Supercritical Fluid Extraction of Halogenated Monoterpenes from the Red Alga *Plocamium cartilagineum*

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Supercritical fluid extraction (SFE) of the marine red alga *Plocamium cartilagineum*, which is known to contain complex mixtures of halogenated monoterpenes, was investigated. *P. cartilagineum* samples were extracted by SFE with carbon dioxide and modified carbon dioxide containing up to 10% methanol at different pressure and temperature conditions to establish the optimum conditions for extraction. These conditions were then used in the extraction of halogenated monoterpenes from 2 different samples of *P. cartilagineum*: one from Davenport, CA, and the other from Casa Beach (San Diego, CA). Several halogenated monoterpenes isolated by conventional solvent extraction with methanol and purified by column chromatography were used as the reference compounds for the determination of the extraction efficiency in the SFE experiments.

*Plocamium cartilagineum* belongs to the red alga family—Plocamiaceae, and has been found to contain a large number of halogenated monoterpenes, whose structures typically contain 1–6 bromine and/or chlorine atoms. *P. cartilagineum* grows along the Pacific coast from Washington to Chile, the British Isles, Australia, and Spain. Interestingly, *P. cartilagineum* collected from different geographical areas in the world are all reported to produce halogenated monoterpenes, but of different structural types and halogen substitution patterns. Most of these halogenated monoterpenes have been found to exhibit varied biological activities, including antifungal, antimicrobial, and molluscidal activity.

Faulkner confirmed structure 2 by X-ray diffraction analysis. Subsequently, a more detailed study showed that *P. cartilagineum* was the dietary source of these 2 monoterpenes due to *A. californica* feeding on this red alga.

At present, there are nearly 50 different polyhalogenated monoterpenes isolated from *P. cartilagineum*, as listed in Figure 2 (3). These halogenated monoterpenes from *P. cartilagineum* can be classified into 3 major structure classes: one alicyclic class and 2 monocyclic classes (Figure 3).

Monoterpenes 2–13 were isolated from *P. cartilagineum* collected at Bird Rock (La Jolla, CA; 4). Crews et al. (5, 6) discovered 2 halogenated monoterpenes 14 and 15 from the algae collected at Davenport, CA. *P. cartilagineum* from the Antarctic produces structures 16–19 (7). Interestingly, the halogenated monoterpenes 20–30 isolated from New Zealand *P. cartilagineum* differed from sample to sample (8). Compounds 20 and 21 were isolated from one sample of this red alga, but others, 22–30, were obtained from a different collection. Monoterpene 31, found to have antifungal and antibacterial activities, was obtained from *P. cartilagineum* collected along the northern Atlantic coast of Spain (9). Recently, Abreu and Galindo (10) isolated 2 new monoterpenes, 32 and 33, from *P. cartilagineum* along the Portuguese coast. Additionally, the monocyclic halogenated monoterpenes 34–50 were reported from *P. cartilagineum* by several research groups (11–15).

In all of the prior studies, extraction of these halogenated monoterpenes from *P. cartilagineum* was performed with a Soxhlet apparatus or by conventional solvent extraction. The extraction process required large volumes of organic solvents, and some extractions took as long as 4 days. The extractions were followed by extensive column chromatography and liquid chromatography (LC) to isolate individual compounds.

The halogenated monoterpenes from *P. cartilagineum* are known to be labile to heat and air oxidation, and thus, readily decompose under conventional solvent extraction and purification. Hence, supercritical fluid extraction (SFE) is an attractive alternative method to conventional solvent extraction because it is more suitable for thermally labile and air-sensitive compounds.
Figure 1. Structures 1 and 2.

Figure 2. Halogenated monoterpenes isolated from the red alga Plocamium cartilagineum.
Figure 2 (continued). Halogenated monoterpenes isolated from the red alga *Plocamium cartilagineum*.

Note: All the structures are taken from Ref. 3, except for structures 32-33 that are taken from Ref. 10.

Figure 3. Classes of halogenated monoterpenes isolated from *P. cartilagineum*. 
Experimental

Collection of Marine Alga—Plocamium cartilagineum

Plocamium cartilagineum (Rhodophyta, Plocamiaceae) was collected intertidally (–1 to –2 m depth) from 3 locations, including (1) Davenport Landing, Santa Cruz County, CA, on March 11, 1998; (2) Casa Beach, San Diego County, CA, on February 28, 1998; (3) Bird Rock, La Jolla, San Diego County, CA, on March 2, 1995. All the P. cartilagineum samples were immediately stored in ice upon collection, transported to the laboratory, and stored frozen at –12°C until analysis.

Chemicals and Supplies

All solvents used in this study, including methanol, methylene chloride, ethyl acetate, hexane, and acetone, were LC grade or Optima™ grade and were obtained from Fisher Scientific (Fair Lawn, NJ). CDC13 and C6D6 solvents containing TMS as an internal standard for NMR were from Aldrich Chemical Co. (Milwaukee, WI). Hydromatrix™ was from Varian, Inc. (Palo Alto, CA) and SFE/SFC-grade carbon dioxide (total hydrocarbon contamination less 0.1 ppb) was from Air Products (Allentown, PA). All other chemicals were reagent grade from Fisher Scientific.

Gas Chromatography/Mass Spectroscopy (GC/MS)

GC/MS analyses were performed on a Hewlett-Packard 5890 Series II gas chromatograph interfaced to a Hewlett-Packard 5971A mass spectrometer MSD/Windows 95 ChemStation (Agilent Technologies, Palo Alto, CA) and equipped with a Hewlett-Packard 5973A autoinjector. For most of the injections, samples were introduced via a 30 m length × 0.25 mm id × 0.25 μm film thickness PTE-5 fused-silica open-tubular column (Supelco, Bellefonte, PA) using helium as a carrier gas at a flow rate of ca 1 mL/min. However, the samples from San Diego P. cartilagineum were introduced via a 30 m length × 0.25 mm id × 0.25 μm film thickness DB-5 fused-silica open-tubular column (J&W Scientific, Folsom, CA). PTE-5 and DB-5 are equivalent columns because the stationary phase in each consists of a dimethylsiloxane containing 5% diphenyl polymer.

The column temperature was held at 40°C for 2 min, then programmed to a final temperature of 280°C at a rate of 12°C/min, where it was held for 12 min. The injection volume was 1 μL, and the injector temperature was 250°C. The injector was set in the splitless mode for 1 min after injection. The electron energy of the mass spectrometer was set at 70 eV, and the electron multiplier voltage held between 2274 to 2356 V. Spectral data were acquired at a rate 0.50 s/scan (scanning range was 35 to 550 amu). The instrument was tuned daily with decafluorotriphenyl phosphine standard introduced via the GC inlet.

Conventional Solvent Extraction and Purification Procedures

Frozen P. cartilagineum (Davenport, CA) was thawed, dried by pressing between several paper towels, and weighed. For conventional extractions, 353 g towel-dried algae was extracted by soaking with LC grade methanol (800 mL each time) 4 times (24 h each) at room temperature. After each extraction, the methanol layer was filtered, and fresh methanol was added to the sample. The filtered methanol solution was evaporated in vacuo on a Buchi rotary evaporator. The sticky residue, remaining after the evaporation of solvent, was partitioned into 100 mL ethyl acetate (EtOAc) in a 250 mL separator funnel. The EtOAc fraction was then separated from the aqueous layer, and the aqueous layer extracted with 20 mL ethyl acetate twice. Then, the combined EtOAc fractions were dried with 2 g anhydrous sodium sulfate for 15 min, filtered, and evaporated to dryness in vacuo. The crude extracts were combined and stored in a freezer at –8°C for future chromatography. Conventional solvent extraction of 353 g towel-dried P. cartilagineum produced 3.68 g of a dark-green oil.

A portion of the crude extract (387.3 mg) was applied to an 18 id × 400 mm length silica gel column (Whatman, 13–24 μm particle size) and eluted with 400 mL hexane followed by 300 mL hexane–EtOAc (95 + 5, v/v). Thirteen fractions were collected and monitored by silica thin-layer chromatography (TLC) plates that were developed with 100 mL hexane–EtOAc (98 + 2, v/v). The detailed purification flow protocol is given elsewhere (16).

Supercritical Fluid Extraction

An ISCO SFE system consisting of 2 Model 260D syringe pumps and an SFX 2–10 extraction module (ISCO, Lincoln, NE) was used for all experiments reported herein. The flow rate of carbon dioxide was maintained at 1.5 mL/min. To prevent plugging during the extraction, the restrictor was heated to 100°C and the 15 mL collection vial (initially filled with 6 mL methylene chloride) was kept in a small beaker with water at room temperature (22°C). A 10 mL disposable extraction cartridge was filled with 2 g towel-dried P. cartilagineum mixed with an equal amount of Hydromatrix (mixing of the algae sample and Hydromatrix was done immediately prior to SFE and was done manually with the help of a glass rod).

Kinetic Study

The towel-dried algae (4.8 g) mixed with 5.2 g Hydromatrix were split equally into two 10 mL disposable extraction cartridges. The extractions with pure CO2 were performed at 400 atm/40°C using a 1.5 mL/min flow rate for 3 h with collection of fractions every 60 min. Each fraction was evaporated in vacuo and weighed. Fraction 1 yielded 8.0 mg of the SFE extract; fraction 2 gave 2.0 mg of the SFE extract; fraction 3 produced 1.0 mg of the SFE extract. Each SFE fraction was diluted with 1 mL LC grade ethyl acetate for TLC analysis. The SFE extracts and the crude extracts obtained from conventional solvent extraction of the same algae were spotted on the same TLC plates, which were developed with hexane–EtOAc (98 + 2, v/v).

Effect of Temperature

The temperature effect upon the SFE was investigated at 40°C, 60°C, 80°C, and 100°C. For all experiments, 4.8 g towel-dried P. cartilagineum mixed with an equal amount of
Figure 4. Time-dependent extraction curves with pure carbon dioxide.

Note 1: *P. cerasiforme* from Davenport (Santa Cruz) was extracted with pure CO$_2$ at 250 atm/40°C with a 1.5 mL/min flow rate for 3 hrs with collection of fractions every 15 min.

Note 2: The total extractable materials and the total concentrations of monoterpene 14 and 15 (3 determinations) after 3-hr extraction were 4800 μg/g, 2400 μg/g and 533 μg/g, respectively.

Note 3: The total concentrations of monoterpene 14 and 15 after 1-hr extraction were 2580 μg/g and 5337 μg/g, respectively.

Figure 5. Time-dependent extraction curves with CO$_2$ modified with 10% methanol.

Note 1: *P. cerasiforme* from Davenport (Santa Cruz) was extracted by CO$_2$ modified with 10% methanol at 250 atm/40°C with a 1.5 mL/min flow rate for 3 hrs with collection of fractions every 15 min.

Note 2: The total extractable material and the total concentrations of monoterpene 14 and 15 (3 determinations) after 3-hr extraction were 9820 μg/g, 565 μg/g and 343 μg/g, respectively.

Note 3: The total concentrations of monoterpene 14 and 15 after 45-min extraction were 565 μg/g and 343 μg/g, respectively.
the Hydromatrix was split equally into two 10 mL extraction vessels. The extraction was performed with CO₂ at 400 atm/40 °C using a 1.5 mL/min flow rate for 3 h, and SFE fractions were collected every 60 min. Each fraction was evaporated in vacuo, weighed, and redissolved in 2 mL methylene chloride. Three SFE fractions produced 9.1 mg (fraction 1), 2.4 mg (fraction 2), and 1.3 mg (fraction 3), respectively, of extractable residue.

A series of extractions was performed under the identical SFE conditions noted above, except at different temperatures (i.e., 60 °C, 80 °C, and 100 °C). A total of 12 fractions of the SFE extracts were obtained and analyzed by TLC using hexane–EtOAc (98 + 2, v/v) as a developing solvent. In addition, these fractions were also analyzed qualitatively by GC/MS.

**Time-Dependent Extraction with Supercritical CO₂ and CO₂ Modified with 10% Methanol**

In a typical extraction, 4 g towel-dried algae were mixed with an equal amount of Hydromatrix. The algae–Hydromatrix mixture was split equally into two 10 mL disposable SFE cartridges. The extractions were performed with either CO₂ alone or CO₂ modified with 10% methanol at 250 atm/40 °C with a 1.5 mL/min flow rate, and were continued under the same conditions for 3 h. The SFE fractions were collected every 15 min. In total, 12 fractions were collected. Each of the SFE fractions was evaporated in vacuo, weighed, and redissolved in 2 mL LC grade methylene chloride. In addition, the residual algae were taken from the extraction vessels and treated with 15 mL methanol for 2 h. The methanol extract was evaporated to dryness, then redissolved in 2 mL CH₂Cl₂ (fraction 13). Each fraction was diluted such that concentration of the total extractable materials was ca 100 μg/mL for GC/MS analysis. The effect of pressure was investigated at 300, 350, and 400 atm and 40 °C.

**Results and Discussion**

**Identification of Halogenated Monoterpenes from Plocamium cartilagineum**

Several purified compounds were needed as reference standards to quantitate the corresponding halogenated monoterpenes in the SFE extracts. Eight halogenated monoterpenes were isolated from *P. cartilagineum*, 2 from the Santa Cruz species, and 6 from San Diego collections. All of the purified monoterpenes are alicyclic compounds. The crude extracts from conventional solvent extraction of the towel-dried algae were purified by a combination of gravity
Figure 7A–B. GC/MS chromatograms of the SFE fractions from Santa Cruz algae. A) First SFE fraction; B) second SFE fraction.
Figure 7C–D. GC/MS chromatograms of the SFE fractions from Santa Cruz algae. C) Third SFE fraction; D) fourth SFE fraction.
Figure 8A–B. GC/MS chromatograms of the fractions obtained by conventional solvent extraction from Santa Cruz algae. A) First fraction; B) second fraction.
Figure 8C–D. GC/MS chromatograms of the fractions obtained by conventional solvent extraction from Santa Cruz algae. C) Third fraction; D) fourth fraction.
chromatography and LC on silica gel with hexane as the eluent. *P. cartilagineum* from Santa Cruz yielded compound 14, (3R, 4S) 3-methyl-3, 4, 8-trichloro-1, 5(E), 7(E)-octatriene-7-al, and compound 15, (3R, 4S) 7-bromochloromethyl-8-chloro-3-methyl-1, 5(E), 7(E)-octatriene.

The other 6 halogenated monoterpenes isolated from San Diego include the following: compound 2, (3R, 4S, 7S) 3, 7-dimethyl-1, 8, 8-tribromo-3, 4, 7-trichloro-1(E), 5(E)-octadiene; compound 16, (3R, 4S, 7S) 1, 8-dibromo-3, 7-dimethyl-3, 4, 7-trichloro-1(E), 5(E)-octadiene; compound 8, (3R, 4R) 7-dichloromethyl-3-methyl-3, 4, 8-trichloro-1, 5(E), 7(Z)-octatriene; compound 4, (3R, 4S) 7-dichloromethyl-3-methyl-3, 4, 8-trichloro-1, 5(E), 7(Z)-octatriene; compound 9, (3R, 4R) 1-bromo-7-dichloromethyl-3-methyl-3, 4, 8-trichloro-1(E), 5(E), 7(Z)-octatriene; compound 7, (3R, 4S) 1-bromo-7-dichloromethyl-3-methyl-3, 4, 8-trichloro-1(E), 5(E), 7(Z)-octatriene. The structures of the purified monoterpenes were confirmed by $^1$H-NMR and $^{13}$C-NMR spectroscopy by comparison with literature spectra. In addition, GC/MS was used in the identification of the purified monoterpenes.

It was observed that during conventional solvent extraction of *P. cartilagineum* from Santa Cruz, compound 14 was unstable and readily decomposed, most likely due to the presence of an aldehyde group. Compound 14 readily oxidized if left standing in air for a time, or even if stored at –4°C in a refrigerator. By comparison with the NMR data in the published reports (5), structures of 14 and 15 were confirmed. The $^{13}$C-NMR spectrum of compound 15 exhibited nearly identical values to those of Crews et al. (5), and its $^1$H-NMR spectrum also showed a characteristic vinylic ABX system ($J = 17.1, 10.6$ Hz). The $^1$H- and $^{13}$C-NMR spectra of compound 14 displayed similar proton and carbon shifts as those of monoterpene 15. The $^1$H signal at 8.9.5, which appeared as a sharp doublet ($J = 2.1$ Hz), clearly indicated the presence of an aldehyde group.

Conventional solvent extraction of *P. cartilagineum* from 2 locations in San Diego produced 6 halogenated monoterpenes, 5 of them from Casa Beach algae, and one from a collection taken at Bird Rock. The $^1$H- and $^{13}$C-NMR spectra of compounds 2 and 16 displayed similar chemical shift values to those in the literature (4, 7), given elsewhere.
Table 1. Comparison of SFEa with conventional solvent extractionb for the Santa Cruz algae

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Compound 14</th>
<th>Compound 15</th>
</tr>
</thead>
<tbody>
<tr>
<td>YSFE, µg/gc</td>
<td>1860</td>
<td>514</td>
</tr>
<tr>
<td>Yconv. extr., µg/gd</td>
<td>782</td>
<td>236</td>
</tr>
<tr>
<td>RSDSFE, %</td>
<td>37.9</td>
<td>41.3</td>
</tr>
<tr>
<td>RSPconv. extr., %</td>
<td>26</td>
<td>40.6</td>
</tr>
<tr>
<td>Recovery (YSFE/YConv.extr.)</td>
<td>238</td>
<td>218</td>
</tr>
</tbody>
</table>

a SFE was performed at 250 atm/40°C/60 min using a 1.5 mL/min flow rate with pure CO2, then the extraction was continued at 250 atm/40°C/30 min with a 1.5 mL/min flow rate with CO2 modified with 2% methanol, 30 min with CO2 modified with 5% methanol, and finally 30 min with CO2 modified with 10% methanol, with collection of fractions in each extraction time.

b Conventional solvent extraction was performed at room temperature with 15 mL hexane for 1 h, then the extraction was continued with 15 mL hexane–methanol (98 + 2, v/v) for 30 min, another 30 min with 15 mL hexane–methanol (95 + 5, v/v) and 30 min with 15 mL hexane–methanol (90 + 10, v/v). At the end, the residual algae was extracted with 15 mL methanol for 2 h.

c YSFE (µg/g) = total yield of the monoterpane obtained by SFE in 2.5 h (3 determinations).

d Yconv. extr. (µg/g) = total yield of the monoterpane obtained by conventional solvent extracts in 4.5 h (3 determinations).

e Recovery = ratio of YSFE and YConv. extr. (YSFE/YConv.extr.).

Diastereoisomer 4 had a proton shift of δ1.78 ppm (CDCl3) at Me9 and a shift of δ6.97 ppm (CDCl3) at H10 and was assigned to be (3R, 4S) 7-dichloromethyl-3-methyl-3,4,8-trichloro-1,5(E),7(Z)-octatriene.

Mass spectra of the purified monoterpenes were obtained by using GC/MS analysis. Although the mass spectra did not provide information about any molecular ions of the halogenated monoterpenes, characteristic fragment ions of these compounds could still be identified. Indeed, the mass spectra of the 8 halogenated monoterpenes from GC/MS indicated 2 types of base peaks, one at m/z = 89, 91 assigned to fragment ion I, the other at m/z = 167, 169, 171 attributed to fragment ion II.

Compounds 4, 8, 14, and 15 yielded fragment ion I, and fragment ion II was produced from compounds 2, 7, 9, and 16. Additionally, fragment ions missing 1 and 2 halogenated atoms from the parent compounds were found in the mass spectra. The NMR spectra and mass spectra of the 8 purified monoterpenes are given elsewhere (16).

Time-Dependent Extraction with CO2

Supercritical CO2 was used at 40°C/250 atm (ρ = 0.89 g/mL for CO2) with a 1.5 mL/min flow rate to extract P. cartilagineum for 3 h. The SFE extracts were collected at 15 min intervals and analyzed by GC/MS. The average concentrations (3 determinations) of compounds 14 and 15 and the total extractable material, as a function of time gives the time-dependent extraction curves as shown in Figure 4. It was found that the concentrations of the total extractable materials, and of the monoterpenes 14 and 15, were 4900 µg/g (RSD = 4.5%), 2460 µg/g (RSD = 26.8%), and 532 µg/g (RSD = 22.4%), respectively, after 3 h of extraction. However, the concentrations of compounds 14 and 15 for a 1 h extraction were 2370 µg/g (RSD = 26%) and 337 µg/g (RSD = 22.3%), and for a 2 h extraction were 2440 µg/g (RSD = 26.5%) and 458 µg/g (RSD = 22.2%). It can be concluded that 1 h of extraction with CO2 at 40°C/250 atm using a 1.5 mL/min flow rate was sufficient for the extraction of P. cartilagineum. A longer extraction time would not improve the overall extraction efficiency of SFE for the halogenated monoterpenes. Further extraction of the residual algae with methanol indicated that compound 15 was not fully extracted with supercritical CO2 in 3 h. It was found that the residual material after SFE still contained 1880 µg/g (RSD = 13.4%) of compound 15. On the other hand, none of compound 14 was detected in the methanol extract of the residual material. Thus, SFE with pure CO2 results in the incomplete extraction of some of the halogenated monoterpenes from P. cartilagineum. However, this situation was found to change when a cosolvent was used in the SFE.

Time-Dependent Extraction with CO2 Modified with 10% Methanol

The time-dependent extraction by CO2 modified with 10% methanol exhibited improved SFE yields for P. cartilagineum. The towel-dried algae were extracted with CO2–CH3OH (90 + 10, v/v) at 40°C/250 atm with a 1.5 mL/min flow rate for 3 h. The SFE extracts collected every 15 min were analyzed by GC/MS. The average concentrations (3 determinations) of
Figure 10A–B. GC/MS chromatograms of the SFE fractions from San Diego algae. A) First SFE fraction; B) second SFE fraction.
Figure 10C–D. GC/MS chromatograms of the SFE fractions from San Diego algae. C) Third SFE fraction; D) fourth SFE fraction.
Figure 11A–B. GC/MS chromatograms of the fractions obtained by conventional solvent extraction from San Diego algae. A) First fraction; B) second fraction.
Figure 11C–D. GC/MS chromatograms of the fractions obtained by conventional solvent extraction from San Diego algae. C) Third fraction; D) fourth fraction.
monoterpenes 14 and 15 and of total extractable material are plotted as a function of time as shown in Figure 5. It was found that an extraction time of 45 min was sufficient to recover over 95% of the halogenated monoterpenes 14 and 15. The concentrations of monoterpenes 14 and 15 after 45 min of extraction were 564 \( \mu g/g\) (RSD = 10.4%) and 342 \( \mu g/g\) (RSD = 36.8%), respectively, and no monoterpenes of interest were found in the remainder of the SFE fractions. Although the use of carbon dioxide with 10% cosolvent in SFE enhanced the solvation strength of supercritical CO2, we still cannot explain why the extraction yields of monoterpenes 14 and 15 were so much lower. It is possible that under these conditions many unwanted compounds (most of them pigments) were extracted that interfered with the GC/MS quantitation of the monoterpenes.

The time-dependent extraction with pure CO2 indicates that an extraction time of 60 min is sufficient for \textit{P. cartilagineum}, but does not give quantitative recoveries for all monoterpenes. The choice of CO2 modified with 10% methanol leads to a higher background, primarily due to pigments, than found with pure CO2. Additionally, the time-dependent extraction with and without a cosolvent indicates that SFE with pure CO2 selectively removed compound 14 more easily than compound 15, even though such a differentiation was not observed when 10% methanol was added.

**Effect of Pressure on SFE**

The purpose of the pressure effect studies was to determine whether the increase of pressure can enhance the solvation strength (extraction efficiency) of supercritical CO2 for the extraction of halogenated monoterpenes from \textit{P. cartilagineum}. The extraction pressure experiments were conducted at 250, 300, 350, and 400 atm with supercritical CO2 alone and \(40^\circ C/60\) min (1.5 mL/min flow rate). The yields of compounds 14 and 15 as a function of the extraction pressure are shown in Figure 6. It was found that an increase of the pressure enhances the extraction efficiency. Above 300 atm, the concentration of compound 14 in the SFE extracts is higher than that at 250 atm. By contrast, the concentration of compound 15 at 250 atm is higher than that above 300 atm. However, only a small amount of compound 15 was extracted at 250 atm of pressure. Although pressure affects the extraction efficiency of SFE, the differences in the recoveries of the compounds are not very significant in this study. It is interesting that SFE at the different pressures exhibits similar selective extraction ability for compound 14. The combined results from the time-dependent studies and the effect of pressure suggest that SFE can selectively extract halogenated monoterpenes from \textit{P. cartilagineum}.  

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**Figure 12. Comparison of SFE with conventional solvent extraction for San Diego algae.**
Table 2. Comparison of SFE\(^a\) and conventional solvent extraction\(^b\) for the San Diego algae

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Compound 2</th>
<th>Compound 7</th>
<th>Compound 8</th>
<th>Compound 4</th>
<th>Compound 9</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Y_{\text{SFE}}), (\mu g/g)(^c)</td>
<td>62.3</td>
<td>196</td>
<td>543</td>
<td>514</td>
<td>568</td>
</tr>
<tr>
<td>(Y_{\text{Conv. extr.}}, \mu g/g)(^d)</td>
<td>134</td>
<td>534</td>
<td>408</td>
<td>511</td>
<td>728</td>
</tr>
<tr>
<td>RSD(^e), %</td>
<td>89.7</td>
<td>51.4</td>
<td>30.4</td>
<td>29.5</td>
<td>52</td>
</tr>
<tr>
<td>RSD(^f), %</td>
<td>64.4</td>
<td>22.1</td>
<td>20.8</td>
<td>21.8</td>
<td>29</td>
</tr>
<tr>
<td>Recovery, %, (^g)</td>
<td>47</td>
<td>37</td>
<td>133</td>
<td>100</td>
<td>78</td>
</tr>
</tbody>
</table>

\(^a\) SFE was performed at 250 atm/40\(^\circ\)C/60 min using a 1.5 mL/min flow rate with pure \(\text{CO}_2\), then the extraction was continued at 250 atm/40\(^\circ\)C/30 min with a 1.5 mL/min flow rate with \(\text{CO}_2\) modified with 2\%, 5\%, and 10\% methanol. Because the solvation strength of supercritical \(\text{CO}_2\) is close to that of pure hexane, the gradual addition of methanol increases the solvation strength of the SFE fluid.

\(^b\) Conventional solvent extraction was performed at room temperature with 15 mL hexane for 1 h, then the extraction was continued with 15 mL hexane–methanol (98 + 2, v/v) for 30 min, another 30 min with 15 mL hexane–methanol (95 + 5, v/v) and 30 min with 15 mL hexane–methanol (90 + 10, v/v). At the end, the residual algae was extracted with 15 mL methanol for 2 h.

\(^c\) \(Y_{\text{SFE}}\) (\(\mu g/g\)) = total yield of the monoterpene obtained by SFE in 2.5 h (3 determinations).

\(^d\) \(Y_{\text{Conv. extr.}}\) (\(\mu g/g\)) = total yield of the monoterpene obtained by conventional solvent extracts in 4.5 h (3 determinations).

\(^e\) RSD = % variance.

\(^f\) Recovery = Ratio of \(Y_{\text{SFE}}\) and \(Y_{\text{Conv. extr.}}\) (\(Y_{\text{SFE}}/Y_{\text{Conv. extr.}}\)).

Comparative Analysis of SFE with Conventional Solvent Extraction

**Santa Cruz Plocamium cartilagineum**

A gradient extraction of *P. cartilagineum* was performed with pure \(\text{CO}_2\) and \(\text{CO}_2\) modified successively with 2\%, 5\%, and 10\% methanol. Because the solvation strength of supercritical \(\text{CO}_2\) is close to that of pure hexane, the gradual addition of methanol increases the solvation strength of the SFE fluid.

SFE was performed by successive extraction of the towel-dried algae mixed with the diatomaceous earth with pure \(\text{CO}_2\) at 250 atm/40\(^\circ\)C/60 min, followed by extraction at 250 atm/40\(^\circ\)C/30 min with \(\text{CO}_2\) modified with 2\%, 5\%, and 10\% methanol, respectively. The residual algae remaining after SFE were further subjected to conventional solvent extraction with methanol for 2 h to check for the presence of any compounds not removed by the processing.

The solvents and time used in the conventional extraction were chosen to be as close as possible to those in SFE, except hexane was the primary solvent instead of supercritical \(\text{CO}_2\). Figures 7A through 7D show the GC/MS chromatograms of the SFE fractions for the selective extraction with the gradient elution. The GC/MS chromatograms of the crude extracts obtained from conventional extraction are presented in Figures 8A through 8D. The compound identified in the first fraction of SFE was compound 14 (\(t_\text{R} = 14.90\) min). The monoterpenes determined in the second and third fractions of SFE were compound 14 (\(t_\text{R} = 14.90\) min) and compound 15 (\(t_\text{R} = 16.40 \pm 0.01\) min). In the final SFE fraction, compound 15 (\(t_\text{R} = 16.39\) min) was found while no compound 14 was observed. The GC/MS chromatograms of the conventional solvent extraction gave similar characteristic peaks as those of SFE. The concentrations of monoterpenes 14 and 15 found in each fraction from SFE and conventional solvent extraction are summarized in Figure 9.

The selective extraction of compound 14 is apparent in the first SFE fraction; a similar result also occurs in the first hour of hexane extraction. However, the yield of compound 14 in SFE reaches 1630 \(\mu g/g\) (RSD = 30\%), and is significantly higher than the yield from conventional extraction (560 \(\mu g/g\), RSD = 30\%) by comparing both methods in the first hour.

It was found that the recoveries of 14 and 15 using SFE reached 238 and 218\% of the same monoterpenes obtained from conventional solvent extraction. Hence, it was concluded that SFE exhibited a significantly higher yield of specific halogenated monoterpenes from Santa Cruz *P. cartilagineum* than conventional solvent extraction.

**San Diego Plocamium cartilagineum**

*Plocamium cartilagineum* from Casa Beach (San Diego, CA) was used to perform the comparative studies of SFE and conventional solvent extraction. Five of the purified halogenated monoterpenes obtained from the algae (compounds 2, 4, 7, 8, and 9) were chosen as the external standards to determine the extraction efficiency of SFE. Compound 16 from *P. cartilagineum* collected at Bird Rock was not found in the SFE extracts and the conventional solvent extracts of the algae from Casa Beach. Thus, it was not used in this comparison.

The San Diego algae were treated exactly the same as the Santa Cruz algae in the SFE and conventional solvent extraction. Figures 10A through 10D show the GC/MS chromatograms of the 4 SFE fractions. Information about the various fractions of conventional solvent extraction is presented in Figures 11A through 11D. The compounds identified in Figures 10A through 10D from the SFE fractions in-
clude 2 (t<sub>r</sub> 21.85 min), 7 (t<sub>R</sub> 20.72 min ± 0.04), 8 (t<sub>R</sub> 17.69 min), 4 (t<sub>R</sub> 17.82 min), and 9 (t<sub>R</sub> 20.18 min). These 5 monoterpenes were observed in both the GC/MS profiles of the conventional solvent extracts and the SFE extracts. The peaks in the GC/MS chromatograms of SFE and conventional solvent extraction appear to be similar. However, the intensity of individual peaks differs slightly.

The concentrations of the monoterpenes 2, 4, 7, 8, and 9 found in each fraction of SFE and conventional solvent extraction of San Diego algae are summarized in Figure 12. It was found that, unlike the result of Santa Cruz P. cartilagineum, the extractions of the halogenated monoterpenes with SFE from San Diego algae are not selective. A further comparison of the recovery in SFE with conventional solvent extraction indicated the different extraction abilities. Table 2 lists the average yields (3 determinations) of monoterpenes 2, 4, 7, 8, and 9 obtained by adding the concentrations of the corresponding monoterpenes found in each fraction of SFE and conventional solvent extraction. The SFE recoveries (3 determinations) gave 46.6% for 2, 36.7% for 7, 133% for 4, 100% for 8, and 78% for 9, relative to the conventional extraction. The yields of some halogenated monoterpenes from SFE are higher than the yields from conventional solvent extraction. In addition, the extraction time using SFE is nearly completed in 2.5 h, compared with 4.5 h to reach the same point using conventional solvent extraction. Hence, SFE provides better overall extraction performance—faster extraction time, less use of organic solvents, cleaner extractions, and higher extraction efficiency—than conventional solvent extraction.

**References**