja, uzorci iz kolovoza su imali dva puta manji udio zasićenih masnih kiselina. Uzorak iz svibnja je također imao i veliki udio arahidinske kiseline, 2,58%. Značajne količine ove kiseline su pronađene i u uzorcima iz lipnja i srpnja, dok u uzorku iz kolovoza nije identificirana. U uzorku iz lipnja su zabilježeni najveći udjeli ostalih masnih kiselina, i to palmitoleinske, miristinske, laurinske, (*Z*)-dodec-5-enske, oleinske, arahidonske i stearinske kiseline. Arahidonska kiselina je jedina višestruko nezasićena masna kiselina identificirana u uzorcima. Iz skupine ugljikovodika, pronađen je visok udio 11-pentan-3-ilhenikozana (0,4 do 1,4%) i heksadekana (0,1 do 1,3%). Pentadec-1-en je identificiran samo u uzorcima iz srpnja i kolovoza s udjelima 0,1 i 2,6%. U uzorku iz kolovoza pronađen je visok udio alkohola, čak 48%, uglavnom fitola (14,2%) i oleilnog alkohola (6,0%). Udio ketona je bio u rasponu od 2 do 17%, a najviši sadržaj je zabilježen u uzorku iz srpnja. Aldehidi su bili najmanje zastupljena skupina sa samo 1-2% udjela u svim uzorcima, s tim da je tridekanal bio dominantan u svim uzorcima s udjelom od 0,4 do 0,8%. Udio estera je iznosio 10% u svibnju i lipnju, dok je u srpnju i kolovozu pao na 6 i 4%. Dominantni ester je bio metil arahidonat s najvišim udjelom u svibnju. Iz skupine terpena, terpen keton farnesil aceton je zabilježen u najvećem udjelu u svim uzorcima. Također, u svim uzorcima pronađen je triterpenoid skvalen, a njegov najveći udio je zabilježen u uzorku iz srpnja. Promjene tijekom sezone rasta u profilu i sadržaju drugih spojeva, koje bi također mogle biti važne za ukupnu aromu i biološku aktivnost eteričnog ulja algi, također su zabilježene tijekom sezone rasta.

5. Rasprava

Kako bi se odredio utjecaj sezone rasta na kemijski sastav i biološki potencijal odabranih vrsta smeđih algi Jadranskog mora bilo je potrebno optimizirati metode sušenja algi kao i parametre ekstrakcije ciljanih spojeva (Rad 1).

Usporedbom različitih metoda sušenja (liofilizacija, sušenje u sušioniku i sušenje na zraku), najboljom metodom sušenja za očuvanje fenolnih spojeva iz algi i njihove antioksidacijske aktivnosti pokazala se liofilizacija koja se provodi pri niskim temperaturama u vakuumu, pri čemu se sprječavaju potencijalne reakcije oksidacije pri visokim temperaturama (Amorim i sur., 2020). Osim sušenja, u ovom istraživanju uspoređeni su konvencionalni i novi zeleni postupci ekstrakcije (ekstrakcija potpomognuta mikrovalovima i ultrazvukom). Ekstrakcijom potpomognutom mikrovalovima su dobiveni ekstrakti s najvišim sadržajem ukupnih fenola i najboljom antioksidacijskom aktivnosti, dok su se oba nova postupka ekstrakcije pokazala najboljima za ekstrakciju ukupnih flavonoida i tanina. Korištenjem navedenih postupaka i optimiziranjem njihovih parametara se skraćuje vrijeme ekstrakcije, smanjuje potrošnja korištenih otapala i povećava prinos ciljanih spojeva (Gullón i sur., 2020; Vernès i sur., 2020). Kod usporedbe otapala, voda se pokazala kao dobro ekstrakcijsko otapalo za ekstrahiranje ukupnih tanina, dok su hidroalkoholnim smjesama dobiveni ekstrakti s najvišim sadržajem ukupnih fenola i flavonoida te najboljom antioksidacijskom aktivnosti.

Alge proizvode sekundarne metabolite kao odgovor na promjene u okolišu u kojem rastu. Njihov kemijski sastav ovisi o zemljopisnom položaju, dubini, salinitetu, temperaturi, razvoju talusa alge, prisutnosti predatora i brojnim drugim čimbenicima (Generalić Mekinić i sur., 2019). Svi navedeni čimbenici variraju tijekom sezone rasta te je potrebno odrediti najbolje vrijeme za prikupljanje algi kako bi se što bolje eksploatirale. Pregledom dostupne literature utvrđeno je kako postoji značajan nedostatak istraživanja utjecaja sezone rasta na kemijski sastav i biološki potencijal algi u svijetu pa tako i u Jadranskom moru. Ovo istraživanje je imalo za cilj istražiti utjecaj sezone rasta na kemijski sastav i biološki potencijal odabranih vrsta smeđih algi iz Jadranskog mora. Određen je utjecaj sezone rasta na kemijski sastav, antioksidacijsku i antimikrobnu aktivnost ekstrakata (Rad 2) i kemijski sastav eteričnog ulja (Rad 5) alge *C. compressa*, kemijski sastav i antioksidacijsku aktivnost ekstrakata alge *P. pavonica* (Rad 4) te na sastav isparljivih organskih spojeva alge *H. scoparia* (Rad 3).

U ovom istraživanju, eterično ulje alge *C. compressa* je izolirano po prvi put i promatran je utjecaj sezone rasta na njegov kemijski sastav. U ulju su identificirane masne kiseline, esteri, alkoholi, ketoni, aldehidi, ugljikovodici, terpeni i terpenoidi i ostali spojevi, te su zabilježene varijacije tijekom sezone rasta. Dominantni spojevi identificirani u ekstraktima alge *C. compressa* su bile masne kiseline, i to oleinska, palmitoleinska i palmitinska kiselina, s najvećim sadržajem u svibnju. U Jadranskom moru *C. compressa* raste i razmnožava se te se razvija gusti talus od svibnja do rujna. Najviše vrijednosti ukupnih fenola i tanina, kao i najveća redukcijska sposobnost ekstrakata je zabilježena u lipnju. Sposobnost ekstrakata da inhibiraju DPPH radikal je bila visoka kroz cijelu sezonu rasta dok je najviša sposobnost ekstrakata da inhibiraju peroksil radikal zabilježena u kolovozu. Ekstrakti su pokazali najbolju antimikrobnu aktivnost u lipnju, srpnju i kolovozu, kada su zabilježene najviše temperature mora, protiv svih testiranih patogenih bakterijskih vrsta. Dominantni spojevi identificirani u ekstraktima alge *P. pavonica* su također bile masne kiseline (oleinska, palmitoleinska i palmitinska kiselina) s najvećim sadržajem u svibnju. Najveći sadržaj ukupnih fenola i najviša antioksidacijska aktivnost zabilježeni su za uzorak iz lipnja. Upravo je stoga ekstrakt iz lipnja izabran za razvoj bioakivnog premaza za PLA filmove. Razvijeni filmovi su pokazali dobru antioksidacijsku aktivnost i svojstva protiv magljenja te potvrdili potencijal korištenja ekstrakata ove alge u prehrambenoj industriji.

Iz dobivenih rezultata je jasno vidljiv utjecaj metode sušenja na sastav isparljivih organskih spojeva alge *H. scoparia*. Uzorci svježih algi su se značajnije razlikovali između mjeseci u odnosu na uzorke suhih algi. Dominantne skupine spojeva u uzorcima vršnih para svježih i suhih algi su bili alifatski spojevi i derivati benzena dok su u hidrodestilatima svježih algi najzastupljeniji bili terpeni u lipnju, srpnju i kolovozu te alifatski spojevi u svibnju i rujnu.

Rezultati su detaljno uspoređeni i obrazloženi u priloženim radovima.

6. Zaključci

Usporedbom različitih metoda sušenja alge *P. pavonica* s ciljem povećanja prinosa fenolnih spojeva i antioksidacijske aktivnosti nakon ekstrakcije odabrana je metoda liofilizacije kao najbolja metoda sušenja.

Uspostavljeni su zeleni postupci za ekstrakciju spojeva iz algi te su uspoređeni s konvencionalnim metodama. Ekstrakcije potpomognute mikrovalovima i ultrazvukom su se pokazale učinkovitijima za dobivanje ekstrakata s većim prinosom fenolnih spojeva i snažnijom biološkom aktivnosti. Optimizirani su i parametri korištenih ekstrakcija te su kao najbolja otapala odabrane hidroalkoholne smjese, odnosno 50%-tni etanol.

Određen je utjecaj sezone rasta na kemijski sastav, antioksidacijsku i antimikrobnu aktivnost ekstrakata i kemijski sastav esencijalnog ulja alge *C. compressa*. Utvrđena je varijabilnost svih parametara tijekom sezone rasta, s najvećim sadržajem masnih kiselina u svibnju, fenolnih spojeva u lipnju te antioksidacijske aktivnosti u lipnju i kolovozu. Najveća antimikrobna aktivnost ekstrakata je zabilježena tijekom mjeseci u kojima su izmjerene najveće temperature mora. Zabilježena je varijacija u sadržaju svih spojeva eteričnog ulja tijekom sezone rasta.

Određen je utjecaj sezone rasta na kemijski sastav i antioksidacijsku aktivnost ekstrakata alge *P. pavonica*. Najveći sadržaj masnih kiselina je zabilježen u svibnju, dok je najviši sadržaj fenolnih spojeva i najsnažnija antioksidacijska aktivnost zabilježena u lipnju. Uspješno je razvijen premaz od hitozana s inkorporiranim ekstraktom alge *P. pavonica* iz lipnja za filmove od polilaktične kiseline te je potvrđen potencijal primjene ekstrakata ove alge u prehrambenoj industriji.

Određen je utjecaj sezone rasta i metode sušenja na sastav isparljivih organskih spojeva alge *H. scoparia* te je zabilježena značajna varijabilnost izoliranih spojeva osobito kod uzoraka svježih algi. U uzorcima vršnih para svježih i suhih algi su dominantne skupine spojeva bili alifatski spojevi i derivati benzena, dok je u hidrodestilatima svježih algi najzastupljenija skupina spojeva u svibnju i rujnu bila alifatski spojevi dok su u ostalim mjesecima to bili terpeni.

Smeđe alge Jadranskog mora su se pokazale kao potencijalni izvor bioaktivnih spojeva s velikim potencijalom primjene u prehrambenoj industriji, međutim kod izbora alge za

eksploataciju, potrebno je voditi računa o vremenu uzorkovanja vrste te o primijenjenim postupcima sušenja i ekstrakcije algalnog materijala.

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8. Radovi objedinjeni u disertaciji

8.1. Rad 1 - Maximizing the antioxidant capacity of *Padina pavonica* by choosing the right drying and extraction methods

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Article

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Maximizing the Antioxidant Capacity of *Padina pavonica* by Choosing the Right Drying and Extraction Methods

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Abstract: Marine algae are becoming an interesting source of biologically active compounds with a promising application as nutraceuticals, functional food ingredients, and therapeutic agents. The effect of drying (freeze-drying, oven-drying, and shade-drying) and extraction methods (shaking at room temperature, shaking in an incubator at 60 °C, ultrasound-assisted extraction (UAE), and microwave-assisted extraction (MAE)) on the total phenolics content (TPC), total flavonoids content (TFC), and total tannins content (TTC), as well as antioxidant capacity of the water/ethanol extracts from *Padina pavonica* were investigated. The TPC, TFC, and TTC values of *P. pavonica* were in the range from 0.44 ± 0.03 to 4.32 ± 0.15 gallic acid equivalents in mg/g (mg GAE/g) dry algae, from 0.31 ± 0.01 to 2.87 ± 0.01 mg QE/g dry algae, and from 0.32 ± 0.02 to 10.41 ± 0.62 mg CE/g dry algae, respectively. The highest TPC was found in the freeze-dried sample in 50% ethanol, extracted by MAE (200 W, 60 °C, and 5 min). In all cases, freeze-dried samples extracted with ethanol (both 50% and 70%) had the higher antioxidant activity, while MAE as a green option reduces the extraction time without the loss of antioxidant activity in *P. pavonica*.

Keywords: *Padina pavonica*; brown algae; ultrasound-assisted extraction; microwave-assisted extraction; antioxidant activity; green extraction; phenolic compounds

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1. Introduction

Among different brown algae from the genus *Padina* (family *Dictyotaceae*, order Dictyotales), *Padina pavonica* is one of the most common species that inhabits coastal intertidal zones. This alga is growing mainly in the Mediterranean Sea and Atlantic Ocean while it is distributed in tropical and temperate seas worldwide [1–3]. Its availability makes it an interesting and suitable choice for research activities, especially considering that brown macroalgae (seaweeds) have been described as unexplored and are rich sources of compounds with different biological activities [4]. It has been reported that marine macroalgae have strong antioxidant, antimicrobial, antiviral, anti-inflammatory, antitumor, and anticancer properties [5–7]. Polyunsaturated fatty acids (comprising docosahexaenoic acid), carbohydrates, pigments, phenolic compounds (phenolic acids, flavonoids, and tannins), peptides, enzymes, lipids, vitamins, and terpenoids are commonly found in macroalgae [8]. Among them, phenolics, a group of secondary metabolites produced by algae as a response to harsh environmental conditions, are the most important bioactive compounds found in brown algae [9]. They are the primary components of algae cell walls while they also have a role as a chemical defence against herbivores, bacteria, and fouling organisms [4]. On the one hand, the biological activity of phenolic compounds from terrestrial plants has been

well studied. On the other hand, the study of compounds from marine origin, especially algal phenolics, has only recently come to focus, as these compounds show wide diversity in biological activity [4]. In algae, these phytochemicals vary from simple molecules such as phenolic acids or flavonoids to highly complex polymers called phlorotannins, a subgroup of tannins formed by polymerization of phloroglucinol units [4,10]. Compared to phlorotannins, studies on flavonoid content in algae are limited. To date, it has been reported that seaweeds are a rich source of catechins, rutin, quercetin, hesperidin, and other flavonoids [10]. Strong antioxidant properties of phenolics make them the functional ingredient for possible applications such as nutraceuticals or pharmaceuticals, as well as in functional food and cosmetic industries. In addition, there is an increased interest in natural antioxidants as a replacement for synthetic ones [11]. Studies on bioactive components from algae have intensified in the last years and most of the focus has been on the identification of new components; the preparations protocols of the material (drying, extraction) have been neglected. To identify macroalgae as a potential source of natural antioxidants requires adaptation of drying and extraction methods. Procedures that are used for terrestrial plants need to be adapted to this new matrix.

Before being subjected to extraction, the macroalgae are traditionally sun-dried, shade-dried, or oven-dried. The selection of the drying method may be a key factor that influences the phytochemical content of algae samples. It has been shown that many parameters, such as temperature, drying time, and UVA-UVB light affect the phytochemical content and antioxidant potential of algae [12]. As low temperature prevents the degradation of heat-sensitive compounds, freeze-drying, a method that is performed under vacuum, has been suggested to prevent the oxidation reactions of functional ingredients [13]. The selection of the best drying method for marine macroalgae remains to be confirmed.

Recently, researchers have investigated novel methods for assisting the extraction of phenolics from macroalgae, such as ultrasound-assisted extraction (UAE) and microwave-assisted extraction (MAE). UAE and MAE are considered to be green methods since they reduce extraction time, decrease high solvent consumption, and increase the yield of targeted compounds as compared with conventional methods such as maceration or shaking [14]. Phenolic compounds have different polarities which indicate that hydro-alcoholic mixtures could be the best suitable solvent for their extraction, plus these mixtures are acceptable for food applications [15]. A hydro-alcoholic mixture has a synergistic effect, water acts as a swelling agent of the matrix, and ethanol induces the rupture of the bond between the solutes and the matrix [16]. In the last few years, the focus of studies has been on *P. pavonica*, analyzing the use of different extraction methods and different solvents to obtain bioactive compounds with specific biological activity (Table 1).

According to recent studies, it is evident that the extraction parameters, solid-to-solvent ratio, and solvent choices in the extraction methods need to be optimized and investigated to find conditions that would yield the highest amount of targeted compounds and preserve their biological activity.

Table 1. Recent studies on different extraction methods and solvents to yield particular biological compound/activity of *Padina pavonica*.

Extraction	Solvent	Targeted Compound/Activity	Reference
MAE	Ethanol and methanol 80%	Antibacterial activity, flavonoids	[17]
Extracted in centrifuge tubes in a 37 °C water bath for 2.5 h	96% Ethanol	Phenolic compounds	[18]
Stirring 12 h	Methanol and dichloromethane	Antioxidant activity, cytoprotectivepotential	[19]
Soxhlet extraction	Acetone	Pro-apoptotic activity, antioxidant activity	[9]
PLE, MAE, SFE, electroporation extraction	Petroleum ether, ethanol, ethyl acetate and water	Anti-hyaluronidase activity	[20]

Table 1. *Cont.*

Extraction	Solvent	Targeted Compound/Activity	Reference
Extracted at 100 °C for 2 h using a reflux condenser under reduced pressure	Acetone and water	Antioxidant activity, antimicrobial activity	[21]
Soxhlet extraction	Acetone	Pro-osteogenic ability, antioxidant activity	[22]
Extracted for 72 h in shaking incubators	80% Acetone, 80% ethanol, 80% methanol and water	Antioxidant activity, antidiabetic activity	[23]
Maceration at room temperature twice for 24 h	Hexane, ethyl acetate, and methanol	Antiparasitic activity, antioxidant activity	[24]
SLE (30 min)	Ethanol 50%	Antioxidant activity, anti-inflammatory activity, and antinociceptive activity	[25]
Percolation at room temperature for 2 days	95% Ethanol	Antimicrobial activity, antioxidant activity and anticancer activity	[26]

MAE, microwave-assisted extraction; PLE, pressurized liquid extraction; SFE, supercritical fluid extraction; UAE, ultrasound-assisted extraction.

Despite the diversity of marine macroalgae in the Adriatic Sea, most of them have not yet been investigated for biological activities and the reports on the total phenolic content (TPC) and antioxidant properties of macroalgae are very limited. Recently, the acetone extracts of *Dictyota dichotoma*, *P. pavonica,* and *Sargassum vulgare* from the Adriatic coast of Montenegro have shown that they have antioxidant, antimicrobial, and cytotoxic potential [27].

The aim of this study was to increase the antioxidant capacity of *P. pavonica* through the optimal drying and extraction methods. Freeze-dried, oven-dried, and shade-dried samples were extracted by four different methods (shaking at room temperature, shaking in an incubator at 60 °C, UAE, and MAE) using water and ethanol (green solvents). A large number of extracts was screened to test the highest yield of total phenolic content (TPC), total flavonoid content (TFC), and total tannins content (TTC). The antioxidant capacity was performed by multiple approaches based on ferric reducing/antioxidant power (FRAP), 2,2-diphenyl-1-picrylhydrazyl radical scavenging ability (DPPH), and the oxygen radical absorbance capacity (ORAC).

2. Materials and Methods

2.1. Chemicals

The following chemicals that were used were analytical grade and obtained from Sigma-Aldrich: GmbH (Steinheim, Germany); Fluka (Buchs, Switzerland); Kemika (Zagreb, Croatia); Merck (Darmstadt, Germany); and Gram-Mol (Zagreb, Croatia).

2.2. Algal Material and Drying Methods

Brown macroalgae, *P. pavonica* was harvested off the coast of the island Čiovo in the Adriatic Sea in May 2020. The sea temperature was 18.3 °C and salinity was 37.4 ppt, measured using a YSI Pro2030 probe (Yellow Springs, OH, USA). Harvested algae were washed thoroughly with tap water to remove epiphytes, and then divided into groups to test the effect of different drying methods on phenolic composition and antioxidant activity. Algae were dried as follows: (i) shade-dried for approximately seven days; (ii) oven-dried in the ventilated oven for 24 h at 60 °C; and (iii) freeze-dried for 2 days using freeze-dryer (FreeZone 2.5, Labconco, Kansas City, MO, USA). All samples were ground (1 min in a high-speed grinder) after drying. A voucher specimen of tested species is deposited in the herbarium at the University Department of Marine Studies in Split, Croatia.

2.3. Extractions

The algal samples, in duplicate, were extracted with water, ethanol 50%, and ethanol 70% using four extraction methods. Based on the preliminary tests (temperature of the sample during extraction, particle size of the samples, and yield of extraction) the algae to solvent ratio were set at 1:10 for all extractions. The following extraction methods were applied: (i) shaking in the dark for 24 h at room temperature (SLE); (ii) shaking in the incubator at 60 °C for 1 and 2 h (MIX); (iii) UAE performed using an ultrasonic bath (Transsonic Tp 310H, Elma Schmidbauer GmbH, Singen, Germany) at 40 kHz frequency and 60 °C for 1 and 2 h; and (iv) MAE was performed in an advanced microwave extraction system (ETHOS X, Milestone Srl, Sorisole, Italy), for 5, 10, 15, and 30 min. The microwave power was kept constant at 200 W and the temperature was set at 60 °C. The extraction conditions used for MAE and UAE were selected during preliminary tests. After the extractions, samples were centrifuged at 5000 rpm for 8 min at room temperature. All extracts were kept in the freezer at −20 °C prior to analyses. Each extraction was done in duplicate.

2.4. Phenolic Composition

All obtained extracts were analysed for TPC, TFC, and TTC.

The TPC of *P. pavonica* extracts was determined by the Folin–Ciocalteu method [28]. Briefly, 25 µL of the extract was mixed with 1.5 mL distilled water and 125 µL Folin–Ciocalteu reagent. The solution was mixed and after one minute 375 µL 20% sodium carbonate solution and 475 µL distilled water was added. The mixture was left in the dark for 2 h at room temperature. The absorbance was read at 765 nm using a spectrophotometer (SPECORD 200 Plus, Edition 2010, Analytik Jena AG, Jena, Germany). The standard calibration (0–500 mg/L) curve was plotted using gallic acid ($y = 0.001x$, $R^2 = 0.9998$). The TPC was expressed as gallic acid equivalents in mg/g of dried algae (mg GAE/g).

The TFCs of *P. pavonica* extracts were measured using the aluminium chloride colorimetric method, previously described by Lovrić et al. [29] with slight modifications. A volume of 250 µL algae extract was mixed with 750 µL 96% ethanol, 50 µL 10% (*w/v*) aluminium chloride, 50 µL 1M sodium acetate, and 1400 µL distilled water. The mixture was kept for 30 min at room temperature before measurement. The absorbance was read at 415 nm using a spectrophotometer. The standard calibration (0–200 µg/mL) curve was plotted using quercetin ($y = 0.0068x - 0.0064$, $R^2 = 0.9989$). The TFC was expressed as mg quercetin equivalents per g of dried algae (mg QE/g).

The TTCs of *P. pavonica* extracts were determined, using the method of Zhong et al. [30] with modifications. The extract (25 µL), 150 µL 4% (*w/v*) ethanolic vanillin solution, and 25 µL 32% (*v/v*) sulfuric acid (diluted with ethanol) were mixed together in a 96-well plate. The mixture was kept for 15 min at room temperature before reading. The absorbance was measured at 500 nm using the microplate reader (Synergy HTX Multi-Mode Reader, BioTek Instruments, Inc., Winooski, VT, USA). The standard calibration (0–1000 µg/mL) curve was plotted using catechin ($y = 0.0005x + 0.0555$, $R^2 = 0.9997$). The TTC was expressed as mg catechin equivalents per g of dried algae (mg CE/g).

2.5. Antioxidant Activity

The antioxidant activity of *P. pavonica* extract was assessed by three different methods that are based on two different mechanisms of action, i.e., hydrogen atom transfer (HAT) (DPPH and ORAC) and electron transfer (ET) (FRAP)) [31].

The reducing activity was measured as FRAP, using a previously described method [32] and the results were expressed as micromoles of Trolox equivalents (µM TE).

The 2,2-diphenyl-1-picrylhydrazyl radical scavenging ability (DPPH) of extracts was measured, according to a previously described method [33] modified to 96-well microplates [34]. The decrease in the absorbance (at 517 nm) was measured after 1 h using the Synergy HTX Multi-Mode micro plate reader (BioTek Instruments, Inc., Winooski, VT,

USA). The antioxidant activity was expressed as inhibition percentages of DPPH radical (% inhibition).

The oxygen radical absorbance capacity (ORAC) method was performed, according to a previously described method [35] modified by Burčul et al. [36]. The reaction was measured every minute during 80 min. Extracts were diluted 100-fold and the results were expressed as μM of Trolox equivalents (μM TE).

2.6. Statistical Analysis

The specific drying conditions and all extractions were replicated, while all analyses (TPC, TFC, TTC, and antioxidant assays) were performed in triplicate. The results are expressed as mean ± standard deviation. Principal component analysis (PCA) was used to determine relations among the studied variables (TPC, TFC, TTC, FRAP, DPPH, and ORAC) and the relationship between them (STATISTICA, version 13, StatSoft Inc, Tulsa, OK, USA).

3. Results and Discussion

3.1. Phenolic Composition

The results of TPC, TFC, and TTC for freeze-dried, oven-dried, and shade-dried extracts of *P. pavonica* are shown in Table 2. Freeze-dried extracts had the highest TPC as compared with other drying methods and, in some cases, freeze-dried samples had from three-fold to seven-fold higher TPC than oven-dried and shade-dried samples. The results showed evidence that freeze-drying was the best drying method for preserving TPC in algal material irrespective of the solvent used. In the comparison of oven-dried and shade-dried samples, oven-dried had a significantly higher content of TPC, with the exception of SLE in water. The application of microwaves resulted in the highest phenolics yield. The MAE yielded higher TPC with a shorter extraction time.

When comparing the three solvents in extractions of freeze-dried samples, those extracted with 50% ethanol had higher TPC than samples extracted with water and 70% ethanol. The highest TPC was in the sample extracted with MAE for 5 min in 50% ethanol (4.32 ± 0.15 mg GAE/g). Freeze-dried samples extracted with water had the lowest results for all extraction methods, but even those results were higher than TPC in the oven and shade-dried samples for the same extractions methods. When comparing the three solvents in extractions of oven-dried and shade-dried samples, those extracted with water had higher TPC than samples extracted with water-ethanol mixtures. The highest TPC among oven-dried and shade-dried samples was in extracts prepared with water using MAE for 30 and 15 min, respectively (2.09 ± 0.01 and 1.84 ± 0.46 mg GAE/g).

Overall, the TPC of *P. pavonica* ranged from 0.44 ± 0.034 to 4.32 ± 0.15 mg GAE/g. As compared to other brown algae such as algae from genus *Cystoseira* and *Fucus* where TPC was above 400 and 150 mg GAE/g dry weight, respectively [4], the TPC of *P. pavonica* was relatively low. Mannino et al. [18] also determined low TPC in oven-dried *P. pavonica*. This may be because this alga calcifies, making it a species that is hardly palatable and possibly not requiring particular high production of phenolic compounds to endure predators and the environmental challenges. Another reason could be the low sea temperature. The TPC increase was correlated with rising sea temperature [37].

Table 2. Total phenolic content (TPC), total flavonoid content (TFC), and total tannin content (TTC) of *P. pavonica* extracts (n = 6).

Extraction Method *	Solvent	Time	FD **	OD	SD	FD	OD	SD	FD	OD	SD
			TPC (mg GAE/g)			TFC (mg QE/g)			TTC (mg CE/g)		
MIX (60 °C)	H_2O	1 h	1.85 ± 0.11	1.79 ± 0.07	1.57 ± 0.03	1.06 ± 0.08	1.59 ± 0.04	1.64 ± 0.11	8.07 ± 0.79	10.02 ± 0.51	6.77 ± 0.52
		2 h	1.94 ± 0.05	1.74 ± 0.01	1.56 ± 0.03	1.01 ± 0.04	2.01 ± 0.08	1.68 ± 0.07	9.02 ± 0.77	9.79 ± 0.85	8.67 ± 0.35
	EtOH 50%	1 h	4.25 ± 0.12	1.29 ± 0.04	0.79 ± 0.06	0.81 ± 0.02	0.71 ± 0.05	0.69 ± 0.01	4.64 ± 0.46	4.06 ± 0.35	3.02 ± 0.28
		2 h	3.90 ± 0.12	1.01 ± 0.04	0.65 ± 0.03	0.80 ± 0.05	0.62 ± 0.04	0.67 ± 0.00	3.25 ± 0.38	1.95 ± 0.12	1.63 ± 0.16
	EtOH 70%	1 h	3.84 ± 0.09	1.01 ± 0.03	0.71 ± 0.04	2.79 ± 0.07	2.21 ± 0.11	1.92 ± 0.08	7.43 ± 0.27	3.00 ± 0.24	2.18 ± 0.09
		2 h	3.48 ± 0.19	0.89 ± 0.01	0.66 ± 0.05	2.21 ± 0.08	2.00 ± 0.06	2.07 ± 0.10	1.88 ± 0.02	2.86 ± 0.05	1.30 ± 0.08
SLE (room temperature)	H_2O	24 h	1.51 ± 0.11	1.31 ± 0.06	1.48 ± 0.05	0.84 ± 0.02	0.49 ± 0.02	0.36 ± 0.02	8.14 ± 0.64	6.23 ± 0.05	3.92 ± 0.35
	EtOH 50%	24 h	3.41 ± 0.12	0.63 ± 0.02	0.49 ± 0.09	0.84 ± 0.02	0.31 ± 0.01	0.43 ± 0.03	2.27 ± 0.10	2.30 ± 0.22	2.22 ± 0.19
	EtOH 70%	24 h	3.31 ± 0.13	0.53 ± 0.06	0.44 ± 0.03	2.22 ± 0.09	0.73 ± 0.05	1.15 ± 0.06	2.19 ± 0.14	1.44 ± 0.06	1.53 ± 0.04
UAE (60 °C)	H_2O	1 h	1.72 ± 0.03	1.62 ± 0.12	1.43 ± 0.03	0.63 ± 0.02	0.32 ± 0.01	0.45 ± 0.01	5.34 ± 0.17	7.05 ± 0.46	5.96 ± 0.26
		2 h	1.70 ± 0.02	1.59 ± 0.12	1.31 ± 0.08	0.78 ± 0.01	0.38 ± 0.02	0.61 ± 0.02	10.41 ± 0.62	7.67 ± 0.03	6.16 ± 0.27
	EtOH 50%	1 h	3.94 ± 0.08	0.96 ± 0.08	0.64 ± 0.08	0.89 ± 0.04	0.48 ± 0.03	0.63 ± 0.02	2.90 ± 0.10	0.62 ± 0.03	0.94 ± 0.14
		2 h	3.97 ± 0.12	0.92 ± 0.00	0.77 ± 0.09	0.92 ± 0.01	0.50 ± 0.04	0.65 ± 0.04	3.63 ± 0.05	1.16 ± 0.08	2.03 ± 0.19
	EtOH 70%	1 h	3.51 ± 0.09	0.82 ± 0.07	0.66 ± 0.06	2.83 ± 0.11	1.31 ± 0.01	1.83 ± 0.08	2.62 ± 0.20	2.12 ± 0.13	2.08 ± 0.07
		2 h	3.45 ± 0.05	1.04 ± 0.05	0.61 ± 0.01	2.75 ± 0.02	2.25 ± 0.12	1.57 ± 0.05	2.41 ± 0.23	2.46 ± 0.08	2.07 ± 0.16
MAE (200 W, 60 °C)	H_2O	5 min	2.06 ± 0.01	1.56 ± 0.04	1.38 ± 0.05	0.51 ± 0.02	0.68 ± 0.02	0.47 ± 0.02	9.03 ± 0.79	0.32 ± 0.02	5.20 ± 0.41
		10 min	1.88 ± 0.19	1.95 ± 0.01	1.77 ± 0.07	0.52 ± 0.02	0.66 ± 0.01	0.54 ± 0.05	7.77 ± 0.45	3.45 ± 0.17	5.48 ± 0.46
		15 min	1.90 ± 0.05	1.90 ± 0.18	1.84 ± 0.05	0.50 ± 0.03	0.68 ± 0.01	0.61 ± 0.04	6.25 ± 0.44	5.60 ± 0.19	3.12 ± 0.22
		30 min	2.11 ± 0.08	2.09 ± 0.01	1.75 ± 0.12	0.62 ± 0.03	0.69 ± 0.02	0.61 ± 0.02	5.86 ± 0.30	6.82 ± 0.64	5.14 ± 0.48
	EtOH 50%	5 min	4.32 ± 0.15	1.41 ± 0.05	1.01 ± 0.06	0.91 ± 0.04	1.06 ± 0.05	0.77 ± 0.04	2.19 ± 0.13	1.09 ± 0.08	1.06 ± 0.10
		10 min	4.32 ± 0.13	1.46 ± 0.07	1.13 ± 0.05	1.48 ± 0.03	0.77 ± 0.05	0.82 ± 0.03	1.89 ± 0.18	0.92 ± 0.06	0.94 ± 0.09
		15 min	3.79 ± 0.07	1.51 ± 0.06	1.06 ± 0.05	1.12 ± 0.04	0.81 ± 0.02	0.77 ± 0.03	2.79 ± 0.13	1.38 ± 0.04	1.40 ± 0.06
		30 min	4.10 ± 0.13	1.64 ± 0.03	1.19 ± 0.05	1.27 ± 0.07	0.92 ± 0.06	0.86 ± 0.00	3.13 ± 0.26	0.93 ± 0.08	1.49 ± 0.13
	EtOH 70%	5 min	3.28 ± 0.02	1.12 ± 0.05	0.82 ± 0.05	2.87 ± 0.01	1.84 ± 0.01	1.79 ± 0.05	3.51 ± 0.16	1.56 ± 0.06	1.76 ± 0.08
		10 min	3.48 ± 0.06	1.17 ± 0.10	0.87 ± 0.08	2.09 ± 0.12	1.82 ± 0.12	1.71 ± 0.05	2.45 ± 0.22	1.14 ± 0.11	1.22 ± 0.11
		15 min	3.46 ± 0.01	1.14 ± 0.05	0.89 ± 0.03	2.16 ± 0.08	1.82 ± 0.13	1.86 ± 0.02	3.30 ± 0.32	1.44 ± 0.04	1.86 ± 0.13
		30 min	2.98 ± 0.04	1.33 ± 0.05	0.95 ± 0.04	1.76 ± 0.03	2.11 ± 0.11	1.97 ± 0.12	3.51 ± 0.24	2.29 ± 0.19	1.87 ± 0.15

* MIX, shaking in the incubator; SLE, shaking in the dark; UAE, ultrasound-assisted extraction; MAE, microwave-assisted extraction; ** FD, freeze-dried; OD, oven-dried; SD, shade-dried.

The freeze-dried samples had the highest TFC and TTC, with some exceptions. Regarding the used solvents, 70% ethanol yielded higher TFC amounts than water and 50% ethanol for all drying and extraction methods. Comparing UAE and MAE, samples extracted with 70% ethanol had two- or three-fold higher TFC than samples extracted with water and 50% ethanol. However, water appears to be the best solvent for extracting tannins among tested solvents. Water extracts prepared using UAE and MAE had TTC more than three-fold higher than those of water-ethanol mixtures for all drying methods. The freeze-dried sample extracted with water using UAE (for 2 h) and MAE (for 5 min) had the highest TTC. These results showed that the two green, novel extraction methods yielded higher TFC and TTC amounts, and it was evident that MAE yielded more TFC in a shorter extraction time.

Magnusson et al. [38] reported a 70% increase in the yield of extracted polyphenols in brown macroalgae (*Carpophyllum flexuosum*) when using MAE (water/biomass solvent ratio of 1:30, 160 °C, and 3 min) as compared with solid-liquid extraction. Yuan et al. [39] reported that the TPC and antioxidant potential of four brown algae increased when using MAE at 110 °C for 15 min as compared with conventional extraction at room temperature for 4 h.

The effect of temperature was not a specific aim of this study; however, all extractions, except SLE, were performed at 60 °C to preserve phenolic compounds susceptible to thermal degradation [4]. The results showed a difference between two similar extraction methods (SLE and MIX), performed at two different temperatures (24 and 60 °C). The higher temperature increased TPC and shortened extraction time 24 times. This could be because a higher temperature increased intermolecular interactions within the solvent which increased the solubility of the compounds [40]. In some studies, authors describe that high temperatures (>60 °C) resulted in a higher yield of TPC from brown algae [39,41,42]. Although the TPC increases (probably due to hydrolysis of complex phlorotannins into simpler compounds), the application of high temperatures brings to question the susceptibility of phenolic compounds to thermal degradation [4].

Treating the samples for 1 and 2 h at the same temperature using UAE resulted in no differences among the TPCs of the extracts. The reason for this could be that the yield of TPC extracted by UAE increases with time and it happens in two phases. The first phase is the "washing" phase in which soluble components on the surfaces of the matrix are being dissolved, it lasts for the first 10–20 min, and up to 90% of the total phenolic content is recovered. In the second phase, i.e., the "slow extraction" phase, diffusion is responsible for mass transfer of the solute from the matrix into the solvent and it can last from 60 to 100 min [15,16]. Apparently, in this experiment, most of the mass transfer occurred in the first 60 min. Some authors have reported that ultrasound frequency does not influence TPC significantly. In fact, Ummat et al. [43] confirmed that UAE frequency of 35 and 130 kHz yielded statistically similar TPC from algae. For this reason, the effect of ultrasound frequency was not tested, and 40 kHz was used. Although studies on flavonoid content in algae are limited, few reports have shown evidence of *P. pavonica* extracts being rich in flavonoids. Alghazeer et al. [17] prepared flavonoids rich extract (70.08 mg/g rutin equivalent) from *P. pavonica* using MAE and demonstrated its potential antibacterial activity, while Bernardini et al. [9] reported high levels of TFC in air-dried *P. pavonica* extracted with acetone using Soxhlet extraction.

3.2. Antioxidant Activity

The antioxidant activity was determined using the FRAP, DPPH, and ORAC assays and the results are shown in Figures 1–3. The FRAP values ranged from 98.46 ± 3.53 to 537.95 ± 9.11 μM TE. The freeze-dried samples prepared with 50% and 70% ethanol had the highest FRAP values for all extraction methods. When water was used as a solvent, freeze-dried samples had lower FRAP than the oven and shade-dried ones. Freeze-drying appears to be the best drying method for obtaining extract with high reducing activity. The highest FRAP was found in freeze-dried samples prepared with 70% ethanol, using

MAE for 30 min (537.95 ± 9.11 µM TE) and using UAE for 1 h (461.28 ± 18.97 µM TE). The highest FRAP result among oven-dried samples was also found in the sample extracted for 30 min in 70% ethanol using MAE.

The free antioxidant ability of *P. pavonica* extracts against DPPH radical ranged from 2.33 ± 1.34% to 62.88 ± 3.13%. The highest free radical scavenging activity was obtained for freeze-dried samples in all extraction methods, with the exceptions of two water extracts (Figure 2). When comparing solvents used for extraction, the results showed that water/ethanol mixtures were more suitable for maintaining high DPPH scavenging activity of investigated algal samples, especially 50% ethanol. In all ethanol extracts, oven-dried and shade-dried samples had significantly lower inhibition capacity than samples obtained by freeze-drying. Novel extraction methods, UAE and MAE, gave high DPPH scavenging activity with inhibition over 50%. Freeze-dried samples prepared in 50% ethanol using UAE for 1 h and MAE for 5 min had 59.42 ± 1.30% and 54.57 ± 1.97% of inhibition, respectively. Water extraction gave better antioxidant capacity in oven-dried and shade-dried samples. As for FRAP, water extracts of freeze-dried samples had lower scavenging activity. Among all water extracts of oven-dried and shade-dried samples, MAE gave the highest DPPH of 41.11 ± 0.83% and 32.99 ± 5.35%, respectively.

The ability of *P. pavonica* extracts to scavenge peroxyl radicals was measured with ORAC assay. While peroxyl radicals are the predominant free radicals found in biological systems, ORAC values are considered to be biologically relevant [44]. The results obtained by the ORAC method ranged from 8.57 ± 0.37 to 71.37 ± 1.97 µM TE. The freeze-dried samples had two- to three-fold higher ORAC values than oven-dried and shade-dried samples when they were extracted with water-ethanol mixtures. Similar to the DPPH method, when water alone was used as a solvent, ORAC values in oven-dried and shade-dried samples were slightly higher than in freeze-dried samples. Among them, samples prepared using MAE for 15 min gave the highest ORAC values for oven-dried (36.40 ± 0.52 µM TE) and shade-dried (35.11 ± 0.73 µM TE) samples. The results suggested that freeze-drying in combination with ethanol as a solvent is the best method for preserving the antioxidant activity of *P. pavonica*. The highest ORAC value was found in a freeze-dried sample extracted with 50% ethanol during MIX for 1 h. However, a similar ORAC value (69.68 ± 1.69 µM TE) was found for freeze-dried sample extracted with MAE for 10 min in 50% ethanol. Using this green extraction method, time was reduced six-fold without losing the oxygen radical absorbance capacity of the alga samples.

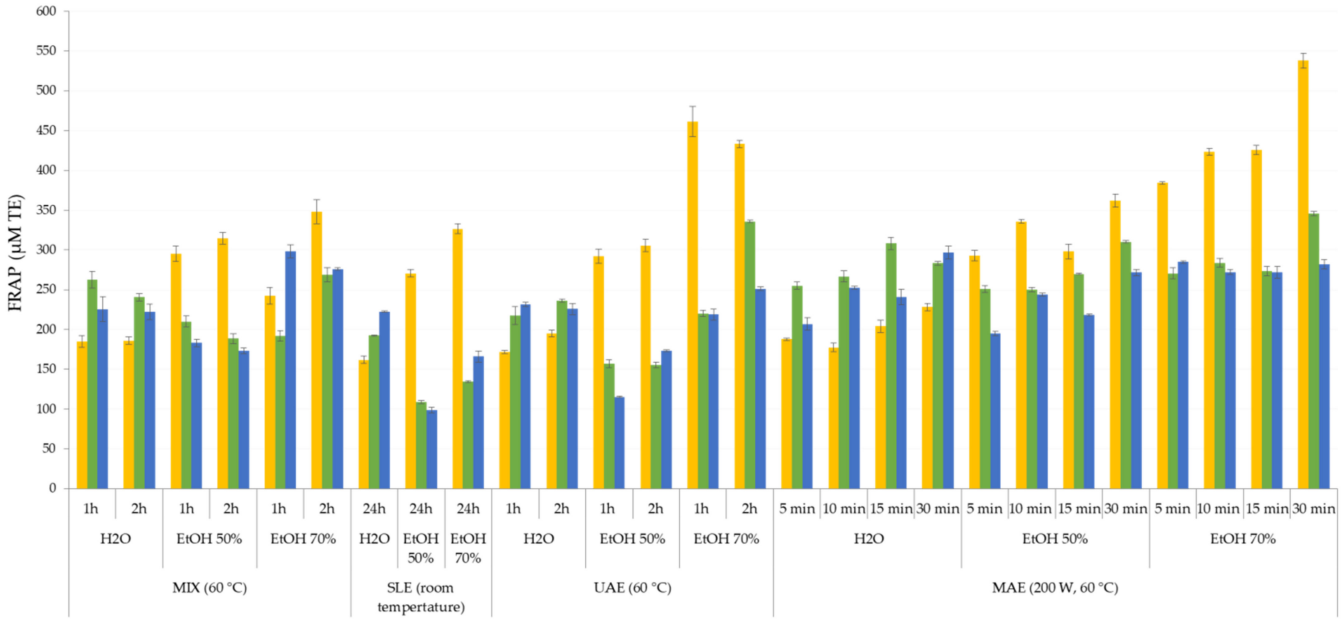

Figure 1. Ferric reducing/antioxidant power (FRAP) of freeze-dried (yellow), oven-dried (green), and shade-dried (blue) extracts of *P. pavonica* (n = 6).

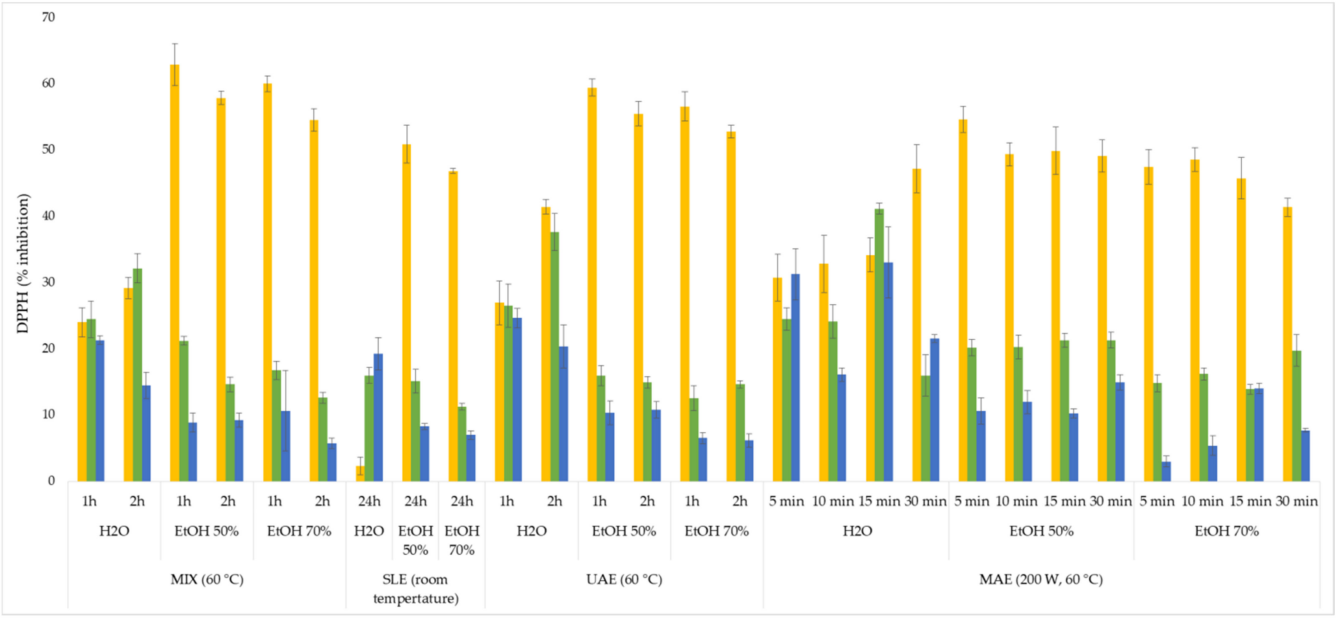

Figure 2. The 2,2-diphenyl-1-picrylhydrazyl radical scavenging ability (DPPH) of freeze-dried (yellow), oven-dried (green) and shade-dried (blue) extracts of *P. pavonica* (n = 6).

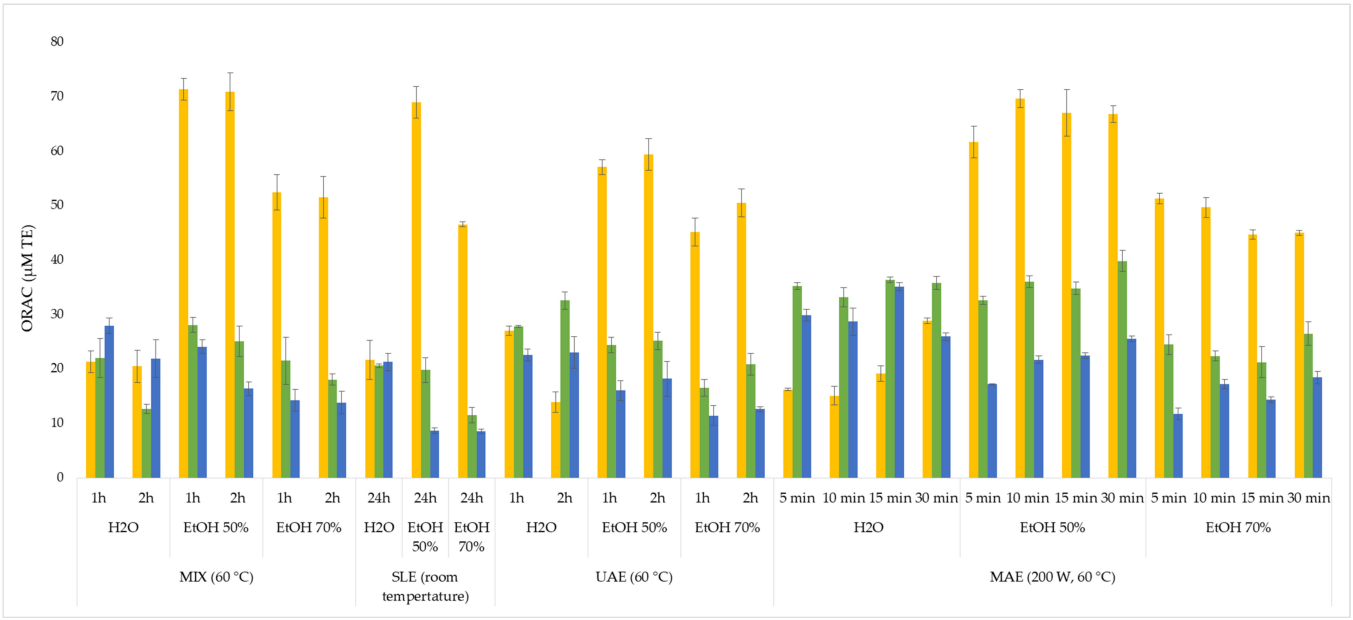

Figure 3. Oxygen radical absorbance capacity (ORAC) of freeze-dried (yellow), oven-dried (green), and shade-dried (blue) extracts of *P. pavonica* (n = 6).

To understand the effect of drying and extraction methods on the TPC, TFC, TTC, and antioxidation assays from *P. pavonica*, all the obtained data were submitted to PCA. The first two factors (Figures 4 and 5) described 79.04% of the initial data variability. Parameters that have the highest values of factor coordinates for the PC1, with the highest variable contributions, based on correlations were DPPH and ORAC. The PC2 was characterized by TPC, TFC, and TTC. The position of the samples in the multivariate space was arranged in two main areas, based on the strong influence of DPPH, ORAC, and FRAP (Factor 1) and total phenolic content (Factor 2), pointing out the clear separation among the samples. The extracts with the highest antioxidant activity were grouped on the left side while those with lower activity were positioned on the right part of the multivariate space.

The freeze-dried samples extracted with water/ethanol mixtures (both 50 and 70%) are grouped as extracts with high antioxidant activity. Water extracts of all samples grouped on the upper right side, while water-ethanol mixture extracts of oven-dried and shade-dried samples remained in the lower part of the graph. Amorim et al. [13] investigated the effect of freeze-drying, oven-drying, and silica-drying on the antioxidant properties of four Brazilian macroalgae (*Gracilariopsis tenuifrons*, *Pterocladiella capillacea*, *Sargassum stenophyllum*, and *Ulva fasciata*) and showed that freeze-drying had the lowest alteration of antioxidant potential in tested algae. However, Ling et al. [12] studied the effect of different drying methods (oven-drying, sun-drying, hang-drying, sauna-drying, shade-drying, and freeze-drying) on the phytochemical content and antioxidant activity of red macroalgae *Kappaphycus alvarezii*. The algae were extracted by shaking in 80% methanol (1:10 ratio) for 2 h at room temperature. They found that oven-drying at 40 °C provided the highest values of phytochemical content and displayed better scavenging and reducing ability. In their study freeze-drying yielded low TPC and TFC, and lower antioxidant activity (FRAP and DPPH).

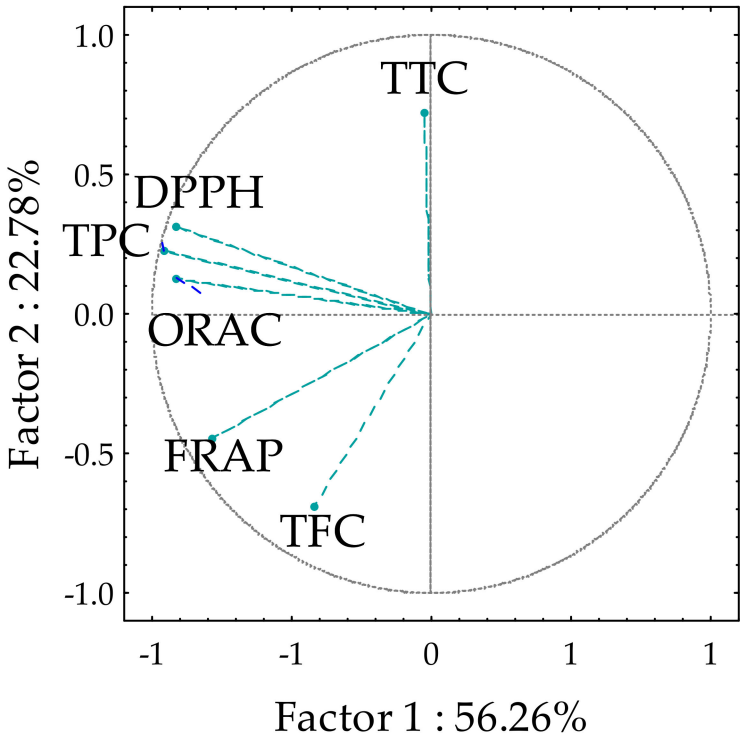

Figure 4. Correlation plots on the TPC, TFC, TTC, and antioxidation assays for *P. pavonica* according to principal component analyses (PCAs) describing all data.

The comparison of results with other studies is difficult in many ways. The TPC of an alga is influenced by environmental factors such as sea temperature, sampling location, salinity, size, age, and reproductive status, which makes the comparison even between the samples of the same species difficult. In many studies, these data are rarely reported. In addition, different standard compounds are used for the expression of the results, and results are reported on the base of wet or dry algae weight, milliliters of extracts, or even dry extracts. In general, in studies on algae, researchers have focused on finding new compounds and their bioactive properties rather than optimizing drying and extraction method which could enhance the activity of the extracts. Bernardini et al. [9] measured the TPC, TFC, and TTC in air-dried *P. pavonica* samples extracted with acetone in a Soxhlet extractor, and obtained 27.0, 54.8, and 54.3 mg per g of extract, respectively. These values correspond to 0.81, 1.64, and 1.63 mg/g dry material. They also reported a FRAP value of 25.6 ± 0.2 µmol of Fe^{2+}/100 mg of extract. The authors aimed to show the anticancer

properties of *P. pavonica* extracts against osteosarcoma cell lines. The TPC, TFC, TTC, and FRAP values are lower in comparison to our results. However, the authors used different drying and extraction methods, and the alga was sampled in the coastal area of Moorea. Hlila et al. [21] reported the TPC of 90.61 and 57.34 mg CE/g in *P. pavonica* acetone and water extract, respectively. Acetone extracts demonstrated a DPPH of 72.92% of inhibition and antimicrobial activity against eight microbial strains. The acetone was not always selected as the best solution. Ismail et al. [23] investigated four extraction solvents (80% acetone, ethanol, methanol, and water) on six different shade-dried seaweeds using shaking for 72 h. The results of TPC and DPPH *for P. pavonica* were the highest in the water extracts, 3.32 mg GAE/g dry weight and antioxidant capacity was 55.2%, respectively. They found more than two-fold higher TPC in water extract than in acetone, ethanol, and methanol extracts. Their results showed a correlation between TPC and antioxidant activity. The TPC of shade-dried *P. pavonica* extracts in our study were lower than reported by Ismail et al., however, freeze-dried water-ethanol extracts had higher TPC for all extraction methods. Another study tested methanol and dichloromethane as suitable solvents for TPC extraction and antioxidant activity of brown algae [19]. *P. pavonica* had TPC of 44.61 mg GAE/g dry extract, and DPPH IC_{50} of 338.8 μg/mL. In addition, ORAC values were 1543.6 μmol TE/g of dry extract. Abdelhamid et al. [25] tested three Mediterranean brown macroalgae (*Cystoseira sedoides*, *P. pavonica,* and *Cladostephus spongeosis*) for phenolic content and antioxidant, anti-inflammatory, and antinociceptive potential. Solid-liquid extraction was performed with 50% ethanol while shaking for 30 min at 50 °C. The extracts were purified in three steps (by using petroleum ether, dichloromethane, and ethyl acetate) to increase the phlorotannin content. *P. pavonica* had the phenolic content of 7.06 ± 2.52 mg PGE/g dry algae and the DPPH IC_{50} of 91.78 ± 1.98 μg/mL. Dang et al. [45] compared phenolic content and antioxidant activities among six freeze-dried brown algae extracted with 70% ethanol using UAE (30 °C, 60′, and 150 W). Collected from Bateau Bay (Australia), *Padina sp.* had high TPC (124.65 ± 0.78 mg GAE/g extract), TFC (20.74 ± 0.49 mg CAE/g extract), and TTC (56.17 ± 0.22 mg CAE/g extract) content.

P. pavonica has also been reported for other biological activities, but studies have shown that biological activities vary with season. Ismail-ben Ali et al. [46] reported seasonal variation of antibacterial activity of the *P. pavonica* collected from the northern coast of Tunisia, with the highest activity in July, August, and September which could be correlated with higher sea temperature. However, the authors reported no data on algae composition. In general, reports on seasonal effect on phytochemical content and their biological potential in brown algae are limited. A recent study showed that different green extractions (pressurized liquid extraction (PLE), MAE, supercritical fluid extraction (SFE), and electrochemical/electroporation extraction) could be used to obtain anti-hyaluronidase water extracts from *P. pavonica* with possible application in cosmetic anti-age products [20].

In this study the chemical analyses of the phenolic profile were not done, however it would be useful to determine the specific compounds responsible for the antioxidant activity. Besides, other molecules, such as pigments, carbohydrates, and proteins are also present in the extract [4] and they can contribute to antioxidant activity.

1, 28, 55	MIX 1h H_2O
2, 29, 56	MIX 2h H_2O
3, 30, 57	MIX 1h EtOH 50
4, 31, 58	MIX 2h EtOH 50
5, 32, 59	MIX 1h EtOH 70
6, 33, 60	MIX 2h EtOH 70
7, 34, 61	SLE H_2O
8, 35, 62	SLE EtOH 50
9, 36, 63	SLE EtOH 70
10, 37, 64	UAE 1h H_2O
11, 38, 65	UAE 2h H_2O
12, 39, 66	UAE 1h EtOH 50
13, 40, 67	UAE 2h EtOH 50
14, 41, 68	UAE 1h EtOH 70
15, 42, 69	UAE 2h EtOH 70
16, 43, 70	MAE 5′ H_2O
17, 44, 71	MAE 10′ H_2O
18, 45, 72	MAE 15′ H_2O
19, 46, 73	MAE 30′ H_2O
20, 47, 74	MAE 5′ EtOH 50
21, 48, 75	MAE 10′ EtOH 50
22, 49, 76	MAE 15′ EtOH 50
23, 50, 77	MAE 30′ EtOH 50
24, 51, 78	MAE 5′ EtOH 70
25, 52, 79	MAE 10′ EtOH 70
26, 53, 80	MAE 15′ EtOH 70
27, 54, 81	MAE 30′ EtOH 70

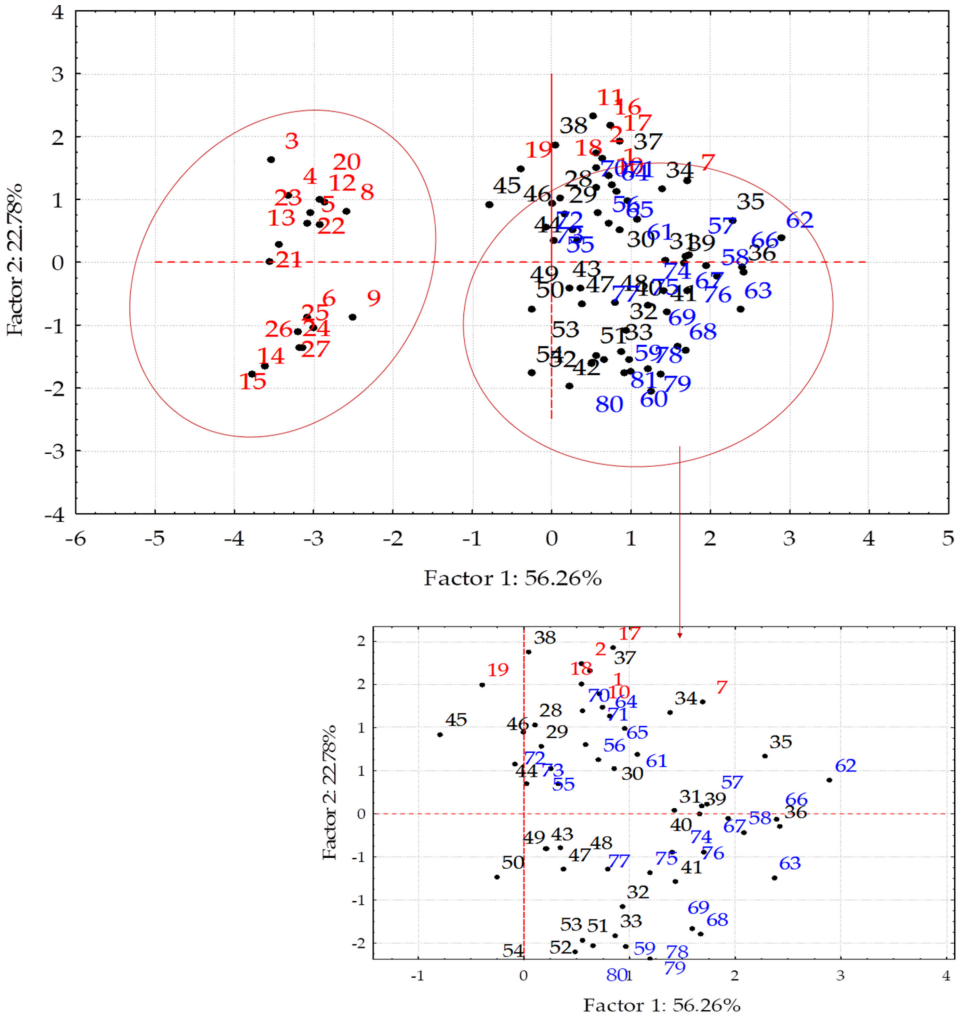

Figure 5. PCA for the antioxidant activity (Factor 1) and TTC (Factor 2) for freeze-dried (red, from 1–27), oven-dried (black, from 28–54), and shade-dried (blue, from 55–81) extracts of *P. pavonica* (lower graph is enlarged part of the main graph).

4. Conclusions

The results of this study contribute to the characterisation of brown algae as a matrix that can yield components with antioxidant potential. The novel, green methods (UAE and MAE) have shown more efficiency than the conventional SLE and MIX.

The TPC *of P. pavonica* was in the range from 0.44 ± 0.03 to 4.32 ± 0.15 mg GAE/g and in high correlation to DPPH and ORAC. The best method for achieving high TPC was freeze-drying while the best extraction method was MAE. In all cases, freeze-drying and extraction with ethanol (both 50% and 70%) maximized the TPC and antioxidant activity. It is an energy consuming method but based on PCA it is obvious that freeze-dried samples and water/ethanol mixtures are the best choices to obtain high reducing activity. MAE as a green option might reduce the extraction time and solvent consumption without loss in the activity of *P. pavonica* extracts.

The results obtained from this study show that *P. pavonica* should have good antioxidant activity despite relatively low TPC, but further analyses should be performed to identify the phenolic profile of the extracts, seasonal effect on phytochemical content, as well as their biological potential.

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8.2. Rad 2 - Variations in the composition, antioxidant and antimicrobial activities of *Cystoseira compressa* during seasonal growth

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Abstract: The underexplored biodiversity of seaweeds has recently drawn great attention from researchers to find the bioactive compounds that might contribute to the growth of the blue economy. In this study, we aimed to explore the effect of seasonal growth (from May to September) on the in vitro antioxidant (FRAP, DPPH, and ORAC) and antimicrobial effects (MIC and MBC) of *Cystoseira compressa* collected in the Central Adriatic Sea. Algal compounds were analyzed by UPLC-PDA-ESI-QTOF, and TPC and TTC were determined. Fatty acids, among which oleic acid, palmitoleic acid, and palmitic acid were the dominant compounds in samples. The highest TPC, TTC and FRAP were obtained for June extract, 83.4 ± 4.0 mg GAE/g, 8.8 ± 0.8 mg CE/g and 2.7 ± 0.1 mM TE, respectively. The highest ORAC value of 72.1 ± 1.2 µM TE was obtained for the August samples, and all samples showed extremely high free radical scavenging activity and DPPH inhibition (>80%). The MIC and MBC results showed the best antibacterial activity for the June, July and August samples, when sea temperature was the highest, against *Listeria monocytogenes*, *Staphylococcus aureus*, and *Salmonella enteritidis*. The results show *C. compressa* as a potential species for the industrial production of nutraceuticals or functional food ingredients.

Keywords: *Cystoseira compressa*; microwave-assisted extraction; green extraction; biological activity; seaweed; seasonal variations; nutraceuticals; fatty acids

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1. Introduction

Among seaweeds, the brown macroalgae (Phaeophyceae) have been identified as an outstanding source of phenolic compounds, from simple phenolic acids to more complex polymers such as tannins (mainly phlorotannins). Algal phlorotannins, a group of phenolic compounds restricted to the polymers of phloroglucinol, present a heterogeneous and high molecular weight group of compounds (from 126 Da to 650 kDa) which are verified in terrestrial plants [1,2]. The phlorotannins play an important role in the cellular and ecological growth and tissue healing of alga but also show strong antioxidant, antimicrobial, cytotoxic, and antitumor properties [3–6].

Brown fucoid algae of the genus *Cystoseira sensu lato* (Sargassaceae) consist of 40 species of large marine canopy-forming macroalgae found along the Atlantic–Mediterranean coasts [7,8]. So far, a total of 214 compounds have been isolated from sixteen *Cystoseira*

species, and the chemical constituents of *Cystoseira* spp. were found to contain fatty acids and derivatives, terpenoids, steroids, carbohydrates, phlorotannins, phenolic compounds, pigments and vitamins [7]. The chemical composition of the alga depends on numerous ecological factors such as temperature, salinity, UV irradiation, collecting season, depth, geographic location, thallus development, etc. However, their individual and synergistic effect on the brown alga chemical profile and biological activity is still relatively unknown. Recent studies showed that seasonality and thallus vegetative parts significantly affect the nutritional and chemical profile of alga [9]. It is considered that higher nutritional and phenolic content, higher polyunsaturated fatty acid (PUFA) content, higher vitamin and mineral content, as well as the antiproliferative properties of brown algae from brown fucoid algae were obtained during hot and dry summer seasons and higher sea temperatures [10–13]. On the other hand, no seasonal effect was recorded for the pigment profile and fucoxanthin content, nor total phenolic content and antimicrobial activity of the genera *Padina*, *Colpomenia*, *Saccharina* or *Dictyota* [14,15]. So far, there are no reports on seasonal variations in chemical profile nor the biological activity of *Cystoseira* spp.

Cystoseira spp. composition suggests their high nutritional value with potential applications in the nutraceutical industry. A range of 29–46% of PUFA, a low *n-6* PUFA/*n-3* PUFA ratio as well as favorable unsaturation, atherogenicity, and thrombogenicity indices were observed in several *Cystoseira* species [16]. Compounds from *Cystoseira* species are important sources of nutraceuticals and may be considered as functional foods, such as extracts of *C. tamariscifolia* and *C. nodicaulis* that were able to protect a human dopaminergic cell line from hydrogen peroxide-induced cytotoxicity and inhibit cholinesterases, while those from *C. crinita* showed significant cytotoxic activity against human breast adenocarcinoma (MCF-7 cells), inducing apoptosis and autophagy [17,18]. Besides non-volatiles, the essential oil constituents of *C. compressa* and their seasonal changes have been identified and among them for a large number of compounds a broad range of biological activities have been already proved [19]. So far, over 50 biological properties have been attributed to compounds found in genus *Cystoseira*, and the most reported are antioxidant, anti-inflammatory, cytotoxic, anticancer, cholinesterase inhibition, antidiabetic, and antiherpetic activities [7,20–24]. Phlorotannins are regarded as responsible for high antioxidant activity (e.g., free radical scavenging ability) [1,25–27]. Besides, there is little information on the antimicrobial activity of *Cystoseira* spp. extracts against major foodborne Gram-positive and Gram-negative bacteria [28].

The aim of this study was to investigate the chemical composition of *C. compressa*, one of the most widely distributed algae in the Adriatic Sea, to determine changes in its antioxidant and antimicrobial activity over the seasonal growth (May–September) when the algae are in the growing and reproductive phases, and the development of dense thallus occurs.

2. Results and Discussion

2.1. Total Phenolic Content, Total Tannin Content and Antioxidant Activity

Seaweed extracts were screened for total phenolic content (TPC), total tannin content (TTC) and antioxidant activity measured by ferric reducing/antioxidant power (FRAP), 2,2-diphenyl-1-picrylhydrazyl radical scavenging ability (DPPH) and the oxygen radical absorbance capacity (ORAC).

The results of TPC and TTC for *C. compressa* are shown in Figure 1. The results for TPC varied from 48.2 ± 0.5 to 83.4 ± 4.0 mg GAE/g. The highest TPC was found in June samples. On the other hand, the TTC values ranged from 2.0 ± 0.3 to 8.8 ± 0.8 mg CE/g with the highest value found also in June, followed by the extract from May. The FRAP values, shown in Figure 2, ranged from 1.0 ± 0.0 to 2.7 ± 0.1 mM TE. Similar to TPC and TTC, the highest FRAP result was obtained for June, showing the reducing activity of >2.5 mM TE. TPC and FRAP results were in high correlation (0.956; $p < 0.01$). The ORAC results are shown in Figure 2. The seaweed extracts were 200-fold diluted for ORAC assay. Among the investigated samples, the highest ORAC value of 72.1 ± 1.2 μM TE was found

in the August extract, with extracts from May having the second best. June and July extracts had the lowest ORAC values, more than 3-fold lower in comparison to the August extract. The DPPH radical inhibitions (in percentages) are shown in Figure 2. The extract from May had the highest inhibition (90.2%) while the August extract had the lowest inhibition (77.3%). The activity of other extracts was similar, around 85%. In the growing season, the sea temperature was the lowest in May (18.3 °C) and it rose every month till August when it peaked at 26.9 °C. Finally, a decrease in the temperature by 2.2 °C was observed in September (Table 1).

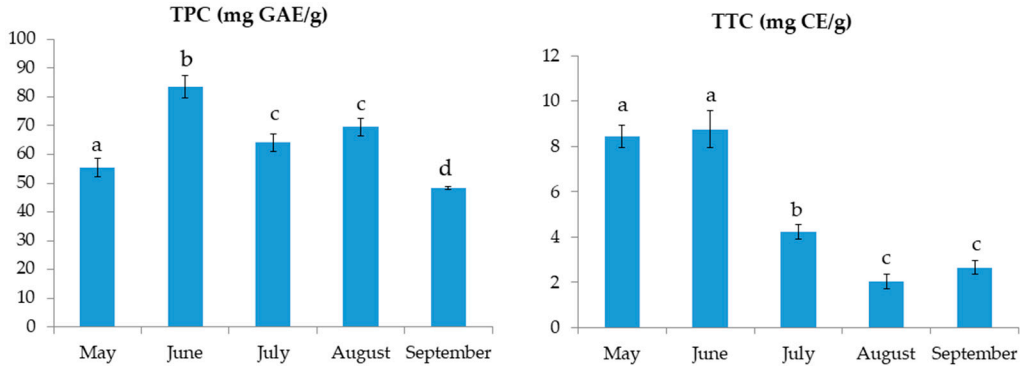

Figure 1. Total phenolic content (TPC) and total tannin content (TTC) of *C. compressa* extracts from May to September. [a–d] different letters denote statistically significant difference (n = 4).

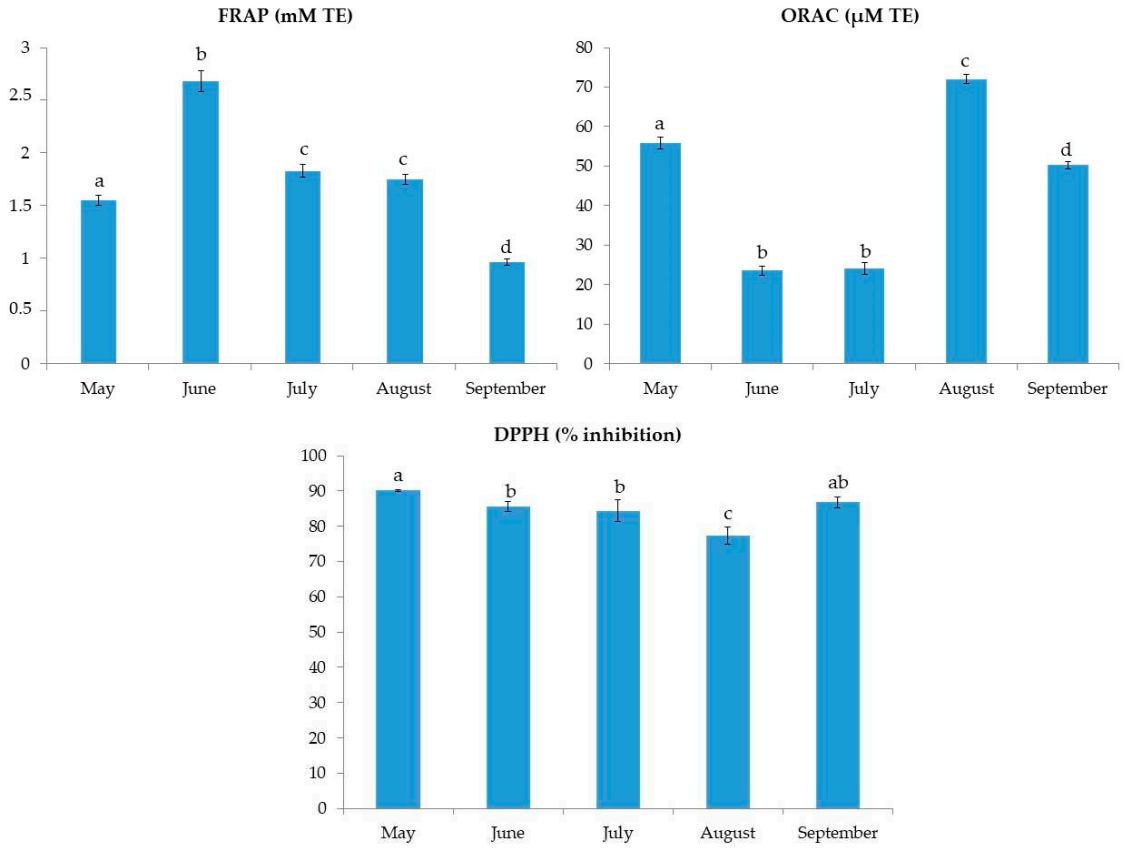

Figure 2. FRAP, ORAC and DPPH inhibition results for *C. compressa* extracts from May to September. [a–d] different letters denote statistically significant difference (n = 4).

Table 1. Sea temperature and salinity recorded during the harvest of the algal samples.

	May	June	July	August	September
Temperature (°C)	18.3	21.8	22.4	26.9	24.7
Salinity (PSU)	37.4	38.1	38.3	38.3	38.3

The TPC of alga varies with seasonal changes of sea temperature, salinity, light intensity, geographical location and depth, as well as other biological factors such as age, size, the life cycle of the seaweed, presence of herbivores [1]. In this study, the geographical location and depth were eliminated as a factor as samples were collected from the same area and depth each month. The TPC, TTC and antioxidant activity results showed no correlation to the sea temperature and salinity. If the growth of alga is considered, in June when the TPC, TTC and FRAP were the highest, *C. compressa* had a fully developed, densely ramified thalli with aerocysts. In May the thalli are not yet fully developed, while in July–September it is less dense, aerocysts appear in fewer numbers [29].

The TPC of *Cystoseira* species was previously investigated and researchers reported a strong effect of harvesting location and seasonal changes, especially temperature. Mancuso et al. [12] investigated TPC in *C. compressa* from eight locations along the Italian coast and confirmed the change of TPC with geographical location. The TPC ranged between different locations from 0.1 to 0.5% of algal dry weight (DW). The authors observed the increase in TPC with the rise of sea temperature (measured at different locations). Accordingly, the highest TPC of 0.53% DW was recorded at 28 °C. In contrast, Mannino et al. [30] investigated the effect of sea temperature seasonal variation on the TPC of *C. amentacea*. They harvested algae once in every season (winter, spring, summer and autumn) and measured the sea temperature. The authors observed the highest TPC in winter (0.8% DW) when the sea temperature was the lowest. In summer and autumn, when the sea temperatures were above 20 °C, the TPCs were the lowest, 0.4 and 0.37% DW, respectively. In their study, the TPC values showed a negative correlation with sea temperature. *Cystoseira compressa* extracts, from Urla (Turkey) [31], were screened for TPC, total flavonoid content (TFC), antioxidant and antimicrobial activity. The highest TPC of 1.5 mg GAE/g and TFC of 0.8 mg QE/g were found for hexane extract while the antioxidant activity of the hexane extract measured by DPPH radical inhibition was only 21.2%, more than four-fold lower than results in our study for hydroalcoholic extracts. In comparison, the methanolic extracts (similar polarity like ethanol) showed the TPC and TFC of 0.2 mg GAE/g and 0.3 mg QE/g, respectively and two-fold lower DPPH inhibition.

Abu-Khudir et al. [18] evaluated the antioxidant, antimicrobial, and anticancer activities of cold methanolic extract, hot methanolic extract, cold aqueous extract, and hot aqueous extract from *C. crinita* and *Sargassum linearifolium*. The highest TPC was found for the cold methanolic extract of *C. crinita*, 15.0 ± 0.58 mg GAE/g of dried extract, which is more than two-fold lower than the amount detected in the September extract from our study which contained the lowest TPC. The authors also found a high content of fatty acids (44%) and their esters in *C. crinita* cold methanolic extract. Both seaweeds showed similar DPPH and ABTS radical scavenging activity with *C. crinita* cold methanolic extract having IC_{50} of 125.6 µg/mL and 254.8 µg/mL, respectively. De La Fuente et al. [32] extracted *C. amentacea* var. *stricta* with dimethyl sulfoxide (DMSO) and 50% ethanol for determining TPC, TFC and antioxidant activity of extracts by DPPH radical scavenging, FRAP, OH scavenging, and nitric oxide (NO) scavenging methods. The TPC and TFC of DMSO extracts were 65.9 µg GAE/mg and 15.8 µg QE/mg, 3.2- and 5.1-fold higher than ethanolic extracts. Similar to our results, both investigated extracts had DPPH radical scavenging activity higher than 90%. Furthermore, the DMSO extracts showed a reducing activity of almost 90% while ethanolic extract showed a higher OH radical scavenging activity. Both extracts showed very low cytotoxicity, enabling their possible use as nutraceuticals.

Oucif et al. [20] screened six seaweed species (including *C. compressa* and *C. stricta*) for TPC, DPPH radical scavenging activity and reducing power. The highest TPCs were found

for *C. compressa* methanolic and ethanolic extracts, 10.24 ± 0.09 and 15.70 ± 0.72 mg GAE/g DW, respectively. *Cystoseira compressa* ethanol extract had over 90% inhibition activity for DPPH radical and the highest reducing power, which can be compared with our results. Mhadhebi et al. [24] determined TPC, DPPH and FRAP in *C. crinita*, *C. sedoides* and *C. compressa* extracts. Among the three alga, *C. compressa* extract had the highest TPC of 61.0 mg GAE/g, which is comparable to our results, the lowest DPPH IC_{50} of 12.0 µg/mL, and the highest FRAP value, 2.6 mg GAE/g.

2.2. Antimicrobial Activity

The results of minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of the *C. compressa* extracts against common foodborne pathogens are shown in Table 2. Gram-positive bacteria were more susceptible to seaweed extracts than Gram-negative bacteria. The lowest MIC results were found against *L. monocytogenes*, for June, July, and August samples with the lowest MBC in June, and against *S. aureus* in July and August with the same MBC. There was no difference in MIC and MBC values for *E. coli* among the investigated months. June, July, and August extracts had the lowest MIC values for *S. enteritidis*. The results showed higher antimicrobial activity from June to August when the sea temperature was the highest, against all bacteria.

Table 2. Results of the minimal inhibitory concentration (MIC, mg/mL) and minimal bactericidal concentration (MBC, mg/mL) of the seaweed extracts against foodborne pathogens (n = 3).

	May		**June**		**July**		**August**		**September**	
	MIC	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**
Escherichia coli	10	10	10	10	10	10	10	10	10	>10
Salmonella enteritidis	10	10	5	10	5	10	5	10	10	>10
Enterococcus faecalis	10	10	10	10	10	10	5	10	10	10
Listeria monocytogenes	10	>10	2.5	2.5	2.5	5	2.5	5	5	>10
Staphylococcus aureus	10	>10	5	5	2.5	2.5	2.5	2.5	10	10
Bacillus cereus	>10	n.d.	10	>10	10	>10	10	>10	>10	n.d.

n.d.—not determined

Alghazeer et al. [33] performed microwave-assisted extraction (MAE) on *P. pavonica* and *C. compressa*. Flavonoid-rich extracts (110.92 ± 11.38 mg rutin equivalents /g for *C. compressa*) were tested for antibacterial activity against multidrug-resistant (MDR) isolates of *S. aureus* subsp. *aureus*, *Bacillus pumilus*, *B. cereus*, *Salmonella enterica* subsp. *enteric*, and enterohemorrhagic *E. coli* using the well diffusion method, MIC and MBC. *Cystoseira compressa* extract showed stronger antibacterial activity than *P. pavonica* with inhibition zones against 14 tested isolates. The largest inhibition zones were 20.5 mm for *S. aureus* and *B. cereus*, 31 mm for *S. enterica* and 17 mm for *E. coli*. Furthermore, *C. compressa* extract had the lowest MIC (31.25 µg/mL) and MBC (62.5 µg/mL) values against *S. aureus* and *S. enterica*. Against *B. cereus*, it had an MIC value of 62.5 µg/mL and an MBC value of 125 µg/mL. The highest MIC (125 µg/mL) and MBC (500 µg/mL) values were found against *E. coli*. Maggio et al. [28] evaluated the antibacterial activity of eight brown seaweeds, six belonging to the genus *Cystoseira* (including *C. compressa*) and two belonging to the Dictyotaceae family, against *E. coli*, *Kocuria rhizophila*, *S. aureus* and a toxigenic and MDR *S. aureus* using the disk diffusion method. None of the seaweed extracts inhibited the growth of *E. coli*. *Cystoseira compressa* and *Carpodesmia amentacea* extracts showed antibacterial activity against *K. rhizophila*, *S. aureus* and MDR *S. aureus*. Abdeldjebbar et al. [34] tested the antibacterial effect of *C. compressa* and *P. pavonica* acetonic extracts against *E. coli* and *S. aureus*. The antibacterial activity was measured by disk diffusion method and MIC determination. *Cystoseira compressa* extract had 14 mm inhibition diameter for *E. coli*, showing better antibacterial activity than *P. pavonica* (12 mm). However, MIC values were not detected for *C. compressa* against both bacteria. *Padina pavonica* extract had an MIC of 50 µL for tested strains. Both extracts had a 10 mm inhibition diameter for *S. aureus*. The authors also tested

the synergy of these two extracts at a 1:1 ratio. The mixture showed significant synergistic effect against *E. coli* and *S. aureus* with 16 and 12 mm inhibition diameters, respectively. The antibacterial activity of a *C. crinita* cold methanolic extract was evaluated by the disk diffusion method [18]. The extract showed the highest inhibition zones for *E. coli*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Bacillus subtilis*, *S. aureus* and *Streptococcus aureus*, with 10.5, 12.8, 10.2, 12.6, 13.3 and 11.2 mm, respectively.

Cystoseira compressa extracts [31] showed moderate activity against *E. coli*, *S. aureus*, *Streptococcus epidermidis*, *E. faecalis*, *Enterobacter cloacae*, *Klebsiella pneumonie*, *B. cereus* and *P. aeruginosa*. In this study, the authors found the lowest MIC value of 32 µg/mL for both methanolic extract against *S. epidermidis* and chloroform extract against *E. cloacae*. Dulger and Dulger [21] tested *C. compressa* water and ethanol extracts against methicillin-resistant *S. aureus* (MRSA). Ethanol extract had the lowest MIC of 3.2 mg/mL and MBC of 6.3 mg/mL.

In the above-mentioned studies, the chemical content of the investigated algae was not correlated with the antimicrobial activity, however, it is evident that *Cystoseira* spp. shows some potential to be used nutraceuticals and therapeutic purposes.

2.3. Chemical Analysis by UPLC-PDA-ESI-QTOF

A quali-quantitative analysis of the polar compounds from *C. compressa* extracts was achieved by LC-ESI-QTOF-MS analysis in negative ion mode. The base peak chromatograms obtained are shown in Figure 3. A total of 49 compounds were identified and the results are shown in Table 3, along with their retention time, observed and theoretical *m*/*z*, error (ppm), score (%), molecular formulae and in source fragments. In all cases, the score remained higher than 90% and the error lower than 5 ppm. All the compounds we tentatively identified according to Bouafif et al. [35] who previously found most of them in *Cystoseira* and PubChem database. Furthermore, the amount of each compound is expressed as a percentage calculated based in the areas for each extract.

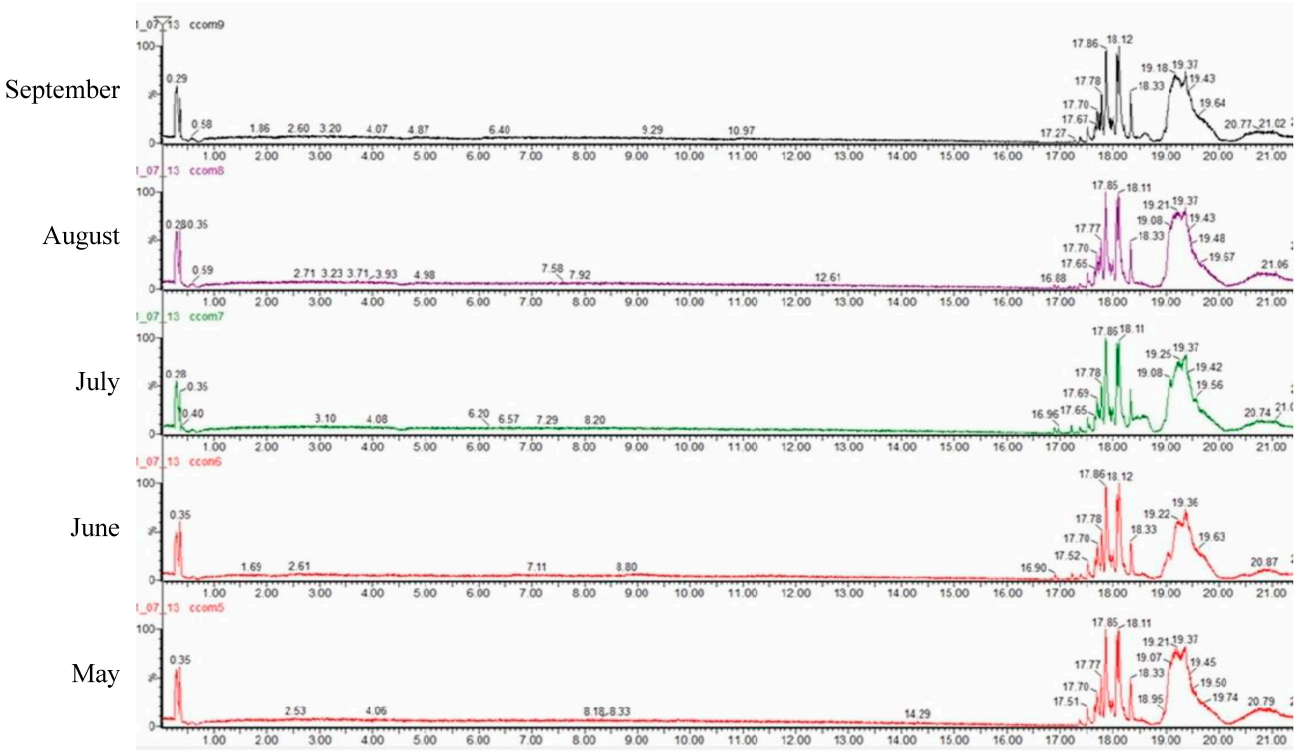

Figure 3. Chromatograms of the HPLC-qTOF-MS analyses of *C. compressa*.

Table 3. The compounds detected in investigated *C. compressa* samples analyzed by UPLC-PDA-ESI-QTOF.

N°	RT (min)	Observed (*m*/*z*)	Theorical (*m*/*z*)	Error (ppm)	Score (%)	Molecular Formula	In Source Fragments	Tentative Compound	May (%)	June (%)	July (%)	August (%)	September (%)
1	0.28	343.0367	343.0368	−0.3	94.18	$C_{20}H_4N_6O$	-	1a,9b-Dihydrophenanthro [9,10-b]oxirene-2,3,4,7,8,9-hexacarbonitrile	4.76	4.50	4.54	4.96	6.51
2	0.29	201.0244	201.0247	−1.5	98.89	$C_4H_{10}O_9$	-	2-(1,2,2,2-Tetrahydroxyethoxy)ethane-1,1,1,2-tetrol	6.67	6.17	6.58	6.72	7.90
3	0.32	141.0162	141.0161	0.7	91.01	$C_2H_2N_6O_2$	-	Diazidoacetic acid	1.97	2.02	2.40	2.01	1.74
4	0.35	181.0707	181.0712	−2.8	100	$C_6H_{14}O_6$	101.0230; 89.0227; 71.0137; 59.0121	D-Sorbitol	3.11	3.57	1.34	2.34	1.83
5	0.40	317.0506	317.0509	−0.9	90.44	$C_{12}H_{14}O_{10}$	209.0890	D-glucaric acid derivate	1.13	0.88	0.57	0.67	0.80
6	0.42	384.1510	384.1519	−2.3	92.29	$C_{15}H_{23}N_5O_7$	-	Threonyl-histidyl-glutamic acid	0.09	0.11	0.06	0.07	0.10
7	16.56	287.2211	287.2222	−3.8	95.91	$C_{16}H_{32}O_4$	-	10,11-Dihydroxy-9,12-dioxooctadecanoic acid	0.19	0.19	0.20	0.17	0.20
8	16.90	275.1999	275.2011	−4.4	96.48	$C_{18}H_{28}O_2$	231.2098; 253.0915	Stearidonic acid (C18:4n-3) isomer a	0.12	0.42	0.49	0.32	0.06
9	16.97	275.2007	275.2011	−1.5	97.68	$C_{18}H_{28}O_2$	231.2092; 177.0854; 255.2322;	Stearidonic acid (C18:4n-3) isomer b	0.21	0.28	0.56	0.32	0.08
10	16.97	293.2112	293.2117	−1.7	92.64	$C_{18}H_{30}O_3$	249.1835; 275.1652	13-ketooctadecadienoic acid isomer a	0.07	0.09	0.24	0.31	0.13
11	17.08	287.2211	287.2222	−3.8	95.91	$C_{16}H_{32}O_4$	271.2083; 253.2157	10,16-Dihydroxyhexadecanoic acid isomer a	0.01	0.01	0.02	0.03	0.03
12	17.13	309.2056	309.2066	−3.2	96.09	$C_{18}H_{30}O_4$	279.2287	6,9-Octadecadienedioic acid	0.02	0.04	0.01	0.08	0.00
13	17.18	295.2276	295.2273	1.0	100	$C_{18}H_{32}O_3$	279.2300; 275.2019; 255.2325	9,10-Epoxyoctadecenoic acid (vernolic acid)	0.02	0.13	0.07	0.13	0.08
14	17.20	277.2159	277.2168	−3.2	91.36	$C_{18}H_{30}O_2$	255.2321; 239.2030; 227.2013	gamma-Linolenic acid isomer a (C18:3n-6)	0.10	0.22	0.29	0.22	0.08
15	17.22	429.3009	429.3005	0.9	91.64	$C_{27}H_{42}O_4$	273.1859; 135.0447	24-Keto-1,25-dihydroxyvitamin D3	0.01	0.57	0.58	0.05	0.02
16	17.26	247.1689	247.1698	−3.6	94.96	$C_{16}H_{24}O_2$	233.0985	2,4,6-Triisopropyl benzoic acid	0.05	0.02	0.02	0.24	0.26
17	17.35	287.2212	287.2222	−3.5	90.62	$C_{16}H_{32}O_4$	271.2082; 253.2158	10,16-Dihydroxyhexadecanoic acid isomer b	0.01	0.01	0.13	0.15	0.09
18	17.37	199.1694	199.1698	−2.0	90.11	$C_{12}H_{24}O_2$	181.1062; 155.0336	Lauric acid	0.90	0.85	0.92	0.81	0.83
19	17.38	297.2426	297.2430	−1.3	98.84	$C_{18}H_{34}O_3$	279.2367; 255.2334	10-Oxooctadecanoic acid isomer a	0.35	0.39	0.34	0.37	0.40
20	17.40	243.1952	243.1960	−3.3	90.78	$C_{14}H_{28}O_3$	197.1907	3-hydroxymyristic acid	0.08	0.07	0.07	0.10	0.09
21	17.42	293.2112	293.2117	−1.7	94.2	$C_{18}H_{30}O_3$	249.1833; 275.1649	13-ketooctadecadienoic acid isomer b	0.04	0.10	0.11	0.29	0.04
22	17.43	427.2827	427.2848	−4.9	90.28	$C_{27}H_{40}O_4$	271.1716; 188.0842; 135.0441	Hydroxyprogesterone caproate	0.00	0.17	0.24	0.02	0.01
23	17.46	429.3009	429.3005	0.9	91.64	$C_{27}H_{42}O_4$	273.1843; 135.0445	24-Keto-1,25-dihydroxyvitamin D3 isomer b	0.00	0.04	0.08	0.01	n.d.
24	17.48	295.2262	295.2273	−3.7	94.13	$C_{18}H_{32}O_3$	279.2295; 275.2023; 255.2321	9,10-Epoxyoctadecenoic acid isomer b (vernolic acid)	0.34	0.43	0.39	0.40	0.41
25	17.51	269.2110	269.2117	−2.6	98.63	$C_{16}H_{30}O_3$	251.2336	3-Oxohexadecanoic acid	0.07	0.16	0.01	0.26	0.11
26	17.51	225.1857	225.1855	−0.9	95.99	$C_{14}H_{26}O_2$	188.0832; 213.1870; 175.0757	Myristoleic acid	2.35	2.14	2.26	2.15	2.22
27	17.53	255.2319	255.2324	−2.0	91.41	$C_{16}H_{32}O_2$	225.1861; 213.1845	Hexadecanoic acid (palmitic acid) isomer a (C16:0)	0.10	0.05	0.04	0.03	0.04
28	17.57	275.2007	275.2011	−1.5	97.68	$C_{18}H_{28}O_2$	231.2093; 255.2326	Stearidonic acid (C18:4n-3) isomer c	0.25	0.35	0.67	0.69	0.33
29	17.58	277.2152	277.2168	−5.8	99.51	$C_{18}H_{30}O_2$	255.2289; 239.2001; 227.1989	gamma-Linolenic acid isomer b (C18:3n-6)	0.00	0.01	0.01	0.01	0.00
30	17.59	213.18458	213.1855	−3.6	92.41	$C_{13}H_{26}O_2$	-	Tridecanoic acid	1.20	1.19	1.15	1.12	1.15
31	17.62	427.2839	427.2848	−2.1	96.07	$C_{27}H_{40}O_4$	271.1659; 188.0827; 135.0442	Hydroxyprogesterone caproate isomer b	n.d.	0.13	0.21	0.02	0.01
32	17.62	257.2108	257.2117	−3.5	95.16	$C_{15}H_{30}O_3$	227.2037; 211.2072	11-Hydroxypentadecanoic acid	0.10	0.09	0.09	0.07	0.11
33	17.63	251.2010	251.2011	−0.4	100	$C_{16}H_{28}O_2$	233.9910; 207.0983	7,10-hexadecadienoic acid	0.77	0.84	0.80	0.80	0.91
34	17.64	297.2429	297.2430	−0.3	97.33	$C_{18}H_{34}O_3$	279.2364; 255.2332	10-Oxooctadecanoic acid isomer b	0.56	0.63	0.58	0.53	0.56
35	17.66	239.2001	239.2011	−4.2	97.7	$C_{15}H_{28}O_2$	227.2002; 159.8926	Myristoleic acid methyl ester	5.22	5.23	5.00	4.89	4.92
36	17.74	277.2162	277.2168	−2.2	99.51	$C_{18}H_{30}O_2$	255.2318; 239.1991; 227.2015	gamma-Linolenic acid isomer c (C18:3n-6)	1.08	1.41	1.92	2.47	2.17

Table 3. *Cont.*

N°	RT (min)	Observed (*m*/*z*)	Theorical (*m*/*z*)	Error (ppm)	Score (%)	Molecular Formula	In Source Fragments	Tentative Compound	May (%)	June (%)	July (%)	August (%)	September (%)
37	17.71	301.2158	301.2168	−3.3	99.56	$C_{20}H_{30}O_2$	283.2283; 275.1972	Eicosapentanoic acid isomer a (C20:5n-3)	0.65	0.65	1.09	0.81	0.75
38	17.73	301.2156	301.2168	−4.0	98.12	$C_{20}H_{30}O_2$	283.2287; 275.1957	Eicosapentanoic acid isomer b (C20:5n-3)	0.62	0.63	1.06	0.80	0.74
39	17.77	227.2001	227.2011	−4.4	93.6	$C_{14}H_{28}O_2$	-	Tetradecanoic acid (C14:0)	5.04	5.19	5.34	5.17	5.22
40	17.81	271.2266	271.2273	−2.6	97.75	$C_{16}H_{32}O_3$	253.0954; 225.2211	Hydroxy-palmitic acid	0.47	0.41	0.45	0.56	0.66
41	17.85	253.2156	253.2168	−4.7	96.47	$C_{16}H_{30}O_2$	-	Palmitoleic acid isomer a (C16:1n-7)	12.65	11.93	11.45	11.57	11.74
42	17.94	241.2170	241.2168	0.8	100	$C_{15}H_{30}O_2$	223.2081	Pentadecanoic acid (C15:0)	3.68	3.89	3.87	3.72	3.67
43	17.97	279.2314	279.2324	−3.6	98.25	$C_{18}H_{32}O_2$	267.2340; 275.2037	Octadeca-10,12-dienoic acid (C18:2n-6)	1.07	1.14	1.29	1.31	1.27
44	18.01	267.2318	267.2324	−2.2	99.96	$C_{17}H_{32}O_2$	249.0437; 223.0291	9-Heptadecenoic acid (C17:1n-8)	3.73	3.99	3.67	3.78	3.60
45	18.08	255.2321	255.2324	−1.2	99.9	$C_{16}H_{32}O_2$	227.2015	Hexadecanoic acid (palmitic acid)(C16:0)	10.46	10.46	10.24	9.92	9.83
46	18.12	281.2486	281.2481	1.8	96.88	$C_{18}H_{34}O_2$	-	Oleic acid (C18:1n-9)	15.87	15.06	15.39	15.33	15.12
47	18.22	269.2476	269.2481	−5.6	99.96	$C_{17}H_{34}O_2$	255.2325	Heptadecanoic acid (C17:0)	5.30	5.23	5.18	5.08	4.95
48	18.33	283.2618	283.2637	−1.9	99.21	$C_{18}H_{36}O_2$	-	Octadecanoic acid (stearic acid) C18:0	5.44	4.92	5.01	5.11	5.24
49	18.54	311.2944	311.2950	−2.0	90.87	$C_{20}H_{40}O_2$	255.2307; 225.0060	Arachidic acid	0.68	0.62	0.67	0.66	0.68

The most dominant compound tentatively identified was oleic acid (C18:1n-9) with a content more than 15% in all tested samples, highest in May. The other two dominant compounds were palmitoleic acid (C16:1n-7) and palmitic acid (C16:0) also showing highest content in the May extract. Except for highly represented fatty acids, ω-3 eicosapentaenoic acid (EPA) was also found, with the highest content in July.

Low molecular weight phenolic compounds were not identified. This does not confirm that the phenolics are not present, but the main phenolics in algae are probably present as tannins (phlorotannins) that cannot be determined by HPLC-ESI-TOF-MS because they cannot be ionized due to their high molecular weight. Maggio et al. [28] reported citric acid, isocitric acid, vanillic acid methyl ester, vanillic acid sulfate, gallic acid, dihydroxybenzoic acid, 2-hydroxy-6-oxo-6-(2-hydroxyphenoxy)-hexa-2,4-dienoate, phloracetophenone, bromo-phloroglucinol, vanillylmandelic acid and exifone in *C. compressa*. The compounds were identified without quantification. Previously, vanilic acid, hydroxybenzoic acid, gallocatechin, carnosic acid, phloroglucinol, and hydroxytyrosol 4-*O*-glucoside were identified as main phenolics in fucoidan algae *Sargassum* sp. [36].

Jerković et al. [37] investigated fucoidal brown alga *Fucus virsoides* and found 42.28% oleic acid, 15.00% arachidonic acid and 10.51% myristic acid in its fatty acid composition. The authors used high performance liquid chromatography–high-resolution mass spectrometry (HPLC-ESI-HRMS) to determine the composition of less polar non-volatile compounds. The major compounds tentatively identified belonged to five groups, steroids, terpenoids, fatty acid glycerides, carotenoids, and chlorophyll derivatives. Fatty acid glycerides were dominant, which is comparable to our study.

Ristivojević et al. [38] identified the bioactive compounds responsible for the radical scavenging and antimicrobial activities of *Undaria pinnatifida* and *Saccharina japonica* methanolic extracts using the high-performance thin layer chromatography (HPTLC)-bioautography assay and ultra-high-performance liquid chromatography (UHPLC)-LTQ-MS/MS combined. They reported eicosapentaenoic, stearidonic and arachidonic acids as major compounds accountable for these activities. Their findings are in accordance with previous reports on PUFAs having antimicrobial activity against bacteria, viruses and fungi [39,40].

PUFAs, such as EPA, docosahexaenoic acid (DHA) and linolenic acid (LNA), showed in vitro antibacterial activity against *Helicobacter pylori*, *S. aureus*, Methicillin-resistant *S. aureus* (MRSA), *Vibrio vulnificus*, and *Streptococcus mutans*, inhibiting bacterial growth or altering their cell morphology [40]. To deactivate microbial cells, PUFAs directly affected the cell membranes, enhanced free radical generation, and increased the formation of cytotoxic lipid peroxides and their bioactive metabolites increasing the leukocytes' and

macrophages' phagocytic action [39]. EPA and DHA extracts showed antimicrobial activity against foodborne pathogenic bacteria, *L. monocytogenes*, *B. subtilis*, Enterobacter aerogenes, *E. coli*, *S. aureus*, *S. enteritidis*, *S. typhimurium*, and *P. aeruginosa* [41]. The authors reported the lowest MIC value of 250 µg/mL for DHA extract against *P. aeruginosa*. A low MIC value of 350 µg/mL was found for EPA extract against *L. monocytogenes*, *B. subtilis* and *P. aeruginosa*, and for DHA extract against *L. monocytogenes* and *B. subtilis*. Besides, Cvitković et al. [42] investigated the extraction of lipid fractions from *C. compressa*, *C. barbata*, *F. virsoides*, and *Codium bursa*. In agreement with our results, the dominant fatty acids in all seaweeds were palmitic, oleic and linolenic fatty acids. *Cystoseira compressa* and *C. barbata* had the highest amounts of omega-3 EPA and DHA. *Cystoseira compressa* had 20.35% oleic acid, 17.66% arachidonic acid, 14.86% linoleic acid, 11.92% palmitic acid and 8.72% linolenic acid. Bacteria *S. aureus* can be inhibited by most free fatty acids: Lacey and Lord [43] seeded this bacterium on human skin and then applied LNA to the skin which resulted in the rapid death of the seeded bacteria. EPA (C20:5n-3) was found to successfully inhibit the growth of *S. aureus* and *B. cereus* with a 64 mg/L MIC value [44]. Oleic acid was confirmed in vitro and in vivo to effectively eliminate MRSA by disrupting its cell wall [45].

3. Materials and Methods

3.1. Sample Collection

Cystoseira compressa samples were collected off the south coast of the island Čiovo in the Adriatic Sea from May to September 2020 (43.493389° N, 16.272505° E). Sampling was done throughout a lagoon at 25 points in a depth range of 20 to 80 cm. The sea temperature and salinity were measured during sampling using a YSI Pro2030 probe (Yellow Springs, OH, USA). A sample of this species is deposited in the herbarium at the University Department of Marine Studies in Split.

3.2. Pre-Treatment and Extraction

Prior to the extraction, harvested algal samples were washed with tap water to remove epiphytes. Samples were then freeze-dried (FreeZone 2.5, Labconco, Kansas City, MO, USA) and ground. Based on the previous research [46] seaweeds were extracted using MAE in the advanced microwave extraction system (ETHOS X, Milestone Srl, Sorisole, Italy). Seaweeds were mixed with 50% ethanol, using 1:10 (*w:v*) algae to solvent ratio and extracted for 15 min at 200 W and 60 °C. The extracts were further centrifuged at 5000 rpm for 8 min at room temperature and the supernatant was filtered. The ethanolic solvent was evaporated at 50 °C and the rest of the extracts freeze dried.

3.3. Determination of Total Phenolics, Total Tannins and Antioxidant Activity

The crude algal extracts were dissolved in 50% ethanol prior to analyses in the concentration of 20 mg/mL. Folin–Ciocalteu method [47] was used for determining the TPC. Briefly, 25 µL of the extract was mixed with 1.5 mL distilled water and 125 µL Folin–Ciocalteu reagent. The solution was stirred and 375 µL 20% sodium carbonate solution and 475 µL distilled water was added after one minute. Samples were left in the dark at room temperature for 2 h. The absorbance was read using a spectrophotometer (SPECORD 200 Plus, Edition 2010, Analytik Jena AG, Jena, Germany) at 765 nm. Results were expressed as gallic acid equivalents in mg/g of freeze-dried extract (mg GAE/g).

The TTC was measured according to Zhong et al. [36] with some modifications. Briefly, 25 µL of the sample, 150 µL 4% (*w/v*) ethanolic vanillin solution, and 25 µL 32% sulfuric acid (diluted with ethanol) were added to the 96-well plate and mixed. The plate was incubated for 15 min at room temperature and absorbance was read at 500 nm using the microplate reader (Synergy HTX Multi-Mode Reader, BioTek Instruments, Inc., Winooski, VT, USA). The TTC results were expressed as mg catechin equivalents per g of dried extract (mg CE/g).

The reducing activity was measured as FRAP (ferric reducing/antioxidant power) [48]. Briefly, 300 µL of FRAP reagent solution was pipetted into the microplate wells, and

absorbance at 592 nm was recorded. Then, 10 µL of the sample was added to the FRAP reagent and the change in absorbance after 4 min was measured. The change in absorbance, calculated as the difference between the final value of the absorbance of the reaction mixture after a certain reaction time (4 min) and the absorbance of FRAP reagent before sample addition, was compared with the values obtained for the standard solutions of Trolox. Results were expressed as micromoles of Trolox equivalents per liter of extract (µM TE).

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging ability of extracts was also measured in 96-well microplates [49]. DPPH radical solution with the initial absorbance of 1.2 (290 µL) was pipetted into microplate wells, and absorbance was measured at 517 nm. Then, 10 µL of the sample was added to the wells and the decrease in the absorbance was measured after 1 h using the plate reader. The antioxidant activity of extracts was expressed as DPPH radical inhibition percentages (% inhibition).

The oxygen radical absorbance capacity (ORAC) method [50,51] was performed to determine the antioxidant capacity of extracts by monitoring the inhibition of the action of free peroxyl radicals formed by the decomposition of 2,2-azobis (2-methylpropionamide)-dihydrochloride (AAPH) against the fluorescent compound fluorescein. Briefly, 150 µL of fluorescein and 25 µL of the sample in 1:200 dilution (or Trolox in the case of standard compound, or puffer in the case of blank) were pipetted into microplate wells and thermostated for 30 min at 37 °C. After 30 min, 25 µL of AAPH was added and measurements were performed at excitation and emission wavelengths of 485 and 520 nm every minute for 80 min. The results were expressed as µM of Trolox Equivalents (µM TE).

3.4. Determination of the Antimicrobial Activity

The foodborne pathogens *Escherichia coli* ATCC 25922, *Salmonella enteritidis* ATCC 13076, *Enterococcus faecalis* ATCC 29212, *Listeria monocytogenes* ATCC 7644, *Staphylococcus aureus* ATCC 25923, and *Bacillus cereus* ATCC 14579 were used in this study.

The microdilution method was used to determine the extracts' MICs against foodborne pathogens. The extracts were dissolved in 4% DMSO (10 mg/mL) and diluted with Mueller–Hinton broth (MHB). Then, 100 µL of the mixture was added to the first well of the 96-well microtiter plate. Two-fold dilutions were done in the next wells (10–0.16 mg/mL). The 50 µL of prepared inoculum (1×10^5 colony forming units (CFU)/mL determined by using the growth curves of bacteria in the *log* phase) was added to each well and plates were mixed on a microtiter plate shaker for 1 min at 600 rpm (Plate Shaker-Thermostat PST-60 HL, Biosan, Riga, Latvia). Positive control (50 µL of inoculum and 50 µL of broth media), negative control (50 µL of broth media and 50 µL of extract), blank (100 µL of broth media) and 4% DMSO were also tested. After 24 h of incubation, 20 µL of the indicator of bacterial metabolic activity, 2-*p*-iodophenyl-3-*p*-nitrophenyl-5-phenyl tetrazolium chloride (INT, in 2 mg/mL concentration) was added to each well. Plates were mixed on a plate shaker and incubated for 1 h in the dark. MIC values were read visually as the lowest concentration of the extract at which there was no detection of bacterial growth seen as the reduction of INT to red formazan [52].

MBC of the seaweed extracts was determined as the lowest concentration at which no microbial growth was detected on agar plates after subcultivation of bacterial suspension pipetted from wells where MIC was determined and from wells with higher extract concentrations [53].

3.5. Compound Analysis by UPLC-PDA-ESI-QTOF

Dried extract (3 mg) of algae was dissolved in 1 mL of MeOH/H_2O 1/1 v/v. The analysis of compounds from algae was carried out with the use of an ACQUITY Ultra Performance LC system equipped with a photodiode array detector with a binary solvent manager (Waters Corporation, Milford, MA, USA) series with a mass detector Q/TOF micro mass spectrometer (Waters) equipped with an electrospray ionization (ESI) source operating in negative mode at the following conditions: capillary voltage, 2300 kV; source temperature, 100 °C; cone gas flow, 40 L/h; desolvation temperature, 500 °C; desolvation gas

flow, 11,000 L/h; and scan range, m/z 50–1500. Separation of individual compounds was carried out using an ACQUITY UPLC BEH Shield RP18 column (1.7 µm, 2.1 mm × 100 mm; Waters Corporation, Milford, MA, USA) at 40 °C. The elution gradient test was carried out using water containing 1% acetic acid (A) and acetonitrile (B), and applied as follows: 0 min, 1% B; 2.3 min, 1% B; 4.4 min, 7% B; 8.1 min, 14% B; 12.2 min, 24% B; 16 min, 40% B; 18.3 min, 100% B, 21 min, 100% B; 22.4 min, 1% B; 25 min, 1% B. The sample volume injected was 2 µL and the flow rate used was 0.6 mL/min. The compounds were monitored at 280 nm. Integration and data elaboration were performed using MassLynx 4.1 software (Waters Corporation, Milford, MA, USA) [54].

3.6. Statistical Analyses

The results of antioxidant analyses were expressed as mean ± standard deviation and antimicrobial results as a mean of 3 replicas. Analysis of variance (one-way ANOVA) was used to assess the difference between TPC, TTC and antioxidant assays, followed by a least significance difference test at 95% confidence level to evaluate differences between sets of mean values [55]. Pearson's correlation coefficient was used to determine the relation between the variables. Analyses were carried out using Statgraphics Centurion-Ver.16.1.11 (StatPoint Technologies, Inc., Warrenton, VA, USA).

4. Conclusions

The results obtained for the brown fucoidal macroalgae *C. compressa* from the Adriatic Sea indicated that it was a good source of compounds. The TPC and TTC content reflected a variation over the growing season, with the highest values in June. The detected FRAP showed high correlation with TPC and TTC content. The DPPH values were >80% inhibition over the whole sampling period, while the highest antioxidant activity with regards to ORAC was in August when the sea temperature was the highest. No evident correlation existed between the temperature and salinity change and TPC, TTC or antioxidant activity. From June to August, higher antimicrobial activity against foodborne pathogens was observed, especially against *L. monocytogenes*, *S. aureus* and *S. enteritidis*. Further investigations are needed to gain insight into the effect of abiotic factors, growth and thallus development of the alga on its biological potential and to discover the compounds responsible for the different biological activities.

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8.3. Rad 3 - Detailed chemical prospecting of volatile organic compounds variations from Adriatic macroalga *Halopteris scoparia*

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Article

Detailed Chemical Prospecting of Volatile Organic Compounds Variations from Adriatic Macroalga *Halopteris scoparia*

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Abstract: The present study aimed to isolate volatile organic compounds (VOCs) from fresh (FrHSc) and air-dried (DrHSc) *Halopteris scoparia* (from the Adriatic Sea) by headspace solid-phase microextraction (HS-SPME) and hydrodistillation (HD) and to analyse them by gas chromatography and mass spectrometry (GC–MS). The impact of the season of growth (May–September) and air-drying on VOC composition was studied for the first time, and the obtained data were elaborated by principal component analysis (PCA). The most abundant headspace compounds were benzaldehyde, pentadecane (a chemical marker of brown macroalgae), and pentadec-1-ene. Benzaldehyde abundance decreased after air-drying while an increment of benzyl alcohol after drying was noticed. The percentage of pentadecane and heptadecane increased after drying, while pentadec-1-ene abundance decreased. Octan-1-ol decreased from May to September. In HD-FrHSc, terpenes were the most abundant in June, July, and August, while, in May and September, unsaturated aliphatic compounds were dominant. In HD-DrHSc terpenes, unsaturated and saturated aliphatic compounds dominated. (*E*)-Phytol was the most abundant compound in HD-FrHSc through all months except September. Its abundance increased from May to August. Two more diterpene alcohols (isopachydictyol A and cembra-4,7,11,15-tetraen-3-ol) and sesquiterpene alcohol gleenol were also detected in high abundance. Among aliphatic compounds, the dominant was pentadec-1-ene with its peak in September, while pentadecane was present with lower abundance. PCA (based on the dominant compound analyses) showed distinct separation of the fresh and dried samples. No correlation was found between compound abundance and temperature change. The results indicate great seasonal variability of isolated VOCs, as well among fresh and dried samples, which is important for further chemical biodiversity studies.

Keywords: brown macroalgae; volatiles; gas chromatography and mass spectrometry (GC–MS); air-drying; benzaldehyde; pentadecane; pentadec-1-ene; (*E*)-phytol; diterpene alcohols

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1. Introduction

Halopteris is a genus of brown seaweeds that currently consists of 14 species. Species belonging to this order usually grow from 3 to 20 cm. Generally, they inhabit the lower intertidal zone and sublittoral in temperate regions. They grow on the rocks and have olive to dark-brown or reddish-brown thalli [1]. The extracts prepared from seaweeds belonging to *Halopteris* genus have shown various biological activities, including antiprotozoal activity [2,3], antibacterial activity [4–8], antifungal activity [7,8], apoptotic/cytotoxic activity [9], antioxidant activity [9–12], anti-inflammatory activity [12], anticoagulant activity [13], anti-acetylcholinesterase activity [14], and antifouling activity [7].

Nunes et al. [15] identified minor and major constituents in *Halopteris scoparia* harvested off the northern coast of the Island of Gran Canaria, Spain. The authors found 5.20 g/100 g dw (dry weight) of moisture, 57.20 g/100 g dw of total minerals, 5.54 g/100 g dw of proteins, 3.64 g/100 g dw lipids, and 29.86 g/100 g dw of total carbohydrates. As

for the chlorophylls, carotenoids, and phenolic compounds, 8.15 mg/g dw, 2.15 mg/g dw, and 1.90 mg gallic acid equivalents (GAE)/g dw, respectively, were found. On the other hand, Uslu et al. [16] found 9.79 g/100 g dw of proteins, 2.85 g/100 g dw of lipids, and 28 g/100 g dw of ash from *H. scoparia* harvested from Iskenderun Bay, Turkey. Furthermore, the authors identified five fatty acids, myristic, palmitic, heptadecanoic, palmitoleic, and oleic acid, with palmitic acid as the predominant one with 37.47% of the total fatty acid content (TFAC) in *H. scoparia*. Pereira et al. [17] identified the fatty acid profile of *H. scoparia* harvested off the Algarve coast, Portugal. Palmitic acid was the predominant saturated fatty acid with 24.36% of TFCA. A high amount (51.01% of TFCA) of polyunsaturated fatty acids (PUFAs) was found. Linoleic, arachidonic, and eicosapentaenoic acids were the predominant PUFAs found with 20.35%, 13.96%, and 14.39% of TFCA, respectively. Docosahexaenoic acid was also identified. A similar fatty acid profile was found by Campos et al. [12] in *H. scoparia* harvested from Azores island, Portugal. Moreover, the authors prepared water extract from seaweed and found 217 mg GAE/100 g dw of phenolic compounds. Güner et al. [9] determined phenolic (33.20 mg GAE/g) and flavonoid (1.26 mg quercetin equivalent (QE)/g) contents in *H. scoparia* methanol extract. This seaweed was harvested off the coast of Urla, Turkey.

To the best of our knowledge, there are no records of *H. scoparia* volatile organic compound (VOC) composition. In our previous study, we identified VOCs from *Halopteris filicina* from the Adriatic Sea [18]. The most dominant individual compounds found were dimethyl sulphide, fucoserratene, benzaldehyde, and octan-1-ol with 12.8%, 9.5%, 8.7%, and 5.1%, respectively. Aliphatic compounds were the most dominant group. Furthermore, Whitfield et al. [19] identified bromophenols in two *Halopteris* species, *Halopteris paniculata* and *Halopteris platycena*, harvested from eastern Australia. Identified compounds were 2- and 4-bromophenol, 2,4- and 2,6-dibromophenol, and 2,4,6-tribromophenol. The methods used for *Halopteris* species VOC isolation were headspace solid-phase microextraction (HS-SPME) [18] and combined steam distillation solvent extraction (SDE) with pentane/diethyl ether (9:1 *v*/*v*) as the solvent after the alga was blended in purified water and acidified to pH 1 for the isolation of bromophenols [19]. Other methods were also used for algal VOC isolation in general such as hydrodistillation (HD) [20], purge and trap [21], simultaneous distillation extraction under reduced pressure, or accelerated solvent extraction [22].

This study aimed to isolate and analyse VOCs from fresh (FrHSc) and air-dried (DrHSc) *H. scoparia* harvested from the Adriatic Sea, by using HD and HS-SPME. These methods were selected since they are complementary, providing isolation of the headspace, volatile, and semi-volatile compounds. The use of HD is appropriate for volatile and semi-volatile compounds (discriminating the most volatile headspace compounds), and HS-SPME is a headspace method (discriminating semi-volatile compounds). Furthermore, the influence of air-drying and season of growth (May–September) on VOC composition was also studied for the first time, and the obtained data were elaborated by principal component analysis (PCA). It was expected to find pentadecane, a marker of brown macroalgae, along with other VOCs from different chemical groups, as well as to observe VOC seasonal changes and determine the impact of air-drying on VOC composition. Seasonal variability of algal VOCs is rarely studied in general, and we previously researched the seasonal variability of volatilome from *Dictyota dichotoma* [20].

2. Results and Discussion

2.1. Headspace Variations of H. Scoparia

Headspace composition was analysed using HS-SPME. To acquire more information about the headspace composition two fibres of different polarities were used: divinylbenzene/carboxene/polydimethylsiloxane (DVB/CAR/PDMS, f1) and polydimethylsiloxane/divinylbenzene (PDMS/DVB, f2). According to our previous research [20,23,24], HS-SPME provides a large number of extracted headspace compounds with more or less diversity within two fibres of different polarity. In FrHSc headspace (HS-FrHSc), great variability was found within the months by two fibres, while the DrHSc headspace (HS-DrHSc)

volatilome was more comparable. From 91.50% (HS-FrHSc, June) to 99.31% (HS-DrHSc, July) of VOCs were identified with f1 and from 89.97% (HS-DrHSc, May) to 100% (HS-DrHSc, September) of VOCs were identified with f2. Identified VOCs could be classified into six different groups: saturated aliphatic compounds, unsaturated aliphatic compounds, benzene derivatives, terpenes, C_{13}-norisoprenoids (carotenoids degradation products), and others (Figures 1 and 2). The dominant VOCs were aliphatic compounds followed by benzene derivatives, except for HS-FrHSc in June and August extracted with f2, where benzene derivatives represented the most prevalent group of compounds (Figures 3 and 4).

The most abundant headspace compounds were benzaldehyde (HS-FrHSc in May (23.85%, f1), June (34.24%, f1; 52.75%, f2), July (32.99%, f2), and August (31.29%, f1; 57.85%, f2), pentadecane (HS-DrHSc from May (22.06%, f1; 14.06%, f2) to September (29.33%, f1; 24.47%, f2)), and pentadec-1-ene (HS-FrHSc in July (25.14%, f1) and September (53.17%, f1; 40.35%, f2)). Benzaldehyde abundance decreased after air-drying. Analysing with f1, the higher drop was in June (8.8 times) and August (8.4 times), while, analysing with f2, this decrease was even more perceived—June (13.8 times) and August (22.1 times). Since benzaldehyde is highly volatile, its loss after air-drying could be assigned to this property [24]. In the period from May to July, an increment in benzyl alcohol area percentage after the drying was noticed, with a peak in May (5.84%, f1; 6.53%, f2). Analysing with f1, it was not detected in HS-FrHSc within all months and also not in HS-DrHSc in August and September (as also seen for f2) (Table 1). This could be the result of polyphenolic compound oxidation rather than lignin-like compound degradation since brown algae seem to not specifically contain lignin, but only phenol compounds [25,26].

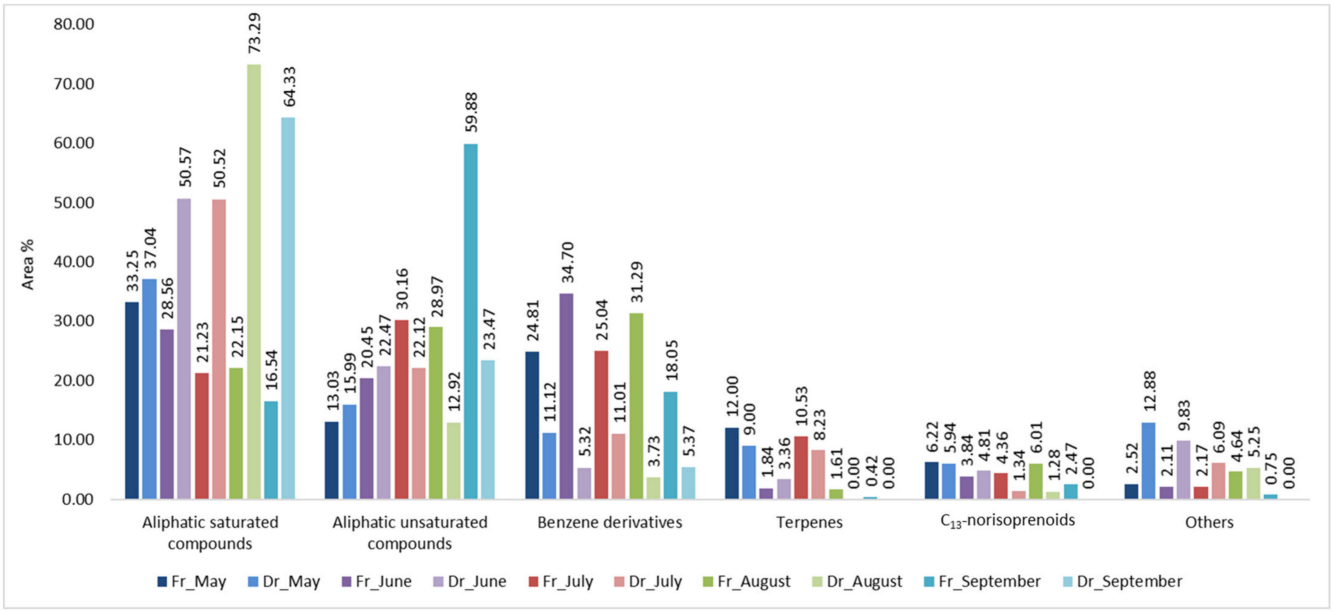

Figure 1. The VOCs from *H. scoparia* sorted by structural groups extracted by headspace solid-phase microextraction (HS-SPME) and analysed by gas chromatography–mass spectrometry (GC–MS) using DVB/CAR/PDMS fibre (f1); Fr (fresh), Dr (dry).

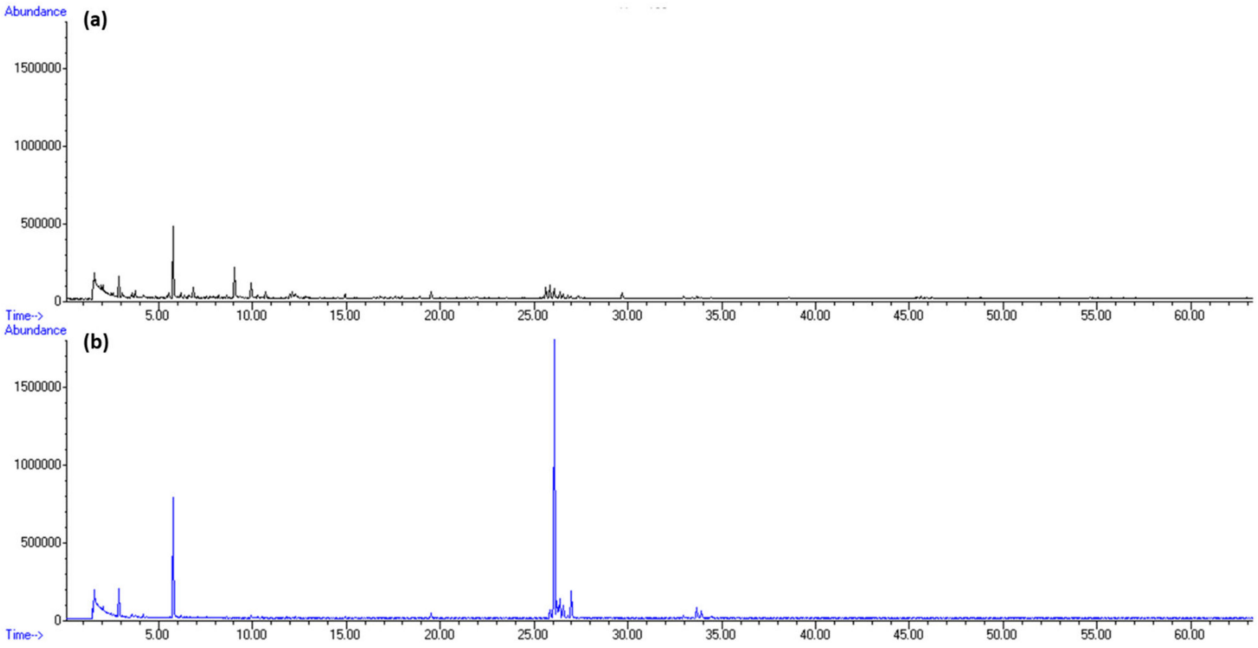

Figure 2. Total ion chromatogram (TIC) comparison of *H. scoparia* isolated by headspace solid-phase microextraction (HS-SPME) and analysed by gas chromatography–mass spectrometry (GC–MS) using DVB/CAR/PDMS fibre (f1): (**a**) in May; (**b**) in September.

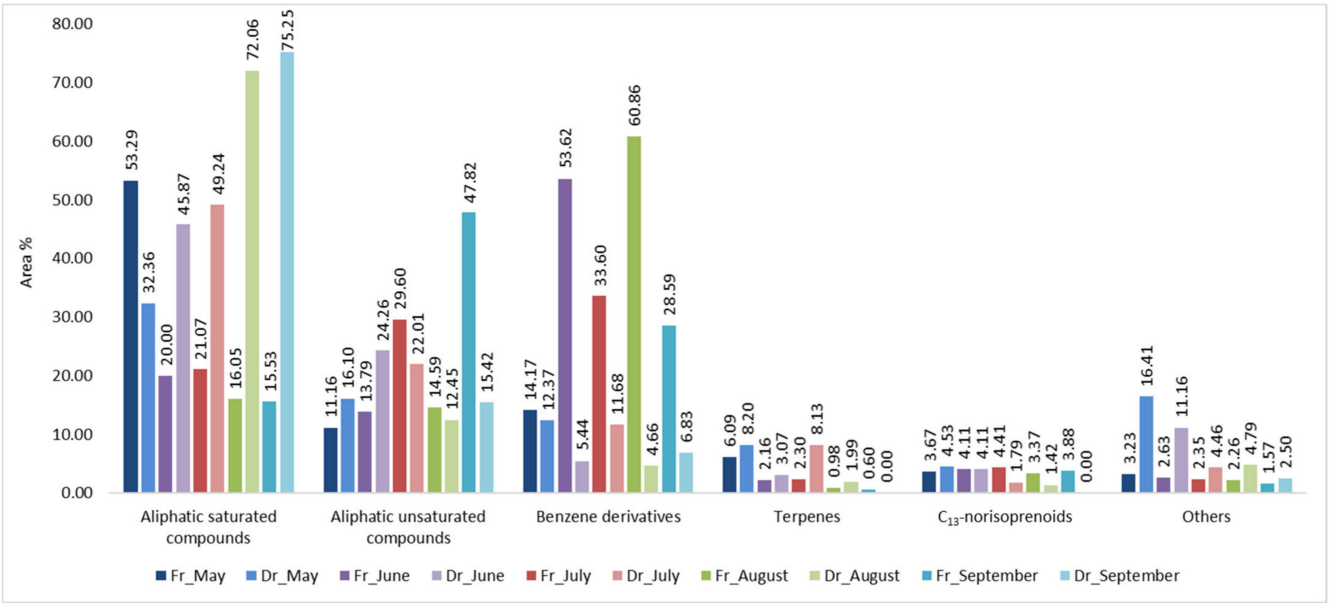

Figure 3. The VOCs from *H. scoparia* sorted by structural groups extracted by headspace solid-phase microextraction (HS-SPME) and analysed by gas chromatography–mass spectrometry (GC–MS) using PDMS/DVB fibre (f2); Fr (fresh), Dr (dry).

Table 1. The volatile compounds from *H. scoparia* isolated by headspace solid-phase microextraction (HS-SPME) and analysed by gas chromatography–mass spectrometry (GC–MS) using DVB/CAR/PDMS fibre (f1); Fr (fresh), Dr (dry).

Compound	RI	Area %									
		May		June		July		August		September	
		Fr	Dr	Fr	Dr	Fr	Dr	Fr	Dr	Fr	Dr
Dimethyl sulfide	<900	-	-	-	-	-	0.76	-	-	-	-
2-Methylpropan-1-ol	<900	-	0.57	-	-	-	-	-	-	-	-
3-Methylbutanal	<900	0.57	-	0.49	-	0.71	-	0.24	-	-	-
Pent-1-en-3-ol	<900	0.97	1.12	1.09	2.34	0.60	1.44	0.48	0.72	0.44	1.54
2-Methylbutanol	<900	-	1.04	-	-	-	-	-	-	-	-
Pentan-1-ol	<900	-	0.24	-	0.55	-	0.47	-	0.79	-	-
(*Z*)-Pent-2-en-1-ol	<900	0.48	-	0.34	0.52	-	0.61	-	-	-	-
Hexanal	<900	3.80	1.83	3.39	1.60	2.03	4.00	1.99	6.11	2.60	4.58
3-Methylbutanoic acid	<900	-	1.13	-	0.90	-	-	-	-	-	-
2-Methylbutanoic acid	<900	-	0.70	-	0.42	-	-	-	-	-	-
(*E*)-Hex-2-enal	<900	1.11	1.32	0.88	0.40	0.39	0.59	0.42	-	0.38	-
Hexan-1-ol	<900	1.26	0.87	0.44	0.95	0.94	3.15	1.29	1.19	-	5.61
Tribromomethane	<900	-	1.80	-	0.77	-	-	0.72	0.93	0.44	-
Heptan-2-one	<900	-	0.39	-	0.16	-	1.16	-	1.70	-	-
Heptanal	907	-	1.12	-	0.67	-	2.69	-	8.83	-	3.53
6-Methylheptan-2-one	962	1.93	-	1.39	0.39	0.23	0.32	-	-	-	-
Benzaldehyde	970	23.85	5.29	34.24	3.91	24.63	6.23	31.29	3.73	18.05	5.37
Heptan-1-ol	975	-	0.69	-	0.56	-	0.51	-	-	-	-
3,5,5-Trimethylhex-2-ene	980	-	-	0.71	-	0.73	-	-	-	-	-
Hexanoic acid	981	-	3.40	-	2.90	-	1.49	-	-	-	-
Sabinene	982	-	-	-	-	0.39	-	-	-	-	-
Oct-1-en-3-ol	984	1.44	3.79	1.00	6.77	0.78	5.36	0.55	2.54	-	2.92
Octan-3-one	991	1.13	-	1.50	-	0.50	-	-	-	-	-
6-Methylhept-5-en-2-one	992	-	1.49	-	1.10	-	1.09	-	-	-	-
Octan-2-one	995	-	-	0.52	-	0.57	-	1.04	-	-	-
2-Pentylfuran	996	-	1.05	-	0.91	-	2.17	-	1.89	-	-
Octanal	1007	3.10	0.99	1.40	-	0.66	2.92	-	3.88	-	1.59
(*E*,*E*)-Hepta-2,4-dienal	1017	-	-	-	0.39	-	1.41	-	-	-	-
2-Ethylhexan-1-ol	1035	-	-	-	0.47	-	2.09	-	1.14	-	-
(*E*)-3-Ethyl-2-methylhexa-1,3-diene	1038	-	1.41	-	1.49	-	1.38	-	-	-	-
Benzyl alcohol	1042	-	5.84	-	1.41	-	4.77	-	-	-	-
(*E*)-β-Ocimene	1044	-	2.64	-	1.31	1.27	1.95	-	-	-	-
Phenylacetaldehyde	1052	0.96	-	0.46	-	0.41	-	-	-	-	-
(*E*)-Oct-2-enal	1064	0.96	-	0.53	0.62	0.62	1.50	0.68	0.98	-	-
(*E*)-Oct-2-en-1-ol	1074	-	-	-	0.81	-	0.76	-	-	-	-
Octan-1-ol	1076	13.35	-	2.84	-	1.17	-	-	-	-	-
(*E*,*E*)-Octa-3,5-dien-2-one	1077	-	1.35	-	1.76	-	3.63	-	3.41	-	4.59
(*E*,*Z*)-Octa-3,5-dien-2-one	1097	-	2.29	-	2.25	-	2.43	-	0.74	-	-
Nonanal	1108	1.08	2.34	0.69	0.46	0.56	5.20	-	5.85	0.24	4.17
2,6-Dimethylcyclohexan-1-ol	1114	-	4.54	0.89	3.66	0.71	1.66	0.52	2.43	0.31	-
4-Ketoisophorone	1150	-	2.63	-	1.11	-	-	-	-	0.27	-
(*Z*)-Non-3-en-1-ol	1155	1.75	-	-	-	-	-	-	-	-	-
6-[(1*Z*)-Butenyl]-cyclohepta-1,4-diene] (Dictyopterene D′)	1158	2.52	0.27	1.21	0.27	1.46	-	2.49	-	-	-
[6-Butyl-cyclohepta-1,4-diene] (Dictyopterene C′)	1175	-	-	-	-	-	-	0.92	-	-	-
Decan-2-one	1196	-	-	-	-	-	-	0.65	-	-	-

Table 1. *Cont.*

Compound	RI	Area %									
		May		June		July		August		September	
		Fr	Dr	Fr	Dr	Fr	Dr	Fr	Dr	Fr	Dr
Decanal	1209	-	-	-	-	-	0.92	-	1.13	-	-
β-Cyclocitral	1226	2.34	1.06	1.06	0.78	1.03	-	1.25	-	0.42	-
Undecan-2-one	1297	-	0.87	-	0.65	-	-	-	-	-	-
Undecanal	1311	-	0.62	-	0.27	-	-	-	-	-	-
(*E*)-Undec-2-en-1-ol	1347	-	0.98	-	1.21	-	-	-	-	-	-
α-Cubebene	1355	-	0.68	-	-	-	-	-	-	-	-
β-Bourbonene	1389	-	0.94	-	-	-	-	-	-	-	-
β-Cubebene	1393	-	0.93	-	-	-	-	-	-	-	-
Tetradecane	1402	-	0.91	-	0.61	-	0.89	-	-	-	-
6,10-Dimethylundecan-2-one	1408	-	0.49	-	0.42	-	-	-	-	-	-
α-Ionone	1432	-	0.82	-	0.69	0.74	-	0.64	-	0.23	-
Germacrene D	1485	5.44	-	-	-	0.85	-	-	-	-	-
β-Ionone	1490	6.22	2.50	3.84	3.01	3.62	1.34	5.38	1.28	1.97	-
Pentadec-1-ene	1495	4.68	2.22	13.82	2.36	25.14	1.93	23.70	4.53	53.17	9.54
(*E*)-Pentadec-7-ene	1498	0.70	-	1.00	0.45	1.29	-	1.96	-	3.56	4.88
Tridecan-2-one	1500	-	-	-	-	-	-	1.29	-	1.94	-
Pentadecane	1500	3.54	22.06	9.66	38.46	9.02	21.22	7.31	24.99	4.10	29.33
Tridecanal	1519	2.78	-	2.64	-	2.02	-	4.11	-	5.92	-
δ-Cadinene	1528	1.10	-	-	-	-	-	-	-	-	-
Dihydroactinidiolide	1533	-	1.26	-	1.27	-	-	-	-	-	-
Gleenol	1589	3.11	-	0.78	-	0.55	-	0.36	-	-	-
Hexadecane	1603	-	-	-	-	0.31	-	-	-	-	-
Heptadec-1-ene	1696	0.94	-	1.09	-	0.61	-	1.19	-	2.34	-
Heptadecane	1700	0.71	2.00	3.58	4.35	2.51	4.97	4.23	17.67	1.75	15.52
1-Phytene (3,7,11,15-Tetramethylhexadec-1-ene)	1791	-	-	-	-	2.49	1.82	-	-	-	-
Phytane	1813	-	-	-	-	3.95	4.46	-	-	-	-
Hexahydrofarnesyl acetone (Phytone)	1850	-	1.48	-	-	-	-	-	-	-	-

The area percentage of two saturated aliphatic hydrocarbons, pentadecane and heptadecane, increased after air-drying in all months (analysed with both fibres; Tables 1 and 2). The largest increment in pentadecane was noticed in May (6.2 times, f1; 7.2 times, f2) and September (7.2 times, f1; 9.1 times, f2), while, for heptadecane, it was in August (4.2 times, f1; 21.8 times, f2) and September (8.9 times, f1; 22.7 times, f2). This could be the consequence of fatty acid degradation [24]. Pentadec-1-ene, as the most abundant unsaturated hydrocarbon, decreased during all months after air-drying, with the top variation in July (13.0 times, f1;12.4 times, f2). Octan-1-ol showed the greatest change through the months. It was detected only in HS-FrHSc with the greatest abundance in May (13.35%, f1; 13.93%, f2). In June, its portion dropped by 4.7 times on f1 and 4.6 times on f2 and even more in July (2.4 times more on f1; 3.8 times more on f2). Similar behaviour was noticed for unsaturated ketones 3-hydroxybutan-2-one (detected with f2) and 6-methylheptan-2-one.

Among terpenes, sesquiterpene germacrene D and diterpene phytane were dominant. Germacrene D was detected only in HS-FrHSc with the peak in May (5.44%, f1; 1.87%, f2). In our previous research, germacrene D was found in *D. dichotoma* headspace as one of the most abundant terpenes at the blooming inception in May [20]. Phytane was detected only in July with the greatest portion in HS-DrHSc (4.46%, f1; 4,19%, f2). The increment in its abundance after drying implies possible chlorophyll degradation. Total ion chromatograms (TICs) of fresh samples from May and September isolated by HS-SPME are presented in Figure 2 for fibre f1 and Figure 4 for fibre f2.

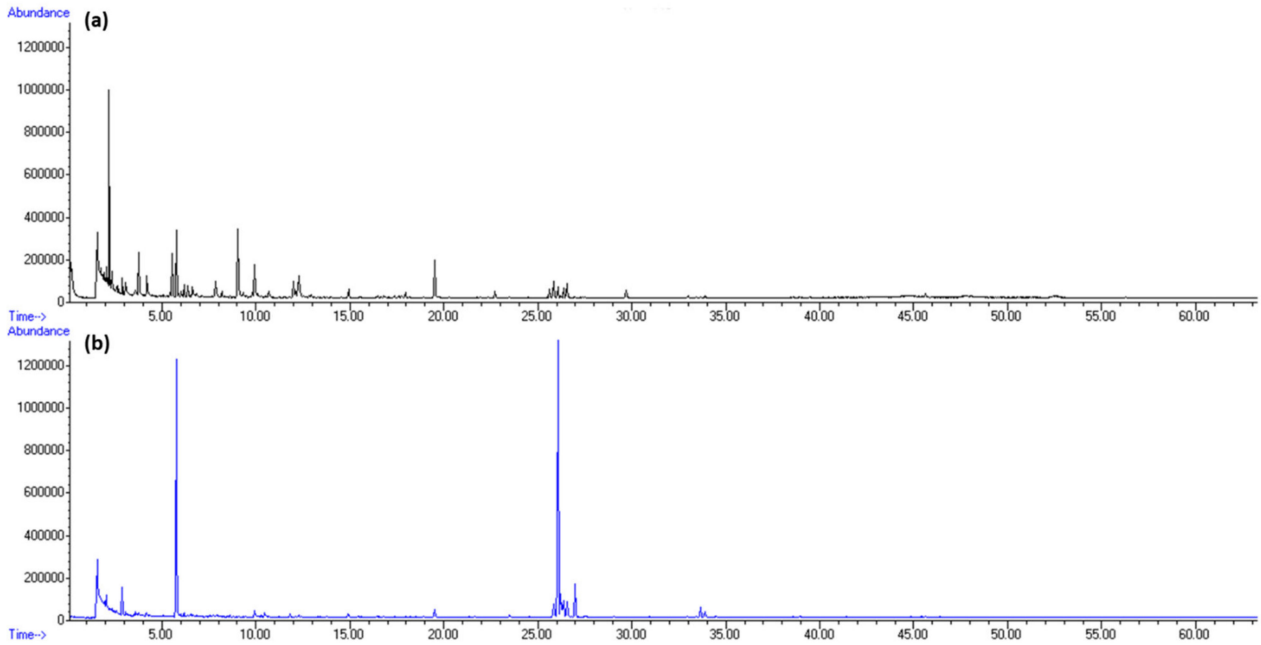

Figure 4. Total ion chromatogram (TIC) comparison of *H. scoparia* isolated by headspace solid-phase microextraction (HS-SPME) and analysed by gas chromatography–mass spectrometry (GC–MS) using PDMS/DVB fibre (f2): (**a**) in May; (**b**) in September.

Table 2. The volatile compounds from *H. scoparia* isolated by headspace solid-phase microextraction (HS-SPME) and analysed by gas chromatography–mass spectrometry (GC–MS) using PDMS/DVB fibre (f2); Fr (fresh), Dr (dry).

Compound	RI	Area %									
		May		June		July		August		September	
		Fr	Dr	Fr	Dr	Fr	Dr	Fr	Dr	Fr	Dr
Dimethyl sulfide	<900	-	-	-	-	-	0.68	-	0.20	-	2.50
Butanal	<900	-	-	0.32	-	0.56	-	0.68	-	0.12	-
3-Methylbutanal	<900	0.51	-	0.77	-	0.61	-	0.70	-	0.16	-
Pent-1-en-3-ol	<900	1.69	1.31	1.35	2.70	0.86	1.74	1.14	1.03	0.72	3.97
Pentanal	<900	-	-	-	0.11	-	0.18	-	0.40	-	1.10
3-Hydroxybutan-2-one	<900	14.16	-	-	-	0.74	-	-	-	-	-
3-Methylbutanol	<900	1.18	-	0.20	-	0.64	-	0.57	-	-	-
2-Methylbutanol	<900	-	1.35	-	-	-	-	-	-	-	-
Pyridine	<900	-	-	-	-	-	-	0.15	-	0.19	-
(*E*)-Pent-2-enal	<900	-	-	-	-	-	0.44	-	-	-	-
Pentan-1-ol	<900	-	0.58	-	0.74	0.14	0.62	0.21	1.12	-	1.25
(*Z*)-Pent-2-en-1-ol	<900	0.42	0.40	0.30	0.80	0.24	0.79	0.32	0.61	0.19	1.82
Hexanal	<900	1.39	2.46	2.27	2.09	1.06	4.98	1.90	8.22	1.89	11.47
3-Methylbutanoic acid	<900	-	1.94	-	2.43	-	-	-	-	-	-
2-Methylbutanoic acid	<900	-	1.58	-	1.58	-	-	-	-	-	-
(*E*)-Hex-2-enal	<900	-	0.93	0.59	1.12	0.19	1.97	0.37	1.48	0.23	-
Hexan-1-ol	<900	4.60	2.47	-	1.08	1.79	0.97	1.58	1.78	-	-
Heptan-2-one	<900	-	0.31	-	0.30	-	0.28	-	-	-	-
Tribromomethane	<900	0.25	-	0.58	-	0.49	-	0.18	-	0.46	-
Heptanal	907	-	1.94	-	1.31	-	3.94	-	10.04	-	8.14
6-Methylheptan-2-one	962	5.85	0.52	0.71	0.43	1.00	0.58	1.08	-	-	-

Table 2. *Cont.*

Compound	RI	Area %									
		May		June		July		August		September	
		Fr	Dr	Fr	Dr	Fr	Dr	Fr	Dr	Fr	Dr
Benzaldehyde	970	9.51	5.11	52.75	3.82	32.99	4.82	57.85	2.62	28.39	6.83
Heptan-1-ol	975	-	0.80	-	0.97	-	0.71	-	0.72	-	-
Oct-1-en-3-one	979	0.87	-	0.80	-	0.60	-	0.37	-	0.39	-
Pentyl propanoate	980	-	-	-	4.12	-	1.61	-	-	-	-
Hexanoic acid	982	-	4.80	-	-	-	-	-	-	-	-
Oct-1-en-3-ol	984	1.81	5.82	1.01	8.56	0.71	5.30	0.68	2.19	0.51	2.74
Octan-3-one	991	1.73	-	0.82	-	0.39	-	0.30	-	-	-
6-Methylhept-5-en-2-one	992	-	1.30	-	1.18	-	0.80	-	-	-	-
2-Pentylfuran	995	-	0.85	0.33	1.01	0.31	1.73	0.59	1.34	0.35	-
Octanal	1007	0.88	1.26	0.66	0.93	-	3.22	-	4.28	-	-
(*E*,*E*)-Hepta-2,4-dienal	1016	-	-	-	0.47	-	1.06	-	-	-	-
2-Ethylhexan-1-ol	1035	-	-	-	0.55	-	2.16	-	2.27	-	-
(*E*)-3-Ethyl-2-methylhexa-1,3-diene	1038	-	1.62	0.69	1.60	1.51	1.39	-	-	0.15	-
Benzyl alcohol	1041	3.67	6.53	0.87	1.61	0.61	5.18	2.86	2.05	-	-
(*E*)-β-Ocimene	1044	-	3.55	-	1.42	-	2.57	-	1.99	-	-
Phenylacetaldehyde	1052	0.99	0.73	-	-	-	1.67	-	-	-	-
γ-Caprolactone	1062	-	1.17	-	0.48	-	-	-	-	-	-
(*E*)-Oct-2-enal	1064	-	-	-	0.65	-	1.74	-	0.90	-	-
(*E*)-Oct-2-en-1-ol	1074	-	0.50	-	0.87	-	0.79	-	-	-	-
Octan-1-ol	1076	13.93	-	3.00	-	0.80	-	-	-	-	-
(*E*,*E*)-Octa-3,5-dien-2-one	1077	-	0.95	-	1.35	-	2.75	-	1.84	-	-
Nonan-2-one	1096	0.53	-	-	-	-	-	-	-	-	-
(*E*,*Z*)-Octa-3,5-dien-2-one	1097	-	0.99	-	1.55	-	1.32	-	0.40	-	-
Nonanal	1108	-	2.52	0.53	0.65	-	4.97	-	4.29	-	5.10
2,6-Dimethylcyclohexan-1-ol	1114	1.75	5.72	1.35	5.34	0.96	2.05	0.62	3.25	0.76	-
4-Ketoisophorone	1150	-	2.27	0.54	1.13	1.01	0.41	-	-	0.61	-
(*Z*)-Non-3-en-1-ol	1155	3.76	-	-	-	-	-	-	-	-	-
6-[(1*Z*)-Butenyl]-cyclohepta-1,4-diene] (Dictyopterene D′)	1158	1.23	0.35	0.37	0.33	0.59	-	0.57	-	-	-
[6-Butyl-cyclohepta-1,4-diene] (Dictyopterene C′)	1175	-	-	-	-	-	-	0.31	-	-	-
Decan-2-one	1196	-	-	-	-	-	-	0.38	-	-	-
Decanal	1209	-	0.33	-	0.21	-	0.90	-	-	-	-
β-Cyclocitral	1226	1.81	0.63	1.09	0.61	0.81	0.70	0.81	-	0.60	-
Undecan-2-one	1296	1.05	0.47	-	0.37	-	0.61	-	-	-	-
Undecanal	1311	-	0.66	-	0.24	-	-	-	-	-	-
(*E*)-Undec-2-en-1-ol	1347	-	0.68	-	0.97	-	-	-	-	-	-
α-Cubebene	1355	-	0.53	-	-	-	-	-	-	-	-
β-Bourbonene	1389	-	0.62	-	-	-	-	-	-	-	-
β-Cubebene	1393	-	0.48	-	-	-	-	-	-	-	-
Tetradecane	1400	-	0.57	-	0.48	-	0.67	-	-	-	-
6,10-Dimethylundecan-2-one	1409	-	0.35	-	0.40	-	-	-	-	-	-
Dodecanal	1412	1.67	-	-	-	-	-	-	-	-	-
α-Ionone	1432	-	0.30	-	0.56	0.53	-	0.41	-	0.43	-
Germacrene D	1485	1.87	-	0.25	-	0.95	-	-	-	-	-
β-Ionone	1489	3.67	1.96	3.57	2.43	2.87	1.39	2.96	1.42	2.84	-
Pentadec-1-ene	1495	2.15	1.59	7.88	2.04	23.77	1.92	10.36	4.00	40.35	6.90
(*E*)-Pentadec-7-ene	1498	0.47	-	0.64	0.41	1.16	-	0.97	-	3.63	-
Tridecan-2-one	1500	-	-	-	-	0.44	-	0.50	-	1.63	-

Table 2. *Cont.*

Compound	RI	Area %									
		May		June		July		August		September	
		Fr	Dr	Fr	Dr	Fr	Dr	Fr	Dr	Fr	Dr
Pentadecane	1500	1.95	14.06	4.91	27.13	6.41	17.01	2.41	20.64	2.68	24.47
Tridecanal	1514	3.29	-	4.34	-	4.44	-	4.89	-	8.00	-
Dihydroactinidiolide	1533	0.18	1.16	0.31	1.04	0.22	-	0.16	-	-	-
Gleenol	1589	2.23	-	0.51	-	0.33	-	-	-	-	-
Hexadecane	1600	-	-	-	-	-	0.88	-	-	-	-
Heptadec-1-ene	1696	-	-	0.53	-	0.57	-	0.38	-	1.64	-
Heptadecane	1703	0.56	1.69	1.48	3.76	2.06	4.95	0.84	18.29	1.05	23.72
Phytane	1813	-	-	-	-	-	4.19	-	-	-	-
Hexahydrofarnesyl acetone (Phytone)	1850	-	1.22	-	-	-	0.67	-	-	-	-

2.2. Statistical Analysis of the H. scoparia Headspace VOCs

The principal component analyses (PCA) were used to describe the variations among the dominant volatiles (>2%) of HS-FrHSc and HS-DrHSc in relation to the material preparation (fresh or dry), seasonal changes, and fibre. The results are shown in Figures 5 and 6.

The PCA analysis of the data obtained by f1 fibre is shown in Figure 2a,b. The first two PCs described 67.4% of the initial data variability. A correlation between certain groups of the compounds was observed (Figure 5a). Pentadecane, nonanal, β-ionone, and tridecanal showed the highest variable contribution to PC1 and factor-variable correlation, while hexan-1-ol and benzyl alcohol contributed to PC2.

The correlation plot and score plot of the dominant components from data obtained by f2 fibre are shown in Figure 5c,d. The first two PCs described 62.38% of the initial data variability. The abundance of benzaldehyde, (*E*,*E*)-octa-3,5-dien-2-one, pentadecane, and tridecanal contributed to PC1, while hexanal and hexanoic acid had the highest contribution to PC2. May samples (fibre f2) were separated in the bottom left part of the plot as a function of the highest octan-1-ol and 3-hydroxybutan-2-one content.

A clear separation between the fresh and dried samples was obtained for both fibres (Figure 5b,d). For f1, fresh samples were positioned on the left part of the multivariate space, while the scores of the dried samples were vertically distributed on the right side of the score plot. Interestingly, the sampling month showed no effect on the fresh samples, while, in dry samples, there were similarities between May and June, and between August and September. Similarly, a clear separation of the dry and fresh samples was observed for f2 fibre. As it can be seen in Figure 5d, the fresh sample harvested in May showed great variation compared to other months. The main reason was the high portion of aliphatic saturated compounds (53.29%), as well as terpenes (6.09%) and others (3.23%).

When the data for f1 and f2 were analysed together, the first two PCs described 53.3% of the initial data variability, but the score plot showed a clear separation between fresh and dried samples (Figure 6). As a function of the higher content of benzaldehyde and octan-1-ol, as well as low content of pentadec-1-ene and pentadecane in the dry samples, the samples from May (both fibres) were segregated in the bottom part of the plot and September samples in the top part. Some separation between the sampling months was seen, but no correlation with temperature change over months was observed.

2.3. Volatile Organic Compounds Variations of H. scoparia Acquired by Hydrodistillation

In the hydrodistillate (HD), the portion of the identified compounds ranged from 90.04% (HD-FrSc, September) to 98.44% (HD-DrHSc, June) of the total detected compounds. Identified VOCs could be classified, as for the headspace, into six different groups: saturated aliphatic compounds, unsaturated aliphatic compounds, benzene derivatives, terpenes, C_{13}-norisoprenoids, and others (Figure 7). In HD-FrHSc, terpenes were the most abundant group in June (32.88%), July (39.80%), and August (51.75%), while, in May (30.85%) and

September (48.16%), the dominant group was unsaturated aliphatic compounds. In HD-DrHSc, three groups of compounds dominated through the months: terpenes (May, 29.26%; July, 37.31%), unsaturated aliphatic compounds (August, 35.65%; September, 39.40%), and saturated aliphatic compounds (June, 32.28%).

Diterpene alcohol (*E*)-phytol was the most abundant compound in HD-FrHSc through all months except September. Its abundance increased from May (11.35%) to August (47.03%) when it was in its highest bloom. Recent studies have shown its antioxidant potential [27], antimicrobial [28], anti-inflammatory [29], antitumour [30], and antidiabetic activities [27]. Its derivative, phytane, was detected in July (6.11%, HD-FrHSc; 2.43%, HD-DrHSc). Two more diterpene alcohols, isopachydictyol A and cembra-4,7,11,15-tetraen-3-ol, and one sesquiterpene alcohol, gleenol, were detected in high abundance. Both diterpene alcohols were the most abundant in May (9.70%, isopachydictyol A; 6.88%, cembra-4,7,11,15-tetraen-3-ol) in the sample after air-drying and were detected in the lowest content in the fresh sample in September. They were detected as the major compounds in HD of *D. dichotoma* [20] and isolated from several species of *Dictyota* genus [31,32]. Both diterpenes have various biological activity [33,34]. Gleenol was detected in the highest portion in May in HD-FrHSc (6.26%) and its portion decreased each month (Table 3).

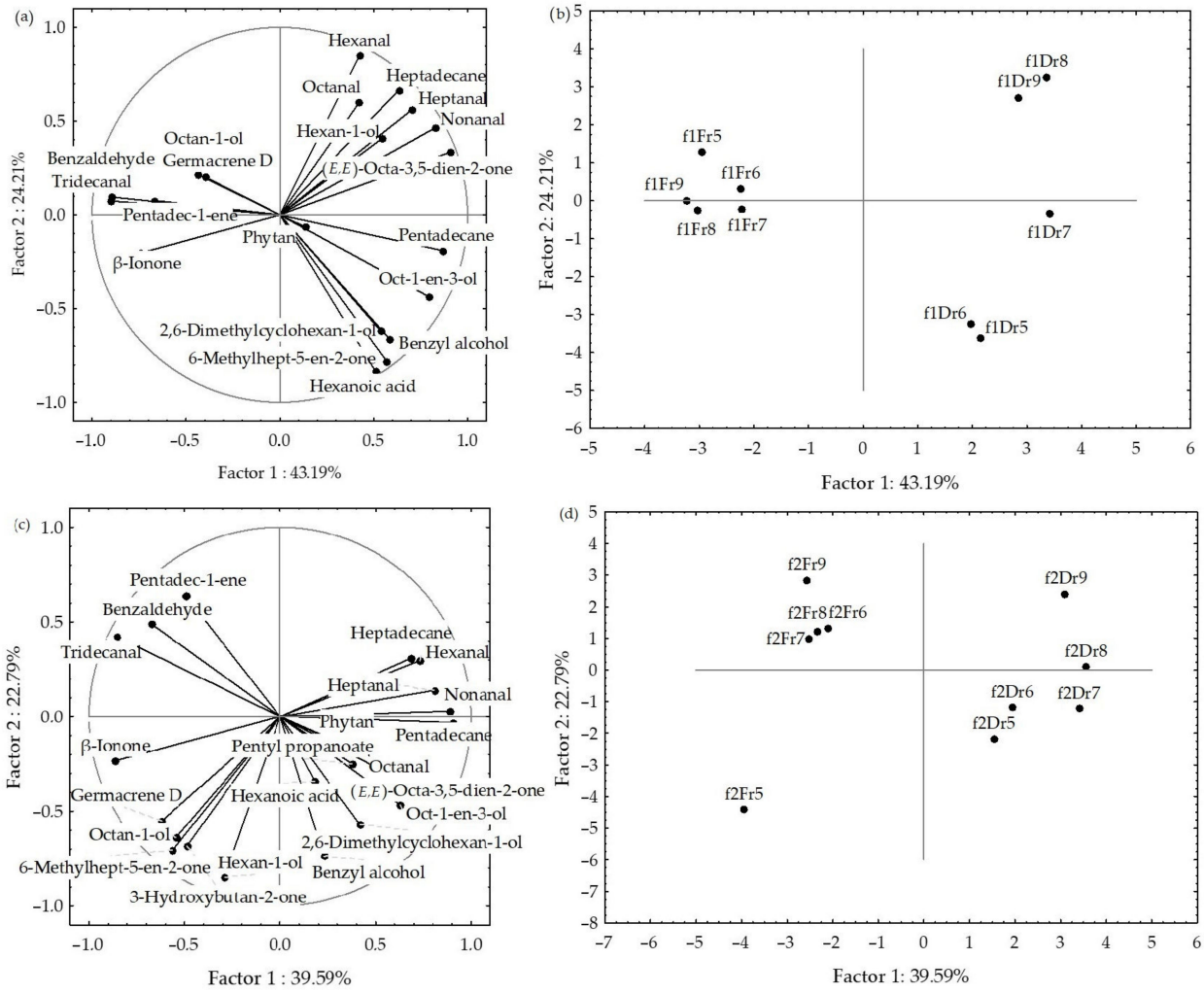

Figure 5. Correlation loadings (**a**,**c**) and score plots (**b**,**d**) of the dominant compounds from the headspace volatiles obtained by two different fibres for HS-SPME/GC–MS: divinylbenzene/carboxene/polydimethylsiloxane (f1) and polydimethylsiloxane/divinylbenzene (f2) of fresh (Fr, **a**,**b**) and dried (Dr, **c**,**d**) *H. scoparia* samples.

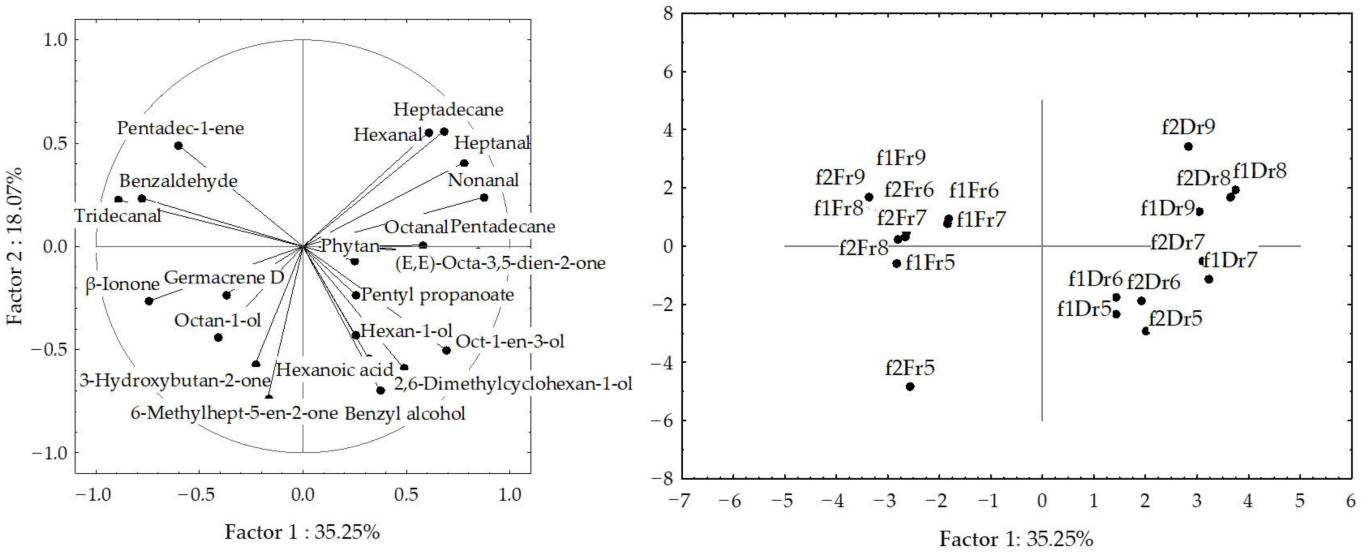

Figure 6. Correlation and score plots of the dominant compounds from headspace volatiles (two different fibres for HS-SPME/GC–MS: divinylbenzene/carboxene/polydimethylsiloxane (f1) and polydimethylsiloxane/divinylbenzene (f2)) of fresh (Fr) and dried (Dr) *H. scoparia* samples.

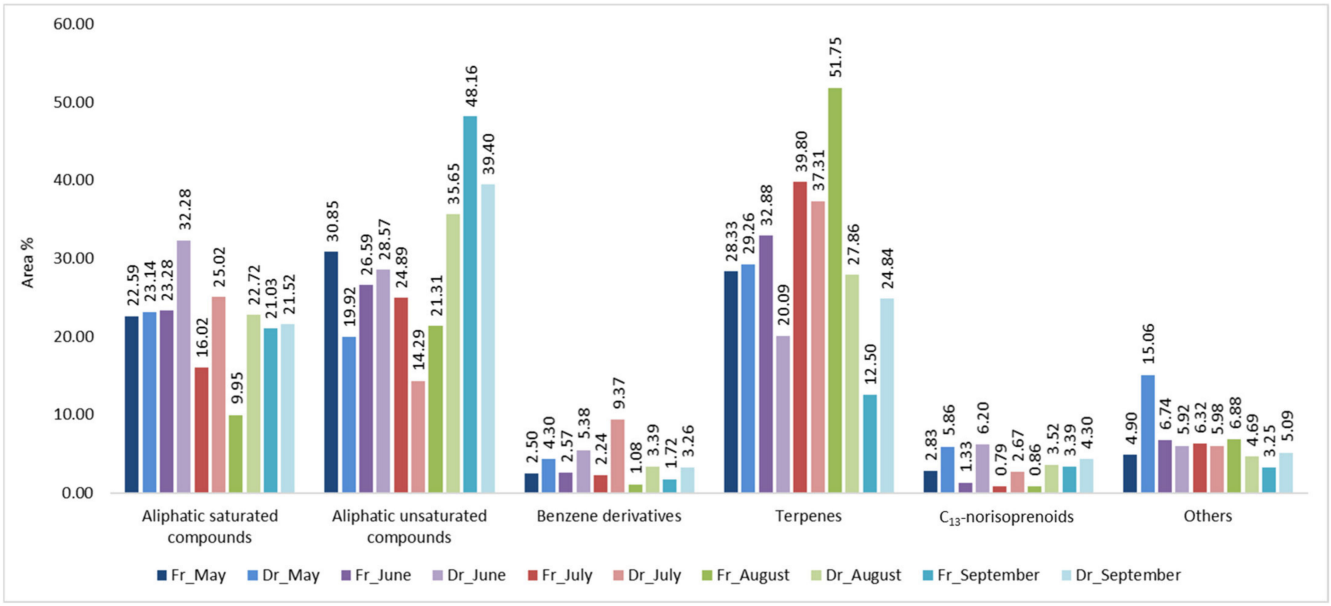

Figure 7. The VOCs from *H. scoparia* sorted by structural groups obtained by hydrodistillation (HD) and analysed by gas chromatography–mass spectrometry (GC–MS); Fr (fresh), Dr (dry).

Among aliphatic compounds, the dominant was unsaturated pentadec-1-ene with its peak in September in both HD-FrHSc (26.46%) and HD-DrHSc (18.98%). Higher aliphatic unsaturated alcohol (*Z*,*Z*,*Z*)-octadeca-9,12,15-trien-1-ol was prevalent in HD-FrHSc in May (8.09%) and decreased each month. Higher aliphatic unsaturated aldehyde (*Z*)-octadec-9-enal was the most abundant in HD-FrHSc in August (8.51%). Both the alcohol and the aldehyde were less abundant in air-dried samples throughout all the months since lipid oxidative degradation probably occurred, which means they were oxidised and further degraded. Six carboxylic acids were detected and identified with hexanoic acid as the most abundant one particularly in HD-DrHSc in May (4.80%). Three C_{13}-norisoprenoids were detected, among which β-ionone was the most abundant. It was higher in air-dried samples, which was the result of carotenoid degradation during the process of air-drying.

Total ion chromatogram (TIC) of fresh samples from May and September obtained by HD are presented in Figure 8.

Table 3. The volatile compounds from *H. scoparia* acquired by hydrodistillation (HD) and analysed by gas chromatography–mass spectrometry (GC–MS); Fr (fresh), Dr (dry).

Compound	RI	Area %									
		May		June		July		August		September	
		Fr	Dr	Fr	Dr	Fr	Dr	Fr	Dr	Fr	Dr
2-Ethyl-5,5-dimethylcyclopenta-1,3-diene	<900	0.06	0.14	0.10	0.23	0.02	0.09	0.02	0.22	0.10	0.19
(*E*)-Hex-2-enal	<900	1.71	1.18	1.13	1.00	0.13	0.31	0.27	0.78	1.76	1.18
Hex-3-en-1-ol	<900	0.06	-	-	-	0.14	-	-	-	-	-
Hexan-1-ol	<900	1.72	0.17	0.81	0.18	0.18	-	0.22	0.04	0.24	0.03
m-Xylene	<900	0.51	-	-	-	0.06	-	-	-	0.13	-
2,3-Dimethylpyridine	<900	0.04	-	-	-	-	-	-	-	-	-
Heptan-2-one	<900	0.16	0.14	0.15	0.30	-	0.12	-	0.24	-	0.28
Tribromomethane	<900	-	-	-	-	0.09	-	0.03	-	0.34	-
Hept-4-enal	902	0.24	0.14	0.08	-	-	-	-	-	0.16	0.10
Heptanal	904	0.06	0.71	0.10	1.11	0.06	0.25	-	0.34	0.07	0.21
(*E*,*Z*)-Hexa-2,4-dienal (Sorbaldehyde)	914	0.06	-	-	-	-	-	0.04	-	-	-
2-Iodopentane	929	0.12	-	0.09	-	-	-	-	-	0.04	-
2,4-Dimethylpyridine	937	0.16	-	0.09	-	0.03	-	-	-	-	-
6-Methylheptan-2-one	960	0.05	-	0.11	-	-	-	-	-	-	-
(*E*)-Hept-2-enal	962	-	0.06	-	0.17	-	-	-	0.14	0.09	0.07
Benzaldehyde	969	0.73	0.69	0.75	0.95	0.17	0.45	0.13	0.41	0.30	0.47
Heptan-1-ol	973	0.13	-	0.09	-	-	-	-	-	-	-
Dimethyl trisulfide	978	0.22	0.22	0.36	0.33	0.39	0.11	0.05	0.11	0.13	0.08
Oct-1-en-3-ol	982	0.34	0.35	0.48	0.73	0.09	0.40	0.04	0.23	0.24	0.28
Octan-2,3-dione	987	0.18	0.34	0.53	0.76	0.15	0.48	0.25	0.57	0.90	0.16
Octan-3-one	989	0.38	0.24	0.67	0.44	0.08	-	-	0.16	0.21	-
Octan-2-one	994	-	-	0.16	-	-	-	-	-	-	-
2-Pentylfuran	995	0.43	0.88	0.17	1.16	0.09	0.38	0.11	0.69	0.90	0.77
Octan-3-ol	998	-	-	0.23	0.22	-	-	-	0.15	-	0.10
(*E*,*Z*)-Hepta-2,4-dienal	1000	-	-	-	-	0.06	-	0.08	-	0.11	-
Octanal	1005	0.25	0.33	0.19	0.33	-	0.17	-	0.11	0.08	0.12
(*E*,*E*)-Hepta-2,4-dienal	1015	0.24	0.30	0.30	0.50	0.14	0.17	0.21	0.12	0.23	0.14
p-Cymene	1030	0.06	-	-	-	-	-	-	-	-	-
2-Ethylhexan-1-ol	1033	-	-	-	-	-	0.07	-	0.23	0.04	-
(3*E*)-3-Ethyl-2-methylhexa-1,3-diene	1039	-	-	-	-	-	-	-	-	0.05	-
Benzyl alcohol	1041	0.18	-	0.19	-	0.06	-	-	-	0.04	-
2,2,6-Trimethylcyclohexan-1-one	1042	-	0.13	-	0.22	-	-	-	-	-	-
Phenylacetaldehyde	1051	0.15	0.77	0.32	0.99	0.20	0.57	0.17	0.33	0.25	0.27
(*E*)-Oct-2-enal	1063	0.14	0.26	0.13	0.52	0.05	0.21	-	0.32	0.32	0.35
2-Methyldecane	1068	0.11	-	0.08	-	0.06	-	0.12	-	0.50	-
(*E*)-Oct-2-en-1-ol	1073	0.20	-	0.15	-	0.08	-	0.05	-	0.13	-
Acetophenone	1073	-	0.21	-	0.25	-	0.16	-	0.17	-	0.20
Octan-1-ol	1075	3.82	-	1.79	-	0.25	-	0.10	-	0.39	-
(*E*,*E*)-Octa-3,5-dien-2-one	1075	-	0.31	-	0.58	-	0.20	-	0.20	-	0.25
1-Methylsulfanylpentan-3-one	1091	-	0.31	-	0.29	-	0.22	-	0.15	-	0.08
(*E*,*Z*)-Octa-3,5-dien-2-one	1096	1.95	0.17	0.91	0.25	0.49	-	0.55	-	1.16	0.18
Linalool	1103	-	-	-	0.11	-	-	-	-	0.13	0.09
Nonanal	1107	0.11	0.44	0.12	0.61	-	0.28	-	0.24	0.18	0.21
2,6-Dimethylcyclohexan-1-ol	1113	0.15	0.34	0.11	0.54	0.08	0.37	0.10	0.21	0.29	0.24

Table 3. *Cont.*

Compound	RI	Area %									
		May		June		July		August		September	
		Fr	Dr	Fr	Dr	Fr	Dr	Fr	Dr	Fr	Dr
2-Phenylethanol	1120	0.10	-	0.10	-	0.73	-	-	-	0.08	-
(*E*,*Z*)-2,6-Dimethylocta-1,3,5,7-tetraene	1141	0.21	-	0.08	-	-	-	-	-	-	-
4-Ketoisophorone	1150	0.56	0.56	0.34	0.24	0.17	0.39	0.09	0.31	0.13	0.26
(*E*,*Z*)-Nona-2,6-dienal	1159	0.30	0.17	0.22	0.32	0.17	0.08	-	0.15	0.22	0.16
6-[(1Z)-Butenyl]-1,4-cycloheptadiene] (Dictyopterene D′)	1161	-	-	-	-	-	-	-	-	0.06	-
Non-2-enal	1165	0.14	0.16	0.11	0.33	0.18	0.09	-	0.14	0.20	0.19
[6-Butyl-1,4-cycloheptadiene] (Dictyopterene C′)	1175	0.14	-	-	-	0.10	-	-	-	-	-
2,4-Dimethylbenzaldehyde	1180	0.10	0.31	0.17	0.53	-	-	0.05	0.13	0.15	0.11
3,4,4-Trimethylcyclohex-2-en-1-one	1194	-	-	-	-	-	-	-	0.19	-	0.19
Dodecane	1200	-	0.12	-	0.25	-	-	-	-	-	-
Decanal	1209	0.38	-	0.21	0.15	-	0.11	-	0.09	0.07	0.09
β-Cyclocitral	1225	0.28	0.36	0.15	0.53	-	0.11	-	0.21	0.24	0.18
β-Cyclohomocitral	1263	0.17	0.21	0.14	0.34	-	-	-	0.11	0.18	0.10
(*E*,*Z*)-Deca-2,4-dienal	1283	0.15	0.16	-	0.23	0.05	0.18	0.08	0.10	0.24	0.10
Indole	1296	0.19	0.36	0.17	0.27	0.13	0.25	0.08	0.26	0.35	0.22
Undecanal	1310	-	0.13	-	0.25	0.06	0.18	-	0.22	0.20	0.28
(*E*,*E*)-Deca-2,4-dienal	1320	0.38	0.80	0.48	1.98	0.15	0.40	0.25	0.75	1.03	0.54
(*E*)-Undec-2-en-1-ol	1347	0.11	0.27	-	0.30	-	0.18	-	0.15	0.07	0.08
β-Bourbonene	1388	0.19	-	0.09	-	-	-	-	-	-	-
β-Cubebene	1393	0.42	0.13	0.46	0.40	0.15	-	-	-	0.16	0.21
4-(2-Methylbutan-2-yl)phenol (*p-tert*-Pentylphenol)	1400	-	-	-	-	0.04	-	-	-	0.07	-
Tetradecane	1400	0.27	0.50	0.26	0.66	0.07	0.23	0.09	1.18	0.91	0.89
6,10-Dimethylundecan-2-one	1408	-	0.20	-	0.21	-	-	-	0.09	-	0.12
Dodecanal	1412	-	0.21	-	0.38	0.04	0.19	-	0.22	0.18	0.38
α-Ionone	1432	0.13	0.26	0.12	0.41	0.05	0.14	0.07	0.27	0.30	0.31
(*E*,*Z*)-Dodeca-2,6-dienal	1452	-	-	-	-	-	-	-	0.04	-	0.10
(*Z*)-Geranylacetone	1458	0.30	0.35	-	0.44	-	-	-	0.13	0.14	0.18
Dodecan-1-ol	1479	1.38	-	0.13	-	-	0.71	-	0.12	0.11	0.35
α-Muurolene	1481	-	-	-	0.37	-	-	-	-	-	0.10
Germacrene D	1485	2.67	0.24	1.71	1.67	0.56	-	0.06	0.34	0.39	0.59
β-Ionone	1489	2.13	5.04	0.86	5.56	0.56	2.14	0.70	2.94	2.96	3.72
Pentadec-1-ene	1495	7.70	2.99	7.56	8.53	4.41	1.68	2.88	17.50	26.46	18.98
(*E*)-Pentadec-7-ene	1499	0.80	0.58	0.66	1.33	0.37	0.35	0.30	2.00	3.26	3.10
Tridecan-2-one	1500	0.18	0.15	-	0.34	0.11	0.08	0.14	0.83	1.41	1.23
Pentadecane	1500	4.26	1.19	4.54	3.80	1.63	0.61	0.74	1.66	2.94	1.47
Tridecanal	1514	0.80	0.99	1.11	1.92	0.79	0.80	0.54	1.88	3.11	2.74
Zonarene	1530	0.42	0.13	0.36	0.46	0.16	0.17	-	0.03	-	0.26
Dihydroactinidiolide	1532	-	0.17	-	-	-	-	-	-	-	-
Tetradecan-2-one	1565	-	0.15	-	-	-	-	-	-	-	-
Dodecanoic acid	1573	-	0.19	0.19	0.27	-	0.18	0.10	0.17	0.19	0.21
Tridecan-1-ol	1580	0.28	0.40	0.04	0.10	0.18	0.22	0.08	0.11	-	0.25
Gleenol	1589	6.26	0.39	3.91	0.81	1.57	0.10	0.33	0.26	0.82	1.30
Hexadec-1-ene (Cetene)	1595	-	-	-	-	-	-	-	-	-	0.11
Diethyl phthalate	1597	-	-	-	-	-	-	0.06	0.05	0.10	0.24
Hexadecane	1600	-	-	-	-	-	0.46	-	-	-	0.30
Tetradecanal	1616	0.25	0.42	0.33	0.73	0.14	0.38	0.14	0.32	0.30	0.48
(*Z*)-Hexadec-7-ene	1622	-	-	0.06	0.14	-	-	0.08	0.35	0.23	0.33

Table 3. *Cont.*

Compound	RI	Area %									
		May		June		July		August		September	
		Fr	Dr	Fr	Dr	Fr	Dr	Fr	Dr	Fr	Dr
Benzophenone	1630	-	-	-	-	0.11	0.42	0.10	0.14	-	-
Cubenol	1647	0.18	-	0.09	-	-	-	-	-	-	-
(*E*)-Heptadec-8-ene	1678	-	0.18	0.24	0.36	-	0.43	0.21	0.89	0.49	0.80
Tetradecan-1-ol	1681	0.59	1.70	0.74	1.87	0.25	1.54	0.18	0.85	0.39	0.94
(*Z*)-Pentadec-6-en-1-ol	1690	3.92	1.40	1.49	2.03	0.60	0.33	0.22	0.84	0.69	0.87
Heptadec-1-ene	1696	2.27	0.68	2.06	1.60	1.57	0.59	1.66	2.91	6.82	5.67
Heptadecane	1700	1.97	1.12	2.74	2.44	2.75	0.93	1.05	2.15	2.58	1.70
2,6,10,14-Tetramethyl-7-(3-methylpentyl)pentadecane	1709	0.18	-	-	-	0.26	-	-	-	-	-
Pentadecanal	1718	1.21	2.03	3.00	2.95	2.53	1.61	2.46	2.12	2.35	1.68
(*Z*)-Heptadec-3-ene	1720	-	-	-	-	-	-	-	-	0.76	0.57
Tetradecanoic acid	1772	-	1.05	-	-	-	-	-	-	-	-
Pentadecan-1-ol	1782	0.29	-	0.20	-	0.26	-	-	-	-	0.20
Octadec-1-ene	1786	-	1.03	-	1.24	-	0.72	-	0.44	-	0.18
Octadecane	1800	-	-	-	-	0.14	-	-	-	-	-
2-Phenoxyethoxybenzene	1807	-	-	0.08	-	-	-	0.07	-	-	0.08
Phytane	1813	-	-	-	-	6.11	2.43	-	-	-	-
Hexadecanal	1820	-	0.17	0.24	0.35	0.15	0.53	0.09	0.15	-	0.12
6,10,14-Trimethylpentadecan-2-one	1850	1.19	6.77	2.27	5.48	3.84	7.58	1.69	5.08	2.68	5.27
p-Cumylphenol	1855	0.34	0.85	0.51	0.61	0.29	0.84	0.23	0.89	0.24	0.62
(*Z*)-Hexadeca-1,9-diene	1864	0.23	1.58	-	1.48	0.18	0.87	0.08	0.66	-	0.25
(*Z*)-Hexadec-11-enal	1868	-	-	0.33	-	0.82	-	0.06	-	-	-
Diisobutyl phthalate	1872	-	0.85	0.21	1.04	0.43	2.40	0.20	0.71	-	0.67
Hexadecan-1-ol	1888	-	2.97	0.73	4.22	-	4.76	0.20	1.97	-	1.04
Nonadec-1-ene	1896	0.67	0.93	2.33	1.38	3.42	1.10	1.21	1.17	0.56	0.80
Nonadecane	1900	0.29	-	-	-	0.52	-	0.15	-	-	-
Heptadecan-2-one	1905	-	0.13	0.20	0.32	-	0.28	0.22	0.24	-	0.31
(*E*,*E*)-Farnesyl acetone	1923	-	0.98	0.40	0.87	0.46	1.07	0.24	0.80	0.25	0.36
Isophytol	1953	-	0.41	0.17	0.27	0.25	0.37	0.11	0.23	-	0.10
Palmitoleic acid	1963	-	0.23	-	0.19	-	0.09	-	-	-	-
Dibutyl phthalate	1967	-	0.26	-	0.74	-	4.29	-	0.29	-	0.37
Hexadecanoic acid	1973	0.19	4.80	0.93	0.20	0.25	0.08	0.45	0.47	0.21	0.61
(*Z*)-Octadecen-9-al	1999	0.76	1.84	1.68	1.29	2.54	1.58	8.51	3.45	3.05	3.52
Octadecanal	2024	0.14	1.26	0.48	1.66	0.63	2.44	0.43	1.39	-	0.39
(*Z*)-Falcarinol	2032	0.26	-	-	-	0.55	0.54	0.73	0.70	0.35	0.85
Methyl (all *Z*) eicosa-5,8,11,14,17-pentaenoate	2038	0.15	0.98	0.48	0.41	0.22	0.38	0.20	0.27	-	0.24
Methyl (all *Z*) eicosa-5,8,11,14-tetraenoate	2044	2.85	2.86	1.86	1.36	1.51	0.91	0.83	0.68	0.46	0.99
(*Z*,*Z*)-Octadeca-9,12-dien-1-ol	2050	1.90	0.66	2.03	0.62	2.51	0.97	1.87	0.55	0.44	0.15
(*Z*,*Z*,*Z*)-9,12,15-Octadecatrien-1-ol	2056	8.09	0.86	4.63	0.66	6.67	0.56	2.84	0.45	0.47	0.10
(*Z*)-Octadec-9-en-1-ol	2061	0.18	0.69	0.25	0.23	0.54	1.01	0.35	0.42	-	0.25
(*Z*,*Z*)-Octadeca-3,13-dien-1-ol	2065	-	0.87	-	0.28	-	1.67	-	0.68	-	-
Heneicos-10-ene	2070	-	1.32	-	0.49	-	0.11	-	-	-	-
Octadecan-1-ol	2088	-	-	0.13	-	0.41	-	0.53	-	-	-
γ-Palmitolactone	2104	-	1.22	0.55	0.39	0.97	1.75	1.73	0.55	-	0.37
(*Z*,*Z*)-Octadeca-9,12-dienoic acid	2110	-	-	0.42	-	1.42	-	1.26	-	0.16	-
(E)-Phytol	2116	11.35	9.31	18.71	8.22	23.25	26.10	47.03	22.53	8.01	12.87
Isopachydictyol A	2127	2.73	9.70	3.07	2.60	2.80	2.25	1.57	1.22	0.96	3.54
(*Z*)-Octadec-9-enoic acid (Oleic acid)	2142	0.65	1.85	1.60	0.56	1.21	1.51	2.01	1.20	0.47	1.32
Cembra-4,7,11,15-tetraen-3-ol	2230	3.06	6.88	3.63	3.00	3.94	4.18	1.67	1.30	0.86	4.11

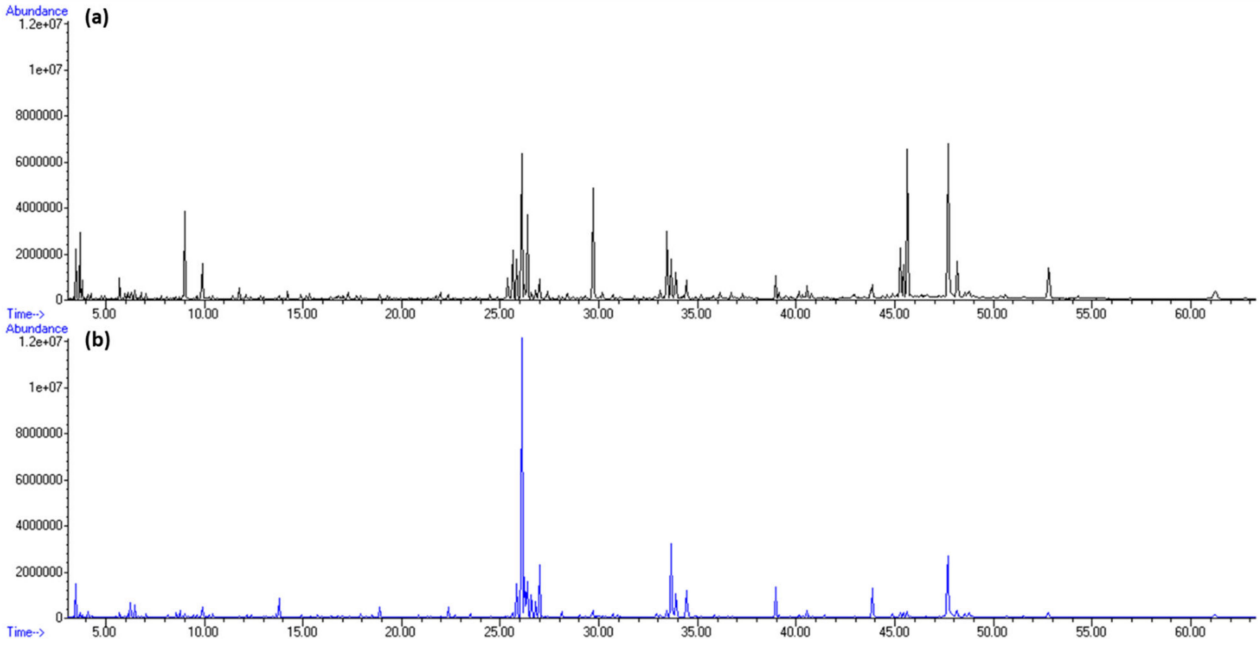

Figure 8. Total ion chromatogram (TIC) comparison of *H. scoparia* obtained by hydrodistillation (HD) and analysed by gas chromatography–mass spectrometry (GC–MS): (**a**) in May; (**b**) in September.

2.4. Statistical Analysis of the H. scoparia VOCs Obtained by Hydrodistillation

The PCA results for VOCs of fresh and air-dried *H. scoparia* obtained by hydrodistillation are shown in Figures 9 and 10.

In fresh samples, the first two PCs described 71.13% of the initial data variability. The correlation loadings of the first two PCs (Figure 9a) showed high correlations between germacrene D and (*Z*)-pentadec-6-en-1-ol and gleenol, between heptadec-1-ene and pentadec-1-ene, and between hexadecan-1-ol and hexadecanoic acid. Germacrene D, gleenol, (*Z*,*Z*,*Z*)-octadeca-9,12,15-trien-1-ol, and isopachydictyol A were the variables with the highest variable contributions, according to the correlations. The PC2 was associated with β-ionone, pentadec-1-ene, heptadec-1-ene, and (*E*)-phytol abundance in the samples. The score plot (Figure 9b) showed the position samples in the multivariate space of the first two PCs. There was a clear separation between the months. The dominant group in the fresh samples from May and September was aliphatic unsaturated compounds, while, in June, July, and August, it was the group of terpenes.

In dried samples, aliphatic compounds (pentadec-1-ene, phytane, and 6,10,14-trimethylpentadecan-2-one) were variables with the highest contribution to PC1, while β-ionone, (*Z*)-pentadec-6-en-1-ol, and (*E*)-phytol, contributed the most to PC2. High correlations were observed among germacrene D, pentadecane, and (*Z*)-pentadec-6-en-1-ol, as well as between phytane and dibutyl phthalate.

When all dominant VOCs obtained by hydrodistillation were analysed, a clear separation of fresh and dried samples was observed (Figure 10). May and June samples were positioned in the lower part of the plot, but clearly separated on the basis of the difference in the content of β-ionone, gleenol, (*Z*,*Z*,*Z*)-octadeca-9,12,15-trien-1-ol, and (*E*)-phytol. There were more similarities in seasonal variation between dry samples. The dried samples appeared in the left part of the plot. The distribution was along the PC1 axis and was in the relation to aliphatic compounds (germacrene D, pentadecane, gleenol, and 6,10,14-trimethylpentadecan-2-one), while the distribution along the PC2 axis was related to sesquiterpene alcohols (cembra-4,7,11,15-tetraen-3-ol and gleenol) and methyl (*Z*)-pentadec-6-en-1-ol, (*Z*)-octadec-9-enal, (*Z*,*Z*,*Z*)-octadeca-9,12,15-trien-1-ol, (*E*)-phytol, isopachydictyol A, and cembra-4,7,11,15-tetraen-3-ol abundance in the samples. No correlation was found between the compound abundance and temperature change.

The impact of HS-SPME and HD on the obtained results was obvious from the composition of the headspace (Tables 1 and 2) and hydrodistillate (Table 3) of the fresh samples. The most abundant headspace compounds were benzaldehyde, pentadecane, and pentadec-1-ene. (*E*)-Phytol was the most abundant compound in HD-FrHSc (except September) followed by diterpene alcohols (isopachydictyol A and cembra-4,7,11,15-tetraen-3-ol) and sesquiterpene alcohol gleenol.

The used statistical test did not correlate compound abundance with the temperature change as the temperature varied.

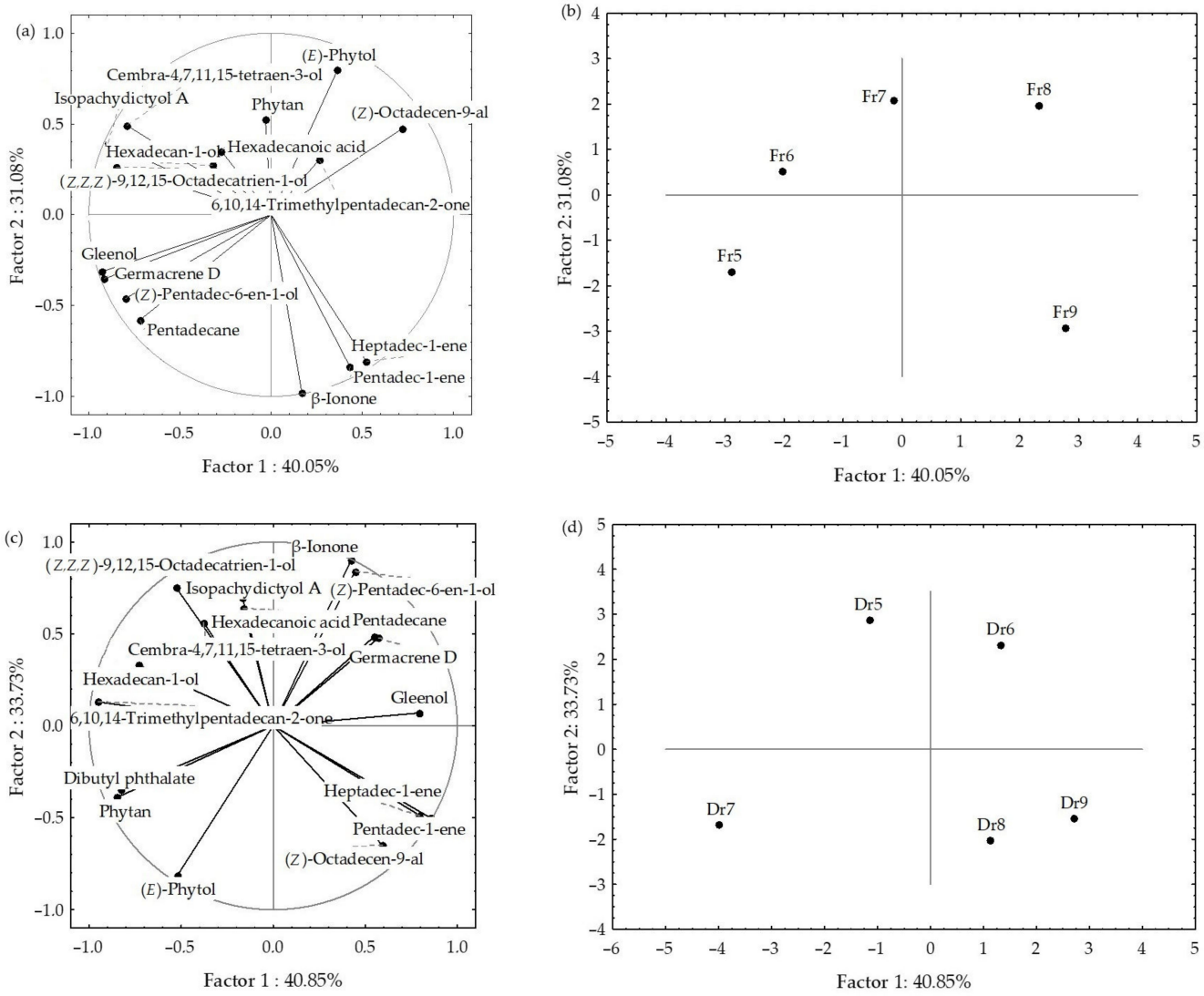

Figure 9. Correlation loadings (**a**,**c**) and score plot (**b**,**d**) of the dominant VOCs of fresh (Fr, **a**,**b**) and air-dried (Dr, **c**,**d**) *H. scoparia* samples obtained by hydrodistillation.

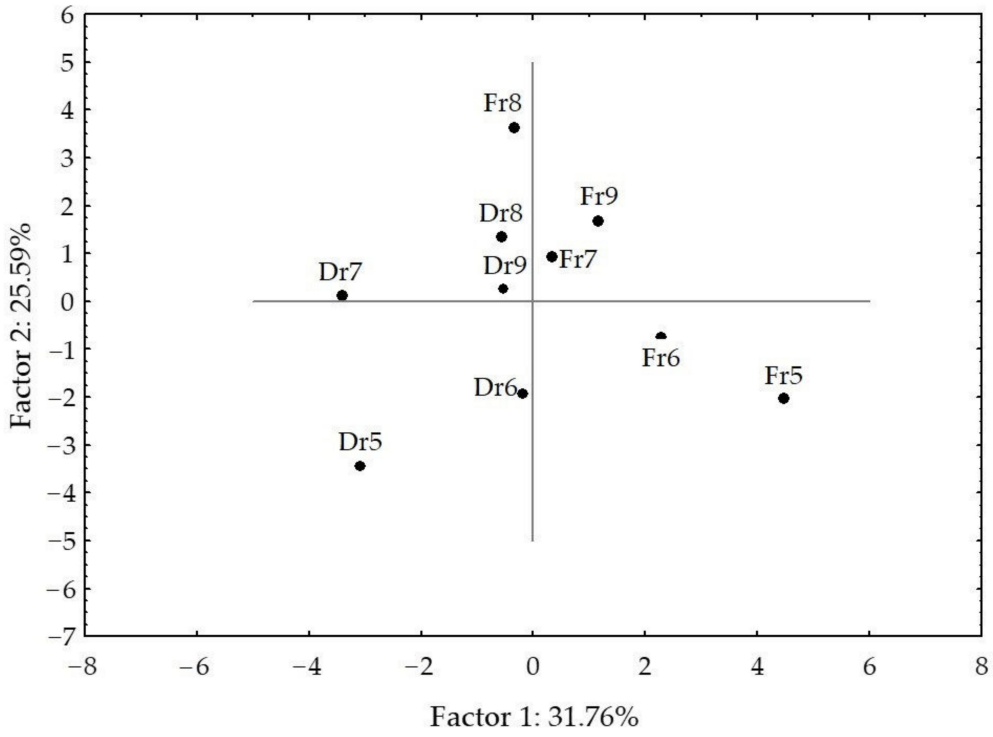

Figure 10. Score plot of the dominant VOCs of fresh (Fr) and air-dried (Dr) *H. scoparia* samples obtained by hydrodistillation.

3. Materials and Methods

3.1. Sample Collection

The seaweed *H. scoparia* (Linnaeus) Sauvageau 1904 samples were harvested in 2021 from May to September. The sampling location was in the Adriatic Sea on the southern side of the island Čiovo (43.493373° N, 16.272519° E). Seaweed samples were harvested at a depth ranging from 20 to 120 cm. A YSI Pro2030 probe (Yellow Springs, OH, USA) was used to measure the sea temperature (Figure 11). The identification was performed by a marine botanist according to seaweed morphological characteristics. The samples were air-dried in the shade at room temperature for 10 days following a similar pattern, which is very common for terrestrial plants and similar to our other studies [20,23,24].

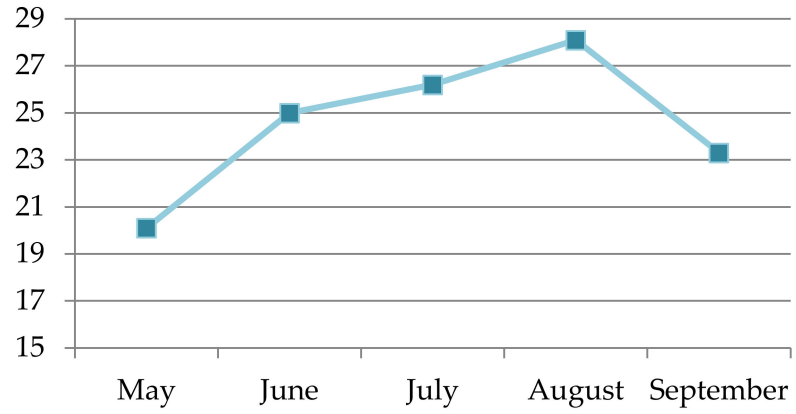

Figure 11. Sea temperature measured during harvesting.

3.2. Headspace Solid-Phase Microextraction (HS-SPME)

Two SPME fibres (Agilent Technologies, Palo Alto, Santa Clara, CA, USA) covered with PDMS/DVB (polydimethylsiloxane/divinylbenzene) or DVB/CAR/PDMS (divinylbenzene/carboxene/polydimethylsiloxane) and placed on the PAL Auto Sampler System

(PAL RSI 85, CTC Analytics AG, Schlieren, Switzerland) were used for HS-SPME. Prior to the extraction, both fibres were conditioned following the manufacturer instructions. The seaweed samples (1 g) were placed into 20 mL glass vials sealed with a stainless-steel cap with polytetrafluorethylene (PTFE)/silicon septa. The equilibration of the sample was conducted at 60 °C for 15 min. After equilibration, the sample was extracted for 45 min. Thermal desorption directly to the GC column was carried out at an injector temperature of 250 °C set for 6 min.

3.3. Hydrodistillation (HD)

Approximately 50 g of fresh and 20 g of air-dried *H. scoparia* samples were hydrodistilled. For HD, a *v/v* ratio 1:2 (3 mL) of pentane and diethyl ether was used as the solvent trap in a modified Clevenger apparatus. HD was performed for 2 h, and the solvent trap with dissolved VOCs was isolated. Concentration of the solvent trap was performed under a slow flow of nitrogen until the final volume was 0.2 mL. A volume of 2 µL was used for GC–MS analyses.

3.4. Gas Chromatography–Mass Spectrometry Analysis (GC–MS)

VOCs isolated from *H. scoparia* were analysed using a gas chromatograph (model 8890 Agilent Technologies, Palo Alto, Santa Clara, CA, USA) equipped with a mass spectrometer detector (model 5977E MSD, Agilent Technologies). For the separation of VOCs, HP-5MS capillary column (30 m × 0.25 mm, 0.25 µm film thickness, Agilent Technologies, Palo Alto, Santa Clara, CA, USA) was used. The conditions for the GC–MS analysis and the process for the identification of the compounds were previously described in detail by Radman et al. [20]. The analyses were carried out in duplicate and shown as the average percentage of peak area.

3.5. Statistical Analyses

The relations between the dominant volatiles (>2%) of fresh and dried alga samples, analysed by HS-SPME and HD were determined using principal component analysis (PCA) [35]. The average percentage peak areas of the dominant volatiles from fresh and dried samples obtained by HS-SPME (two fibres) and HD were used for the analysis. For the PCA analyses, STATISTICA® (version 13, StatSoft Inc, Tulsa, OK, USA) was used, and the data were log-transformed prior to analyses.

4. Conclusions

The present study reported complementary isolation of VOCs from FrHSc and DrHSc by headspace solid-phase microextraction (HS-SPME) and hydrodistillation (HD) and their analysis by gas chromatography and mass spectrometry (GC–MS). The use of both methods was justified for detailed chemical prospection of the headspace, volatile, and less volatile organic compounds from *H. scoparia*. The impact of the season of growth (May–September) and air-drying on VOC composition was also studied for the first time, and the obtained data were elaborated by principal component analysis (PCA).

The most abundant headspace compounds of *H. scoparia* were benzaldehyde, pentadecane (a chemical marker of brown macroalgae), and pentadec-1-ene. Benzaldehyde abundance decreased after air-drying, while an increment in benzyl alcohol after the drying was noticed. The percentage of pentadecane and heptadecane increased after air-drying while pentadec-1-ene abundance decreased. Octan-1-ol decreased from May to September. In HD-FrHSc, terpenes were the most abundant group in June, July, and August, while, in May and September, the dominant group was unsaturated aliphatic compounds. In HS-FrHSc, great variability was found within the 5 months by two fibres, while the HS-DrHSc volatilome was more comparable.

In HD-DrHSc terpenes, unsaturated and saturated aliphatic compounds dominated. (*E*)-phytol was the most abundant compound in HD-FrHSc through all months except September. Its abundance increased from May to August. Since phytol is a bioactive

compound, the obtained hydrodistillates could be attractive for further bioactivity studies. Two more diterpene alcohols, isopachydictyol A and cembra-4,7,11,15-tetraen-3-ol, and one sesquiterpene alcohol, gleenol, were also detected in high abundance. However, due to lower volatility, phytol and other diterpene alcohols could not be isolated completely by HD. Among aliphatic compounds, the dominant was pentadec-1-ene with its peak in September, while pentadecane was present with lower abundance.

PCA (based on the dominant compound analyses) showed a distinct separation of the fresh and dried samples. However, the used statistical test did not correlate compound abundance with the temperature change as the temperature varied. The results indicate great seasonal variability of isolated VOCs, as well among fresh and dried samples, which is important for further chemical biodiversity studies.

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Seasonal Changes in Chemical Profile and Antioxidant Activity of *Padina pavonica* Extracts and Their Application in the Development of Bioactive Chitosan/PLA Bilayer Film

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Abstract: Seaweeds are a potentially sustainable source of natural antioxidants that can be used in the food industry and possibly for the development of new sustainable packaging materials with the ability to extend the shelf-life of foods and reduce oxidation. With this in mind, the seasonal variations in the chemical composition and antioxidant activity of brown seaweed (*Padina pavonica*) extracts were investigated. The highest total phenolic content (TPC) and antioxidant activity (measured by ferric reducing/antioxidant power (FRAP), 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging, and oxygen radical absorbance capacity (ORAC)) were found for *P. pavonica* June extract. The TPC of 26.69 ± 1.86 mg gallic acid equivalent/g, FRAP of 352.82 ± 15.41 µmole Trolox equivalent (TE)/L, DPPH of 52.51 ± 2.81% inhibition, and ORAC of 76.45 ± 1.47 µmole TE/L were detected. Therefore, this extract was chosen for the development of bioactive PLA bilayer film, along with chitosan. Primary or quaternary chitosan was used as the first layer on polylactic acid (PLA) films. A suspension of chitosan particles with entrapped *P. pavonica* extract was used as the second layer. X-ray photoelectron spectroscopy confirmed the presence of layers on the material surface. The highest recorded antioxidant activity of the newly developed films was 63.82% inhibition. The developed functional films exhibited antifogging and antioxidant properties, showing the potential for application in the food industry.

Keywords: functional PLA films; seaweed and chitosan bilayer; sustainable natural antioxidants; microwave-assisted extraction

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1. Introduction

Seaweed *Padina pavonica* belongs to the genus *Padina*, family Dictyotaceae, order Dictyotales, and class Phaeophyceae. Currently, there are 60 taxonomically recognized species names in the genus *Padina*. The species of this genus are widely distributed from tropical to temperate seas [1]. Due to its availability and proven biological potential [2–4], *P. pavonica* is a good choice for the production of biologically active extracts that can be used in many food industry processes, including packaging. Moreover, the chemical composition and biological activity of seaweeds vary depending on the season, growth phase, and other environmental factors [5,6]. In this regard, *P. pavonica* extracts were found to have the strongest antibacterial activity against human and fish pathogens during the period from April to September [7]. The highest antitumor activity of fucoidans was found in samples collected in June [8]. Since there are no reports of seasonal changes in the chemical profiles or antioxidant activity of this species, it is important to determine the best time to harvest

seaweeds and prepare extracts for further exploitation. It is also important to choose the right extraction method to extract higher amounts of bioactive compounds and obtain stronger bioactivity [3].

In the food industry, new sustainable packaging materials should be developed and integrated with new packaging solutions to reduce the environmental footprint (both in terms of biodegradability and the origin of raw materials for packaging production). Apart from environmental aspects, new packaging solutions should also be functional and provide direct benefits to consumers by extending food shelf-life, ensuring food safety, and monitoring food quality [9]. New bio-based coatings are the trend for implementing active packaging concepts [10]. Active coated films with antioxidant properties interact with food, change its conditions, and control its quality [11].

On the other hand, biopolymers used for the development of new packaging solutions should be economical, abundant, and come from renewable sources. Polylactic acid (PLA), a thermoplastic polyester that is biodegradable, is obtained from renewable sources. PLA is one of the most widely used bioplastics due to its mechanical and physical properties. To achieve the active functionality and biodegradability of food packaging, the most commonly used biopolymers are polysaccharides, such as chitosan [12–15]. Chitosan, which is derived from the waste of marine crustaceans (shrimp, shellfish, crabs, and lobsters), is abundant, natural, and biodegradable [16]. However, chitosan itself has some limitations, such as low antioxidant capacity. Therefore, it has been shown that colloidal complexes based on the combination of chitosan and other substances, such as surfactants, polyphenols, etc., can overcome these drawbacks [17,18].

The variations in the chemical profile and biological activity of *P. pavonica* during seasonal growth, as well as the use of seaweed extracts for the development of functional PLA films alone or in combination with chitosan have not yet been investigated. In our previous research, the general concept of packaging materials with layer-by-layer colloidal chitosan-extract-polyphenol coatings for polypropylene and polyethylene was developed [18–20]. Therefore, the aim of this study was: (i) to determine the changes in total phenolic content (TPC) and chemical profile of *P. pavonica* during seasonal growth (from May to September); (ii) to determine the antioxidant activity of *P. pavonica* extracts; (iii) to develop and physicochemically characterize PLA films with bilayers of primary or quaternary chitosan in combination with *P. pavonica* extract—for the first time in this formulation; and (iv) to determine the antioxidant activity of the developed active packaging.

2. Materials and Methods

2.1. Collection, Extraction, and Compound Analyses

Padina pavonica was sampled in the Adriatic Sea off the southern coast of the island Čiovo (43.493389° N, 16.272505° E) from May to September 2020. The depth range was from 20 to 80 cm. A YSI Pro2030 probe (Yellow Springs, OH, USA) was used for the sea temperature and salinity measurements during the sampling (Figure 1). Seaweed samples were washed with tap water and freeze-dried (FreeZone 2.5, Labconco, Kansas City, MO, USA).

Freeze-dried seaweed samples were pulverized and extracted based on our previous research [3]. The seaweed powder was mixed with 50% ethanol (Gram-Mol, Zagreb, Croatia) in a 1:10 (*w*/*v*) seaweed-to-solvent ratio. Microwave-assisted extraction (MAE) was performed in an advanced microwave extraction system (ETHOS X, Milestone Srl, Sorisole, Italy) at 200 W and 60 °C for 15 min. After the extraction, samples were centrifuged for 8 min at 5000 rpm and room temperature and filtered. The ethanol was evaporated at 50 °C in a rotary evaporator and the remaining water extract was freeze-dried.

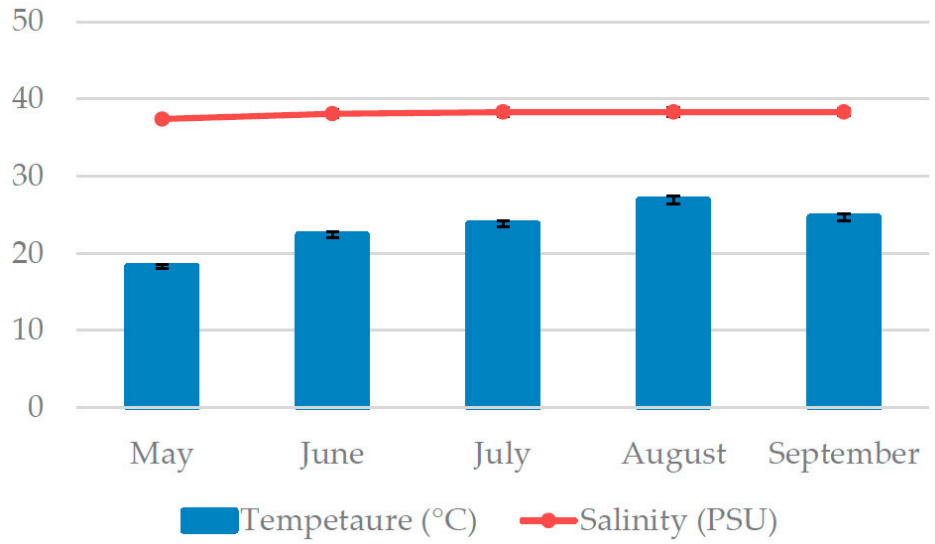

Figure 1. Sea temperature and salinity measured during harvesting of *P. pavonica*.

Total phenolic content (TPC) was determined by the Folin–Ciocalteu method [21], previously described by Čagalj et al. [5]. In brief, 125 µL of Folin–Ciocalteu reagent and 1.5 mL of distilled water were combined with 25 µL of the sample. After stirring the mixture for a minute, 375 µL of a 20% sodium carbonate solution and 475 µL of distilled water were added. Samples were kept at room temperature in the dark for two hours. Absorbance was measured at 765 nm. The results were expressed as mg gallic acid equivalents (GAE)/g of freeze-dried extract.

The analysis of compounds from *P. pavonica* was performed by dissolving 3 mg of freeze-dried extract in 1 mL of methanol/water (*v/v*) (Merck KGaA, Darmstadt, Germany) using the ACQUITY Ultra Performance LC system equipped with a photodiode array detector with binary solvent manager (Waters Corporation, Milford, MA, USA) series with a mass detector Q/TOF micromass spectrometer (Waters) with electrospray ionization (ESI) source operating in negative mode (UPLC-PDA-ESI-QTOF). The conditions were as follows: capillary voltage, 2300 kV; source temperature, 100 °C; cone gas flow, 40 L/h; desolvation temperature, 500 °C; desolvation gas flow, 11,000 L/h; and scan range, *m/z* 50–1500. Individual compounds' separation was performed using an ACQUITY UPLC BEH Shield RP18 column (1.7 µm, 2.1 mm × 100 mm; Waters Corporation, Milford, MA, USA) at 40 ○C. Water containing 1% acetic acid (A) and acetonitrile (B) (Merck KGaA, Darmstadt, Germany) was used for the elution gradient test and applied as follows: 0 min, 1% B; 2.3 min, 1% B; 4.4 min, 7% B; 8.1 min, 14% B; 12.2 min, 24% B; 16 min, 40% B; 18.3 min, 100% B, 21 min, 100% B; 22.4 min, 1% B; 25 min, 1% B. The injected sample volume was 2 µL and the used flow rate was 0.6 mL/min. The wavelength of 280 nm was used to monitor the compounds. MassLynx 4.1 software (Waters Corporation, Milford, MA, USA) was used for the integration and data elaboration [5,22].

2.2. Collection, Extraction, and Compound Analyses

The antioxidant activity of *P. pavonica* extracts was measured by using three different methods with different mechanisms of action. Ferric-reducing/antioxidant power (FRAP) method is based on electron transfer, while 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging ability and oxygen radical absorbance capacity (ORAC) methods are based on hydrogen atom transfer [23]. The freeze-dried extracts were dissolved in 50% ethanol prior to the antioxidant activity assays.

The used FRAP method was previously described by Benzie and Strain [24] with modifications described in Čagalj et al. [5]. In brief, the microplate wells were filled with 300 µL of FRAP reagent solution and the absorbance at 592 nm was measured. Four minutes after adding 10 µL of the sample to the microplate wells, the absorbance

change was measured. Measured absorbances were compared with the readings obtained for the standard Trolox solutions. The FRAP results were expressed as micromoles of Trolox equivalents/liter of extract (μmole TE/L).

The ability to scavenge DPPH radicals was measured using the method previously described by Milat et al. [25]. DPPH radical solution (290 μL) was pipetted to microplate wells and absorbance was measured at 517 nm. One hour after addition of 10 μL of the sample, the decrease in the absorbance was measured. The results were expressed as the percentage of DPPH radical inhibition (% inhibition).

The extracts were diluted 200-fold before performing the ORAC method. The method used was previously described by Burčul et al. [26]. In brief, the mixture of 150 μL of fluorescein and 25 μL of the sample (or Trolox for standard or puffer for blank) was pipetted to microplate wells and thermostated at 37 °C for 30 min. Following the addition of 25 μL of 2,2′-Azobis(2-amidinopropane) dihydrochloride (AAPH), measurements were made every minute for 80 min at excitation and emission wavelengths of 485 and 520 nm. ORAC results were expressed in μmole TE/L.

The TPC and antioxidant assays were performed in triplicate. The absorbance of the extracts' color was subtracted before the calculations.

The extract with the highest antioxidant activity was selected for chitosan/PLA bilayer film development.

2.3. Development of Chitosan/Polylactic Acid Bilayer Films

2.3.1. Preparation of Bilayer Solutions

For this study, the solutions listed in Table 1 were prepared. Primary (low-molecular-weight chitosan (50 to 190 kDa), poly (D-glucosamine); Sigma-Aldrich, St. Louis, MO, USA) and quaternary (Chitosan Quaternary Ammonium Salt; CD Bioparticles, Shirley, NY, USA) chitosan solutions of 1% and 2% (*w/v*) were prepared by adding deionized water to chitosan powder with a few drops of absolute acetic acid (≥99.8%; Sigma-Aldrich, St. Louis, MO, USA) to dissolve the powder. The solutions were stirred overnight, and the pH was adjusted to 4.0 with acetic acid. Sodium tripolyphosphate (TPP; Sigma-Aldrich, St. Louis, MO, USA) was suspended in deionized water and stirred overnight to prepare a 0.2% (*w/v*) solution. The main purpose of setting pH = 4 was to ensure the same conditions for both chitosans. It should be taken into account that there are still 20% (the degree of substitution is 80%) of the primary groups in quaternary chitosan, which could be protonated in an acidic environment.

Table 1. Sample description of prepared solutions.

Solutions	Abbreviation
1% primary chitosan	CH
1% quaternary chitosan	QCH
2% primary chitosan	2%CH
2% quaternary chitosan	2%QCH
Primary Chitosan particles	CHP's
Quaternary Chitosan particles	QCHP's
P. pavonica June extract	PPAV
Primary chitosan particles with captured extract	CHP'sPPAV
Quaternary Chitosan particles with captured extract	QCHP'sPPAV
Sodium tripolyphosphate	TPP

Before preparation of the chitosan particles, the freeze-dried *P. pavonica* June extract was dissolved in 50% ethanol (99.8%, GC; Sigma-Aldrich, St. Louis, MO, USA) to prepare a solution with a concentration of 10 mg/mL. The chitosan particles with the captured

extract were then prepared using the ionic gelation technique. The prepared TPP and *P. pavonica* extract solutions were simultaneously added to a fixed volume of 1% (*w/v*) chitosan solution (primary and quaternary) to obtain a 5:1 weight ratio between chitosan and TPP, which was chosen according to our previous research [27]. The particles formed spontaneously under continuous stirring for 1 h at room temperature. The final pH of the chitosan particle dispersions with PPAV was adjusted to 4.0 with acetic acid under slow mixing.

2.3.2. Application of Chitosan Solutions and Particles to PLA Films

The prepared chitosan solutions and chitosan particle dispersions were applied to the PLA films (Optimont® PLA-Folie, Bleher Folientehnik GmbH, Ditzingen, Germany), which were cleaned and air-dried before application. The layers were applied directly to the surfaces of PLA in a roll-to-roll printing process using a printing table and a magnetic roller (Johannes Zimmer, Kufstein, Austria) at rolling speed level 3 and magnetic strength level 1. All previously prepared solutions were stirred before application and applied to the film surfaces in a fixed volume (primary and quaternary chitosan: 4 mL, chitosan particles with embedded extracts: 4 mL). Both layers were, thus, prepared under the same conditions and air-dried after each individual layer. The first layer consisted of 2% primary or 2% quaternary chitosan and the second layer consisted of chitosan particle dispersions (CHPs or QCHPs) with captured *P. pavonica* June extract. The description of the samples and their names are listed in Table 2.

Table 2. PLA film samples description.

Description of the Samples	Sample Name
References	
PLA with no layers	PLA
PLA applicated with 2% CH	PLA + 2%CH
PLA applicated with 2% QCH	PLA + 2%QCH
Samples with primary chitosan as first layer	
PLA applicated with 2% CH and CHP'sPPAV	P3
PLA applicated with 2% CH and QCHP'sPPAV	P4
Samples with quaternary chitosan as first layer	
PLA applicated with 2% QCH and CHP'sPPAV	P9
PLA applicated with 2% QCH and QCHP'sPPAV	P10

2.3.3. Physical and Chemical Properties of Chitosan/PLA Bilayer Films

The zeta potential (ZP) and hydrodynamic diameter (HD) of the prepared particle dispersions were determined using the particle size analyzer (Litesizer 500, Anton Paar, Graz, Austria) at 25 °C. ZP was measured by electrophoretic light scattering (ELS), which measures the velocity of particles in the presence of an electric field. HD was measured by dynamic light scattering (DLS). The speed of this motion depends on the size of the particles; smaller particles move faster than larger ones. Before analysis, the dispersion was stirred and, if necessary, adjusted to pH 4 with acetic acid. To perform the measurements, the diluted sample was placed in an Omega cuvette for ZP and size. Data were collected using Kalliope software (Anton Paar, Graz, Austria).

Contact angles were measured using a goniometer (Data Physics, Fidelstadt, Germany) to estimate the surface wettability of layered molding compounds. Milli-Q water (5 µL) was carefully placed on the test film surface. A goniometer with static contact angle (SCA) 20 software was used to determine SCA at room temperature. Analyses were performed in triplicates.

The chemical composition of the chitosan/PLA bilayer films was analyzed using the X-ray photoelectron spectroscopy (XPS) instrument model TFA-XPS (Physical Electronics, Munich, Germany). The spectrometer was equipped with a hemispherical electron analyzer

and a monochromatic X-ray source with Al Kα1.2 radiation with a photon energy of 1486.6 eV. The excitation area of the sample was 400 μm^2. The emitted photoelectrons were measured at a departure angle of 45°. During the XPS measurements, an electron gun was used to neutralize the surface charge. The survey spectra were measured at a transit energy of 187 eV and an energy step of 0.4 eV. MultiPak v8.1c software (Physical Electronics, Munich, Germany) was used to analyze the measured spectra.

Chitosan/PLA bilayer films were examined with a scanning electron microscope (SEM) using the JSM-IT800 instrument (Jeol, Tokyo, Japan). The PLA films were cut and glued to a double-sided conductive carbon tape, placed on a holder, and sprayed with gold to ensure conductivity and prevent charging effects. The samples were examined with an accelerating voltage of 5 kV and a variable working distance at comparable magnification. Images were acquired using a secondary electron detector.

2.4. Antioxidant Potential of Chitosan/PLA Bilayer Films

The antioxidant activity of the chitosan/PLA bilayer films was tested using 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid (ABTS) reagent. The assay is based on the spectrophotometric determination (UV-VIS) of the decolorization of the reagent in the presence of an antioxidant. The ABTS reagent (7 mM; Sigma-Aldrich, St. Louis, MO, USA) was prepared in 2.45 mM potassium persulfate (Sigma-Aldrich, St. Louis, MO, USA) and diluted with phosphate-buffered saline (PBS; Gibco, Life Technologies, Grand Island, NY, USA). Absorbance was measured at 734 nm and 25 °C at time points of 0 min, 15 min, and 60 min after addition of 0.1 g of film sample to 3.9 mL of ABTS solution. The solution was shaken during the extraction. The results are given as percentage of inhibition [18].

2.5. Statistical Analysis

Results are expressed as mean ± standard deviation. The statistically significant difference between *P. pavonica* extracts' TPC and antioxidant activity over the months was determined by analysis of variance (one-way ANOVA), followed by a least significant difference test at the 95% confidence level [28]. Analyses were performed using Statgraphics Centurion-Ver.16.1.11 (StatPoint Technologies, Inc., Warrenton, VA, USA).

3. Results and Discussion

Padina pavonica was harvested from May to September during the period when its thallus grows in the Adriatic Sea. In particular, *P. pavonica*'s thallus detaches every winter and regrows in spring. After September and during the winter, this seaweed is in the form of rhizoids, filamentous thalli, or sporelings until spring comes and the conditions are suitable for its full regrowth [29]. Thus, its seasonal growth is from May till September. For this reason, we aimed to investigate the difference during thallus growth to see if there are significant changes in chemical profile and antioxidant activity during this algae's seasonal growth. Knowing the perfect harvesting time can contribute to the knowledge needed for the possible cultivation or farming of this species and its potential exploitation.

3.1. Compound Analyses of P. pavonica Extracts

The results of the TPC for *P. pavonica* harvested from May to September are shown in Figure 2. The highest TPC value was determined for the extract of *P. pavonica* harvested in June. Overall, the results varied between 11.88 ± 0.51 and 26.69 ± 1.86 mg GAE/g. The lowest TPC was found for the May sample. There are many factors that can affect the TPC in seaweeds. TPC can vary due to seasonal variations in salinity, sea temperature and light intensity, different geographic locations, and biological factors, such as algal life cycle, size, age, and the presence of predators [6]. To avoid the effects of geographic location, the seaweed samples in this study were collected from the same location and depth each month. The sea temperature and TPC results showed no correlation. The results of this study are in accordance with Bernardini et al. [30], who reported a TPC of 27.0 mg GAE/g

in *P. pavonica* extract. In addition, Sofiana et al. [31] reported a TPC value of 20.34 mg GAE/g in the ethanolic extract of *P. pavonica*.

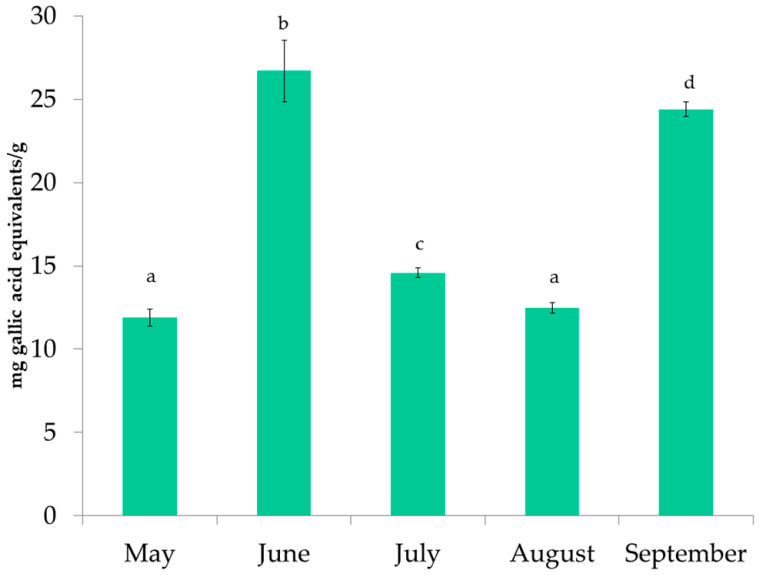

Figure 2. Total phenolic content of *P. pavonica* extracts. a–d different letters denote statistically significant difference.

Padina pavonica extracts were subjected to quali-quantitative analysis of polar compounds using LC-ESI-QTOF-MS in negative ion mode. The chromatograms of the basic peaks are shown in Figure 3. The results are listed in Table 3, along with their retention time, score (%), molecular formulae, observed and theoretical *m/z*, and error (ppm). Forty-seven compounds were tentatively identified. For all compounds, the error was lower than 5 ppm and the score was higher than 90%. All compounds were identified considering previous research [5] and the PubChem database. The amount of each compound was calculated based on the peak areas and expressed as a percentage.

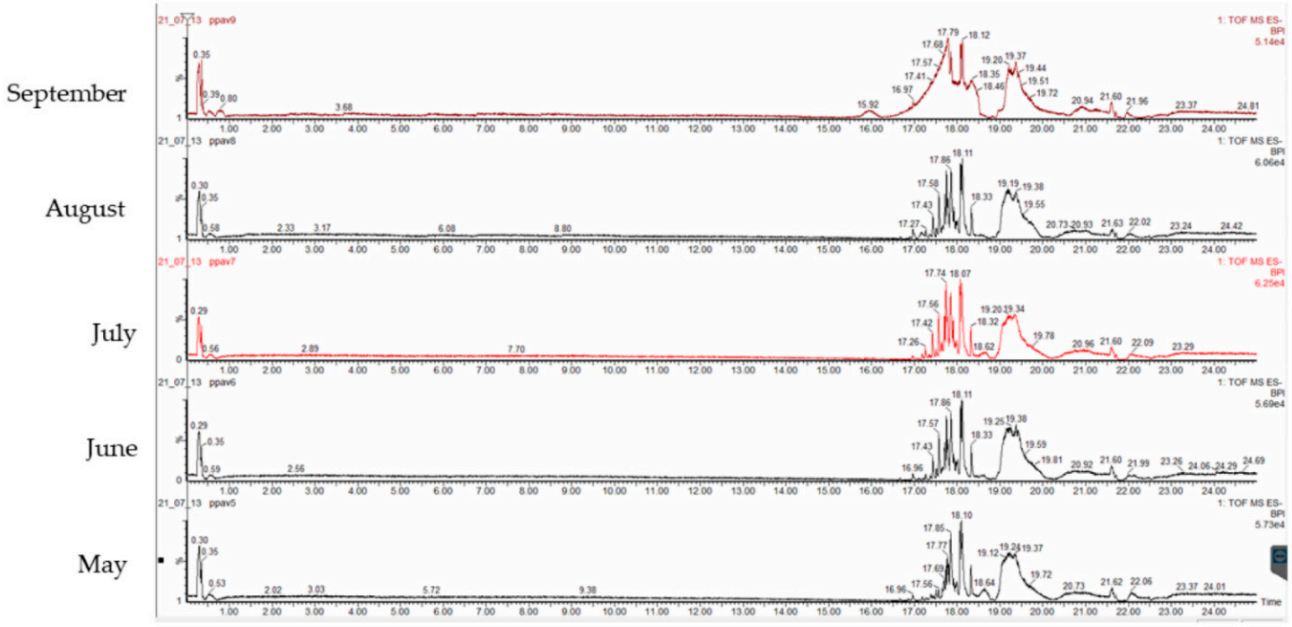

Figure 3. Chromatograms of the UPLC-PDA-ESI-QTOF analyses of *P. pavonica*.

Table 3. The list of compounds detected in *P. pavonica* extracts analyzed by UPLC-PDA-ESI-QTOF.

No.	RT (min)	Observed *m/z*	Theorical *m/z*	Error (ppm)	Score (%)	Molecular Formulae	Tentative Compound	May (%)	June (%)	July (%)	August (%)	September (%)
1	0.27	343.0367	343.0368	−0.3	94.07	C_{20} H_4 N_6 O	1a,9b-Dihydrophenanthro [9,10-b]oxirene-2,3,4,7,8,9-hexacarbonitrile	7.61	6.45	4.78	6.42	6.63
2	0.29	201.0239	201.0247	−4.0	98.89	C_4 H_{10} O_9	2-(1,2,2,2-Tetrahydroxyethoxy)ethane-1,1,1,2-tetrol	7.62	7.13	5.76	6.72	7.05
3	0.34	141.0157	141.0161	−2.8	91.01	C_2 H_2 N_6 O_2	Diazidoacetic acid	0.82	1.27	1.30	0.80	1.05
4	0.35	181.0709	181.0712	−1.7	100	C_6 H_{14} O_6	D-Sorbitol	1.56	1.14	1.35	1.12	2.44
5	0.39	317.0516	317.0509	2.2	90.44	C_{12} H_{14} O_{10}	D-glucaric acid derivate	0.50	0.45	0.55	0.52	1.62
6	16.60	343.2122	343.2121	0.3	95.77	C_{18} H_{32} O_6	10,11-Dihydroxy-9,12-dioxooctadecanoic acid	0.15	0.14	0.12	0.13	0.13
7	16.84	487.3426	487.3423	0.6	96.96	C_{30} H_{48} O_5	Esculentic acid	0.36	0.00	0.00	0.00	0.00
8	16.96	275.2012	275.2011	0.4	100	C_{18} H_{28} O_2	Stearidonic acid (C18:4*n-3*) isomer a	0.08	0.08	0.04	0.16	0.41
9	17.10	309.2056	309.2066	−3.2	96.09	C_{18} H_{30} O_4	6,9-Octadecadienedioic acid	0.20	0.33	0.17	0.26	0.32
10	17.16	285.2066	285.2066	0.0	90.36	C_{16} H_{30} O_4	Hexadecanedioic acid	0.32	0.14	0.07	0.14	0.21
11	17.17	277.2168	277.2168	0.0	90.14	C_{18} H_{30} O_2	gamma-Linolenic acid isomer a (C18:3*n-6*)	0.12	0.11	0.08	0.30	0.53
12	17.19	295.2276	295.2273	1.0	100	C_{18} H_{32} O_3	9,10-Epoxyoctadecenoic acid isomer a (vernolic acid)	0.45	0.20	0.47	0.29	0.14
13	17.22	429.30090	429.3005	0.9	91.64	C_{27} H_{42} O_4	24-Keto-1,25-dihydroxyvitamin D3 isomer a	n.d.*	0.02	n.d.	0.01	0.03
14	17.27	247.1712	247.1698	5.7	100	C_{16} H_{24} O_2	2,4,6-Triisopropyl benzoic acid	0.17	0.60	1.09	0.77	0.48
15	17.30	297.2426	297.2430	−1.3	98.84	C_{18} H_{34} O_3	10-Oxooctadecanoic acid isomer a	1.14	0.43	n.d.	0.34	0.29
16	17.34	287.2212	287.2222	−3.5	90.62	C_{16} H_{32} O_4	10,16-Dihydroxyhexadecanoic acid isomer a	0.07	0.09	0.10	0.09	0.16
17	17.35	287.2211	287.2222	−3.8	90.73	C_{16} H_{32} O_4	10,16-Dihydroxyhexadecanoic acid isomer b	0.03	0.16	0.67	0.58	1.40
18	17.37	199.16890	199.1698	−4.5	92.54	C_{12} H_{24} O_2	Lauric acid	0.60	0.63	0.57	0.57	0.43
19	17.38	243.1952	243.1960	−3.3	90.78	C_{14} H_{28} O_3	3-hydroxymyristic acid	0.68	0.32	0.37	0.34	0.40
20	17.42	293.2117	293.2117	0.0	80.96	C_{18} H_{30} O_3	13-ketooctadecadienoic acid isomer a	0.33	0.22	0.57	0.29	0.12
21	17.43	293.2117	293.2117	0.0	87.56	C_{18} H_{30} O_3	13-ketooctadecadienoic acid isomer b	0.46	1.93	2.47	1.95	3.15
22	17.44	295.2276	295.2273	1.0	100	C_{18} H_{32} O_3	9,10-Epoxyoctadecenoic acid isomer b (vernolic acid)	1.00	0.34	n.d.	0.29	0.24
23	17.51	269.2110	269.2117	−2.6	98.63	C_{16} H_{30} O_3	3-Oxohexadecanoic acid	1.29	1.05	1.11	1.12	2.11
24	17.51	225.1847	225.1855	−3.6	95.99	C_{14} H_{26} O_2	Myristoleic acid	1.53	1.44	1.27	1.33	1.03
25	17.56	275.2007	275.2011	−1.5	36.37	C_{18} H_{28} O_2	Stearidonic acid (C18:4*n-3*) isomer b	0.72	0.66	0.40	1.09	2.19
26	17.58	275.2010	275.2011	−0.4	93.59	C_{18} H_{28} O_2	Stearidonic acid (C18:4*n-3*) isomer c	1.13	4.46	4.38	3.85	2.88
27	17.59	277.2159	277.2168	−3.2	91.36	C_{18} H_{30} O_2	gamma-Linolenic acid isomer b (C18:3*n-6*)	0.01	0.08	0.03	0.07	0.04
28	17.60	213.18450	213.1855	−4.7	92.41	C_{13} H_{26} O_2	Tridecanoic acid	0.82	0.75	0.62	0.72	0.54
29	17.61	257.2108	257.2117	−3.5	95.16	C_{15} H_{30} O_3	11-Hydroxypentadecanoic acid	0.43	0.16	n.d.	0.17	0.22
30	17.63	251.2010	251.2011	−0.4	100	C_{16} H_{28} O_2	7-cis,10-cis-hexadecadienoic acid	0.72	0.94	1.07	1.12	0.61
31	17.64	297.2429	297.2430	−0.3	97.33	C_{18} H_{34} O_3	10-Oxooctadecanoic acid isomer b	0.88	0.42	n.d.	0.55	0.39

Table 3. *Cont.*

No.	RT (min)	Observed *m*/*z*	Theorical *m*/*z*	Error (ppm)	Score (%)	Molecular Formulae	Tentative Compound	May (%)	June (%)	July (%)	August (%)	September (%)
32	17.66	239.2004	239.2011	−2.9	98.8	C_{15} H_{28} O_2	Myristoleic acid methyl ester	3.69	3.23	2.67	3.06	2.39
33	17.70	301.2156	301.2168	−4	98.12	C_{20} H_{30} O_2	Eicosapentanoic acid isomer a (C20:5*n-3*)	0.96	3.26	3.49	3.39	2.36
34	17.75	277.2171	277.2168	1.1	50.48	C_{18} H_{30} O_2	gamma-Linolenic acid isomer c (C18:3*n-6*)	2.75	5.28	6.90	5.60	5.16
35	17.77	227.2005	227.2011	−2.6	94.05	C_{14} H_{28} O_2	Tetradecanoic acid (C14:0)	5.16	4.13	4.47	4.01	3.13
36	17.80	271.2266	271.2273	−2.6	97.75	$C_{16}H_{32}O_3$	Hydroxy-palmitic acid	3.43	1.40	1.76	1.46	1.91
37	17.85	253.2159	253.2168	−3.6	99.61	C_{16} H_{30} O_2	Palmitoleic acid (C16:1*n-7*)	10.10	9.25	9.71	9.28	8.84
38	17.91	279.2319	279.2324	−1.8	98.14	C_{18} H_{32} O_2	Octadeca-10,12-dienoic acid (C18:2*n-6*) isomer a	1.49	1.68	2.55	2.08	2.32
39	17.93	241.2168	241.2168	0.0	100	C_{15} H_{30} O_2	Pentadecanoic acid (C15:0)	2.89	2.65	2.39	2.57	2.49
40	17.97	279.2324	279.2324	0.0	91.12	C_{18} H_{32} O_2	Octadeca-10,12-dienoic acid (C18:2*n-6*) isomer b	1.93	1.84	1.91	1.73	1.58
41	18.00	267.2329	267.2324	1.9	100	C_{17} H_{32} O_2	9-Heptadecenoic acid (C17:1*n-8*)	2.81	2.88	2.47	2.70	2.86
42	18.07	255.2318	255.2324	−2.4	99.95	C_{16} H_{32} O_2	Hexadecanoic acid (palmitic acid) isomer a (C16:0)	0.24	0.31	0.39	0.35	0.45
43	18.08	255.2318	255.2324	−2.4	99.44	C_{16} H_{32} O_2	Hexadecanoic acid (palmitic acid) isomer b (C16:0)	9.40	8.43	9.19	8.67	8.11
44	18.10	281.2472	281.2481	−3.2	99.96	C_{18} H_{34} O_2	Oleic acid (C18:1*n-9*)	13.85	12.59	11.68	11.81	11.48
45	18.21	269.2474	269.2481	−2.6	97.24	C_{17} H_{34} O_2	Heptadecanoic acid (C17:0)	3.78	3.64	3.27	3.59	3.33
46	18.33	283.2629	283.2637	−2.8	99.97	C_{18} H_{36} O_2	Octadecanoic acid (stearic acid) C18:0	4.33	3.76	3.74	3.65	3.40
47	18.54	311.2944	311.295	−1.9	92.83	C_{20} H_{40} O_2	Arachidic acid	0.55	0.46	0.53	0.49	0.49

* n.d.—not detected.

Overall, most of the compounds detected were fatty acids. Oleic acid (C18:1*n-9*) was the predominant compound detected in *P. pavonica* extracts, with a content over 11% in all harvesting months. The highest content of oleic acid was detected in May, followed by June samples. Palmitic (C16:0) and palmitoleic (C16:1*n-7*) acids were also found in high amounts. Two ω-3 fatty acids, stearidonic acid (C18:4*n-3*) and eicosapentaenoic acid (EPA) (C20:5*n-3*), were also detected. Low molecular weight phenolic compounds were not identified. However, this does not exclude the presence of phenolics in the extracts. The reason could be the presence of high molecular weight phlorotannins in the extracts, which cannot be ionized and determined by HPLC-ESI-TOF-MS [5]. However, in our previous study, we identified phenolic acids (protocatechuic acid, *p*-hydroxybenzoic acid, *p*-coumaric acid, *t*-ferulic acid, and *o*-coumaric acid) in *P. pavonica* extracts using a different methodology [32]. Palmitic acid, palmitic acid ethyl ester, phytol, and phthalic acid were previously identified as major compounds in *P. pavonica* [33]. In addition, the same authors identified five phenolic compounds (kaempferol, ferulic acid, naringenin, delphinidin-3-oglucoside, and ellagic acid) in *P. pavonica* extracts [34]. Uslu et al. [35] identified myristic, palmitic, heptadecanoic, stearic, palmitoleic, and oleic acids in *P. pavonica*. The major fatty acids were margaric, palmitic, and oleic acids accounting for 39.55, 29.84, and 6.49% of the fatty acid composition, respectively. El Shoubaky and El Rahman Salem [36] identified palmitic acid (28.10%) and oleic acid (19.80%) as major fatty acids in the fatty acid composition of *P. pavonica*.

3.2. Antioxidant Activity of P. pavonica Extracts

The antioxidant activity of *P. pavonica* extracts was measured by the FRAP, DPPH, and ORAC methods. The results of FRAP are shown in Figure 4. The values ranged from 221.54 ± 13.41 to 352.82 ± 15.41 µmole TE/L. The highest reducing activity was observed in the June samples, followed by the September samples. In addition, the extracts from June had the highest DPPH inhibition (Figure 5) and the highest ORAC results (Figure 6). DPPH radical inhibition varied from 21.70 ± 1.34% to 52.51 ± 2.81%. The lowest inhibition was observed for the August sample, which was more than two times lower than the highest inhibition result. Prior to ORAC analyses, the extracts were diluted 200-fold. ORAC values ranged from 21.81 ± 0.71 to 76.45 ± 1.47 µmole TE/L. The lowest ORAC value was also found in the August sample.

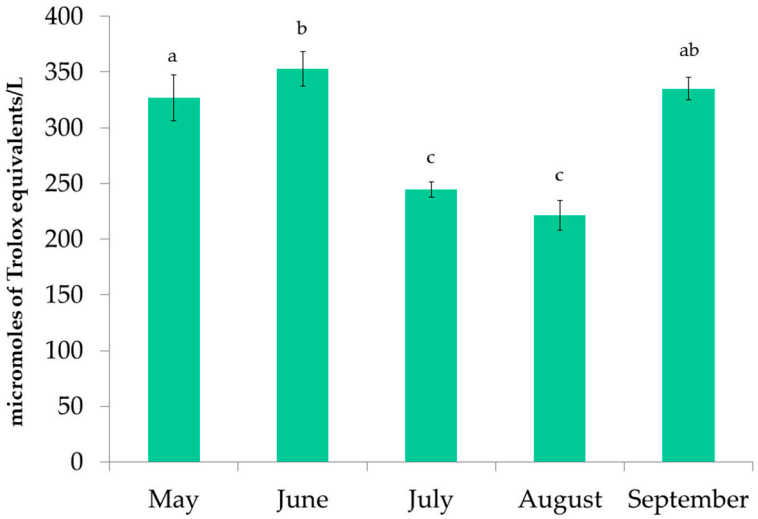

Figure 4. FRAP results for extracts of *P. pavonica* harvested from May to September. a–c different letters denote statistically significant difference.

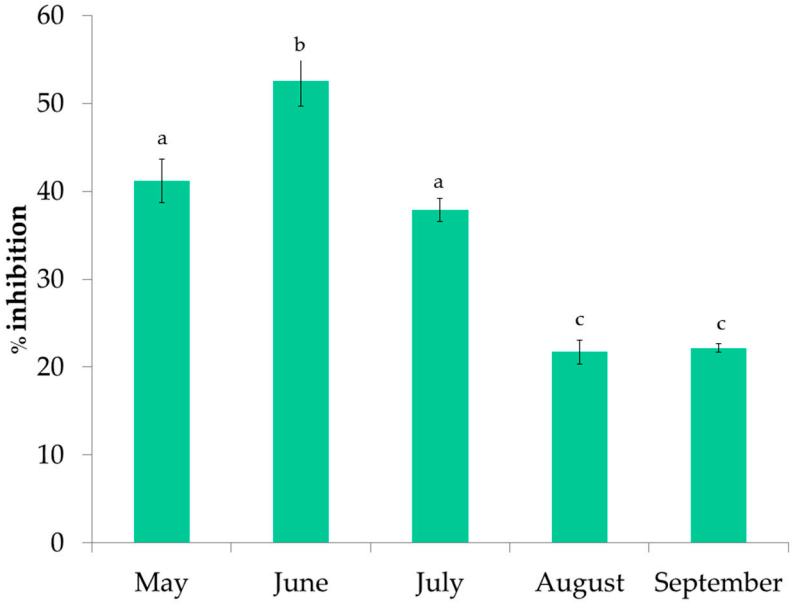

Figure 5. DPPH inhibition results for extracts of *P. pavonica* harvested from May to September. a–c different letters denote statistically significant difference.

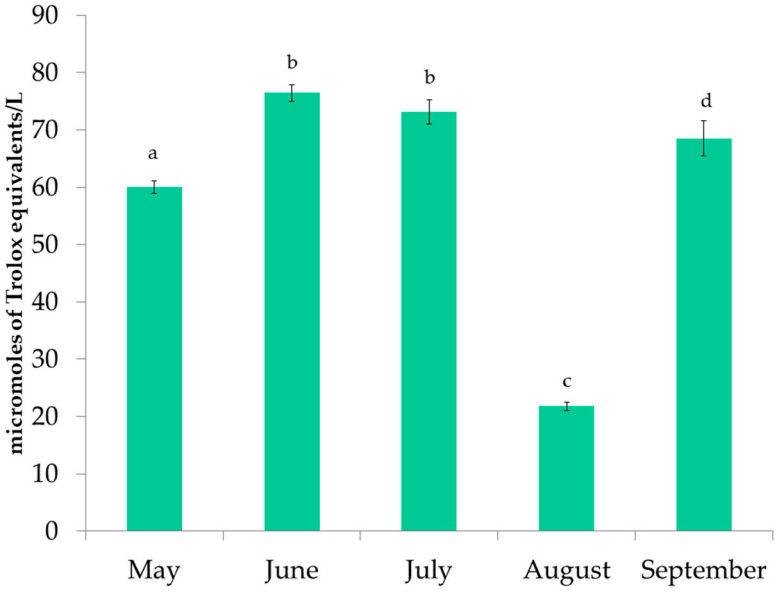

Figure 6. ORAC results for extracts of *P. pavonica* harvested from May to September. a–d different letters denote statistically significant difference.

Al-Enazi et al. [4] determined higher inhibition of DPPH radical (77.60%) for ethanolic extracts of *P. pavonica* harvested from the Red Sea (sampling season was not reported). Generalić Mekinić et al. [32] investigated the antioxidant activity of *P. pavonica* harvested from the Adriatic Sea in August 2020 but at a different location. Extracts were prepared in water and ethanol using ultrasound-assisted extraction. The highest FRAP (231 µmole TE) and ORAC (55.8 µmole TE) results were reported for the ethanolic extract. These results are comparable to the results of this study for the August samples. However, in this study, higher antioxidant activity measured by FRAP and ORAC was recorded for all months except for August. Kosanić et al. [37] investigated the antioxidant, antimicrobial and anticancer potential of *P. pavonica*, *Dictyota dichotoma*, and *Sargassum vulgare* acetone extracts. *Padina pavonica* extracts showed higher DPPH radical scavenging activity (IC_{50} of 691.56 µg/L) than other seaweed species.

3.3. Development of Chitosan/PLA Bilayer Films

Hydrodynamic diameter and zeta potential for all solutions used for PLA film layers are given in Table 4.

Table 4. Hydrodynamic diameter (HD) and zeta potential (ZP) with standard deviation (SD) of different formulations.

Sample	HD (nm)	ZP ± SD (mV)	pH
CHP'sPPAV	4873	18.8 ± 0.4	4
QCHP'sPPAV	6928	23.6 ± 0.4	4
CHPs	358	36 ± 0.2	4
QCHPs	239.9	33 ± 0.3	4

The average particle size for the CHPs was 358 nm and, for the QCHPs, 240 nm, whereas, for both chitosan particles with embedded *P. pavonica* extract (the June extract was selected for its highest antioxidant activity), the average particle size was 4873 and 6928 nm, respectively. The increase in particle size was evident because the incorporation of the active compounds from the extracts into or onto the surface of the chitosan particles increased the hydrodynamic diameter from colloidal size to micron scale. The increase in particle size confirms the successful incorporation of the extracts and the binding of the active compounds to the interior and/or surface of the chitosan particles [18,19].

According to the literature, dispersions/suspensions with a zeta potential greater than 30 mV are defined as stable suspensions with minimal sedimentation tendency. Thus, both chitosan's dispersions are stable. No particle formulation with incorporated *P. pavonica* extract exceeded the zeta potential limit of 30 mV. The lowest zeta potential value was found in the CHP'sPPAV formulation at 18.8 mV. This could indicate that the extract is also bound to the surface of the chitosan particles or that the phenolic compounds interact with the amino groups, reducing the amount of available (free) amino groups and, thus, lowering the positive zeta potential [18,19].

The measured contact angles of the functionalized films are shown in Table 5. The results are shown as average angle and the percentage of angle reduction compared to the reference, an untreated PLA film.

Table 5. Contact angles for PLA, reference samples, and P3, P4, P9, and P10.

Samples	Average Angle (α/°)	Difference (%)
PLA	77.56 ± 1.61	/
PLA + 2%CH	80.39 ± 2.23	−3.66 ± 2.23
PLA + 2%QCH	78.69 ± 4.02	−1.47 ± 4.02
P3	41.38 ± 0.98	46.65 ± 0.98
P4	32.50 ± 0.25	58.10 ± 0.25
P9	24.93 ± 0.43	67.86 ± 0.43
P10	32.54 ± 1.91	58.18 ± 1.91

The reference PLA film had an average value of 77.56°, proving the hydrophilicity of the film, but with a rather high contact angle. The same is true for the chitosan layers. Functionalization of the films with chitosan and *P. pavonica* extract layers (in particle suspensions) decreased the contact angle in all samples. In all cases, the contact angle was reduced. The reduction was 46%, 58%, 68%, and 58% for P3, P4, P9, and P10, respectively.

For all two-layered functionalized films, the contact angle decreased compared to the PLA reference, regardless of the type of layer. The application of the developed layers on the surface of the PLA films improved hydrophilicity, which is of outstanding added value for practical applications in the food industry, while improving the transparency of water droplets and eliminating the fogging effect.

The XPS composition results for the layers of *P. pavonica* extract and chitosan applied onto PLA film are shown in Table 6. Compared to the unlayered PLA film, a slightly lower oxygen content (containing: 66.4%, 32.9%, and 0.7% of carbon, oxygen, and silicon, respectively) and an additional presence of nitrogen and several microelements at low concentrations, such as Na, Mg, Si, P, Cl, Ca, S, and K, were observed. Nitrogen can be correlated with the presence of chitosan polymers, P to particles, while other microelements should belong to the *P. pavonica* extract, clearly confirming the presence of the layers on the PLA surface. One possible reason that P was not detected in all samples, even though all samples are layered with nanoparticles, could be the nonuniformity of the layers, as seen at SEM, and the fact that XPS analysis is limited due to the very thin surface layer of about 50–100 nm (which does not necessarily mean that P is available; as an ion, it can penetrate deeper). After desorption, some microelements are no longer observed; however, other microelements remain on the surface. The inhomogeneity of the layers leads to illogical concentration variations, i.e., for some elements, the concentration increases after desorption.

Table 6. XPS analysis of functionalized PLA films with *P. pavonica* extract before and after desorption (in %).

Sample	C	N	O	Na	Mg	Si	P	Cl	Ca	S	K
Before Desorption											
P3	65.8	1.2	30.3	0.3	1.2	0.3	0	0.8	0.2	0.3	0
P4	62.4	2.6	31.9	0.4	0.5	0.3	0.2	1.7	0.1	0	0
P9	67.3	2.2	26.3	0.3	0.8	1.5	0	1.3	0.2	0	0
P10	62.8	5.6	26.1	0.7	1	0.4	0	3.3	0	0.2	0
After Desorption											
P3	66.6	4.0	26.7	0.3	0	1.5	0.4	0.2	0	0.4	0
P4	65.0	1.7	32.5	0	0	0.4	0	0.2	0	0.2	0
P9	60.9	4.2	30.2	1.9	0	0.8	0	0.8	0.2	0.4	0.7
P10	66.1	4.4	26.7	0	0	1.3	0.3	0.5	0	0.6	0

Potrč et al. [18] and Zemljič et al. [19] used a very similar deposition of formulations on films and found that the low desorption was due to a sequential deposition strategy: a 2% solution of primary chitosan was used as the first layer, followed by a dispersion of chitosan particles with the active ingredient—the extract—as the second layer. The same deposition strategy was used in this study. This could increase the stability, as the macromolecular chitosan solution serves to improve the adhesion of the chitosan nanoparticles with the incorporated extract, thus increasing the stability of the phenolic compounds at the surface of the film [18,19].

The results of the SEM analyses are shown in Figures 7 and 8. To further investigate the morphology of the PLA films after the two-layers application, SEM was used to visualize the coverage of the films. The untreated PLA film shows a typical flat surface with some imperfections, possibly due to impurities, as also shown by XPS (Si). The layered samples show changes in morphology. Samples P3 and P4, containing a top layer with CH and QCHPs with embedded PPAV extract, show, in the case of P3, that the particles are embedded in the applied first layer of macromolecular chitosan solutions and particles forming dendrite clusters are clearly visible, the size of the particles being also below 100 nm. In the applications with the 2% QCH first layer, i.e., samples P9 and P10, similar morphologies are observed as in the samples with the first layer of 2% CH. However, due to the presence of fewer agglomerates and evenly distributed particles, some differences can be observed. This is especially true for samples P9 and P10, which contain particles with entrapped PPAV extract.

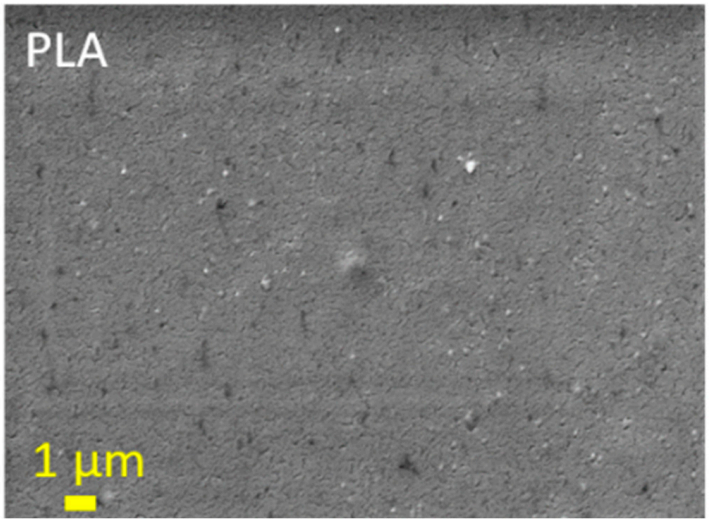

Figure 7. SEM image of uncoated PLA film that was used as reference.

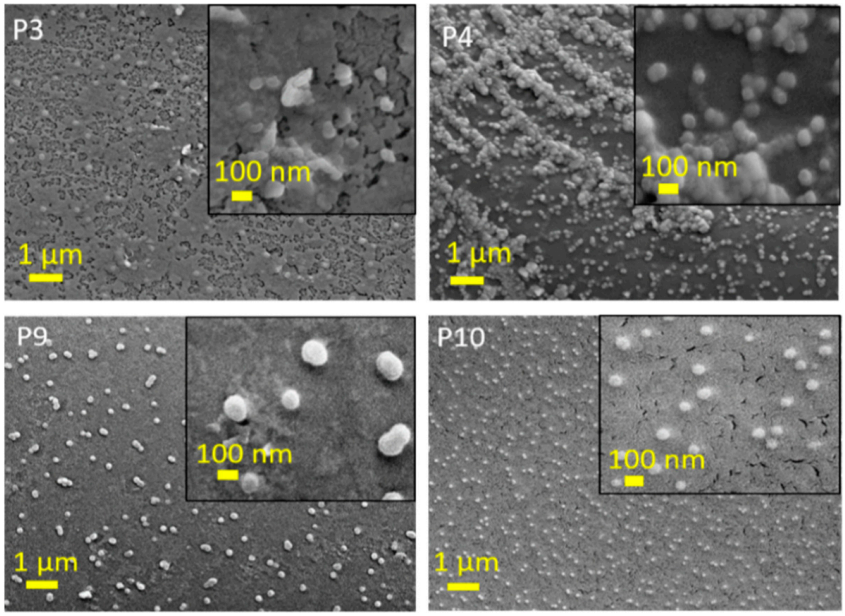

Figure 8. SEM images for PLA films layered first with 2% CH or QCH, followed by second layer in form of micro/nanoparticles of CH or QCH with embedded *P. pavonica* extract.

The results of the analyses of SEM are shown in Figures 7 and 8. To further investigate the morphology of the PLA films after bilayer application, SEM was used to visualize the coverage of the films. The original PLA film shows a typical flat surface with some impurities, possibly due to contamination, as also shown by XPS analysis (Si). The layered samples show a change in morphology. Samples P3 and P4, containing a first layer of primary chitosan and a top layer with CH and QCHPs with embedded PPAV extract, show that nanoparticles are embedded in the applied first layer or particles forming dendrite assemblies are clearly visible, with the size of the particles also below 100 nm. In samples P9 and P10, where the first layer consists of 2% QCH, similar morphologies are observed as in the samples with the first layer composed of 2% CH. Nevertheless, some differences can be seen due to the presence of fewer agglomerates and uniformly distributed particles.

3.4. Antioxidant Potential of Chitosan/PLA Bilayer Films

The antioxidant activity of the developed films was investigated using the ABTS method to determine how the layers on the surface of the PLA films affect the inhibition of the oxidation process that commonly occurs during food storage. The ABTS inhibition results of the PLA film samples after 0, 15, and 60 min are shown in Figure 9.

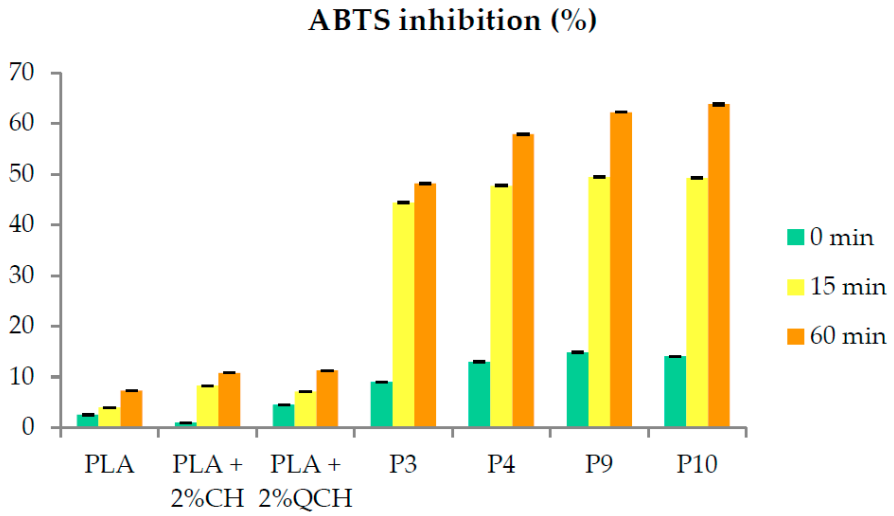

Figure 9. ABTS inhibition after 0, 15, and 60 min for all films.

The results show that the lowest antioxidant activity was obtained for the PLA films and the PLA films layered with chitosans alone. Chitosans alone as layers on the films did not show good antioxidant activity. It can be seen that, in both chitosans, the first layers generally showed antioxidant activity below 10%. For the two-layer PLA films based on chitosans and *P. pavonica* extract, the inhibition immediately after addition of the ABTS solution was low and ranged from 8.99 to 14.84%. After 15 min, inhibition was generally 47% and was highest for samples P9 and P10, where it reached 49.5%. However, after 60 min, the inhibition for the film samples with *P. pavonica* extract was 63% for P9 and P10. The lowest inhibition was observed for P3, with 48%, and for P4, with almost 58%. It is evident that the antioxidant capacity of *P. pavonica* extracts was preserved in these film formulations.

There are several studies that investigated film-forming properties and application of seaweed polysaccharides for the development of edible coatings and films [38,39]. However, only few studies have investigated the effectiveness of seaweed extracts for the coatings or films development. Albertos et al. [40] developed edible chitosan films with added seaweeds (*Himanthalia elongata* and *Palmaria palmata*) and seaweed extracts. The TPC of the extracts ranged from 46.72 to 206.69 mg GAE/g sample and extracts with higher TPC exhibited higher antioxidant activity. The antioxidant activity of the formulated edible films was consistent with the antioxidant activity of the extracts. The films were then tested on rainbow trout (*Oncorhynchus mykiss*) burgers, where the authors observed a reduction in lipid oxidation and microbial growth, as well as an improvement in the antioxidant capacity of the burgers during the storage. Andrade et al. [41] developed film based on whey protein with incorporated extract of *Fucus vesiculosus*. The TPC of the extract was 45.21 ± 0.21 mg phloroglucinol equivalents/g extract and the inhibition of DPPH radical was 78.26 ± 0.21%. The developed film inhibited lipid oxidation of vacuum-packed chicken breasts for 25 days under refrigerated conditions.

4. Conclusions

The most dominant compounds of *P. pavonica* extracts, determined by HPLC-ESI-TOF-MS, were fatty acids (oleic, palmitic, and palmitoleic). In addition, ω-3 fatty acids, stearidonic and EPA, were identified. *Padina pavonica* harvested in June showed the highest TPC and

antioxidant activity. This extract was selected for the development of chitosan/PLA bilayer films. This is the first report of testing such a formulation. A macromolecular solution of chitosan (primary and quaternary chitosan) was applied as the first layer on PLA films, and a second layer consisted of a suspension of chitosan particles with entrapped PPAV extract. XPS spectra confirmed that both layers, first as chitosan macromolecular solutions and second as PPAV extract embedded into chitosan particles, were successfully deposited on the film surface, with some desorption. In addition, the hydrophilicity of the films was reduced, which is very important for ensuring food safety and quality due to the anti-fog effect. An increase in antioxidant activity in the range of 48–65% was observed for all functionalized films. The developed films exhibited anti-fogging and antioxidant properties, confirming the development of the active concept and the potential use of these films for food packaging solutions.

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8.5. Rad 5 - Seasonal changes in essential oil constituents of *Cystoseira compressa*: first report

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Seasonal Changes in Essential Oil Constituents of *Cystoseira compressa*: First Report

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Seasonal Changes in Essential Oil Constituents of *Cystoseira compressa*: First Report

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Abstract: Marine macroalgae are well known to release a wide spectrum of volatile organic components, the release of which is affected by environmental factors. This paper aimed to identify the essential oil (EO) compounds of the brown algae *Cystoseira compressa* collected in the Adriatic Sea monthly, from May until August. EOs were isolated by hydrodistillation using a Clavenger-type apparatus and analyzed by gas chromatography coupled with mass spectrometry (GC–MS). One hundred four compounds were identified in the volatile fraction of *C. compressa*, accounting for 84.37–89.43% of the total oil. Samples from May, June, and July were characterized by a high share of fatty acids (56, 69, and 34% respectively) with palmitic acid being the dominant one, while in the August sample, a high content of alcohols (mainly phytol and oleyl alcohol) was found. Changes in the other minor components, which could be important for the overall aroma and biological activities of the algal samples, have also been noted during the vegetation periods. The results of this paper contribute to studies of algal EOs and present the first report on *C. compressa* EOs.

Keywords: *Cystoseira compressa*; brown algae; essential oil; harvest period; GC–MS

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1. Introduction

More than 70% of the Earth's surface is covered with oceans and seas, so it is not surprising that marine ecosystems are extremely complex with tremendous biodiversity. Recently, there is a growing trend in the investigation of new, inexpensive, and valuable sources of biologically active compounds, and marine origin products, like algae, are one of the most interesting sources, due to their production of a great variety of unique secondary metabolites [1]. Algae are vegetative organisms widely distributed throughout the world. Although many of them are of commercial importance in some parts of the world due to their nutritional, biological, and functional properties, only a small number of species are currently exploited for industrial food applications [2]. Studies on marine algae are usually focused on the isolation of structurally different bioactive compounds like polysaccharides (e.g., fucoidan, alginate, and laminarin), photosynthetic pigments (carotenoids, chlorophylls, and phycobilins), sterols, polyphenolics, etc. [3–9]. In comparison to the research on these non-volatile compounds, studies on volatiles of marine origin are still scarce.

Essential oils (EOs), as a special chemical group of algal metabolites, play an important role in communication in marine ecosystems, both interspecies and intraspecies, as well as in interactions with the surrounding environment. These compounds are involved in various algal ecological functions: they are defenses against predators and herbivores; they

act as pheromones (allelochemicals; take part in the adaptation to abiotic stresses; and are important for the inhibition of bacterial and/or fungal fouling [1,10–12]. The essential oil metabolites present in marine algae species contain a mixture of different chemical classes such as hydrocarbons, fatty acids, esters, alcohols, carboxylic acids, aldehydes, ketones, terpenes, polyphenols, furans, pyrazines, pyridines, halogenated amines, and sulphur compounds [1,2]. The production of algal EOs is closely related to the physiology of the species [11,12]. Studies on EOs of green and red algae mainly report the presence of monoterpenoids, halogenated compounds, and sulphur compounds that have a low impact on their aroma perception. In contrast to those species, brown algae is responsible for strong and pleasant marine odors (the so-called "beach note"), which is usually related to the presence of C11-hydrocarbons. Among other aroma compounds, these species contain a wide range of monoterpenoids and sesquiterpenoids [13]. Although the functions of algal EOs are similar to those in terrestrial plants, studies dealing with algal EOs and their role are still in the primary stage, and there is a lack of reports on this subject [12]. EO profiles differ between species, but they are also influenced by various factors as age, geographical origin, growth and nutrition conditions, season, temperature, light, salinity, and processing/extraction parameters [2,12].

There are about 40 species of algae from the genera *Cystoseira* (Phaeophyta), which are widely distributed along the Eastern Atlantic and Mediterranean coasts [14], and *C. compressa*, is one of the most widespread brown algae in the Adriatic Sea. *C. compressa* is attached to the substratum by a small disc and its thallus shows morphological plasticity. Changes are most evident in the spring/summer period, when the winter rosette shape of the branches shifts to dense and ramified branches with aerocysts [15]. These changes might be related to the length of the photoperiod and sea temperature, and their effect on the EOs or other chemical components of the algae (phenolic profile, pigments, etc.) is unknown.

Compounds from *C. compressa* were characterized from extracts and associated with various biological activities, e.g., polysaccharides and phlorotannins with antioxidant activity [16,17], phlorotannins with antidiabetic activity [17], and phenolic compounds with antibacterial activity [5]. Furthermore, a connection between total phenolic content and the seawater temperature was observed, showing that the amount of phenolics is influenced by the temperature [18]. However, characterization of EO components has been done for *C. sedoides* [13], *C. barbata* [19,20], *C. crinita* [19], and *C. tamariscifolia* [21], but to our knowledge, there are no reports on compounds of *C. compressa* and their comparison over the spring/summer period, when the algae are under the influence of the thallus change, a rise in sea temperature, and an intensive photoperiod. For these reasons, this work aimed to study the EO profiles of *C. compressa*, collected in the Adriatic Sea monthly from May until August, to identify the molecules characterizing this species.

2. Results and Discussion

Seaweeds are widespread around the world, being of commercial importance in some parts, where they are consumed fresh, dry, or as an ingredient. Although in some regions they are widely used in the human diet, only a small number of species are currently exploited for food applications. One of the main limitations of the use of algal materials in the food industry is their flavor, which is the main parameter of quality directly related to consumers' acceptance of food [2]. In comparison to the terrestrial odoriferous plants, only some algae possess an attractive, pleasant odor and characteristic marine flavor, and, therefore, great potential to be used in various food and cosmetic preparations [1,13].

Different extraction methods like hydrodistillation, solvent extraction, microwave-assisted extraction, supercritical fluid extraction, headspace extraction, etc., are commonly used for the isolation of volatile analytes from algal materials. In recent times, the conventional extraction procedures are usually being replaced by novel techniques that are less time-consuming, often (fully) automated, more environmentally friendly, require less solvent, and are more efficient [8]. However, despite all its disadvantages (duration, high

temperatures, low efficiency, potential degradation of compounds, etc.), hydrodistillation is still the most used method. On the other hand, identification of the EO components is usually performed using capillary gas chromatography coupled with mass spectrometry (GC–MS), as this method of characterization covers a wide spectrum of compounds, from non-polar to polar ones [11,13].

The chemical profile of volatile fractions and the relative content of detected components obtained by hydrodistillation of *C. compressa* are reported in Table 1. One hundred four compounds were identified, accounting for 84–89% of the total chemical composition. Figure 1 presents the relative share of the sum of compounds from the same chemical class to get better insight into the algal EOs profile. The GC–MS chromatograms of the essential oils obtained from *C. compressa* collected in different months are shown in Figure 2.

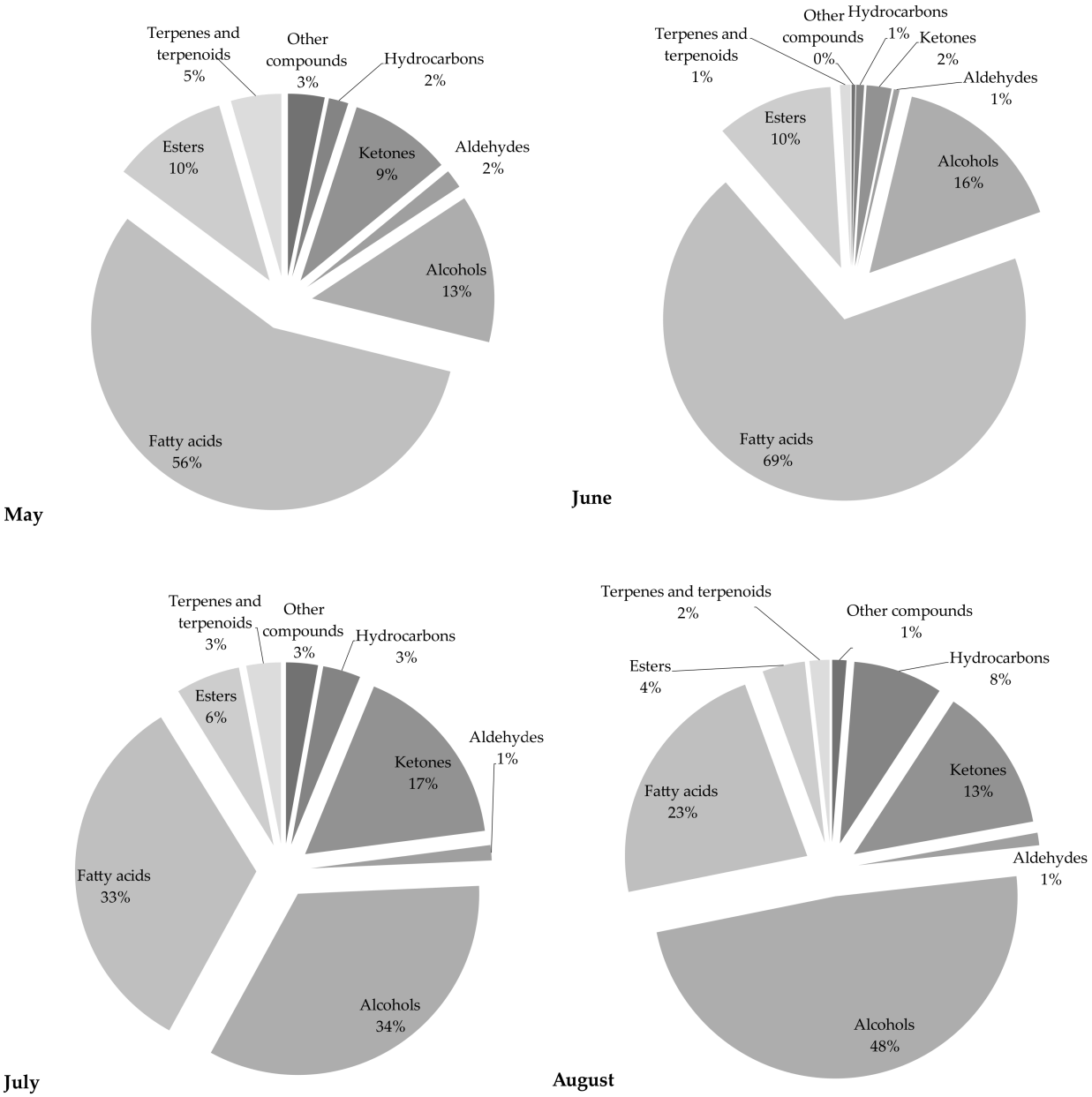

Figure 1. The relative content of each chemical class of compounds detected in *C. compressa* samples.

Table 1. Differences in the chemical composition of essential oils of *C. compressa* harvested in different periods. The data are expressed as relative percentages of each single peak area with respect to the total peak area.

Compound							*C. compressa* Samples			
No.	**Rt (min)**	**Name**	**Similarity (%)**	**MW**	**Formula**	**CAS**	**May**	**June**	**July**	**August**
1	7.10	Pent-1-en-3-one	95	84.12	C_5H_8O	1629-58-9	0.07	n.d.	n.d.	n.d.
2	**7.55**	**Tetrahydro-2,5-dimethyl-furan**	**97**	**100.15**	**$C_6H_{12}O$**	**1003-38-9**	**0.88**	0.06	0.35	0.16
3	10.24	Tetrahydro-2-furanmethanol	92	102.13	$C_5H_{10}O_2$	97-99-4	0.06	n.d.	0.02	0.01
4	10.85	Hex-5-enal	93	98.14	$C_6H_{10}O$	764-59-0	n.d.	0.01	0.01	0.01
5	11.07	Hexan-2-one	93	100.16	$C_6H_{12}O$	591-78-6	0.05	n.d.	0.03	0.01
6	11.33	Hexan-3-ol	90	102.17	$C_6H_{14}O$	623-37-0	0.07	n.d.	0.03	0.03
7	11.48	Hexanal	95	100.16	$C_6H_{12}O$	66-25-1	0.10	0.04	0.07	0.07
8	14.16	(*E*)-Hex-2-enal	91	98.14	$C_6H_{10}O$	6728-26-3	0.04	0.01	0.04	0.04
9	16.75	Heptanal	92	114.19	$C_7H_{14}O$	111-71-7	0.04	0.01	0.01	0.01
10	19.81	1-methyl-pentyl hydroperoxide	88	118.17	$C_6H_{14}O_2$	24254-55-5	0.11	0.01	0.05	0.02
11	20.97	Oct-1-en-3-one	90	126.19	$C_8H_{14}O$	4312-99-6	0.03	0.02	0.05	0.01
12	24.17	Propylcyclohexane	82	126.24	C_9H_{18}	696-29-7	0.01	n.d.	0.02	n.d.
13	25.31	(*E*)-Oct-2-enal	90	126.20	$C_8H_{14}O$	2548-87-0	n.d.	0.01	n.d.	n.d.
14	25.79	1-Phenylethanone	95	120.15	C_8H_8O	98-86-2	n.d.	0.01	n.d.	n.d.
15	26.05	Octan-1-ol	94	130.23	$C_8H_{18}O$	111-87-5	n.d.	n.d.	n.d.	0.03
16	27.30	Non-1-en-4-ol	85	142.24	$C_9H_{18}O$	35192-73-5	n.d.	n.d.	0.03	0.01
17	27.58	Linalool	90	154.25	$C_{10}H_{18}O$	78-70-6	n.d.	n.d.	n.d.	0.01
18	27.78	Nonanal	92	142.24	$C_9H_{18}O$	124-19-6	0.03	n.d.	0.02	0.02
19	29.85	2,6,6-trimethyl-2-cyclohexene-1,4-dione	92	152.19	$C_9H_{12}O_2$	1125-21-9	n.d.	n.d.	0.03	0.02
20	32.95	Decanal	91	156.26	$C_{10}H_{20}O$	112-31-2	n.d.	n.d.	0.02	0.01
21	33.64	2,6,6-trimethyl-1-cyclohexene-1-carboxaldehyde	83	152.23	$C_{10}H_{16}O$	4884-24-6	0.02	n.d.	n.d.	n.d.
22	35.67	2-(2,6,6-trimethylcyclohexen-1-yl) acetaldehyde	89	166.26	$C_{11}H_{18}O$	472-66-2	n.d.	n.d.	0.02	n.d.
23	35.80	Ethyl 5-methyl-3,4-dihydro-2H-pyran-4-carboxylate	83	170.21	$C_9H_{14}O_3$	38858-64-9	n.d.	n.d.	0.05	n.d.
24	35.92	3,5,5-trimethyl-hex-1-ene	86	126.24	C_9H_{18}	4316-65-8	n.d.	0.02	0.29	0.03
25	36.18	Decan-1-ol	93	158.28	$C_{10}H_{22}O$	112-30-1	n.d.	0.01	0.09	0.36
26	36.58	Dec-1-en-3-one	92	154.22	$C_{10}H_{18}O$	56606-79-2	0.42	0.02	0.10	0.17
27	37.17	Undecan-2-one	95	170.29	$C_{11}H_{22}O$	112-12-9	n.d.	n.d.	0.14	0.02
28	37.80	Undecanal	96	170.29	$C_{11}H_{22}O$	112-44-7	n.d.	n.d.	n.d.	0.09
29	38.26	(2*E*,4*E*)-deca-2,4-dienal	94	152.23	$C_{10}H_{16}O$	25152-84-5	n.d.	n.d.	0.05	0.02
30	**38.37**	**Ethyl cyclohexanecarboxylate**	**87**	**156.22**	**$C_9H_{16}O_2$**	**3289-28-9**	0.25	n.d.	**0.54**	0.07
31	38.76	Bicyclo(3.3.1)nonane-2,6-dione	83	152.19	$C_9H_{12}O_2$	16473-11-3	0.17	n.d.	0.35	0.08
32	**39.00**	**(*R*)-5,7-dimethyl-1,6-octadiene (Isocitronellene)**	**87**	**138.25**	**$C_{10}H_{18}$**	**85006-04-8**	**0.88**	n.d.	0.04	0.03
33	39.42	Hexacosan-1-ol	81	382.7	$C_{26}H_{54}O$	506-52-5	n.d.	n.d.	0.30	n.d.
34	**40.20**	Eugenol	95	164.20	$C_{10}H_{12}O_2$	97-53-0	0.19	n.d.	n.d.	n.d.
35	41.98	Nona-3,5-dien-2-one	84	138.21	$C_9H_{14}O$	80387-31-1	0.11	n.d.	0.45	0.16

Table 1. *Cont.*

Compound							*C. compressa* Samples			
No.	**Rt (min)**	**Name**	**Similarity (%)**	**MW**	**Formula**	**CAS**	**May**	**June**	**July**	**August**
36	42.18	6,10-dimethylundecan-2-one	95	198.34	$C_{12}H_{26}O$	1604-34-8	0.05	n.d.	0.32	0.09
37	**44.29**	**Geranylacetone**	**84**	**194.31**	**$C_{13}H_{22}O$**	**689-67-8**	0.14	n.d.	**0.70**	n.d.
38	42.37	Dodecanal	92	184.32	$C_{12}H_{24}O$	112-54-9	n.d.	n.d.	n.d.	0.05
39	43.40	(*E*)-4-[(1*R*,5*S*)-2,5,6,6-tetramethylcyclohex-2-en-1-yl]but-3-en-2-one	85	206.32	$C_{14}H_{22}O$	79-69-6	n.d.	n.d.	n.d.	0.15
40	44.08	3,7,11-trimethyldodecan-1-ol (hexahydrofarnesol)	92	228.41	$C_{15}H_{32}O$	6750-34-1	n.d.	0.14	n.d.	0.24
41	44.29	(*Z*)-6,10-dimethyl-5,9-undecadien-2-one	93	194.31	$C_{13}H_{22}O$	3879-26-3	n.d.	n.d.	n.d.	0.17
42	44.73	3,7,11-trimethyl-dodecan-1-ol	83	228.31	$C_{15}H_{32}O$	6750-34-1	0.32	0.07	0.18	0.29
43	45.08	(*Z*)-dec-3-enyl acetate	89	198.30	$C_{12}H_{22}O_2$	81634-99-3	n.d.	n.d.	0.18	n.d.
44	**45.17**	**Dodecan-1-ol**	**94**	**186.33**	**$C_{12}H_{26}O$**	112-53-8	**1.06**	**1.02**	**1.03**	**3.65**
45	**45.91**	**(*E*)-4-(2,6,6-trimethyl-1-cyclohexen-1-yl)-3-buten-2-one**	**85**	**192.30**	**$C_{13}H_{20}O$**	14901-07-6	**2.76**	**0.53**	**5.41**	**4.34**
46	**46.07**	**Tridecan-2-one**	88	198.34	$C_{13}H_{26}O$	593-08-8	0.12	0.36	**0.67**	0.31
47	**46.22**	**Hexadecane**	**96**	**226.44**	**$C_{16}H_{34}$**	544-76-3	**0.58**	0.08	**0.84**	**1.30**
48	**46.34**	**Dimethyl 1,4-benzenedicarboxylate**	**88**	**194.18**	**$C_{10}H_{10}O_4$**	120-61-6	n.d.	0.06	0.19	**1.17**
49	46.89	*n*-pentadecan-1-ol	94	228.41	$C_{15}H_{32}O$	629-76-5	n.d.	n.d.	0.02	0.23
50	47.33	(*E*)-Octadec-5-ene	91	252.47	$C_{18}H_{36}$	7206-21-5	n.d.	0.09	n.d.	n.d.
51	47.86	5,6,7,7a-Tetrahydro-4,4,7a-trimethyl-2(4H)-benzofuranone	90	180.24	$C_{11}H_{16}O_2$	15356-74-8	0.27	n.d.	0.45	0.28
52	**48.31**	**(*Z*)-dodec-5-enoic acid**	95	198.30	$C_{11}H_{22}O_2$	2430-94-6	n.d.	**2.79**	n.d.	n.d.
53	48.92	(*E*)-3,7,11-Trimethyl-1,6,10-dodecatrien-3-ol	89	222.36	$C_{15}H_{26}O$	40716-66-3	n.d.	n.d.	0.08	n.d.
54	**49.12**	**Dodecanoic acid (lauric acid)**	97	200.31	$C_{12}H_{24}O_2$	143-07-7	n.d.	**3.78**	0.22	0.08
55	49.31	Tetradecan-1-ol	84	214.38	$C_{14}H_{30}O$	112-72-1	n.d.	0.26	0.37	0.31
56	49.90	2-O-(4-methylpentyl) 1-O-octadecyl oxalate	84	426.70	$C_{26}H_{50}O_4$	29590-28-1	0.04	n.d.	0.11	0.05
57	50.16	Heptadecane	92	240.46	$C_{17}H_{36}$	629-78-7	0.08	n.d.	0.05	0.05
58	50.62	Tetradecanal	94	212.37	$C_{14}H_{28}O$	124-25-4	0.14	n.d.	0.08	0.20
59	51.26	2-methylhexadecan-1-ol	90	256.46	$C_{17}H_{36}O$	2490-48-4	n.d.	0.35	0.18	0.39
60	51.42	Diphenylmethanone	95	182.21	$C_{13}H_{10}O$	119-61-9	0.34	0.29	0.39	n.d.
61	52.16	2-ethyldodecan-1-ol	88	214.38	$C_{14}H_{30}O$	19780-33-7	0.24	n.d.	0.23	0.18
62	**52.30**	**alpha-Cadinol**	**87**	**222.36**	**$C_{15}H_{26}O$**	**481-34-5**	**1.24**	0.12	n.d.	n.d.
63	**52.65**	**Hexadecan-1-ol**	**92**	**242.44**	**$C_{16}H_{34}O$**	**36653-82-4**	**1.56**	**2.60**	**7.20**	**7.99**
64	52.91	(*E*)-heptadec-8-ene	77	238.45	$C_{17}H_{34}$	54290-12-9	n.d.	n.d.	0.11	0.09
65	**53.03**	**Pentadec-1-ene**	92	210.40	$C_{15}H_{30}$	13360-61-7	n.d.	n.d.	0.14	**2.62**
66	**53.22**	**11-pentan-3-ylhenicosane**	**89**	**366.70**	**$C_{26}H_{54}$**	**55282-11-6**	**0.65**	0.43	**1.11**	**1.41**
67	53.38	2-tetradecoxyethanol	81	258.44	$C_{16}H_{34}O_2$	2136-70-1	0.24	0.32	0.44	0.37
68	**53.64**	**Tridecanal**	**93**	**198.34**	**$C_{13}H_{26}O$**	**10486-19-8**	**0.60**	0.37	**0.81**	**0.43**
69	53.72	2-hexadecoxyethanol	87	286.49	$C_{18}H_{38}O_2$	2136-71-2	0.25	n.d.	n.d.	n.d.
70	**54.12**	**2-octadecoxyethanol**	**90**	**314.54**	**$C_{20}H_{42}O_2$**	**2136-72-3**	n.d.	0.42	0.41	**0.50**
71	54.30	*n*-Nonadecan-1-ol	88	284.52	$C_{19}H_{40}O$	1454-84-8	n.d.	0.11	n.d.	n.d.
72	54.52	(*E*)-icos-3-ene	86	280.53	$C_{20}H_{40}$	74685-33-9	n.d.	0.14	n.d.	n.d.

Table 1. *Cont.*

Compound							*C. compressa* Samples			
No.	**Rt (min)**	**Name**	**Similarity (%)**	**MW**	**Formula**	**CAS**	**May**	**June**	**July**	**August**
73	**54.99**	**Myristic acid**	**97**	**228.37**	**$C_{14}H_{28}O_2$**	**544-63-8**	n.d.	**7.60**	0.31	0.18
74	**55.22**	***n*-Pentadecanol**	**93**	**228.41**	**$C_{15}H_{32}O$**	**629-76-5**	0.46	**0.85**	**0.72**	**1.19**
75	**55.63**	**Eicosanoic acid**	**87**	**312.53**	**$C_{20}H_{40}O_2$**	**506-30-9**	**2.58**	**0.51**	**1.14**	**n.d.**
76	55.69	(Z)-Undec-4-enal	87	168.27	$C_{11}H_{20}O$	68820-32-6	0.36	n.d.	n.d.	n.d.
77	56.17	Isopropyl myristate	93	270.45	$C_{17}H_{34}O_2$	110-27-0	0.30	n.d.	n.d.	n.d.
78	**56.63**	**6,10,14-Trimethyl-2-pentadecanone**	**96**	**268.5**	**$C_{18}H_{36}O$**	**502-69-2**	**2.99**	**0.75**	**5.98**	**5.72**
79	**56.89**	**2,3-diisopropyl-naphthalene**	**77**	**212.33**	**$C_{16}H_{20}$**	**94133-81-0**	**1.47**	0.36	**0.55**	0.42
80	**56.98**	**Oleyl alcohol**	93	268.5	$C_{18}H_{36}O$	143-28-2	n.d.	**0.68**	**4.41**	**5.96**
81	**57.18**	**bis(2-methylpropyl) benzene-1,2-dicarboxylate**	**97**	**278.34**	**$C_{16}H_{22}O_4$**	**84-69-5**	**2.11**	**0.51**	**1.11**	n.d.
82	**57.52**	**Nonadec-1-ene**	**93**	**266.50**	**$C_{19}H_{38}$**	**18435-45-5**	n.d.	n.d.	0.20	**1.30**
83	**58.11**	**Farnesyl acetone**	**90**	**266.43**	**$C_{18}H_{30}O$**	**1117-52-8**	**0.93**	**0.57**	**1.28**	**1.05**
84	**58.62**	**Palmitoleic acid**	**95**	**254.40**	**$C_{16}H_{30}O_2$**	**373-49-9**	**2.79**	**11.94**	n.d.	n.d.
85	**58.90**	**Palmitic acid**	**93**	**256.42**	**$C_{16}H_{32}O_2$**	**57-10-3**	**40.15**	**31.92**	**26.81**	**18.62**
86	**60.14**	**Arachidonic acid**	**90**	**304.46**	**$C_{20}H_{32}O_2$**	**506-32-1**	**0.96**	**1.45**	0.14	0.27
87	**60.30**	**Methyl Arachidonate**	**83**	**318.49**	**$C_{21}H_{34}O_2$**	**2566-89-4**	**4.00**	**2.49**	**1.55**	**1.74**
88	**60.81**	***n*-Nonadecan-1-ol**	**89**	**284.52**	**$C_{19}H_{40}O$**	**1454-84-8**	**1.67**	**3.13**	**4.13**	**4.34**
89	**60.84**	**(*Z*,*Z*)-2,13-Octadecadien-1-ol**	85	266.46	$C_{18}H_{34}$	123551-47-3	0.21	n.d.	**1.67**	n.d.
90	**61.21**	**5-dodecyloxolan-2-one**	**90**	**254.41**	**$C_{16}H_{30}O_2$**	**730-46-1**	**1.44**	0.21	**1.94**	**0.88**
91	**61.29**	**Phytol**	**96**	**296.53**	**$C_{20}H_{40}O$**	**150-86-7**	**3.97**	**2.90**	**5.76**	**14.20**
92	**61.69**	**Oleic acid**	91	282.46	$C_{18}H_{34}O_2$	112-80-1	0.23	**1.63**	0.12	0.29
93	61.81	Heptadecyl heptadecanoate	81	508.92	$C_{34}H_{68}O_2$	n.a.	0.22	n.d.	0.12	0.40
94	61.99	Ascorbyl palmitate	70	414.53	$C_{22}H_{38}O_7$	137-66-6	0.32	n.d.	n.d.	0.12
95	62.39	Octadecyl propan-2-yl sulphite	82	376.60	$C_{21}H_{44}O_3S$	n.a.	0.22	n.d.	n.d.	0.19
96	**62.58**	**Tetradecyl benzoate**	**78**	**318.50**	**$C_{21}H_{34}O_2$**	**70682-72-3**	**1.10**	**4.37**	**0.53**	0.09
97	**63.04**	**Pentadecyl benzoate**	80	332.50	$C_{22}H_{36}O_2$	n.a.	0.07	**0.50**	0.10	0.04
98	**63.98**	**Tridecyl benzoate**	**88**	304.50	$C_{20}H_{32}O_2$	29376-83-8	0.16	**1.28**	0.40	0.05
99	**64.22**	**2-(Octadecyloxy)ethanol**	**91**	**314.55**	**$C_{20}H_{42}O_2$**	**9005-00-9**	**0.28**	**1.04**	**0.76**	**1.46**
100	64.39	Stearic acid	80	284.48	$C_{18}H_{36}O_2$	57-11-4	0.36	0.09	n.d.	n.d.
101	64.96	1-Decylsulfonyldecane	79	346.61	$C_{20}H_{42}O_2S$	111530-37-1	0.13	n.d.	n.d.	0.14
102	66.14	Icosane	94	282.55	$C_{20}H_{42}$	112-95-8	0.16	n.d.	0.30	0.11
103	67.28	Nonacosane	93	408.79	$C_{29}H_{60}$	630-03-5	0.34	0.05	0.09	0.14
104	70.37	Squalene	90	410.73	$C_{30}H_{50}$	111-02-4	0.18	0.04	0.20	0.07
TOTAL IDENTIFIED COMPOUNDS (from peak total area)							**84.37**	**89.43**	**85.44**	**87.41**

Compounds detected in amounts higher than 0.5% are written in bold; n.d.—not detected; n.a.—not available.

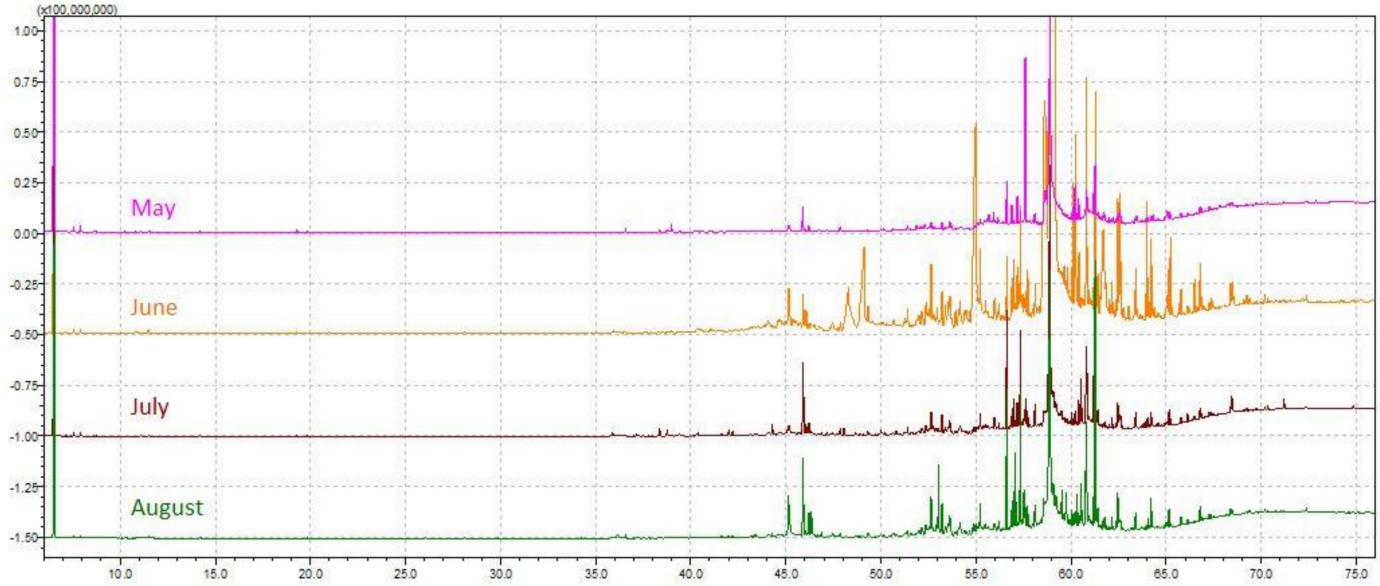

Figure 2. GC–MS chromatograms of the essential oils obtained from *Cystoseira compressa* collected from May to August.

Samples from May and June were characterized by a high share of fatty acids, while in the July and August samples the dominant chemical class of compounds were alcohols (34 and 48%, respectively). EOs from May and June were characterized by an extremely high content of fatty acids, 56 and 69%, respectively, while almost two-fold lower results were obtained for the July extract. The major acid in all samples was palmitic acid (C16:0), with the highest amount found in the May extract (40.15%), and shares of 31.92%, 26.81%, and 18.62%, in the June, July, and August samples, respectively. It is interesting to note that this saturated fatty acid was present in high amounts in all samples and followed a regular trend characterized by a continued decrease in content during the collecting months. In comparison to the May samples, there was a more than two-fold lower amount detected in the August samples. This compound was also previously reported as an abundant fatty acid in different *Cystoseira* species [3,4,8,14,21,22]. The May extract also contained the highest share of eicosanoic acid (2.58%). Significant amounts of this acid were also found in June (0.51%) and July (1.14%), while it was not detected in the August sample. The content of all other fatty acids was the highest in the June fraction: palmitoleic acid (11.94%) > myristic acid (7.60%) > lauric acid (3.78%) > (*Z*)-dodec-5-enoic acid (2.79%) > oleic acid (1.64%) > arachidonic acid (1.45%) > stearic acid (0.36%). It is well known that fatty acids with >12 carbon atoms are odorless, so although present in high amounts they do not affect significantly the flavor of the samples [2].

Among monounsaturated fatty acids, the presence of (*Z*)-5-dodecenoic acid was confirmed only in the June sample, where the content of oleic acid was also the highest in comparison with the others. Arachidonic acid was the only detected polyunsaturated acid, with the highest amount again found in the June sample, but significant amounts were also detected in May (0.96%). Cvitković et al. [8] reported the domination of total unsaturated fatty acids in the lipid fraction of different Adriatic brown algae species and two *Cystoseira* species, *C. barbata* and *C. compressa*. These authors also reported the domination of oleic acid among unsaturated fatty acids, as well as the presence of arachidonic acid in high amounts in brown algae samples. Similar results were also reported by Oucif et al. [4]. Kord et al. [22] also identified fatty acids (14 to 20 carbon atoms) of which palmitic acid was the major compound in *C. sauvageauana* lipid fractions, while among polyunsaturated fatty acids, arachidonic acid was the found in highest concentration.

Compounds from the chemical class of hydrocarbons, alkanes, and alkenes are common compounds in the majority of marine macroalgae EOs [1]. Although unsaturated hydrocarbons from C8 to C19 with the presence of 1 to 4 degrees of unsaturation are

common, our study mainly reported the presence of compounds with one double bond. From the class of hydrocarbons, the straight chain saturated hydrocarbon 11-pentan-3-ylhenicosane was found in high amounts (from 0.43% to 1.41%), as well as hexadecane (from 0.08% to 1.30%). Both of these compounds followed similar trends, with the lowest concentrations found in the July sample, while their content significantly increased in next two collecting months, with the highest concentration in August. Also, pentadec-1-en was found in July (0.14%) and in even higher amounts in August (2.62%), while in the first two collection months this compound was not detected. The presence of squalene, which is the biosynthetic precursor of triterpenes and steroids, was confirmed in all samples, with the highest amounts detected in July.

Previous studies on volatile components from *Cystoseira* species confirmed the domination of hydrocarbons in *C. barbata*, while this class of compounds was found only in traces in *C. crinite*, where the majority of compounds were monoterpenoids [19]. The domination of hydrocarbons in the volatile oil of *C. barbata* was also reported by Ozdemir et al. [20], while Bouzidi et al. [13] reported that the most important class of VOCs obtained by hydrodistillation in *C. sedoides* were fatty acids and derivatives, with a content of 53.1%. Gressler et al. [11] reported the identification of hexadecane in different algae, among which were two *Cystoseira* species: *C. barbata* and *C. mediterranea*. Furthermore, heneicosan was also detected in *C. barbata* [20]. In their study, Bouzidi et al. [13] confirmed the presence of hexadecane and pentadec-1-en in samples of the Algerian endemic algae *C. sedoides*. It is interesting to note that these compounds were found in samples obtained by hydrodistillation, while they were not present in fractions obtained by focused microwave hydrodistillation and supercritical fluid extraction, which could be confirmation that aggressive isolation conditions (e.g. high temperature, long extraction duration, oxidation, and contact with water) cause the degradation of volatiles.

Samples from July and August contained high percentages of alcohols, 34% and 48%, respectively. Phytol, an acyclic diterpene alcohol, also known as a precursor of vitamin E and a degradation product of chlorophyll, was found in all samples at the highest percentage, especially in the August sample, where its content was 14.20% of all detected compounds [1]. This compound was detected in the lowest concentration in the June sample (2.9%), but in the next two months its content was almost 2 and 5-fold greater. El Amrani Zerrifi et al. [21] confirmed the domination of phytol in *C. tamariscifolia* from their study, as well as Bouzidi et al. [13] in *C. sedoides*. Other dominant components from the chemical class of alcohols were oleyl alcohol and *n*-nonadecan-1-ol, for which the regular amount increase during the collecting months was recorded. The presence of oleyl alcohol in the May sample was not confirmed, while its content in June was 0.68%, in July 5.76%, and in August almost 6%. On the other hand, the share of *n*-nonadecan-1-ol was 1.67% in May, 3.13% in June, 4.13% in July, and 4.34% in August, and an increase in its concentration during the collection periods could be noted. The great impact of unsaturated alcohols on the overall aroma and sensory perception of food has been previously reported [2].

The share of ketones was 9% in the May sample, 13% in the August sample and 17% in the July sample, while the lowest amount was found in the June sample (only 2%). Among detected compounds, (*E*)-4-(2,6,6-trimethyl-1-cyclohexen-1-yl)-3-buten-2-one (ranging from 0.53% to 5.41%) and 6,10,14-trimethyl-pentadecan-2-one (ranging from 0.75% to 5.98%), were found in the highest amounts. It is interesting to note that the amounts and the variations in their content among samples for both compounds followed the same trend: July > August (5.72% and 5.41%, respectively) > May (2.76% and 2.58%, respectively) > June. Bouzidi et al. [13] also reported the identification of 6,10,14-trimethyl-pentadecan-2-one in *C. sedoides.* Among other detected ketones, significant amounts of tridecan-2-one and dec-1-en-3-one were found. The first component was detected in the highest amount in the July sample (0.67%), while the other one was found in the May sample (0.42%). The July sample was also rich in monoterpene ketone geranyl acetone (0.70%).

Among all detected compounds, aldehydes, which are important odor compounds, were detected in the lowest percentages in all samples (1–2%), with only a few compounds

present at a percentage above 0.10%. Aldehydes with low molecular weight are associated with unpleasant aroma, while those with higher molecular weight are responsible for sweet and fruity notes [2]. Tridecanal was dominant in all samples ranging from 0.37% in June to 0.81% in July. Tetradecanal was found in the highest amount in August (0.20%), while its presence in June was not confirmed. On the other hand, (*Z*)-undec-4-enal was found in the May sample at a percentage of 0.36%, while in other samples it was not detected.

The share of esters in the first two collecting months was equal (10%), while in July and August it was significantly lower, at 6% and 4%, respectively. The dominant ester was methyl arachidonate, with the highest amount found in the May sample (4%). Its content was significantly lower in June (2.49%), July (1.55%), and August (1.74%). Other benzoic acid esters were also found in high amounts in all samples, especially tetradecyl ester in the June sample (4.37%). The highest content of other esters, namely pentadecyl and tridecyl benzoate, were detected in samples harvested in June. Finally, it is interesting to note that all these compounds—tri, tetra, and penta-decyl esters—showed a similar trend across the collecting months: June > July > May > August.

Terpenes are a class of compounds that play an important role as chemical defense agents, but are also involved in some metabolic processes and functions, like the stability of cell membranes and photosynthesis [1]. It has been reported that terpenes are responsible for the distinctive ocean smell of algae, particularly acyclic and cyclic non-isoprenoid C11-hydrocarbons, while the disagreeable odor is related to amines and halogenated, sulphurous, and other specific compounds [1]. However, for the detection of polycyclic aromatic hydrocarbons, substituted phenols, and sulphur compounds, liquid chromatography is required, as they are semi-volatile [11]. From the group of terpenes, a terpene ketone farnesyl acetone (6,10,14-trimethylpentadeca-5,9,13-trien-2-one) was found in the highest amount in all samples (from 0.57% in June to 1.28% in July). The joint FAO/WHO Expert Committee on Food Additives put this compound on its list of flavoring agents, as it is characterized by an intensely sweet and floral odor, which makes it interesting for further applications [23]. Among others, alpha-cadinol was dominant in the May sample at 1.24%; its content was significantly lower in June, while in samples from other to collecting months it was not detected. Bouzidi et al. [13] also reported the presence of this compound in their study, though again, only in samples prepared by hydrodistillation.

Previous studies on *Cystoseira* species confirmed the potential health benefits of algae extracts and present individual compounds. Bruno de Sousa et al. (2017) in their review paper reported various biological activities of the *Cystoseira* algae samples, among which properties like antioxidant, antimicrobial (antibacterial, antifungal, antiviral), cytotoxic, antiproliferative, anticancer, antifouling, anti-inflammatory, antileishmanial, cholinesterase inhibitory, anti-diabetic, anti-obesity, hepatoprotective, etc. were confirmed by different studies.

Among recent studies, Hentati et al. [16] detected good antioxidant activity of water-soluble polysaccharides (fucoidan and a sodium alginate), while antidiabetic and antioxidant activity of phlorotannins extracted from *C. compressa* were reported by Gheda et al. [17]. Abu-Khudir et al. [24] investigated and confirmed the good free radical scavenging activity of the *C. crinita* extracts, antimicrobial activity against various pathogenic microorganisms, and strong cytotoxic effects against a panel of cancer cells. The authors, using GC–MS analysis, also confirmed the presence of a vast array of medicinally valuable phytochemical compounds belonging to various classes. Ahmed et al. [25] investigated the antimicrobial and cytotoxic activity of the extract, fractions, and pure compounds from *C. trinodis*, and their results pointed out the good activity of the samples.

Although the yield of EOs obtained from algal samples is low, *C. compressa* could be an interesting subject of further analysis on algae biological activities, due to the results of previous studies and the interesting chemical profiles of isolates (EOs and extracts from our other study).

3. Materials and Methods

3.1. Algal Material

The wild-growing populations of *C. compressa* (Phaeophyceae) were collected monthly from May to August 2020 on the coast of Čiovo Island, Central Dalmatia, Croatia (43.493389° N, 16.272505° E). Samples were collected throughout a lagoon at 25 points in depth, ranging from 20 to 120 cm. During every sampling, the sea parameters (temperature in °C and salinity in Practical Salinity Unit, PSU) were measured using an YSI Pro2030 probe (YSI Inc., Yellow Springs, OH, USA) and the obtained results are shown in Figure 3. The sea temperature rose during the months of sampling, while the salinity changed under the influence of water springs (typical only in periods with sufficient rainfall, while in periods of drought the springs cease to flow). Pre-treatment of the algal material involved removal of sand, epiphytes, and other organisms from the surface by washing it with tap water. The algal materials were air-dried (for 7 days at room temperature in a shaded and aerated place) and dried algal materials were used for the isolation of the volatile organic compounds.

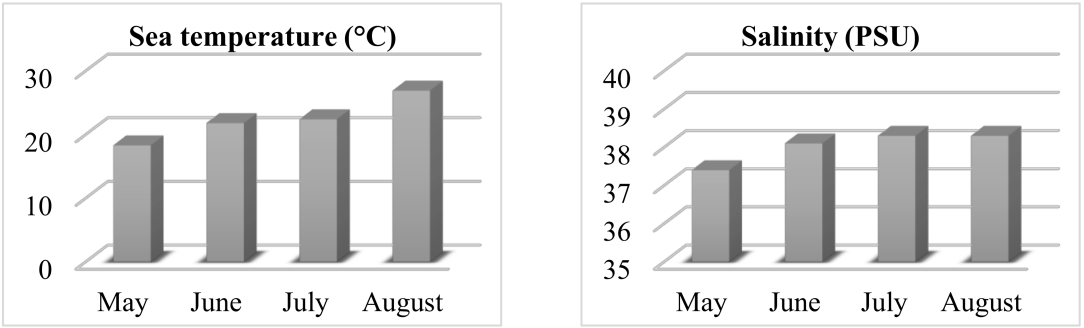

Figure 3. Sea parameters during the algal material sampling; (**a**) Temperature and (**b**) Salinity.

3.2. Extraction of Essential Oils

C. compressa essential oils were obtained by hydrodistillation of dried algal material (100 g) that was immersed in a flask with distilled water (1000 mL). The extraction process was performed in a Clavenger apparatus (Deotto Lab, Zagreb, Croatia) for 3 h. Pentane and diethyl ether (1:1, *v*/*v*) in the inner tube of the apparatus were used for trapping the volatile compounds carried through the system by vapor. Finally, after hydrodistillation, the distillate was dried over anhydrous sodium sulphate while nitrogen was used to evaporate the organic solvent. The samples of essential oils were stored at +4 °C in the dark until analysis [21,26,27].

3.3. GC–MS Analysis of Volatiles

The seaweed EOs were analyzed by GC–MS (Shimadzu QP2010, Shimadzu, Kyoto, Japan) using an autosampler and a DB-5 60 m × 0.25 mm × 0.25 µm column (Agilent Technologies Italia Spa, Milano, Italy). The EOs were resuspended in hexane and 1 µL was injected in the following gas chromatographic conditions: injection temperature 260 °C, interface temperature 280 °C, ion source 220 °C, carrier gas (He) flow rate 30 cm/s, splitting ratio 1:10. The oven temperature was programmed as follows: 40 °C for 4 min, from 40 °C to 175 °C with a 3 °C/min rate of increase, from 175 °C to 300 °C with a 7 °C/min increase, then holding for 10 min. EO constituents were identified by comparing their mass spectra with those reported in literature and the NIST Mass Spectral Database (NIST 08, National Institute of Standards and Technology, Gaithersburg, MD, USA). For each sample, the volatile profile composition was expressed as the relative percentage of each single peak area with respect to the total peak area.

4. Conclusions

This paper is the first report that provides information about the influence of the harvest period on essential oil aromatic compounds in *C. compressa*, and to obtain insight into the impact of individual components on the general sensory perception of the algae. According to the results obtained, *C. compressa* could be considered as a source of novel chemical entities with great potential to be used as an ingredient in different industrial applications such as functional foods, pharmaceuticals, and/or cosmeceuticals. The increase in the content of some of the key aroma compounds during the vegetation periods has been noted, while some detected compounds are probably products of degradation or modifications caused by aggressive isolation conditions. As new extraction methods have greatly developed in the last few years and have been widely used in the field of natural compounds due to their numerous benefits in comparison to conventional ones, this scientific research is still ongoing and opens a wide spectrum of possibilities for future research.

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Sample Availability: Samples of the EOs are not available from the authors.

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2023 - 2023 - Suradnica na projektu INNODAGNJA, Sveučilište u Splitu

2020 - 2023 - Doktorandica na H2020 PRIMA projektu BioProMedFood, Sveučilište u Splitu

2018 - 2020 - Stručna suradnica za znanost/Voditeljica projekta, Sveučilište u Splitu

Područje interesa

Bioaktivni spojevi morskih algi i nusproizvoda agro-prehrambene industrije, zelene metode ekstrakcije, antioksidacijsko i antimikrobno djelovanje, produljenje roka trajanja hrane, razvoj biopakiranja za prehrambenu industriju

Sudjelovanje na projektima

Godina	Naziv	Financira
2020-2023	Bio-protective cultures and bioactive extracts as sustainable combined strategies to improve the shelf-life of perishable Mediterranean food (BioProMedFood ID. 1467) (Voditeljica projekta: Izv. prof. dr. sc. Vida Šimat)	O2020 EU PRIMA
2022-2023	Marine natural compounds: an untapped source of biomass for biotechnological applications in the human health sector (MarHealth) (Voditeljica projekta: Prof. dr. sc. Claire Helio)	SeaEU-SEARCH
2022-2023	Inovativni, ekološki pristup uzgoju dagnje na konopima od recikliranih materijala uz eDNA barkodiranje i pasterizaciju konzumnih školjki s ciljem podizanja kvalitete i vrijednosti finalnog proizvoda te zaštita okoliša – „INNODAGNJA“ (Voditeljica projekta: Izv. prof. dr. sc. Mirela Petrić)	Ministarstvo poljoprivrede, Mjera 2.1. „Inovacije“
2023-2026	Innovative sustainable solutions for ready-to-eat traditional Mediterranean products and non-conventional healthy foods (InnoSol4Med) (Voditeljica projekta: Izv. prof. dr. sc. Vida Šimat)	O2020 EU PRIMA

Popularizacija znanosti

2017 – danas - član organizacijskog odbora Festivala znanosti Split

2018 – 2019 - logistički koordinator Europske noći istraživača u Splitu 2019.

Popis radova

1. Šimat, Vida; Skroza, Danijela; **Čagalj, Martina**; Soldo, Barbara; Generalić Mekinić, Ivana. Effect of plant extracts on quality characteristics and shelf-life of cold-marinated shrimp (*Parapenaeus longirostris*, Lucas, 1846) under refrigerated storage // Food bioscience, 102673 (2023), 102673, 10 doi:10.1016/j.fbio.2023.102673
2. Režić Mužinić, Nikolina; Veršić Bratinčević, Maja; Grubić, Marina; Frleta Matas, Roberta; **Čagalj, Martina**; Visković, Tanja; Popović, Marijana. Golden Chanterelle or a Gold Mine? Metabolites from Aqueous Extracts of Golden Chanterelle (*Cantharellus cibarius*) and Their Antioxidant and Cytotoxic Activities // Molecules, 28 (2023), 5; 2110, 12 doi:10.3390/molecules28052110
3. **Čagalj, Martina**; Fras Zemljič, Lidija; Kraševac Glaser, Tjaša; Mežnar, Eva; Sterniša, Meta; Smole Možina, Sonja; Razola-Díaz, María del Carmen; Šimat, Vida. Seasonal Changes in Chemical Profile and Antioxidant Activity of *Padina pavonica* Extracts and Their Application in the Development of Bioactive Chitosan/PLA Bilayer Film // Foods, 11 (2022), 23; 3847, 18 doi:10.3390/foods11233847
4. Zemljič, Lidija Fras; Glaser, Tjaša Kraševac; Plohl, Olivija; Anžel, Ivan; Šimat, Vida; **Čagalj, Martina**; Mežnar, Eva; Malin, Valentina; Sterniša, Meta; Možina, Sonja Smole. Biomass-Derived Plant Extracts in Macromolecular Chitosan Matrices as a Green Coating

for PLA Films // Journal of Functional Biomaterials, 13 (2022), 4; 228, 19 doi:10.3390/jfb13040228

5. Barbieri, Federica; Montanari, Chiara; Šimat, Vida; Skroza, Danijela; **Čagalj, Martina**; Smole-Možina, Sonja; Bassi, Daniela; Gardini, Fausto; Tabanelli, Giulia; Effects of *Rubus fruticosus* and *Juniperus oxycedrus* derivatives on culturability and viability of *Listeria monocytogenes* // Scientific Reports, 12 (2022), 13158, 12 doi:10.1038/s41598-022-17408-4
6. **Čagalj, Martina**; Radman, Sanja; Šimat, Vida; Jerković, Igor. Detailed Chemical Prospecting of Volatile Organic Compounds Variations from Adriatic Macroalga *Halopteris scoparia* // Molecules, 27 (2022), 15; 4997, 22 doi:10.3390/molecules27154997
7. Skroza, Danijela; Šimat , Vida; Vrdoljak, Lucija; Jolić, Nina; Skelin, Anica; **Čagalj, Martina**; Frleta, Roberta; Generalić Mekinić, Ivana. Investigation of Antioxidant Synergisms and Antagonisms among Phenolic Acids in the Model Matrices Using FRAP and ORAC Methods // Antioxidants, 11 (2022), 9; 1784, 19 doi:10.3390/antiox11091784
8. Šimat, Vida; Skroza, Danijela; Tabanelli, Giulia; **Čagalj, Martina**; Pasini, Federica; Gómez-Caravaca, Ana Maria; Fernández-Fernández, Carmen; Sterniša, Meta; Smole Možina, Sonja; Ozogul, Yesim; Generalić Mekinić, Ivana. Antioxidant and Antimicrobial Activity of Hydroethanolic Leaf Extracts from Six Mediterranean Olive Cultivars // Antioxidants, 11 (2022), 9; 1656, 13 doi:10.3390/antiox11091656
9. Radman, Sanja; **Čagalj, Martina**; Šimat, Vida; Jerković, Igor. Seasonal Variability of Volatilome from *Dictyota dichotoma* // Molecules, 27 (2022), 3012, 17 doi:10.3390/molecules27093012
10. Šimat, Vida; Bhojraj Rathod, Nikheel; **Čagalj, Martina**; Hamed, Imen; Generalić Mekinić, Ivana. Astaxanthin from Crustaceans and Their Byproducts: A Bioactive Metabolite Candidate for Therapeutic Application // Marine drugs, 20 (2022), 3; 206, 22 doi:10.3390/md20030206
11. **Čagalj, Martina**; Skroza, Danijela; Carmen Razola-Díaz, Maria; Verardo, Vito; Bassi, Daniela; Frleta, Roberta; Generalić Mekinić, Ivana; Tabanelli, Giullia; Šimat, Vida. Variations in the Composition, Antioxidant and Antimicrobial Activities of *Cystoseira compressa* during Seasonal Growth. // Marine drugs, 20 (2022), 64, 14 doi:10.3390/md20010064
12. Šimat, Vida; **Čagalj, Martina**; Skroza, Danijela; Gardini, Fausto; Tabanelli, Giulia; Montanari, Chiara; Hassoun, Abdo; Özogul, Fatih. Sustainable sources for antioxidant and

antimicrobial compounds used in meat and seafood products // Advances in Food and Nutrition Research / Toldra, Fidel (ur.). Cambridge, MA, United States: Elsevier Inc., 2021. str. 1-64 doi:10.1016/bs.afnr.2021.03.001

13. **Čagalj, Martina**; Skroza, Danijela; Tabanelli, Giulia; Ozogul, Fatih; Šimat, Vida. Maximizing the Antioxidant Capacity of *Padina Pavonica* by Choosing the Right Drying and Extraction Methods. // Processes, 9 (2021), 4; 587, 16 doi:10.3390/pr9040587
14. Polat, Sevim; Trif, Monica; Rusu, Alexandru; Šimat, Vida; **Čagalj, Martina**; Alak, Gonca; Meral, Raciye; Özogul, Yesim; Polat, Abdurahman; Özogul, Fatih. Recent advances in industrial applications of seaweeds. // Critical Reviews in Food Science and Nutrition, (2021), 1-30 doi:10.1080/10408398.2021.2010646
15. Ozogul, Fatih; **Čagalj, Martina**; Šimat, Vida; Ozogul, Yesim; Tkaczewska, Joanna; Hassoun, Abdo; Kaddour, Abderrahmane Ait; Kuley, Esmeray; Rathod, Nikheel Bhojraj; Phadke, Girija Gajanan. Recent developments in valorisation of bioactive ingredients in discard/seafood processing by-products. // Trends in Food Science & Technology, 116 (2021), 559-582 doi:10.1016/j.tifs.2021.08.007
16. Generalić Mekinić, Ivana; **Čagalj, Martina**; Tabanelli, Giulia; Montanari, Chiara; Barbieri, Federica; Skroza, Danijela; Šimat, Vida. Seasonal Changes in Essential Oil Constituents of *Cystoseira compressa*: First Report. // Molecules, 26 (2021), 21; 6649, 12 doi:10.3390/molecules26216649
17. Generalić Mekinić, Ivana; Šimat, Vida; Botić, Viktorija; Crnjac, Anita; Smoljo, Marina; Soldo, Barbara; Ljubenkov, Ivica; **Čagalj, Martina**; Skroza, Danijela. Bioactive Phenolic Metabolites from Adriatic Brown Algae *Dictyota dichotoma* and *Padina pavonica* (Dictyotaceae). // Foods, 10 (2021), 6; 1187, 10 doi:10.3390/foods10061187
18. Šimat, Vida; Elabed, Nariman; Kulawik, Piotr; Ceylan, Zafer; Jamroz, Ewelina; Yazgan, Hatice; **Čagalj, Martina**; Regenstein, Joe M.; Özogul, Fatih. Recent Advances in Marine-Based Nutraceuticals and Their Health Benefits. // Marine drugs, 18 (2020), 12; 627, 40 doi:10.3390/md18120627
19. Šimat, Vida; Vlahović, Jelena; Soldo, Barbara; Generalić Mekinić, Ivana; **Čagalj, Martina**; Hamed, Imen; Skroza, Danijela. Production and characterization of crude oils from seafood processing by-products. // Food Bioscience, 33 (2020), 100484, 8 doi:10.1016/j.fbio.2019.100484
20. Generalić Mekinić, Ivana; Skroza, Danijela; Šimat, Vida; Hamed, Imen; **Čagalj, Martina**; Popović Perković, Zvjezdana. Phenolic content of brown algae (Pheophyceae)

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