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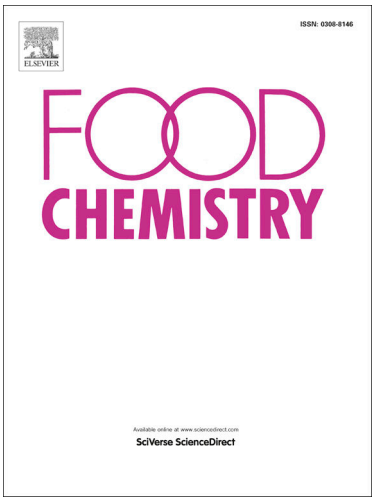

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Rapid analytical approach for bioprofiling compounds with radical scavenging and antimicrobial activities from seaweeds

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Abstract

Brown seaweeds are traditionally used as food in Asian countries, and they are a valuable source of bioactive compounds. Herein, a novel high-throughput methodological approach was developed for the tracing of compounds with radical scavenging and antimicrobial activities in *Saccharina japonica* and *Undaria pinnatifida* methanol extracts. The seaweed metabolites were separated by a novel high-performance thin-layer chromatography method, the bioactive bands were identified by bioautography assays. The bioactive compounds were characterized with ultra-high-performance liquid chromatography coupled with linear trap quadrupole tandem mass spectrometry. Stearidonic, eicosapentaenoic, and arachidonic acids were identified as major components having radical scavenging and antimicrobial activities. The suggested method provides a fast identification and quantification of bioactive compounds in multicomponent biological samples.

Keywords: Bioautography, Gas chromatography-mass spectrometry, Seaweeds, Planar chromatography, Ultra-high performance liquid chromatography-mass spectrometry

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1. Introduction

Edible seaweeds provide a rich and sustainable source of macro- and micronutrients to the human diet, particularly in regions where seaweed makes a significant contribution to regular meals, such as in China, Japan, and Korea, where approximately one-fifth of meals contain seaweed (Peng et al., 2015).

The recent global surge of interest in this type of food source and the increased global seaweed aquaculture production in the last 20 years (FAO 2016b) is fueled by the discovery of bioactive compounds in seaweed. Seaweed is a diverse source of raw material for the manufacture of food, beverages, cosmetics, fertilizers, and chemicals, and was proposed as a food source that will ensure future food security (Wells at al., 2017). Beyond the traditional considerations with respect to nutrition, seaweeds are also considered as functional foods or nutraceuticals, because they contain bioactive compounds or phytochemicals that may contribute to improved health (Wells et al., 2017).

The most studied bioactive compounds in seaweeds include polysaccharides (*e.g.*, alginate, laminarans and fucoidans, (Alves, Sousa, & Reis, 2013)), proteins (*e.g.*, phycobiliproteins), polyphenols (*e.g.*, phlorotannins and bromophenols), carotenoids (*e.g.*, fucoxanthin and astaxanthin), and n-3 long-chain polyunsaturated fatty acids (LC PUFAs) (Holdt & Kraan, 2011; Wells et al., 2017). The reported *in vitro* bioactivities of the aforementioned bioactive compounds include antibacterial, anticoagulant, antiviral, anti-tumor, anti-hyperlipidemic, anti-toxic, immunoregulatory, hepatoprotective, anti-aging, and antioxidant effects, and in addition the reduced risk of hypertensions (DHA) and cardiac heart disease (DHA) (Wells et al., 2017). To date, research is focused on brown seaweeds because they provide sufficient biomass along with various bioactive substances (Vijay, Balasundari, Jeyashakila, Velayathum, Masilan, & Reshma, 2017), and are the most common class of seaweed used by humans (Garson, 1989). Next to this seaweed species, it is expected that future research focus will be directed to many other seaweed species in an attempt to find new bioactive compounds or new species suitable for human consumption and/or industrial application. The total number of existing species worldwide (more

than 12,000) is also far higher compared to that used by humans (around 250) (Krishnamurthy Chennubhotla, Umamaheswara Rao, & Rao, 2013).

Since seaweeds are a valuable source of bioactive compounds and raw material for various industries, and in addition could contribute to future global food security either in their original form or as extracts, the development of effective and economical analytical technologies is crucial to assess their quality (Wells et al., 2017).

To date, many studies focus on pharmacological investigations of crude seaweed extracts, without clarifying the biological activities of the individual constituents or their contributions to the total biological activity (Shanmughapriya, Manilal, Sugathan, Selvin, Kiran, & Kalimuthusamy, 2008; Chakraborty, Maneesh, & Makkar, 2017). High-performance liquid chromatography–high-resolution mass spectrometry–solid-phase extraction–nuclear magnetic resonance spectroscopy (HPLC–HRMS–SPE–NMR) proved to be successful for the direct structural verification of individual α-glucosidase inhibitors in crude seaweed extracts (Liu, Kongstad, Wiese, Jäger & Staerk, 2016). Additionally, microplate-based high-resolution bioassays coupled to HPLC–HRMS–SPE–NMR were applied for the fast and simultaneous identification of bioactive compounds in a complex natural matrix (Wubshet, Moresco, Tahtah, Brighente, Ines & Staerk, 2015). However, these procedures are time- and solvent-consuming and require highly trained personnel and expensive equipment (Wubshet et al., 2015; Liu et al., 2016).

As an alternative to these sophisticated techniques, bioautography, a simple and high-throughput (HT) technique was applied for the rapid screening of biological active molecules in complex biological extracts (Agatonović-Kustrin, Morton & Ristivojević, 2016; Agatonović-Kustrin & Morton, 2017). Bioautography was also successfully applied for the screening of molecules from complex algae extracts which show radical scavenging and inhibition activity with respect to various enzymes such as amylase, acetylcholinesterase, and aldose reductase (Agatonović-Kustrin et al., 2016; Agatonović-Kustrin et al, 2017).

The main objective of the current study was to develop a HT environmentally friendly and simple method which combines the high-performance thin layer chromatography (HPTLC)-bioautography assay with ultra-high-performance liquid chromatography (UHPLC)-LTQ-MS/MS to identify bioactive metabolites from seaweeds that exhibit radical scavenging and antimicrobial activities. To the best of our knowledge, there is no study that reports the fast identification of bioactive compounds from seaweed extracts using HPTLC/bioautography/UHPLC-LTQ-MS^2/GC-MS. The applied methodology was used as a model system in this study to identify metabolites with radical scavenging properties and antimicrobial activities in brown seaweeds

2. Materials and methods

2.1. Chemicals and solvents

All reagents and solvents were of analytical grade. Methanol, ethyl acetate (EtOAc), HPTLC silica gel 60 F_{254} plates, hydrochloric acid, chloroform, and *n*-hexane were purchased from Merck, Darmstadt, Germany. Reagents used for GC-MS analyses were of LC-MS grade. Formic acid (HCOOH) was procured from VWR International, Paris, France. Thiazolyl blue tetrazolium bromide (MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was obtained from Fluka, Sigma-Aldrich Chemie GmbH, Schnelldorf, Germany. 2,2-Diphenyl-1-picrylhydrazyl (DPPH), *p*-anisaldehyde, Triton X-100, and Luria-Bertani (LB) broth were acquired from Sigma-Aldrich, Vienna Austria. Supelco 37 component fatty acid methyl ester (FAME) mix, the internal standard (IS) (13:0), and butylated hydroxytoluene (BHT) were secured from Sigma-Aldrich, Seoul, South Korea. Sulfuric acid (95–98.6%) was bought from Gatt-Koller GmbH, Absam, Austria. Bacteria cultures (*Bacillus subtilis* and *Escherichia coli*) were provided by the Zotchev research group, University of Vienna, Vienna, Austria. Deionized water for the preparation of extraction solutions was prepared using a Milli-Q purification system (Merck Millipore, Darmstadt, Germany).

2.2. Sample collection and extraction of bioactive compounds

Samples of five seaweed cultivars, namely three *Saccharina japonica* (samples **1**, **2**, **5**) and two *Undaria pinnatifida* samples (samples **3** and **4**), were collected from a farm on Deokjeok Island, South Korea (Figure S1). Sample **1** is an early form of *S. japonica*. Voucher samples were deposited in the Herbarium of the Department of Environmental Technology, Food Technology and Molecular Biotechnology, Ghent University Global Campus, Incheon, South Korea. The samples were identified by morphology (Keown et al., 2018). The seaweed samples were harvested and immediately transported in an ice box before further processing. The samples were washed with tap and demineralized water and then dried at 40 °C for 24 h. Approximately 1 g of seaweed was milled for 60 s using a home mill, and extracted with 10 mL of methanol by ultrasonication for 45 min. Subsequently, the extract was filtered, evaporated to dryness at a temperature below 40 °C, and dissolved in 5 mL of methanol. The obtained methanolic solutions were stored at –20 °C prior to analysis.

2.3. HPTLC analysis of seaweed extracts

Seaweed extracts (4 µL) were applied to 10 cm×10 cm HPTLC plates as an 8 mm band using an automatic TLC sampler (ATS4, CAMAG, Muttenz, Switzerland). The first position was at 20.0 mm with a 12.0 mm distance between bands, and the development was performed to a distance of 7 cm in a twin trough developing chamber (CAMAG, Muttenz, Switzerland). A mixture of n-hexane/EtOAc/HCOOH = 3:5:0.1 (*v*/*v*/*v*) was used as the mobile phase. The developed plates were dried in a stream of cold air for 15 min using a hair dryer, derivatized using an anisaldehyde–sulfuric acid reagent (1.5 mL of anisaldehyde was mixed with 210 mL of ethanol, 25 mL acetic acid and 13 mL conc. sulfuric acid), and heated for 3 min at 120 °C. The obtained HPTLC chromatograms were documented using a TLC Visualizer 2 (CAMAG, Muttenz, Switzerland) under 366 nm (with and without derivatization) and visible light (with derivatization).

2.4. HPTLC-Bioautography antimicrobial assays

For the HPTLC-bioautography antimicrobial assays, *E. coli* and *B. subtilis* cultures were cultivated in LB broth. Briefly, 100 µL of bacteria suspension was added to 10 mL of LB broth, then, the mixture was

placed on a shaker (37 °C, 200 rpm) for 16 h. Subsequently, 1 mL of overnight bacterial culture was added to 200 mL of LB broth and incubated in an incubator shaker at 37 °C. The bacteria growth during incubation was monitored using a spectrophotometer (Evolution 260 Bio UV-Visible Spectrophotometer, Thermo Scientific, USA). The *B. subtilis* and *E. coli* cultures were used for the bioassay when the optical density at 600 nm reached 0.539 and 0.541, respectively (Ristivojević & Morlock, 2018).

For the *B. subtilis* assay, 1 µL of crude extract was applied to an HPTLC plate. After development, the HPTLC chromatogram was dried for 30 min under cold air and then manually immersed in cell suspension for 6 s and further incubated for 1 h and 30 min at 37 °C. The zones with an antibacterial effect were visualized by dipping the bioautogram into an aqueous solution of MTT (1 mg/mL) and then incubated for another 15 min. For the *E. coli* assay, 4 µL of crude extract was applied to an HPTLC plate. The developed HPTLC plate was dried, dipped in an *E. coli* suspension, and further incubated at 37 °C for 1 h and 40 min. For visualization, the bioautogram was immersed into a solution of MTT dye with Triton X-100 (1 mg/mL). To enhance the intensity of the yellow bands, one drop of Triton X-100/10 mL MTT aqueous solution was added (Ristivojević et al., 2018; Ristivojević, Tahir, Malfent, Milojkovic, Opsenica & Rollinger, 2019). The obtained bioautograms were documented using TLC Visualizer 2 under white light.

2.5. HPTLC-Bioautography DPPH assay

After development, the HPTLC chromatogram was dipped into a methanolic solution of DPPH radicals at a speed of 3.5 cm/s for an immersion time of 2 s using a TLC chromatogram immersion device (CAMAG, Muttenz, Switzerland). After derivatization with DPPH solution (1 mg/mL), the plate was dried for 90 s in the dark at ambient temperature. Then, the obtained bioautogram was documented every 3 min for 30 min using TLC Visualizer 2 under white light (Ristivojević et al., 2018; Ristivojević et al., 2019).

2.6. Identification of bioactive compounds using UHPLC-LTQ-MS/MS analysis

The chromatography separation and identification of bioactive compounds was performed using a Dionex Ultimate 3000 ultra-high-performance liquid chromatography (UHPLC) coupled LTQ-XL linear ion trap mass spectrometer (Thermo Fisher Scientific, Darmstadt, Germany) with an ESI source. A HPLC-LTQ-MS/MS method with some modifications was described in literature (Takahashi et al., 2013). The mobile phase consisted of A (0.1% aqueous formic acid) and B (0.1% formic acid in acetonitrile). Bioactive compounds were separated using a reversed-phase C18 Phenomenex column (2.1 mm × 15 cm, 2.6 μm, C18, 100 Å). The rate was set to 0.5 μL/min, and a gradient was applied (0−2 min, 5%; 2–25 min, 5–98%; 25–30 min, 98%; 30–35 min, 5% mobile phase B). Mass spectrometry run time was 35 min. MS detection was performed using an ESI source (275 °C heater temperatures and 3.7 kV spray voltage at 275 °C capillary temperature, and 25.0 kV collision energy) to achieve both a positive and negative ion mode ionization. MS scans were performed with an *m/z* range from 50 to 2000.

2.7. Preparation of fatty acid methyl esters (FAMEs)

To determine the FAs composition of seaweed samples using GC/EI-MS, FAMEs were prepared by transmethylation according to the following procedure. Briefly, 25 mg of the dry seaweed sample was placed in a Pyrex test tube with a Teflon-lined screw cap. Subsequently, 3.3 mL of a 2 M hydrochloric acid solution in methanol was added and the mixture was vortexed for 5–10 s. Then, 0.3 mL of chloroform with IS 13:0 and BHT (50 μL of IS (13:0) in 100 mg/mL chloroform and 50 μL of antioxidant (BHT) in 100 mg/mL chloroform) was added to 10 mL of chloroform and the tube tightly closed. After vortexing for 30 s, the tube was heated at 90 °C for 2 h. Once cooled to room temperature, the FAMEs were extracted by adding 0.9 mL of Milli-Q water, vortexing for 5–10 s, adding 1.8 mL of *n*-hexane, and vortexing for 20–30 s. After centrifugation for 5 min at 4000 rpm, the upper *n*-hexane phase-containing the FAMEs was transferred to a sample vial for GC/EI-MS analysis.

2.8. Analysis and identification of fatty acids by GC/ESI-MS

The FAME composition was analyzed using an Agilent 6890 gas chromatograph equipped with a DB-23 capillary column (30 m × 0.25 mm id, film thickness 0.25 μm, Agilent Technologies Inc., Santa Clara,

196 USA). The capillary column was directly coupled to an Agilent 5973 mass spectrometer (Agilent
197 Technologies Inc., Santa Clara, USA). The sample (1 μL) was injected onto the capillary column with a
198 split ratio of 10:1. Helium (high-purity 5.0 grade) was used as the carrier gas with a flow rate of 0.6
199 mL/min. The oven temperature was maintained at 50 °C for 1 min, increased from 50 to 175 °C at a ramp
200 rate of 25 °C/min, held at 175 °C for 1 min, increased from 175 to 235 °C at a ramp rate of 4 °C/min,
201 and then held at 235 °C for 5 min. The injector and detector temperatures were 250 and 150 °C,
202 respectively. A solvent delay of 3 min was applied. The electron energy was 70 eV and the temperature
203 of the ion source was 230 °C.
204 The FAMEs were identified by comparing their retention times with those of the FAME standard
205 (Supelco 37 component FAME mix) at the same conditions, as well as by comparing their mass spectra
206 with those stored in the National Institute of Standards and Technology (NIST) Mass Spectral Library.
207 Only the FAs whose spectra overlapped with a probability of more than 90 % with the spectra from the
208 NIST base were considered. After integration of the GC-MS spectra, the content of each FA was
209 expressed as perecntage of the total FAs content.

210 **2.9. Preparative HPTLC**

211 For structural identification of the bioactive compounds in the seaweed extracts, 75 μL of extract (sample
212 **3**) was applied to a 10 × 20 cm HPTLC plate as an 18 cm wide band. The plate was developed to a
213 distance of 10 cm with n-hexane/EtOAc/HCOOH = 3:5:0.1 (*v*/*v*/*v*) as the mobile phase and dried for 20
214 min at room temperature under a cold air flow. From the developed 18 cm HPTLC bands, the part 1.5
215 cm away from the left was derivatized with the anisaldehyde–sulfuric acid reagent (Materials and
216 methods 2.3.). The identified positions of bioactive bands on the plate were observed under visible light.
217 The remaining part of non-derivatized bands were marked, scraped into microcentrifuge tubes, and
218 extracted with acetone/methanol = 1:1. The composition of the acetone-methanol extract of the bioactive
219 band was analyzed using UHPLC-LTQ-MS/MS (Jesionek, Fornal, Majer-Dziedzic, Móricz, Nowicky &
220 Choma, 2016).

3. Results and discussion

3.1. HPTLC fingerprint of brown seaweeds

The HPTLC fingerprinting was used as an initial, simple and HT screening tool to verify the differences between *S. japonica* and *U. pinnatifida* (2–3 min/sample). The seaweed metabolites were separated using a newly developed mobile phase consisting of a mixture of *n*-hexane/EtOAc/HCOOH (3:5:0.1,*v*/*v*/*v*). Under 366 nm light, the HPTLC chromatogram (without derivatization) showed different chemical patterns for the two seaweed species: *S. japonica* displayed red bands at hR_F 32, 44, 48, 58, 64, 70, and 78 (samples **2** and **5**) of which the bands at hR_F 44, 58 and 78 were the most intense, whereas *U. pinnatifida* exhibited two intense red bands at hR_F of 48 and 78 (samples 3 and 4). *S. japonica* sample **1** showed a slightly different chemical profile compared to f samples **2** and **5** two red bands at hR_F s of 48 and 78 were the most intense (Figure 1A). This profile variation resulted from the different life cycle stages of *S. japonica*, because sample **1** was analyzed at an earlier stage. The red bands on the chromatograms were carotenoids, which are abundant pigments in seaweeds. This conclusion agrees with literature (Agatonović-Kustrin et al., 2016; Agatonović-Kustrin et al., 2017; Hynstova, Sterbova, Klejdus, Hedbavny, Huska & Adam, 2018).

After derivatization with the anisaldehyde–sulfuric acid reagent, the plates were observed under visible and 366 nm light and other compounds beside carotenoids, such as phenols, sugars, steroids, and FAs, were identified. The chromatograms obtained for the two seaweed species were quite similar (Figures 1B and 1C), and under 366 nm light six distinct bands at hR_F 5, 8, 48, 70, 78, and 89 (Figure 1B) were revealed. These HPTLC profiles suggested that the methanol extract of *S. japonica* is richer in pigments (carotenoids) than that of *U. pinnatifida*. The obtained results show that the newly developed HPTLC mobile phase can effectively be used for the rapid carotenoid profile screening of seaweeds, especially to verify carotenoids with a specific biological activity. Furthermore, the most intense band in all samples after derivatization was observed at hR_F 78.

3.2. Identification of bioactive compounds using bioautography/HPTLC-UHPLC-LTQ-MS/MS workflow

There are a few papers published that are related to bioautography-based methods for bioprofiling of seaweed extracts (Agatonović-Kustrin et al., 2016; Agatonović-Kustrin et al., 2017), in which authors used reference compounds to identify compounds in the bioactive band(s). The workflow applied in this study includes an improved highly specific bioautography/HPTLC-UHPLC-MS/MS-based procedure providing an unambiguous identification of compounds in the biologically active band(s), prior to any attempt to isolate the pure compounds. To distinguish the molecules in each seaweed extract with radical scavenging and antimicrobial activities, the developed HPTLC chromatograms were derivatized with a $DPPH^{\cdot}$ solution and bacterial cells, respectively (Material and methods 2.4 and 2.5).

After dipping the HPTLC chromatograms in a DPPH solution, compounds with radical scavenging activity appeared as a yellow band against a purple background (Figure 1D). Potential radical scavenging compounds were present in all seaweed samples. Beside the most intense band observed at $h\mathrm{R_F}$ 78 additional less intense bands were present in all samples at $h\mathrm{R_F}$ 11, 48, and 89, while only sample **3** displayed an additional intense band at $h\mathrm{R_F}$ 8 (Figures 1D and 2). Based on these results, it was concluded that both seaweed species exhibited several bands that contained compounds with radical scavenging activity.

In an attempt to determine compounds with antibacterial activity in the seaweed extracts, *E. coli* as a Gram-positive and *B. subtilis* as a Gram-negative bacterium were chosen because of their distinct activities and sensitivities against antibacterial compounds. After incubation of the chromatograms in the presence of *B. subtilis* or *E. coli* cell suspensions, HPTLC chromatograms were additionally incubated and visualized using an MTT solution. The bands which included compounds with potential antibacterial activity appeared in yellow against a purple background. In all samples, a single yellow band was observed at $h\mathrm{R_F}$ 78 in the presence of both bacteria (Figures 1E, 1F, and 2). Thus, this band contains compounds that have significant inhibitory effects against both Gram-negative *E. coli* and Gram-positive

B. subtilis bacteria. Comparing the intensities of this band against all five extracts for both bacteria species, it is observed that extracts prepared from sample 1 and 3 had the highest antimicrobial activity.

After bioautography, the next step of the proposed methodology involved the UHPLC-LTQ-MS/MS analysis to identify compounds with antimicrobial and radical scavenging activity. To determine the structure of the compound in the most active band with hR_F at 78, sample **3** (*U. pinnatifida*) was chosen as a representative, because its band intensity was the highest of all bioassays (Figure 2). To provide a sufficient amount of sample for this analysis, preparative TLC of sample **3** was performed. The most active band was scraped from the plate (Figure S2), and compounds present in this band extracted with a mixture of acetone/methanol (1:1, *v*/*v*), and analyzed using UHPLC-LTQ-MS/MS (Figure S2). The targeted compounds were tentatively identified according to their retention time, molecular mass in both positive $[M+1]^+$ and negative $[M-1]^-$ modes, and fragmentation patterns, as reported in literature. The obtained chromatogram from the UHPLC-LTQ-MS/MS analysis displayed three major peaks, which were marked as compounds ***1*** (t_{R1} = 22.08 min), ***2*** (t_{R2} = 23.08 min), and ***3*** (t_{R3} = 24.40 min)(Figure S3). In the negative ionization mode, compounds ***1***, ***2***, and ***3*** with molecular ions at *m/z* 275.23, 301.28, and 303.28, respectively, were tentatively assigned as stearidonic acid (SDA, 18:4 n-3), eicosapentaenoic acid (EPA, 20:5 n-3), and arachidonic acid (AA, 20:4 n-6), respectively (Table 1, Figures 3 and S4). SDA, EPA, and AA were finally confirmed after fragmentation of the molecular ions of these FAs (Figure S5). For example, the molecular ion of EPA, and AA showed fragment ions corresponding to the losses of -44 Da and -98 Da (Table 1, Figure S5). Additionally, in the positive ionization mode, SDA, EPA, and AA produced molecular ions at *m/z* 277.23, 303.28, and 305.28, respectively. The molecular ion of SDA at *m/z* 277.23 led to a characteristic fragment ion at *m/z* 259 (Figures S4 and S5), whereas EPA produced fragment ions *m/z* 285, 257, and 203 (Table 1, Figures 3B and S5). Furthermore, AA produced fragment ions at *m/*z 259, 241, 221, and 195, which is in agreement with literature (Figure S5) (Al-Mubarak, Vander Heiden, Broeckling, Marivić, Brennan & Varalakshmi, 2011; Dhananjay et al., 2012; Serafim et al., 2019).

295 Furthermore, the identification of these compounds as bioactive components is consistent with
296 literature results. It was reported that free PUFAs such as EPA, SDA and AA show antimicrobial activity
297 not only against different Gram-positive and Gram-negative bacteria, but also against fungi, viruses and
298 parasites.
299 Several mechanisms are described in the literature how PUFAs exert their antibacterial activity on
300 bacteria or in the human body (Sivagnanam, Yin, Choi, Park, Woo, & Chun, 2015; Richard, Kefi, Barbe,
301 Bausero & Visioli, 2008; Król & Kiełtyka-Dadasiewicz, 2015, Das, 2018; Chandra et al. 2018). The
302 PUFAs identified in our study are known to inhibit the bacterial enoyl-acyl carrier protein reductase
303 (FabI), an essential component of bacterial FAs synthesis that is critical for bacteria to survive. The
304 inhibition of the FabI enzyme can alter the membrane viscosity and hydrophobicity by changing the FA
305 composition of the membrane, the cell surface charge by the leakage of electrons and ions through the
306 membrane, and the membrane permeability by the disruption of the active and passive transport (Das,
307 2018; Chandra et al. 2018).
308 Beside this, various compounds with antimicrobial activity have also antioxidant activity, and it was
309 reported that these two activities are directly proportional (Mattos, Tonon, Furtado, & Cabral, 2016;
310 Chanda at al. 2018). Inhibition or competition with the electron donor within the cell, leakage of
311 intracellular proteins and alteration of vital FAs in the organisms are some of suggested mechanism by
312 which antioxidants can interfere antimicrobial growth (Chanda et al. 2018). PUFAs from the most active
313 band can effectively donate a hydrogen atom to stabilize a free DPPH radical, which demonstrates their
314 radical scavenging or antioxidant activity due to the presence of several double bonds in their structure.
315 This ability of PUFAs to remove free radicals is characteristic of their innate functions but is depending
316 on environmental conditions, meaning PUFAs inherently have both antioxidant and pro-oxidant
317 properties (Das, 2018; Chanda et al. 2018).
318 The identification of these three FAs in brown seaweed extracts, providing antibacterial and radical
319 scavenging activities, could encourage further investigations into this field and help to determine the best

quality of seaweed materials. This could include the study of seaweed species, season, location and stage of their life cycle as health promoting food sources endowed with antibacterial and radical scavenging properties. Also, the suggested HPTLC/bioautography UHPLC-MS/MS-based methodology could further be used as a rapid dereplication method for natural products and/or to identify unknown bioactive plant metabolites. In addition, the different extraction conditions, mobile phases for HPTLC separation, and HPTLC /bioautography assays can be easily combined during application.

3.3 GC-MS analysis of FAs in brown algae

GC-MS was used as a complementary technique to determine the ratio of the identified bioactive FAs in brown seaweed samples. The combination of HPTLC/bioautography/UHPLC-LTQ-MS/MS with GC-MS delivered the complete information about other FAs in the investigated seaweeds. Brown seaweeds are considered as a valuable source of essential LC-PUFAs, such as EPA, and DHA, which are not only important because of their nutritional value, but also because of their biological activities (Schmid, Guihéneuf & Stengel, 2014). In addition, the determination of seaweed FAs profiles is valuable because it provides a signature profile for organic lipid chemistry and food research along with the algal taxonomic location as a potential chemo-taxonomic biomarker (Kendel, Wielgosz-Collin, Bertrand, Roussakis, Bourgougnon, & Bedoux, 2015).

Analyzing the FA profiles of the seaweed samples with GC-MS showed the presence of 17FAs, of which six FAs were saturated, three were monounsaturated FAs and eight were PUFAs, whose mass spectra overlapped with a probability of more than 91% with the spectra from the NIST base (Table 2). According to the data obtained for the content of each FA (expressed as a percentage of the total FAs content) (Table 2), it can be concluded that the eight most abundant FAs (myristic acid, palmitic acid, oleic acid, linolenic acid, α-linolenic acid, SDA, AA and EPA) contributed to 91.93–93.63% and 90.66 –94.96% of the total FA contents of *U. pinnatifida* and *S. japonica*, respectively.

Among saturated FAs, palmitic (C16:0) and myristic acid (C14:0) were the most abundant in all samples. The palmitic and myristic acid contents were 17.3 (sample **2**) and 34.10% (sample **4**), and 2.73

(sample **1**) and 14.30% (sample **5**), respectively. These results are in line with those of Hwang, Kim, Woo, Rha, Kim, and Shin, (2014), who reported that palmitic acid and myristic acid were the most abundant saturated FAs in *S. japonica*, the concentrations varying with location and time of harvest. The most abundant PUFAs in both species were EPA and SDA as n-3 FAs, and AA as Table 2 shows that there are significant differences in the content of each of these three FA between samples of the same species as well as between the species. The highest EPA, SDA and AA contents were observed for samples **1** and **3**. Linoleic acid as n-6 FA and α-linolenic acid as n-3 FA were present in lower quantity compared to EPA, SDA and AA. The total content of these five PUFAs in samples 1 to 5 were 62.26, 49.01, 66.20, 40.90, and 33.40%, respectively. These results agree with literature, considering brown seaweeds (Boulom, Robertson, Hamid, Ma, & Lu, 2014; Hwang et al., 2014) as a valuable source **of important LC-PUFAs such as EPA, γ-linolenic acid, AA, *α*-linolenic acid and SDA, *α*-linolenic acid being the first product in the synthesis pathway to C20-22 PUFAs** (Hwang et al., 2014; Wells et al. 2017). All investigated seaweed samples showed a higher content of n-3 FAs in comparison to n-6 FAs, with n-6:n-3 ratios ranging from 1:1 to 1:10.

In both seaweed species SDA, EPA and AA were identified in our study as major compounds present in the band hR_F 78, having abundant antimicrobial and free radical scavenging activities (Table 2). The total content of these three PUFAs in samples **1** to **5** were 47.58, 34.60, 51.70, 30.00 and 23.36%, respectively. Comparing the total contents of these FAs with the intensities of bands obtained at hR_F 78 for both bioassays (radical scavenging and antimicrobial assays) in all samples, a direct proportionality between them was revealed. The most intense bands and the highest total SDA, EPA, and AA contents were determined for samples **1** and **3**, while the least intense band and the lowest content of these FAs were observed for sample **5** (Figure 1 D, E and F). According to these results, each of these FAs contributes to the total antimicrobial and radical scavenging activity. This confirms literature data that reported the antimicrobial and radical scavenging activities of SDA, EPA and AA (Shin et al., 2007; Richard et al., 2008; Sivagnanam et al., 2015; Das, 2018; Yoon et al., 2018).

4. Conclusion

HPTLC/bioautography provides a simple, and high-throughput screening method for the rapid and economically friendly evaluation of potentially health-promoting seaweed samples. The identification of bioactive constituents in seaweed samples was achieved through the different chemical metabolite profiles of the samples (2–3 min per sample) with a minimal organic solvent consumption (200–500 µL/ per sample), and derivatization of the obtained HPTLC plates with DPPH solution or bacteria, allowing the simultaneous identification of bands containing compounds with radical scavenging and antibacterial activities (9–10 min/sample is required for these bioassays). In the last step, the preparative TLC in combination with UHPLC-LTQ-MS/MS identified SDA, EPA, and AA as major bioactive compounds in seaweed extracts. GC-MS, used as a complementary technique in this study, confirmed the existence of a direct proportionality between the total content of these three FAs in seaweed extracts and the biological activity of bands containing these FAs.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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Human and animal rights and informed consent

The article does not contain any studies with animals or human performed by any of the authors.

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Figure caption

Figure 1. HPTLC profiles of seaweed samples: A) under 366 nm light without derivatization; B) under visible light, and C) under 366 nm light after derivatization with anisaldehyde–sulfuric acid; D) after the DPPH assay; E) after the *B. subtilis* bioassay; and F) after the *E. coli* bioassay. From left to right, the lines **1**, **2**, and **5** are *S. japonica* samples and the lines **3** and **4** are *U. pinnatifida* samples.

Figure 2. HPTLC profiles of *U. pinnatifida* sample **3**: A) under 366 nm light, and B) under visible light without derivatization; C) under visible light after derivatization with anisaldehyde–sulfuric acid; D) after the DPPH· assay; E) after the *B. subtilis* bioassay; and F) after the *E. coli* bioassay.

Figure 3. The HPTLC-UPLC-MS^2 mass spectra of multipotent compounds of *U. pinnatifida* sample **3**: A) stearidonic acid, B) eicosapentaenoic acid, C) arachidonic acid.

518 **Table 1.** The molecular mass of ions obtained after UHPLC-MS/MS analysis of compounds **1**, **2** and **3**

519 and after their fragmentation in positive and negative ionization modes.

520

No.	Compounds	Molecular ions		Fragmentions	
		$[M-1]^-$	$[M+1]^+$	MS/MS (-)	MS/MS (+)
1	Stearidonic acid	275.23	277.23	231, 177	259
2	Eicosapentaenoic acid	301.28	303.28	257, 203	285, 257, 203
3	Arachidonic acid	303.28	305.28	259, 205	259, 241, 235, 221, 195

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Table 2. The fatty acid (FA) profiles of the brown seaweed *S. japonica* (samples **1**, **2**, and **5**) and *U. pinnatifida* (samples **3** and **4**), and the probability (%) of overlapping mass spectra of FA with the spectra from the NIST base using GC-MS analysis. The content of the each FA is expressed as percentage of the total FA content and is given as a mean values ± SD of triplicate determinations.

				1	**2**	**3**	**4**	**5**
No.	**Retention time (min)**	**Fatty acid**	**NIST (%)**	**Fatty acid content (% of the total fatty acids content)**				
1	9.72	Myristic acid	99	2.73 ± 0.04	8.90 ± 0.60	3.01 ± 0.08	5.80 ± 0.20	14.30 ± 0.20
2	10.66	Pentadecylic acid	98	0.28 ± 0.02	0.32 ± 0.04	0.39 ± 0.09	0.77 ± 0.08	0.28 ± 0.02
3	11.72	Palmitic acid	99	22.01 ± 0.02	17.30 ± 0.40	19.30 ± 0.50	34.10 ± 0.20	25.60 ± 0.10
4	12.14	Palmitoleic acid	99	0.60 ± 0.10	1.90 ± 0.20	0.54 ± 0.05	1.30 ± 0.30	1.37 ± 0.08
5	12.90	Margaric acid	99	0.11 ± 0.01	0.06 ± 0.01	0.07 ± 0.01	0.18 ± 0.03	0.04 ± 0.01
6	14.20	Stearic acid	99	0.97 ± 0.01	0.55 ± 0.01	0.61 ± 0.02	1.64 ± 0.01	0.51 ± 0.01
7	14.6	Oleic acid	99	7.90 ± 0.20	16.70 ± 0.50	6.10 ± 0.20	10.00 ± 0.20	20.30 ± 0.30
8	14.72	Vaccenic acid	99	0.01 ± 0.01	0.06 ± 0.04	0.01 ± 0.01	0.46 ± 0.02	0.03 ± 0.01
9	15.31	Linoleic acid (LA)	95	7.69 ± 0.07	8.60 ± 0.10	6.60 ± 0.10	5.70 ± 0.20	6.40 ± 0.10
10	15.80	γ-Linolenic acid	95	1.18 ± 0.01	3.06 ± 0.02	1.00 ± 0.07	1.14 ± 0.06	1.47 ± 0.04
11	16.30	α-Linolenic acid	98	6.99 ± 0.03	5.81 ± 0.06	7.90 ± 0.30	5.20 ± 0.20	3.64 ± 0.09
12	16.74	Stearidonic acid	91	18.18 ± 0.09	12.70 ± 0.20	22.30 ± 0.10	12.10 ± 0.40	7.30 ± 0.10
13	17.00	Arachidic acid	99	0.55 ± 0.01	0.18 ± 0.01	0.50 ± 0.02	0.79 ± 0.01	0.25 ± 0.01
14	18.22	Eicosadienoic acid	93	0.21 ± 0.01	0.06 ± 0.03	0.17 ± 0.03	0.14 ± 0.01	0.07 ± 0.01
15	19.05	Arachidonic acid (AA)	95	17.90 ± 0.20	14.20 ± 0.20	17.30 ± 0.20	11.70 ± 0.40	11.16 ± 0.04
16	19.20	Eicosatrienoic acid	95	0.06 ± 0.08	0.03 ± 0.03	0.20 ± 0.20	0.03 ± 0.04	0.02 ± 0.01
17	20.05	Eicosapentaenoic acid (EPA)	95	11.50 ± 0.20	7.70 ± 0.10	12.10 ± 0.20	6.20 ± 0.20	4.90 ± 0.40

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Highlights

- A novel HPTLC method to separate seaweed metabolites was developed.
- A bioautography-based method identified bioactive compounds in seaweeds.
- Complementary GC-MS analysis verified the presence of three bioactive fatty acids.
- The developed methodology offers a novel and rapid dereplication strategy.

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Credit Author Statement

Petar Ristivojević: Conceptualization; Data curation; Formal analysis , Investigation; Methodology; Project administration; Visualization, Writing - original draft; Writing - review & editing

Vesna Jovanović: : Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Validation; Visualization; Writing - original draft; Writing - review & editing

Dušanka Milojković Opsenica: Formal analysis; Methodology; Project administration; Resources; Supervision; Writing - original draft; Writing - review & editing.

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Judith M. Rollinger: : Conceptualization; Formal analysis; Funding acquisition; Methodology; Project administration; Resources; Supervision; Visualization; Roles/Writing - original draft; Writing - review & editing.

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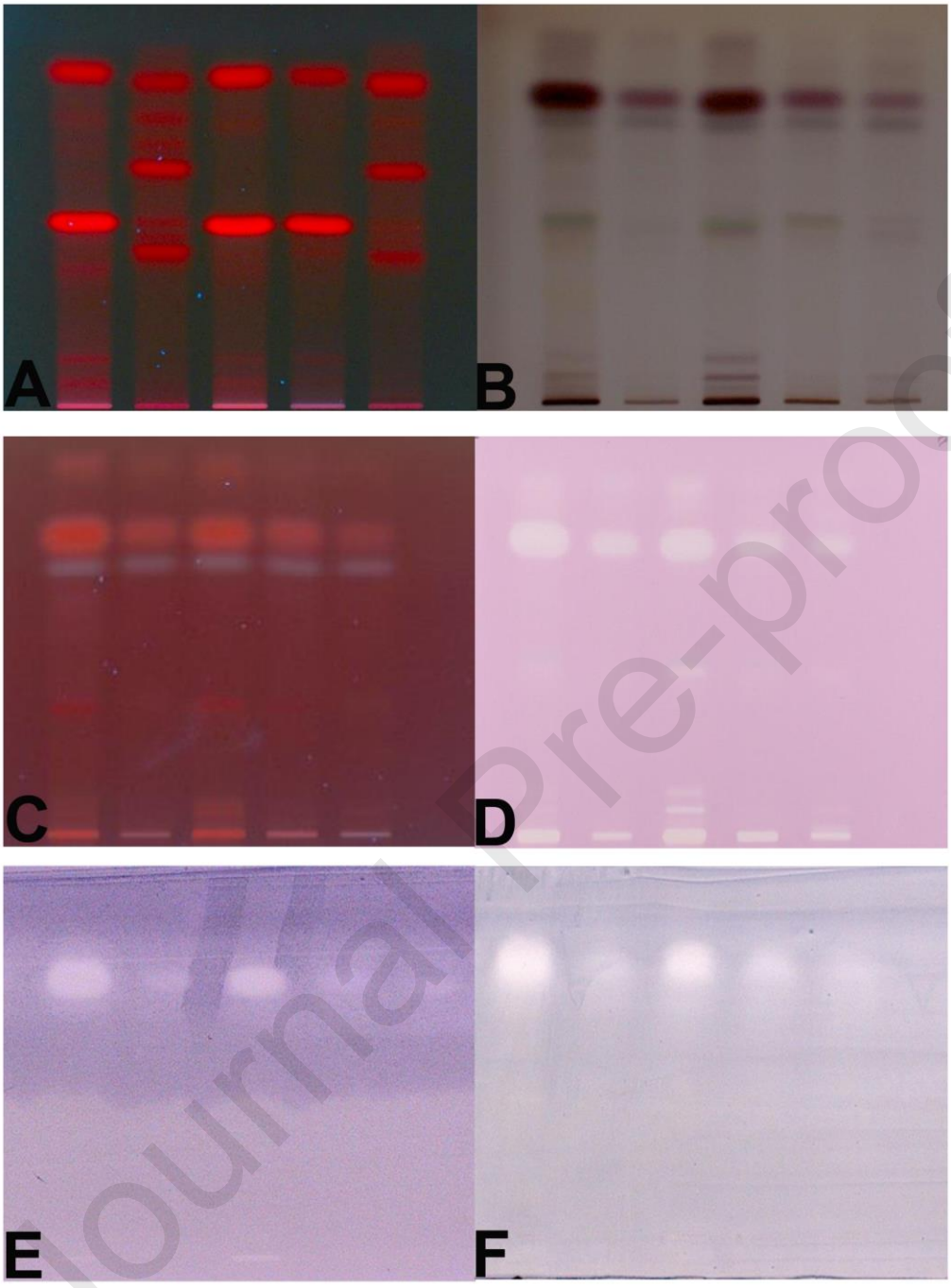

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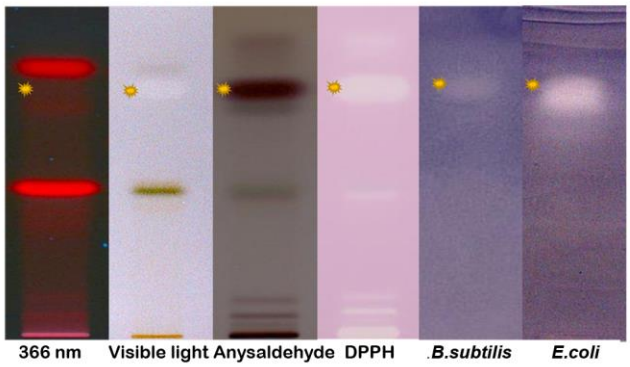

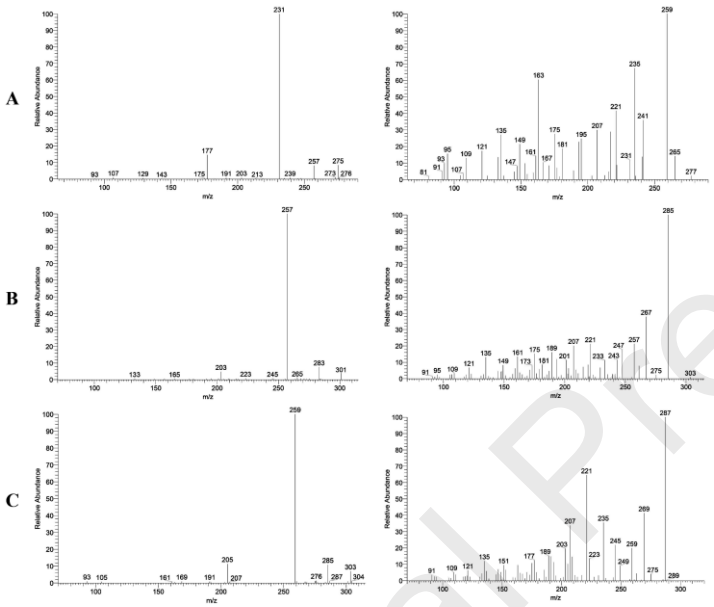

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