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**SUPERCRITICAL FLUID EXTRACTION OF MONOTERPENES FROM THE
RED ALGA *PLOCAMIMUM CARTILAGINEUM***

A Thesis

Presented to

The Faculty of the Department of Chemistry

San Jose State University

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

by

Donghui Gao

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ABSTRACT

SUPERCRITICAL FLUID EXTRACTION OF MONOTERPENES FROM THE RED ALGA *PLOCAMIUM CARTILAGINEUM*

by Donghui Gao

Supercritical fluid extraction (SFE) was evaluated using the marine red alga *Plocamium cartilagineum*, which is known to contain complex mixtures of halogenated monoterpenes. *Plocamium cartilagineum* from Davenport (north of Santa Cruz) was chosen for the development of a protocol for the application of SFE, including the time-dependent extraction with carbon dioxide and modified carbon dioxide containing up to 10% methanol, and the effect of pressure on SFE. The protocol developed for the extraction of halogenated monoterpenes from the alga was then used to extract two different samples of *Plocamium cartilagineum*: one from Davenport (Santa Cruz) and the other from Casa Beach (San Diego). Furthermore, SFE has been compared with conventional solvent extraction and workup in terms of yields and selectivity. Several halogenated monoterpenes isolated by conventional solvent extraction and purified by column chromatography are used as the standard compounds for the determination of the extraction efficiency in the SFE studies.

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CHAPTER 1

INTRODUCTION

A. Natural Product and Sample Preparation Techniques

Natural products are a large group of organic compounds that are produced by living organisms. They have been utilized by man to produce perfumes, fragrances, and drugs since before recorded time. For example, one of the newest weapons against cancer is the novel natural product Taxol™ isolated from the bark of the Pacific yew tree (*Taxus brevifolia*). It has been approved by the FDA as a potent antitumor agent for the treatment of metastatic breast and refractory ovarian cancers (1). One of its analogues, baccatin III, is separated from the needles of the European yew (*Taxus baccata*), and has been used to synthesize taxol and its analogues in large scale. However, the extraction and isolation of natural products from the biological source are time-intensive processes. A number of different sample preparation techniques that are dependent on the physical-chemical properties of each sample are used today, including solvent extraction, steam distillation, and supercritical fluid extraction.

Conventional liquid-solid extraction is the oldest sample preparation technique for the isolation of natural products. It requires large volumes of organic solvents to complete the extraction in several hours to several days and the high-volume of dilute extract must then be concentrated before the constituents can be further purified and studied. Thus, conventional solvent extraction is a time- and labor- intensive process. In

addition, the high cost of organic solvents adds a considerable expense to this extraction method. Solvent extractions are nonselective, often resulting in highly complex mixtures that must be purified to yield individual compounds for further study.

Steam distillation is another widely used sample preparation method for natural products that is suitable for the extraction of volatile compounds. The use of steam distillation can directly produce certain compounds of interest without the accompaniment of a high-volume, dilute solution. However, thermally labile compounds and nonvolatile compounds cannot be extracted with steam distillation. The problems associated with conventional solvent extraction and steam distillation have stimulated natural products chemists to search for alternative extraction methods.

Supercritical fluid extraction (SFE) is an emerging alternative technique for the extraction of natural products. SFE is a sample preparation method in which a supercritical fluid (SCF) acts as a solvent to remove analytes from the sample matrices. In 1971, Zosel reported the use of supercritical fluid extraction for the decaffeination of green coffee (2). However, the application of SFE developed slowly in the following 10 years. In 1981, the use of SFE in the extraction of hops, coffee beans, and spices was published by Williams (3). Since then, the number of reports on the application of SFE has steadily increased. To date, SFE has gradually substituted the conventional solvent extraction and has become an attractive sample preparation technique for environmental, food, polymer, and natural products.

B. Theory and Principle of Supercritical Fluid Extraction

1. Physical State of Supercritical Fluid:

SFE uses a supercritical fluid (SCF) as the solvent to extract the sample matrices. The SCF is neither a gas nor a liquid. The physical state of a typical SCF can be illustrated by the pressure-temperature (PT) phase diagram of carbon dioxide, as shown in Figure 1 (4). In this PT-phase diagram, there are two lines and one curve defining melting, sublimation, and boiling processes, respectively. The triple point represents the equilibrium among the three phases: solid, liquid, and gas. The boiling curve starts at the triple point (TP) and has an upper limit at the critical point (CP). Along the boiling curve, if the pressure above a liquid is increased, the temperature at which the liquid evaporates to a gas is also raised. Above the critical point, the physical property of the substance changes, and a new phase exists, which is called a supercritical fluid. Therefore, the SCF is obtained from either a gas or a liquid by raising the temperature and pressure above the critical point.

Supercritical fluids possess both gas-like and liquid-like physicochemical properties (5). The ranges of solvation strengths of available SCFs are close to those of organic solvents, and thus SCFs can dissolve many classes of chemical compounds. The solvation strengths of SCFs are highly dependent on temperature and pressure, as shown in Figure 2 (6). For example, the solubility of naphthalene in supercritical CO₂ at 100 bar of pressure drops with an increase in temperature, but solubility above 150 bar of pressure grows with an increase in temperature. Furthermore, the diffusion coefficients of supercritical fluids

Figure 1. Phase Diagram of Supercritical Fluid CO₂ (Based on *Ref. 4*).

approach those of gases so that SCFs can carry the dissolved solutes through materials very quickly and efficiently. In fact, many solutes in SCFs exhibit coefficients approximately 10 times greater than those found in organic solvents (7). SCFs also display lower viscosity than organic solvents, which allows them to penetrate easily through materials. Hence, as a result of the unique properties of the SCF to extract chemical compounds, SFE is an attractive technique for application in the field of natural products.

Figure 2. Solubility (mole fraction) of Naphthalene in Supercritical CO₂ as a Function of Temperature and Pressure (Based on Ref. 6).

2. Choice of Supercritical Fluid:

Most applications of SFE systems choose supercritical CO₂ due to its low critical temperature (31.3°C) and pressure (73.0 bar). Although some pure supercritical fluids have better solvation strengths than CO₂, unsatisfactory critical parameters (associated with temperature and pressure), toxicity, or chemical properties reduce their usefulness in SFE systems. Table 1 (8) lists the critical temperatures, pressures, and densities for some supercritical fluids. Supercritical H₂O and methanol can be considered as powerful SCFs due to their extremely polar properties, which can dissolve a large range of compounds, but the requirement of high pressure and high temperature limits their practical application in SFE. From a safety standpoint, high pressure or high temperature SCFs require an apparatus that is very expensive and potentially unsafe (9). High temperature SCFs are also incompatible with thermally labile compounds. Supercritical NH₃, N₂O, and NO₂ are polar SCFs and display low critical pressures and temperatures, but they exhibit acute toxicity and reactivity which limit their application in SFE (10). For example, supercritical NH₃ requires that components of the SFE system that come in contact with supercritical NH₃, must be plated with gold metal to prevent dissolution by NH₃. N₂O has the potential of violent explosion if mixed with some organic compounds. Alkanes and alkenes of low molecular weight (3 carbons or less) are non-polar SCFs, which are similar to CO₂, and have low critical temperature and pressure. However, they are very flammable and not practical fluids for SFE system. Furthermore, supercritical alkanes and alkenes of high molecular weight (4 carbons and above) exhibit high critical temperature and pressure,

Table 1. Critical Temperatures, Pressures, and Densities of Various Fluids (Based on *Ref. 8*).

Fluid	Critical Temperature (°C)	Critical Pressure (bar)	Critical Density (g/mL)
CO ₂	31.1	73.0	0.47
N ₂ O	36.5	70.6	0.45
NO ₂	158	98.7	0.27
NH ₃	132.5	109.8	0.23
H ₂ O	374.2	214.8	0.32
CH ₃ OH	239	78.9	0.27
C ₂ H ₅ OH	243.4	72.0	0.276
CH ₄	-82	46.0	0.169
C ₂ H ₆	32.3	47.6	0.2
C ₃ H ₈	96.7	42.4	0.22
C ₂ H ₄	11	50.5	0.2
C ₃ H ₆	92	45.4	0.22

which also limit their application in the SFE system. Supercritical CO₂ has low critical pressure and temperature and can be easily delivered by standard HPLC pumping systems. Although CO₂ exhibits a solvation strength similar to a hydrocarbon solvent (e.g., hexane), the temporarily induced-dipole moment allows it to dissolve moderately polar compounds. Consequently, carbon dioxide is considered as the most popular and safest supercritical

fluid for SFE systems. In addition, supercritical CO₂ has the benefit of being nontoxic, nonflammable, environmentally “friendly”, and relatively inexpensive (11). For simplicity, the remaining discussion will focus only on supercritical CO₂.

3. Modified Supercritical Fluid in SFE:

Supercritical CO₂ is the most commonly used SCF for SFE systems. The solvation strength of supercritical CO₂ is proportional to its density, which can be controlled by adjusting the pressure and temperature. Table 2 (12) displays the density ranges of supercritical CO₂ at various temperatures and pressures. Compared with polar organic solvents such as water and methanol, supercritical CO₂ still shows lower polarity and cannot extract most highly polar compounds. However, the extraction ability of supercritical CO₂ is enhanced by the addition of a cosolvent, or a modifier, such as methanol, ethanol, acetonitrile, or any other polar organic solvents. The cosolvent modifies the polarity of CO₂ and improves the solvation strength of supercritical CO₂. Methanol, which is miscible with CO₂, is a common cosolvent used in SFE. The addition of the cosolvent only minimally changes the diffusion coefficient of supercritical CO₂. Therefore, the use of the cosolvent not only enhances the extraction ability of CO₂, but also improves the application range of SFE to polar samples.

Table 2. Density, Temperature, and Pressure Relationship for CO₂ (Based on *Ref. 12*).

Density (g/mL)	40°C	50°C	60°C	70°C	80°C	90°C	100°C
1.000	526 bar	618 bar					
0.95	383 bar	463 bar	544 bar	644 bar	680 bar		
0.90	281 bar	350 bar	420 bar	489 bar	518 bar		
0.85	211 bar	269 bar	329 bar	401 bar	447 bar		
0.80	164 bar	213 bar	264 bar	314 bar	365 bar	416 bar	467 bar
0.75	134 bar	175 bar	218 bar	261 bar	305 bar	348 bar	392 bar
0.70	115 bar	150 bar	187 bar	223 bar	260 bar	297 bar	334 bar
0.65	104 bar	133 bar	165 bar	196 bar	227 bar	259 bar	290 bar
0.60	97 bar	122 bar	149 bar	176 bar	203 bar	229 bar	256 bar
0.55	93 bar	115 bar	138 bar	161 bar	183 bar	206 bar	230 bar
0.50	91 bar	109 bar	129 bar	148 bar	168 bar	188 bar	207 bar
0.45	89 bar	104 bar	122 bar	138 bar	155 bar	172 bar	188 bar
0.40	87 bar	100 bar	115 bar	129 bar	143 bar	157 bar	171 bar

4. Supercritical Fluid Extraction Instrumentation:

The basic configuration of a SFE system is shown in Figure 3. The major components include a high pressure pumping system, an extraction vessel, an oven, and a collection device (13). The sample matrix is put into the extraction vessel of the SFE system, and then the supercritical fluid under specific temperature and pressure passes through the samples and removes the soluble compounds. This “working” supercritical fluid carrying the extracted analytes is trapped in the collection device containing a liquid

solvent, where the supercritical fluid is decompressed and vented to the atmosphere so that only the extracted compounds are left in the liquid solvent of the collection device. The extraction process can be performed in a static or dynamic mode. A static extraction is performed by recycling the supercritical fluid through the sample matrix several times before the extraction is completed. In a dynamic extraction, fresh supercritical fluid is continually passed through the sample matrix and carries the analytes to the collection device. If a cosolvent is used, two pumps are combined together to deliver supercritical fluid, with one pump for neat supercritical CO₂ and the other one for a cosolvent (e.g., methanol). The oven is designed to control the temperature of the extraction vessel, and hence solvation strength of the supercritical fluid is under control. Additionally, SFE can be run in on-line and off-line modes. In the off-line mode, the extracts are collected for further separation and analysis. The off-line SFE mode is also used to prepare large scale extracts. In the on-line mode, a collection device is connected to an analytical detector, such as GC, MS, UV/Vis or IR instrument, and directly yields structural information on the extracts (14).

Compared with conventional solvent extraction, SFE offers higher efficiency, shorter extraction time, and less usage of organic solvents. The use of SFE to extract thermally labile compounds and air-sensitive compounds, which are easily decomposed under conventional solvent extraction, represents a significant advantage of SFE. Modern SFE instruments provide the chemist a significant range of extraction parameters, which may be modified to suit each individual sample.

Figure 3. Basic Configuration of Supercritical Fluid Extraction System.

5. Applications of SFE:

In recent years, the range of applications in SFE has increased rapidly and has been extended successfully and extensively for extraction of environmental, food, polymer, and natural product samples. In environmental analysis, SFE has been widely used to study the extraction of petroleum hydrocarbons, PCBs, PAHs, organochlorine pesticides, organotin compounds, and aromatic amines from soils, sediments, and similar samples (15). The extraction of cholesterol from butter and the fractionation of vegetable and cotton seed oils are among the successful applications of SFE in the food industry (16). In the chemical synthesis of polymers, SFE has been used to remove residual monomers, solvents, and oligomeric materials, and leave behind the purified materials (17). The most common applications of SFE are to extract the natural products from biological matrices, such as steroids, alkaloids, terpenes, and flavors.

In the practical application of SFE, it is necessary to consider not only the characteristics of the SCF but also the nature of analytes. Although a change of temperature, pressure, extraction time, and flow rate affects the extraction efficiency, the nature of the samples also plays an important role in real-world extraction with SFE. Analytical SFE is mainly applied to solid, dry samples and is generally not suitable for aqueous samples. Water in the sample can interfere with the performance of SFE (18). For example, high water content in the matrix prevents the penetration of supercritical CO₂ through the samples, so that the analytes may not be extracted to any appreciable extent. In addition, water in the extract can freeze at the outlet of the flow restrictor line

that connects to the collection device. It is thus very important to reduce the presence of water in an SFE system when carbon dioxide is acting as the SCF. For a sample with high water content, water is generally removed before it is subjected to SFE. Usually the sample is dried in air, an oven, or microwave, as well as freeze dried. However, such techniques can also cause the loss of volatile and semi-volatile analytes. In some cases, a small amount of water existing in the sample matrix can act as a modifier to improve the extraction efficiency of supercritical CO₂. In order to reduce the effect of water and increase the extraction efficiency of SFE, desiccants, or drying agents, are used to absorb and bind water from the samples. Desiccants used in SFE include sodium sulfate, calcium chloride, molecular sieves, silica, and Hydromatrix™ (diatomaceous earth). The use of desiccants not only decreases the possibility of loss of volatile and semi-volatile compounds but also extends the application range of SFE to "wet" sample matrices, i.e. not subjected to any rigorous dehydration steps.

C. Marine Red Alga *Plocamium cartilagineum*

A wide variety of unique natural products have been derived from marine plants and animals. Many of these marine secondary metabolites are rich in halogen atoms (chlorine, bromine, and iodine) which are rarely found in natural products from terrestrial organisms. Presumably, the marine organisms gain some advantage by absorbing these elements from seawater and utilizing them in natural product biosynthesis.

Table 3. Taxonomy of *Plocamium cartilagineum* (Based on Ref. 19).

Division — Rhodophyta
Class — Florideophyceae
Order — Gigartinales
Family — Plocamiaceae
Genera — <i>Plocamium</i> <i>Plocamiacolax</i>
Species — <i>Plocamium cartilagineum</i> (Dixon) <i>Plocamium violaceum</i> (Farlow) <i>Plocamium oregonum</i> (Doty) <i>Plocamiocolax pulvinata</i> (Setchell)

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. The complete classification of Plocamiaceae is shown in Table 3 (19). *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 molluscicidal activity.

The first two halogenated monoterpenes (structure 1 and 2), reported by Faulkner and Stallard (20) and Faulkner *et al* (21) in 1973, were isolated from the digestive gland of the sea hare, *Aplysia californica*.

Faulkner confirmed structure 2 by X-ray diffraction analysis. Subsequently, a more detailed study showed that *P. cartilagineum* was the dietary source of these two 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 4 (22). In fact, all of these halogenated monoterpenes from *Plocamium cartilagineum* can be classified into three major structure classes: one alicyclic type and two monocyclic types.

The monoterpenes 2–13 were isolated from *Plocamium cartilagineum* collected at Bird Rock (La Jolla, San Diego) (23). Crews and Kho (24) and Crews *et al* (25) discovered two halogenated monoterpenes 14–15 from the algae collected at Davenport (north of Santa Cruz). *P. cartilagineum* from the Antarctic produces structures 16–19 (26). Interestingly, the halogenated monoterpenes 20–30 (27) isolated from New Zealand *P. cartilagineum* differed from sample to sample. The compounds 20 and 21 were

isolated from one sample of this red alga, but others, 22–30, were obtained from a different collection. The monoterpene 31, found to have antifungal and antibacterial activities, was obtained from *P. cartilagineum* collected along the northern Spanish Atlantic coast (28). Recently, Abreu and Galindro (29) isolated two new monoterpenes 32–33 from *Plocamium cartilagineum* along the Portuguese coast. Additionally, the monocyclic halogenated monoterpenes 34–50 were reported from *P. cartilagineum* by several research groups (30, 31, 32, 33, 34).

In all prior studies, the extraction of these halogenated monoterpenes from *Plocamium cartilagineum* was performed with a Soxhlet apparatus or conventional solvent extraction. The extraction process required a large volume of organic solvents to complete the extraction in 3–4 days. This was followed by extensive column and high performance liquid chromatography 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. Supercritical fluid extraction is an attractive, alternative method to conventional solvent extraction, since it is suitable for thermally labile and air-sensitive compounds. Hence, the development of SFE for the extraction of halogenated monoterpenes offers another method to study *Plocamium cartilagineum*.

Figure 4. Halogenated Monoterpenes Isolated from the Red Alga *Plocamium cartilagineum*.

Note: All the structures are based on *Ref. 22*, except for structures 32-33 that are based on *Ref. 29*.

Figure 4. Halogenated Monoterpenes Isolated from the Red Alga *Plocamium cartilagineum* (continued).

D. Research Objective

Although SFE has been successfully applied for the extraction of natural products, relatively few reports of the application of SFE with marine natural products have been published. The purpose of this research is to determine the feasibility of using SFE to extract halogenated monoterpenes from *Plocamium cartilagineum*. The choice of *Plocamium cartilagineum* lies in its availability and convenience to collect on a large scale. Additionally, *P. cartilagineum* contains structurally similar halogenated monoterpenes, which are mainly lipophilic compounds and are readily dissolved in supercritical CO₂. Therefore, a specific objective was to develop a supercritical fluid extraction protocol for the extraction of the halogenated monoterpenes from the marine alga *Plocamium cartilagineum*.

CHAPTER 2

EXPERIMENTAL

A. Collection of Marine Alga *Plocamium cartilagineum*

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

B. Chemicals and Supplies

All solvents used in this study, including methanol, methylene chloride, ethyl acetate, hexane, and acetone, were HPLC grade or Optima™ grade and purchased from Fisher Scientific (Fair Lawn, NJ). CDCl₃ and C₆D₆ solvents containing TMS as an internal standard for NMR were purchased from Aldrich Chemical Company (Milwaukee, WI). Hydromatrix™, composed of diatomaceous earth, was purchased from Varian, Inc (Palo Alto, CA). SFE/SFC grade carbon dioxide (total hydrocarbon contamination less 0.1 ppb) was obtained from Air Products (Allentown, PA). All other chemicals were reagent grade and purchased from Fisher Scientific.

C. TLC Visualization

The Silica gel (F₂₅₄) TLC plate (20 cm × 20 cm × 250 μm layer) on aluminum was purchased from Whatman Ltd. (Germany). Unless otherwise noted, TLC plates were developed in mixtures of hexane and ethyl acetate. Once developed, UV at 254 nm was first used to visualize UV-sensitive monoterpenes, then heated on a hot plate. The plate was sprayed with a reagent consisting of a solution of 1 g vanillin dissolved with glacial CH₃COOH: CH₃OH: 18 M concentrated H₂SO₄ (15: 80: 5, v/v).

D. Instrumentation

1. High Performance Liquid Chromatography (HPLC)

HPLC was performed on a Rainin HPLX solvent delivery system fitted with an Alltech Econosil silica column (250 mm × 4.6 mm ID × 10 μm) and a Rainin Dynamax RI-1 refractive index (RI) detector. HPLC separation was performed at room temperature, with the corresponding mobile phase as the reference solvent for the RI detector. The injection volume was 10 μL at a flow rate of 0.7 mL/min. Prior to analysis, the refractive index detector was equilibrated using the elution solvent.

2. Nuclear Magnetic Resonance (NMR) Spectrometry

The NMR spectra were recorded on a General Electric QE-300 plus NMR spectrometer operating at 300.152 MHz for ¹H NMR and 75.038 MHz for ¹³C NMR at 20°C (TMS = 0 as the reference peak).

3. 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/Windows95 ChemStation (Hewlett-Packard, Palo Alto, CA) and equipped with a Hewlett-Packard 5973A autoinjector. For most of the injections, samples were introduced *via* a 30 m length $\times$ 0.25 mm ID $\times$ 0.25 μ m film thickness PTE-5 fused-silica open-tubular column (Supelco, Bellefonte, PA) with helium as a carrier gas at a flow rate of *ca* 1 mL/min. However, the samples from San Diego *Plocamium cartilagineum* were introduced *via* a 30 m length $\times$ 0.25 mm ID $\times$ 0.25 μ m film thickness DB-5 fused-silica open-tubular column (J&W Scientific, Folsom, CA). PTE-5 and DB-5 are equivalent columns since 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 μ L and the injector temperature was 250°C. The injector was set to the splitless mode for 1 min after injection. The electron energy was set at 70 eV, and the electron multiplier voltage was between 2274 to 2356 V. Spectral data were acquired at a rate of 0.50 s/scan (scanning range was 35 to 550 amu). The instrument was tuned daily with decafluorotriphenyl phosphine introduced *via* the GC inlet.

4. Supercritical Fluid Extraction (SFE)

An ISCO SFE system consisting of two 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 of towel-dried *Plocamium cartilagineum* mixed with an equal amount of Hydromatrix™.

E. Conventional Solvent Extraction and Purification Procedures

The isolation and identification of some halogenated monoterpenes from *Plocamium cartilagineum* are presented herein. *P. cartilagineum* was collected from three locations: Davenport (north of Santa Cruz), Casa Beach (San Diego), and Bird Rock (San Diego). Two compounds were isolated from the Davenport algae, five compounds from the Casa Beach algae, and one compound from the Bird Rock algae.

1. Purification and Extraction of Santa Cruz *Plocamium cartilagineum*

Frozen *Plocamium cartilagineum* (Davenport, Santa Cruz) was thawed, dried by pressing between paper towels, and weighed. For conventional extractions, 353 g of towel-dried algae was extracted by soaking with HPLC grade methanol (800 mL for each time) four times (24 hours for each time) at room temperature. After each extraction, the

methanol layer was filtered and fresh methanol was added to the beaker. The filtered methanol solution was evaporated *in vacuo* using a Buchi rotary evaporator to obtain a sticky residue that was partitioned with 100 mL of ethyl acetate in a 250 mL separator funnel. The EtOAc fraction was separated from the aqueous layer and the aqueous layer was extracted with 20 mL of ethyl acetate twice. Then, the combined EtOAc fractions were dried with 2 g of anhydrous sodium sulfate for 15 min, filtered, and evaporated to dryness *in vacuo*. The crude extracts were combined and stored frozen at -8°C for future chromatography. Conventional solvent extraction of 353 g towel-dried *Plocamium cartilagineum* produced 3.68 g of a dark-green oil.

A portion of the crude extract (387.3 mg) was applied to a 18 mm ID $\times$ 400 mm length silica gel column (Whatman, 13–24 μm size) and eluted with 400 mL of hexane followed by 300 mL of hexane: EtOAc (95: 5, v/v). Thirteen fractions were collected and monitored by silica TLC plates that were developed with 100 mL of hexane: EtOAc (98: 2, v/v). The detailed purification flow protocol is shown in Scheme 1. Fraction 1 ($R_f = 0.70$), fraction 2 ($R_f = 0.66$), fraction 3 ($R_f = 0.63$), fraction 4 ($R_f = 0.60$), fraction 5 ($R_f = 0.56$), and fraction 6 ($R_f = 0.53$) contained a small amount of mixtures (0.4–5.7 mg) which were too complex and of insufficient mass for further purification. Fraction 8 ($R_f = 0.44$), fraction 9 ($R_f = 0.40$), fraction 10 ($R_f = 0.36$), fraction 11 ($R_f = 0.32$), and fraction 13 ($R_f = 0.24$) were also difficult to separate due to the complexity of the mixtures. A portion of fraction 7 (33.1 mg, $R_f = 0.48$) was purified by silica gel HPLC (hexane as the mobile phase), and yielded 17.6 mg of pure compound 15. Fraction 12 (24.8 mg, $R_f = 0.28$) was

separated by silica gel HPLC in which hexane: EtOAc (97: 3, v/v) was chosen as the mobile phase, and 11.2 mg of compound 14 was purified.

2. Purification and Extraction of San Diego *Plocamium cartilagineum*

2.1 *Plocamium cartilagineum* from Casa Beach in San Diego

Frozen *Plocamium cartilagineum* was thawed and dried with paper towels. The towel-dried algae (121.5 g) was placed into a 500 mL beaker and was extracted with HPLC-grade methanol (4 × 250 mL) over 3 days. After each extraction, the supernatant was filtered and fresh methanol was added to the beaker. The filtered supernatant was evaporated to produce an aqueous suspension that was partitioned with 80 mL of ethyl acetate. The EtOAc fraction was separated from the aqueous layer, dried with 1 g of anhydrous sodium sulfate for 15 min, and evaporated to dryness *in vacuo*. The combined crude extracts were stored frozen at -8°C. Conventional solvent extraction of 121.5 g of towel-dried algae yielded 1.01 g of a dark-green oil.

A portion of the crude extracts (678.8 mg) were chromatographed on a 18 mm ID × 380 mm length column packed with silica gel (Whatman, 13–14 µm size) and eluted with 300mL of hexane followed by 100 mL of hexane: EtOAc (50: 50, v/v). The purification protocol is presented in Scheme 2. A total of 6 fractions were collected according to silica TLC analysis in which hexane: EtOAc (98: 2, v/v) was chosen as the developing solvent. Fraction 1 ($R_f = 0.70$), fraction 5 ($R_f = 0.23$), and fraction 6 ($R_f = 0.18$) were difficult to purify due to the complexity of the mixtures. Fraction 4 (28.4 mg,

$R_f = 0.32$) was purified by silica gel HPLC (hexane as the mobile phase) and produced 17.0 mg of pure compound 2. In the same manner, 23.6 mg of fraction 3 ($R_f = 0.38$) yielded 9.8 mg of pure 7 by silica gel HPLC. A portion of fraction 2 (27.1 mg, $R_f = 0.47$) was separated and yielded pure 8 (2.0 mg), 4 (2.0 mg), and 9 (6.0 mg).

2.2 *Plocamium cartilagineum* from Bird Rock in San Diego

In this research, *P. cartilagineum* from Casa Beach in San Diego and Davenport in Santa Cruz was chosen as the primary subjects for most of the experiments. However, the separation of monoterpene 16 from Bird Rock in San Diego is briefly reported herein. The purification flow protocol is displayed in Scheme 3.

Scheme 1. Purification Scheme of Halogenated Monoterpenes from Santa Cruz *Plocamium cartilagineum*.

Scheme 1. Purification Scheme of Halogenated Monoterpenes (Continued).

Scheme 2. Purification Scheme of Halogenated Monoterpenes from Casa Beach (San Diego) *Plocamium cartilagineum*.

Scheme 2. Purification Scheme of Halogenated Monoterpenes (Continued).

Scheme 3. Purification of Halogenated Monoterpenes from Bird Rock (San Diego) *Plocamium cartilagineum*.

Scheme 3. Purification Scheme of Halogenated Monoterpenes (Continued).

F. Supercritical Fluid Extraction Studies

The *Plocamium cartilagineum* samples used in all SFE studies were thawed, dried by paper towel, and mixed with the inert Hydromatrix™ (diatomaceous earth) before extraction by SFE, except as stated otherwise. *Plocamium cartilagineum* from Bird Rock (La Jolla, San Diego) was chosen as the primary subject for the preliminary studies.

1. Preliminary Studies to Establish the SFE Conditions

1.1 Time-Dependence of SFE

The towel-dried algae (4.8 g) mixed with 5.2 g of diatomaceous earth 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 hours 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 HPLC-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).

1.2 Temperature-Dependence of SFE

In this study, the temperature of the SFE system was tested at 40°C, 60°C, 80°C and 100°C. For all experiments, 4.8 g of towel-dried *P. cartilagineum* mixed with an

equal amount of the diatomaceous earth 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 hours, and the SFE fractions were collected every 60 min. Each fraction was evaporated *in vacuo*, weighed, and diluted with 2 mL of methylene chloride. Three SFE fractions produced 9.1 mg (fraction 1), 2.4 mg (fraction 2), and 1.3 mg (fraction 3) of extractable residues respectively.

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 with GC/MS.

2. Comparative Time-Dependent Extraction of SFE with and without 10% Methanol

Plocamium cartilagineum from Davenport in Santa Cruz County was chosen as the subject for the studies of time-dependent extraction and the effect of pressure (discussed in the next section). The time-dependent studies were used to determine the optimum extraction time for the SFE process. SFE with carbon dioxide with and without 10% methanol was used to determine the extraction efficiency with a cosolvent. Experiments under the same conditions of SFE in the time-dependent extraction were performed in triplicate to determine the reproducibility.

2.1 Time-Dependent Extraction with Supercritical CO₂

In a typical extraction, 4 g of towel-dried algae was 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 CO₂ at 250 atm/40°C with a 1.5 mL/min flow rate and were continued under the same conditions for 3 hours. The SFE fractions were collected every 15 min. In total, 12 fractions of the SFE extracts were collected. Each of the SFE fractions was evaporated *in vacuo*, weighed, and diluted with 2 mL of HPLC grade methylene chloride. Additionally, the residual algae was taken from the extraction vessels and treated with 15 mL of methanol for 2 hours. The methanol extract was evaporated to dryness, then dissolved to a volume of 2 mL in CH₂Cl₂ (fraction 13). Each fraction was diluted such that concentration of the total extractable materials was approximately 100 µg/mL for GC/MS analysis. Tables 4, 5, and 6 summarize the data from three replicate experiments.

2.2 Time-Dependent Extraction with Supercritical CO₂ Modified with 10% Methanol

A typical time-dependent extraction with 10% cosolvent was performed by placing 2 g of towel-dried algae with an equal amount of Hydromatrix™ into one 10 mL extraction vessel. The extractions were performed with CO₂ modified with 10% methanol at 250 atm/40°C using a 1.5 mL/min flow rate for 3 hours with collection of the SFE fractions every 15 min. A total of 12 fractions were collected. Each fraction was

evaporated *in vacuo*, weighed, and diluted with 2 mL of HPLC grade methylene chloride. The residual algae in the SFE system was removed from the extraction vessels, and extracted with 15 mL of methanol for 2 hours. The methanol extract was then evaporated to dryness and dissolved to a 2 mL solution in CH₂Cl₂ (fraction 13). The concentration in each fraction was adjusted so that the total extractable materials were approximately 100- μ g/mL for GC/MS analysis. Tables 7–9 summarize the data from three replicate experiments.

3. Effect of Pressure on SFE

In this study, the extractions were performed with CO₂ at different pressures to determine the extraction efficiency of SFE for *Plocamium cartilagineum*. SFE was tested at 250 atm, 300 atm, 350 atm, and 400 atm of supercritical CO₂. Each experiment was conducted three times under the same conditions. A typical extraction was performed by placing 2 g of towel-dried algae and an equal amount of the HydromatrixTM into a 10 mL extraction vessel. The extraction was performed with CO₂ at 250 atm/40°C/60 min using a 1.5 mL/min flow rate. A vial filled with 6 mL methylene chloride was used to collect the SFE extracts. After 60 min, the collection vial was removed from the SFE system and the extract was concentrated to 1 mL in CH₂Cl₂, then transferred to a 2 mL GC/MS vial. In a similar manner, SFE was performed under 300 atm, 350 atm, and 400 atm at 40°C using a 1.5 mL/min flow rate for one hour. A total of 12 SFE solutions were obtained and each was subjected to GC/MS analysis as shown in Table 10.

Table 4. Time-Dependent Extraction of Santa Cruz *Plocamium cartilagineum*^a with Pure CO₂ (SC-A).

Fraction	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	A11	A12	A13 ^b
T (min)	15	15	15	15	15	15	15	15	15	15	15	15	120°
W (mg)	7.7	3.6	2.1	2.2	1	0.7	0.6	0.5	0.1	0.5	0.2	0.4	24.3 ^d
V _{extract} (mL)	2	2	2	2	2	2	2	2	1	2	2	2	2
V ₁ (μL)	30	60	100	90	200	300	340	400	1000	400	1000	500	10
DF	33.33	16.67	10	11.11	5	3.33	2.94	2.5	1	2.5	1	2	100
C (μg/mL)	115.5	108	105	99	100	105	102	100	100	100	100	100	121.5

Note: Towel-dried algae (4.2 g) was extracted under 250 atm/40°C at 1.5 mL/min flow rate for 3 hours with collection of the SFE fractions every 15 min.

^a – *Plocamium cartilagineum* from Davenport (north of Santa Cruz);

^b – one fraction of the extracts obtained from the conventional solvent extraction of the residual algae after SFE;

T – Extraction Time of SFE, (° – Extraction Time of Conventional Solvent Extraction);

W – Weight of the SFE extracts after evaporation, (^d – Weight of the Conventional Solvent Extracts);

V_{extract} – Volume of CH₂Cl₂ for dilution of the extracts (mL);

V₁ – Volume of the extracts taken from V_{extract} and diluted to 1 mL solution with CH₂Cl₂ (μL);

DF – Dilution Factor, DF = 1000/V₁;

C – Concentration of the extracts in 1 mL solution for the GC-MS analysis, C = 1000 × W / (V_{extract} × DF).

Table 5. Time-Dependent Extraction of Santa Cruz *Plocamium cartilagineum*^a with Pure CO₂ (SC-B).

Fraction	B1	B2	B3	B4	B5	B6	B7	B8	B9	B10	B11	B12	B13 ^b
T (min)	15	15	15	15	15	15	15	15	15	15	15	15	120°
W (mg)	7	5	1.7	1	0.8	0.9	0.5	0.5	0.5	0.5	0.3	0.4	30.5 ^d
V _{extract} (mL)	2	2	2	2	2	2	2	2	2	2	2	2	2
V ₁ (μL)	30	40	120	200	250	230	400	400	400	400	670	500	10
DF	33.33	25	8.33	5	4	4.35	2.5	2.5	2.5	2.5	1.49	2	100
C (μg/mL)	105	100	102	100	100	103.5	100	100	100	100	100.5	100	152.5

Note 1: Towel-dried algae (3.9 g) was extracted under 250 atm/40°C at 1.5 mL/min flow rate for 3 hours with collection of the SFE fractions every 15 min.

Note 2: The definitions of^a , , , , T, W, V_{extract}, V₁, DF, C here are the same as those in Table 4.

Table 6. Time-Dependent Extraction of Santa Cruz *Plocamium cartilagineum*^a with Pure CO₂ (SC-C).

	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12	C13 ^b
T (min)	15	15	15	15	15	15	15	15	15	15	15	15	120 ^c
W (mg)	7	5.1	2.2	1.2	0.5	0.5	0.6	0.6	0.4	0.3	0.2	0.3	25.2 ^d
V _{extract} (mL)	2	2	2	2	2	2	2	2	2	2	2	2	2
V ₁ (μL)	30	40	90	170	400	400	350	350	500	670	1000	670	10
DF	33.33	25	11.11	5.88	2.5	2.5	2.86	2.86	2	1.49	1	1.49	100
C (μg/mL)	105	102	99	102	100	100	105	105	100	100.5	100	100.5	126

Note 1: Towel-dried algae (3.7 g) was extracted under 250 atm/40°C at 1.5 mL/min flow rate for 3 hours with collection of the SFE fractions every 15 min.

Note 2: The definitions of^a , , , , T, W, V_{extract}, V₁, DF, C here are the same as those in Table 4.

Table 7. Time-Dependent Extraction of *Plocamium cartilagineum*^a with CO₂ Modified with 10% CH₃OH (SC-D).

	D1	D2	D3	D4	D5	D6	D7	D8	D9	D10	D11	D12	D13 ^b
T (min)	15	15	15	15	15	15	15	15	15	15	15	15	120 ^c
W (mg)	5.8	2.4	3.2	2	1.7	1.4	1	1	0.5	0.5	0.3	0.3	6.2 ^d
V _{extract} (mL)	2	2	2	2	2	2	2	2	2	2	2	2	2
V ₁ (μL)	40	80	60	100	120	140	200	200	400	400	660	660	30
DF	25	12.5	16.67	10	8.33	7.14	5	5	2.5	2.5	1.51	1.51	33.33
C (μg/mL)	116	96	96	100	102	98	100	100	100	100	99	99	93

Note: Towel-dried algae was extracted under 250 atm/40°C at 1.5 mL/min flow rate for 3 hours with collection of the SFE fraction every 15 min.

^a – *Plocamium cartilagineum* from Davenport (north of Santa Cruz);

^b – One fraction of the extracts obtained from the conventional solvent extraction of the residual algae after SFE;

T – Extraction Time of SFE, (° – Extraction Time of Conventional Solvent Extraction);

W – Weight of the SFE extracts after evaporation, (^d – Weight of the Conventional Solvent Extracts);

V_{extract} – Volume of CH₂Cl₂ for dilution of the extracts (mL);

V₁ – Volume of the extracts taken from V_{extract} and diluted to 1 mL solution with CH₂Cl₂ (μL);

DF – Dilution Factor, DF = 1000/V₁;

C – Concentration of the extracts in 1 mL solution for the GC-MS analysis, C = 1000 × W / (V_{extract} × DF).

Table 8. Time-Dependent Extraction of *Plocamium cartilagineum*^a with CO₂ Modified with 10% CH₃OH (SC-E).

	E1	E2	E3	E4	E5	E6	E7	E8	E9	E10	E11	E12	E13 ^b
T (min)	15	15	15	15	15	15	15	15	15	15	15	15	120 ^c
W (mg)	2.6	5.4	2.3	2	1.1	1.5	0.8	0.5	0.2	0.5	0.1	0.2	9.4 ^d
V _{extract} (mL)	2	2	2	2	2	2	2	2	2	2	1	2	2
V ₁ (μL)	80	40	90	100	180	130	250	400	1000	400	1000	1000	20
DF	12.5	25	11.11	10	5.55	7.69	4	2.5	1	2.5	1	1	50
C (μg/mL)	104	108	103.5	100	99	97.5	100	100	100	100	100	100	94

Note 1: Towel-dried algae (1.9 g) was extracted under 250 atm/40°C at 1.5 mL/min flow rate for 3 hours with collection of the SFE fractions every 15 min.

Note 2: The definitions of ^a , ^b , ^c , ^d T, W, V_{extract}, V₁, DF, C here are the same as those in Table 7.

Table 9. Time-Dependent Extraction of *Plocamium cartilagineum*^a with CO₂ Modified with 10% CH₃OH (SC-F).

	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10	F11	F12	F13 ^b
T (min)	15	15	15	15	15	15	15	15	15	15	15	15	120 ^c
W (mg)	4	2.5	2.4	2	1.5	2	1.5	0.4	0.6	1	0.9	0.9	8.9 ^d
V _{extract} (mL)	2	2	2	2	2	2	2	2	2	2	2	2	2
V ₁ (μL)	50	80	80	100	130	100	130	500	340	200	220	220	20
DF	20	12.5	12.5	10	7.69	10	7.69	2	2.94	5	4.54	4.54	50
C (μg/mL)	100	100	96	100	97.5	100	97.5	100	102	100	99	99	89

Note 1: Towel-dried algae (1.9 g) was extracted under 250atm/40°C at 1.5 mL/min flow rate for 3 hours with collection of the SFE fractions every 15 min.

Note 2: The definitions of ^a , ^b , ^c , ^d T, W, V_{extract}, V₁, DF, C here are the same as those in Table 7.

Table 10. Effect of Supercritical CO₂ Pressure (from SC-G to SC-R).

	SC-G	SC-H	SC-I	SC-J	SC-K	SC-L	SC-M	SC-N	SC-O	SC-P	SC-Q	SC-R
W (g)	2.2	2.1	2.1	2.1	2.1	2.1	2.1	2.1	2.1	2.1	2	2.1
T (min)	60	60	60	60	60	60	60	60	60	60	60	60
P (atm)	250	250	250	300	300	300	350	350	350	400	400	400
V _{extract} (mL)	1	1	1	1	1	1	1	1	1	1	1	1
V ₁ (μL)	40	40	80	40	40	40	40	40	40	40	40	40
DF	25	25	12.5	25	25	25	25	25	25	25	25	25

Note: Towel-dried algae^a was extracted at 40°C/60 min using a 1.5 mL/min flow rate. SC-G, SC-H, and SC-I were extracted at 250 atm of CO₂. SC-J, SC-K, and SC-L were extracted at 300 atm of CO₂. SC-M, SC-N, and SC-O were extracted at 350 atm of CO₂. SC-P, SC-Q, and SC-R were extracted at 400 atm of CO₂.

^a – *Plocamium cartilagineum* from Davenport (north of Santa Cruz);

T – Extraction Time of SFE (min);

W – Weight of the Towel-Dried Algae (g);

V_{extract} – Volume of CH₂Cl₂ for dilution of the SFE extracts (mL);

V₁ – Volume of the SFE extracts taken from V_{extract} and diluted to 1 mL solution with CH₂Cl₂ (μL);

DF – Dilution Factor, DF = 1000/V₁.

G. Comparative Studies of SFE and Conventional Solvent Extraction

Plocamium cartilagineum from Davenport (north of Santa Cruz) and Casa Beach (San Diego) were used in the comparative studies of SFE and conventional solvent extraction. In these comparative studies, SFE was compared with conventional solvent extraction to determine the extraction ability of SFE for specific halogenated monoterpenes. All the experiments were repeated three times under identical extraction conditions.

1. *Plocamium cartilagineum* from Davenport (Santa Cruz)

In a typical experiment, SFE was performed by placing 2 g of towel-dried algae mixed with 2 g of the diatomaceous earth into one extraction vessel. The extraction was conducted with CO₂ at 250 atm/40°C/60 min using a 1.5 mL/min flow rate and was continued with CO₂ modified with 2% methanol under the same conditions (except the time was 30 min). After 30 min, CO₂ modified with 5% methanol at 250 atm/40°C/30 min was used to continue the extraction. Finally, the extraction was continued using CO₂ modified with 10% methanol at 250 atm/40°C for 30 min. A total of four fractions of the SFE extracts were obtained, and each fraction was diluted to a final volume of 1 mL in CH₂Cl₂ for GC/MS analysis. Following the SFE process, the residual algae in the extraction vessel was further extracted with 15 mL of methanol for 2 hours. The methanol extract was concentrated to dryness, then diluted with 1 mL of CH₂Cl₂ for

GC/MS analysis. A portion of each fraction was used for GC/MS analysis as summarized in Tables 11–13.

Conventional solvent extraction was performed by soaking 2 g of towel-dried algae in 15 mL of hexane for 1 hour. After 60 min, the hexane layer was filtered, and 15 mL of fresh hexane: methanol (98: 2, v/v) was added for another 30 min extraction. After 30 min, the organic layer was again filtered and 15 mL of hexane: methanol (95: 5, v/v) was added for the next 30 min extraction. The extraction process was continued with the use of 15 mL of hexane: methanol (90: 10, v/v) for 30 min. Finally, the algae was extracted with 15 mL of methanol for 2 hours. The methanol extract was concentrated to dryness, then diluted with 1 mL of CH_2Cl_2 for GC/MS analysis. A total of five fractions of the crude extracts were collected and a portion of each fraction was used for GC/MS analysis as summarized in Tables 14–16.

2. *Plocamium cartilagineum* from Casa Beach (San Diego)

SFE and conventional solvent extraction of 2 g of towel-dried algae (Casa Beach, San Diego) were performed under the same conditions as those mentioned above. A total of 5 fractions were obtained from SFE and conventional solvent extraction, respectively. Tables 17–19 summarizes the GC/MS analysis of the SFE extracts and Tables 20–22 shows the same analysis of conventional solvent extracts.

Table 11. Comparative Studies of SFE for Santa Cruz *Plocamium cartilagineum*^a.

	S1	S2	S3	S4	S5 ^b
T (min)	60	30	30	30	120 ^d
CO ₂ (%)	100	98	95	90	N/A
CH ₃ OH (%) ^c	0	2	5	10	100
V _{extract} (mL)	1	1	1	1	2
V ₁ (μL)	40	100	100	25	60
DF	25	10	10	40	16.67

Note: Towel-dried algae (2.0 g) was extracted at 250 atm/40°C using a 1.5 mL/min flow rate with CO₂ (one hour), followed by a gradient elution using CO₂ modified with 2%, 5% and 10% methanol for 30 min.

^a – *Plocamium cartilagineum* from Davenport in Santa Cruz;

^b – One fraction of the extracts from conventional solvent extraction of the residual algae after 2.5 hours of SFE;

^c – The composition of methanol in SFE increased from 0, 2%, 5% to 10% (v/v) successively;

T – Extraction Time of SFE, (^d – Extraction Time of conventional solvent extraction);

V_{extract} – Volume of CH₂Cl₂ for dilution of the extracts (mL);

V₁ – Volume of the extracts taken from V_{extract} and diluted to 1 mL solution with CH₂Cl₂ (μL);

DF – Dilution Factor, DF = 1000/V₁.

Table 12. Comparative Studies of SFE for Santa Cruz *Plocamium cartilagineum*^a.

	T1	T2	T3	T4	T5 ^b
T (min)	60	30	30	30	120 ^d
CO ₂ (%)	100	98	95	90	N/A
CH ₃ OH (%) ^c	0	2	5	10	100
V _{extract} (mL)	1	1	1	1	2
V ₁ (μL)	40	100	100	25	40
DF	25	10	10	40	25

Note 1: Towel-dried algae (2.0 g) was extracted at 250 atm/40°C using a 1.5 mL/min flow rate with CO₂ (one hour), followed by a gradient elution using CO₂ modified with 2%, 5% and 10% methanol for 30 min.

Note 2: The definitions of ^a, ^b, ^c, ^d, T, V_{extract}, V₁, DF here are the same as those in Table 11.

Table 13. Comparative Studies of SFE for Santa Cruz *Plocamium cartilagineum*^a.

	U1	U2	U3	U4	U5 ^b
T (min)	60	30	30	30	120 ^d
CO ₂ (%)	100	98	95	90	N/A
CH ₃ OH (%) ^c	0	2	5	10	100
V _{extract} (mL)	1	1	1	1	2
V ₁ (μL)	40	100	100	50	40
DF	25	10	10	20	25

Note 1: Towel-dried algae (2.0 g) was extracted at 250 atm/40°C using a 1.5 mL/min flow rate with CO₂ (one hour), followed by a gradient elution using CO₂ modified with 2%, 5% and 10% methanol for 30 min.

Note 2: The definitions of ^a, ^b, ^c, ^d, T, V_{extract}, V₁, DF here are the same as those in Table 11.

Table 14. Comparative Studies of Conventional Solvent Extraction for Santa Cruz *Plocamium cartilagineum*^a.

	V1	V2	V3	V4	V5
T (min)	60	30	30	30	120
C ₆ H ₁₄ (%)	100	98	95	90	N/A
CH ₃ OH (%) ^b	0	2	5	10	100
V _{extract} (mL)	1	1	1	1	2
V ₁ (μL)	50	100	100	100	30
DF	20	10	10	40	33.33

Note 1: Towel-dried algae (2.0 g) was extracted with hexane (one hour), followed by a gradient elution using hexane modified with 2%, 5% and 10% methanol for 30 min.

^a – *Plocamium cartilagineum* from Davenport in Santa Cruz;

^b – The composition of methanol in conventional solvent extraction increased from 0 to 2% to 5% to 10% to 100% (v/v) successively;

T – Extraction Time of conventional solvent extraction;

V_{extract} – Volume of CH₂Cl₂ for dilution of the extracts (mL);

V₁ – Volume of the extracts taken from V_{extract} and diluted to 1 mL solution with CH₂Cl₂ (μL);

DF – Dilution Factor, DF = 1000/V₁.

Table 15. Comparative Studies of Conventional Solvent Extraction for Santa Cruz *Plocamium cartilagineum*^a.

	W1	W2	W3	W4	W5
T (min)	60	30	30	30	120
C ₆ H ₁₄ (%)	100	98	95	90	N/A
CH ₃ OH (%) ^b	0	2	5	10	100
V _{extract} (mL)	1	1	1	1	2
V ₁ (μL)	50	100	100	100	30
DF	20	10	10	40	33.33

Note 1: Towel-dried algae (2.0 g) was extracted with hexane (one hour), followed by a gradient elution using hexane modified with 2%, 5% and 10% methanol for 30 min.

Note 2: The definitions of ^a, ^b, T, V_{extract}, V₁, DF here are the same as those in Table 14.

Table 16. Comparative Studies of Conventional Solvent Extraction for Santa Cruz *Plocamium cartilagineum*^a.

	X1	X2	X3	X4	X5
T (min)	60	30	30	30	120
C ₆ H ₁₄ (%)	100	98	95	90	N/A
CH ₃ OH (%) ^b	0	2	5	10	100
V _{extract} (mL)	1	1	1	1	2
V ₁ (μL)	50	100	100	100	40
DF	20	10	10	40	25

Note 1: Towel-dried algae (2.0 g) was extracted with hexane (one hour), followed by a gradient elution using hexane modified with 2%, 5% and 10% methanol for 30 min.

Note 2: The definitions of ^a, ^b, T, V_{extract}, V₁, DF here are the same as those in Table 14.

Table 17. Comparative Studies of SFE for San Diego *Plocamium cartilagineum*^a.

	A1	A2	A3	A4	A5 ^b
T (min)	60	30	30	30	120 ^d
CO ₂ (%)	100	98	95	90	N/A
CH ₃ OH (%) ^c	0	2	5	10	100
V _{extract} (mL)	1	1	1	1	2
V ₁ (μL)	80	100	100	50	50
DF	12.5	10	10	20	20

Note: Towel-dried algae (1.8 g) was extracted at 250 atm/40°C using a 1.5 mL/min flow rate with CO₂ (one hour), followed by a gradient elution using CO₂ modified with 2%, 5% and 10% methanol for 30 min.

^a – *Plocamium cartilagineum* from Casa Beach in San Diego;

^b – One fraction of the extracts from conventional solvent extraction of the residual algae after 2.5 hours of SFE;

^c – The composition of methanol in SFE increased from 0, 2%, 5% to 10% (v/v) successively;

T – Extraction Time of SFE, (^d – Extraction Time of conventional solvent extraction);

V_{extract} – Volume of CH₂Cl₂ for dilution of the extracts (mL);

V₁ – Volume of the extracts taken from V_{extract} and diluted to 1 mL solution with CH₂Cl₂ (μL);

DF – Dilution Factor, DF = 1000/V₁.

Table 18. Comparative Studies of SFE for San Diego *Plocamium cartilagineum*^a.

	B1	B2	B3	B4	B5 ^b
T (min)	60	30	30	30	120 ^d
CO ₂ (%)	100	98	95	90	N/A
CH ₃ OH (%) ^c	0	2	5	10	100
V _{extract} (mL)	1	1	1	1	2
V ₁ (μL)	80	100	100	50	40
DF	12.5	10	10	20	25

Note 1: Towel-dried algae (1.8 g) was extracted at 250 atm/40°C using a 1.5 mL/min flow rate with CO₂ (one hour), followed by a gradient elution using CO₂ modified with 2%, 5% and 10% methanol for 30 min.

Note 2: The definitions of ^a, ^b, ^c, ^d, T, V_{extract}, V₁, DF here are the same as those in Table 17.

Table 19. Comparative Studies of SFE for San Diego *Plocamium cartilagineum*^a.

	C1	C2	C3	C4	C5 ^b
T (min)	60	30	30	30	120 ^d
CO ₂ (%)	100	98	95	90	N/A
CH ₃ OH (%) ^c	0	2	5	10	100
V _{extract} (mL)	1	1	1	1	2
V ₁ (μL)	40	100	100	50	50
DF	25	10	10	20	20

Note 1: Towel-dried algae (1.8 g) was extracted at 250atm/40°C using a 1.5 mL/min flow rate with CO₂ (one hour), followed by a gradient elution using CO₂ modified with 2%, 5% and 10% methanol for 30 min.

Note 2: The definitions of ^a, ^b, ^c, ^d, T, V_{extract}, V₁, DF here are the same as those in Table 17.

Table 20. Comparative Studies of Conventional Solvent Extraction for San Diego *Plocamium cartilagineum*^a.

	D1	D2	D3	D4	D5
T (min)	60	30	30	30	120
C ₆ H ₁₄ (%)	100	98	95	90	N/A
CH ₃ OH (%) ^b	0	2	5	10	100
V _{extract} (mL)	1	1	1	1	2
V ₁ (μL)	80	100	100	50	30
DF	12.5	10	10	20	33.33

Note 1: Towel-dried algae (2.0 g) was extracted with hexane (one hour), followed by a gradient elution using hexane modified with 2%, 5% and 10% methanol for 30 min.

^a – *Plocamium cartilagineum* from Casa Beach in San Diego;

^b – The composition of methanol in conventional solvent extraction increased from 0 to 2% to 5% to 10% to 100% (v/v) successively;

T – Extraction Time of conventional solvent extraction;

V_{extract} – Volume of CH₂Cl₂ for dilution of the extracts (mL);

V₁ – Volume of the extracts taken from V_{extract} and diluted to 1 mL solution with CH₂Cl₂ (μL);

DF – Dilution Factor, DF = 1000/V₁.

Table 21. Comparative Studies of Conventional Solvent Extraction for San Diego *Plocamium cartilagineum*^a.

	E1	E2	E3	E4	E5
T (min)	60	30	30	30	120
C ₆ H ₁₄ (%)	100	98	95	90	N/A
CH ₃ OH (%) ^b	0	2	5	10	100
V _{extract} (mL)	1	1	1	1	2
V ₁ (μL)	80	100	100	100	30
DF	12.5	10	10	40	33.33

Note 1: Towel-dried algae (2.0 g) was extracted with hexane (one hour), followed by a gradient elution using hexane modified with 2%, 5% and 10% methanol for 30 min.

Note 2: The definitions of ^a, ^b, T, V_{extract}, V₁, DF here are the same as those in Table 20.

Table 22. Comparative Studies of Conventional Solvent Extraction for San Diego *Plocamium cartilagineum*^a.

	F1	F2	F3	F4	F5
T (min)	60	30	30	30	120
C ₆ H ₁₄ (%)	100	98	95	90	N/A
CH ₃ OH (%) ^b	0	2	5	10	100
V _{extract} (mL)	1	1	1	1	2
V ₁ (μL)	80	100	100	50	40
DF	12.5	10	10	20	25

Note 1: Towel-dried algae (2.0 g) was extracted with hexane (one hour), followed by a gradient elution using hexane modified with 2%, 5% and 10% methanol for 30 min.

Note 2: The definitions of ^a, ^b, T, V_{extract}, V₁, DF here are the same as those in Table 20.

H. Quantitative Analysis with GC/MS

All of quantitative measurements in this research were performed on the Hewlett-Packard GC/MS using external standard calibration. The monoterpenes 2, 4, 7, 8, 9, 14, 15, and 16 obtained from *Plocamium cartilagineum* as described above were used as the external standards in which the concentration of each compound was set to 30 $\mu\text{L/mL}$, 50- $\mu\text{L/mL}$, 100 $\mu\text{L/mL}$, and 150 $\mu\text{L/mL}$ for GC/MS calibration. All samples from the SFE extracts and the conventional solvent extracts were diluted with CH_2Cl_2 for GC/MS analysis, as shown in Tables 4–22. The concentrations of the monoterpenes in the SFE and conventional solvent extracts were calculated using known concentrations of the corresponding standards according to the following relationship (Equation 1).

$$C_{\text{analyte}} = \frac{A_{\text{analyte}} \times C_{\text{standard}}}{A_{\text{standard}}} \quad \text{Equation 1}$$

A_{standard} – Chromatographic peak area of the standard found in the TIC of GC/MS;

A_{analytes} – Chromatographic peak area of the monoterpene in the extracts from its TIC of GC/MS;

C_{standard} – Concentration of the standard used for the instrument calibration ($\mu\text{g/mL}$);

C_{analyte} – Concentration of the monoterpene in the analyzed solution ($\mu\text{g/mL}$).

In Equation 1, the corresponding monoterpenes of the standards found in the analyzed solutions were determined by comparison of the total ion chromatogram (TIC) of the

analytes with the TIC of the standards. Then, the total concentration of the corresponding monoterpene extracted from the algae was determined on the following Equation 2.

$$Y_{\text{analyte}} = \frac{C_{\text{analyte}} \times DF \times V_{\text{extract}}}{W} \quad \text{Equation 2}$$

Y_{analyte} – Total concentration of the analyzed monoterpene of the corresponding standard obtained from the alga ($\mu\text{g/g}$);

C_{analyte} – Concentration of the monoterpene calculated from Equation 1 ($\mu\text{g/mL}$).

V_{extract} – Volume of the SFE extracts or conventional extracts (mL);

DF – Dilution factor (volume taken from V_{extract} and diluted to 1 mL with CH_2Cl_2), see Tables 4–22 for dilution factor;

W – Weight of the towel-dried algae for extraction (g).

All the GC/MS data from the quantitation of the monoterpenes in the time-dependent extraction, effect of pressure, and comparative studies are listed in Appendix A.

CHAPTER 3

RESULTS AND DISCUSSION

A. Preliminary Experiments to Establish SFE Conditions

Plocamium cartilagineum from Bird Rock (San Diego) was chosen for the preliminary studies. The purpose of the preliminary studies was to determine whether SFE was able to extract the halogenated monoterpenes from the marine algae. Although several parameters including pressure, temperature, the cosolvent and the flow rate can affect the performance of SFE, the ultimate choice of the SFE conditions is based on the properties of the sample involved. In the case of *P. cartilagineum*, the halogenated monoterpenes are lipophilic compounds which dissolve in non-polar solvents so that supercritical CO₂ is expected to readily extract them from the sample matrices. Second, *Plocamium cartilagineum* was not air-dried or lyophilized. Since water is not miscible with CO₂, it has to be removed from *P. cartilagineum* prior to SFE. Furthermore, the solvation strength of supercritical CO₂ is proportional to the density of CO₂ which is dependent on its pressure and temperature. Supercritical CO₂ under 400 atm/40°C has a density close to 0.96 g/mL and was selected for the extraction.

Frozen *P. cartilagineum* was thawed and carefully dried with a paper towel to minimize loss of monoterpenes from the sample. The towel-dried algae were further mixed with an equal amount of the diatomaceous earth to absorb water remaining in the algae. SFE was then performed with CO₂ at 400 atm/40°C using a 1.5 mL/min flow rate

for 3 hours with collection of the fractions every 60 min. The SFE fractions and conventional solvent extracts collected from the same algae were analyzed by TLC as shown in Figure 5. Some of the spots developed in the TLC plate can be observed with UV light at 254 nm, and such spots correspond to monoterpenes with conjugated double bonds. All monoterpenes, whether they are visible with UV light or not, are visualized by spraying the TLC plate with a developing reagent then heating. The TLC analysis indicated that the SFE extracts contained similar monoterpenes as the conventional solvent extracts because of the identical spots developed on the TLC plates.

Additional studies of SFE for the extraction of *Plocamium cartilagineum* at different temperatures (60°C, 80°C, and 100°C) but holding all other conditions constant (400 atm at a 1.5 mL/min flow rate for 3 hours with collection of fractions every 60 min) produced similar results. Table 23 lists the total extractable material recovered in the SFE extracts at the different temperatures. The small difference in the total amount of the extractable materials indicates that an increase of temperature gave no dramatic effect on the performance of SFE. In addition, the TLC analysis revealed that the SFE fractions at each temperature contained similar monoterpenes which was consistent with GC/MS analysis. The GC/MS chromatograms of these SFE fractions exhibited the same peaks as the conventional solvent extracts of the same sample of algae. Although GC/MS in this study was used as a qualitative tool, it indicated that SFE could extract the same halogenated monoterpenes from *P. cartilagineum* as conventional solvent extraction.

Furthermore, the TLC analysis provides a convenient way to rapidly determine the extraction ability of SFE even though it only gives general information.

The preliminary studies at different extraction times and different temperatures indicated that the halogenated monoterpenes could be extracted from *Plocamium cartilagineum* with SFE. However, further determination of the extraction efficiency of SFE required the purification of some halogenated monoterpenes from *P. cartilagineum* which were then used as the external standards for more detailed studies.

- SFE of halogenated monoterpenes from *Plocamium cartilagineum* (Bird Rock) carried out at 400-atm/40°C using a 1.5 mL/min flow rate for 3 hours with collection of SFE fractions every 60 min.
- Spot 1 represents the first SFE fraction; spot 2 represents the second SFE fraction; spot 3 represents the third SFE fraction.
- Spot 4 represents the crude extracts obtained from the conventional solvent extraction of the same algae.

Figure 5. TLC Analysis of SFE Fractions and Conventional Solvent Extracts.

Table 23. Preliminary Studies of the Change of Extraction Temperature.
(SFE conditions: 400 atm, pure CO₂, 1.5 mL/min flow rate)

	40°C	60°C	80°C	100°C
Weight of Algae (g)	4.8 g	4.8 g	4.7 g	4.7 g
Fraction 1 (first hour)	9.1 mg	6.8 mg	7.2 mg	6.7 mg
Fraction 2 (second hour)	2.4 mg	0.4 mg	2.8 mg	1.0 mg
Fraction 3 (third hour)	1.3 mg	3.1 mg	3.8 mg	0.4 mg
Total Extractable Materials	12.8 mg	10.3 mg	13.8 mg	8.1 mg

- *Plocamium cartilagineum* from Bird Rock (San Diego) was chosen for the preliminary studies.
- SFE is performed 3 hours with collection of the fractions every 60 min at each temperature.

B. Identification of Some Halogenated Monoterpenes from *P. cartilagineum*

Several purified compounds were needed as the external standards to quantitate the corresponding halogenated monoterpenes in the SFE extracts. As part of this project, eight halogenated monoterpenes were isolated from *Plocamium cartilagineum* – two from the Santa Cruz species, and six 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 chromatography and HPLC on silica gel with hexane as the eluent. *Plocamium 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 six halogenated monoterpenes isolated from San Diego include the following metabolites: 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 with ¹H NMR and ¹³C NMR spectroscopy by comparison with literature values. In addition, GC/MS was used in the identification of the purified monoterpenes.

1. Spectroscopic Identification of the Halogenated 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 its aldehyde group. It was readily oxidized if left standing in air for a time, or even if stored in the refrigerator. By comparison to the NMR data in the published reports (24, 25), 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* (25), and its ^1H NMR spectrum also showed a characteristic vinylic ABX system ($J = 17.1, 10.6$ Hz) as listed in Tables 24 and 25. The ^1H and ^{13}C NMR spectra of compound 14 displayed similar proton and carbon shifts as those of monoterpene 15 (Tables 24 and 25). The ^1H signal at $\delta 9.5$, 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 two locations in San Diego produced six halogenated monoterpenes, five from Casa Beach algae, and one from a collection taken at Bird Rock. The ^1H and ^{13}C NMR spectra of compounds 2 and 16 displayed similar chemical shift values to those in the literature (23, 26), as listed in Tables 26 and 27. Compound 7 exhibited proton shifts (in CDCl_3) close to those (in CCl_4) observed by Mynderse and Faulkner (23), and its ^{13}C NMR spectrum was similar to that of compound 2, except for three carbon positions (C_7 , C_8 , and C_{10}). The differences are likely due to differences in the solvents used for NMR analysis (CDCl_3 vs. CCl_4).

Compounds 9 and 7 are geometrical isomers, however, the confirmation of monoterpene 9 was not clear due to the overlap of two protons (protons at C₅ and C₆) in its ¹H NMR spectrum in CDCl₃. With the aid of anisotropic benzene-*d*₆, the AMX pattern of the chloromethine proton (H₄) and the two vinyl protons (H₅ and H₆) of compound 9 was easily observed. Additionally, compound 9 was determined to be a Z isomer across C₇ = C₈ according to the chemical shift of H₈ (δ6.97, CDCl₃), which was higher than that (δ6.77, CDCl₃) of compound 15. The assignment of (3R, 4R) to monoterpene 9 was based upon its proton shift, δ1.82, at Me₉ vs. the proton shift, δ1.79, at Me₉ of monoterpene 15 (3R, 4S). Mynderse and Faulkner (23) reported that the Me₉ shift (CCl₄) in the (3R, 4R) isomer was shifted 0.05 ppm upfield from that of the (3R, 4S) isomer (¹H shift of δ1.79 for 3R, 4R and δ1.74 for 3R, 4S).

Compounds 4 and 8, a pair of diastereoisomers, were similar to compound 9. The ¹H NMR spectra (CDCl₃) of 4 and 8 clearly exhibited ABX patterns for their terminal vinyl groups, but not AMX patterns for the chloromethine proton (H₄) and the two vinyl protons (H₅ and H₆). With the help of the anisotropic solvent – C₆D₆, the ¹H NMR spectra of structures 4 and 8 show not only the ABX patterns but also the AMX signals. Moreover, the ¹H NMR spectrum (CDCl₃) of compound 8 displays a proton shift of δ1.81 ppm for Me₉ and a shift of δ6.96 ppm for H₈. Thus, it was assigned to be (3R, 4R) 7-dichloromethyl-3-methyl-3, 4, 8-trichloro-1, 5(E), 7(Z)-octatriene. Diastereoisomer 4 had a proton shift of δ1.78 ppm (CDCl₃) at Me₉ and a shift of δ6.97 ppm (CDCl₃) at H₈ 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 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 eight halogenated monoterpenes from GC/MS indicated two 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.

Fragment I

Fragment 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 one and two halogenated atoms from the parent compounds were found in the mass spectra. Table 28 lists the retention time and key m/z peaks of the eight halogenated monoterpenes. All of the actual NMR spectra and mass spectra of the eight purified monoterpenes are shown in Appendix B.

GC/MS was used as the tool for calculating the concentration of the monoterpenes found in the SFE extracts and the conventional solvent extracts by comparison to the

known concentrations of the identified standard compounds (i.e., 2, 4, 7, 8, 9, 14, 15, and 16). Therefore, the extraction efficiency of SFE can be quantitatively determined by comparison to the results obtained from conventional solvent extraction.

Table 24. ^{13}C NMR Data of Monoterpenes 14 – 15.
(CDCl_3 , 75 MHz; chemical shift, ppm)

Structure	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10
14	116.41	139.59	71.54	69.62	134.15	122.54	137.32	143.65	24.75	189.16
15	116.51	139.44	71.69	69.34	124.19	123.89	136.01	131.92	25.10	69.41

Table 25. ^1H NMR Data of Monoterpenes 14 – 15.
(CDCl_3 , 300 MHz; Chemical shift, ppm; J, Hz)

	H_{1a}	H_{1b}	H_2	H_4	H_5	H_6	H_8	H_9	H_{10}
14	5.30 (d) $J_{1a2} = 10.62$	5.42 (d) $J_{1b2} = 17.03$	6.09 (dd) $J_{1a2} = 10.63$ $J_{1b2} = 17.11$	4.55 (d) $J_{45} = 8.84$	7.09 (dd) $J_{45} = 8.84$ $J_{56} = 15.84$	6.56 (d) $J_{56} = 15.75$ $J = 2.1$	7.13 (s)	1.78 (s)	9.55 $J = 2.1$
15	5.31 (d) $J_{1a2} = 10.63$	5.42 (d) $J_{1b2} = 17.11$	6.10 (dd) $J_{1a2} = 10.62$ $J_{1b2} = 17.04$	4.60 (d) $J_{45} = 8.37$	6.49 (dd) $J_{45} = 8.34$ $J_{56} = 16.22$	6.63 (d) $J_{56} = 16.24$	6.40 (s)	1.79 (s)	6.77 (s)

Note: s - singlet; d - doublet; dd - double doublet.

Table 26. ^1H NMR Data of Monoterpenes 2, 4, 7, 8, 9, and 16 (CDCl_3 , 300 MHz; Chemical shift, ppm; J, Hz).

	H_1	H_2	H_4	H_5	H_6	H_8	H_9	H_{10}
2	6.56 (d) $J_{12} = 13.59$	6.43 (d) $J_{12} = 13.6$	4.49 (d) $J_{45} = 7.19$	6.05 (m) $J_{45} = 7.20, J_{56} = 15.07$	6.13 (m) $J_{56} = 15.07$	5.76 (s)	1.77 (s)	1.97 (s)
16	6.55 (D) $J_{12} = 13.62$	6.44 (d) $J_{12} = 13.6$	4.46 (d) $J_{45} = 7.04$	5.91 (m) $J_{45} = 7.02, J_{56} = 14.77$	5.98 (m) $J_{56} = 14.77$	3.70 (s)	1.76 (s)	1.84 (s)
7	6.58 (dd) $J_{12} = 13.6$	6.46 (d) $J_{12} = 13.52$	4.59 (d) $J_{45} = 8.41$	6.47 (dd) $J_{45} = 8.25, J_{56} = 16.44$	6.56 (d) $J_{56} = 16.21$	6.40 (s)	1.78 (s)	6.77 (s)
8	H_{1a} 5.33 (d) $J_1 = 10.67$ H_{1b} 5.47 (d) $J_2 = 16.92$	6.10 (dd) $J_1 = 10.66$ $J_2 = 16.91$	4.55 (t) $J_{45} = 8.77$ (C_6D_6)	6.34 (m) $J_{45} = 8.77, J_{56} = 15.6$ (C_6D_6)	6.34 (m) $J_{56} = 15.6$ (C_6D_6)	6.30 (s)	1.81 (s)	6.96 (s)
4	H_{1a} 5.30 (d) $J_1 = 10.66$ H_{1b} 5.42 (d) $J_2 = 17.04$	6.08 (dd) $J_1 = 10.63$ $J_2 = 17.06$	4.55 (t) $J_{45} = 8.79$ (C_6D_6)	6.35 (m) $J_{45} = 8.74, J_{56} = 15.69$ (C_6D_6)	6.35 (m) $J_{56} = 15.63$ (C_6D_6)	6.31 (s)	1.78 (s)	6.97 (s)
9	6.60 (d) $J_{12} = 13.42$	6.46 (d) $J_{12} = 13.44$	4.54 (d) $J_{45} = 8.48$ (C_6D_6)	6.32 (m) $J_{45} = 8.51, J_{56} = 15.65$ (C_6D_6)	6.32 (m) $J_{56} = 15.68$ (C_6D_6)	6.32 (m)	1.82 (s)	6.97 (s)

Note: The J constants of structures 8, 4, and 9 are difficult to determine at the H_4 , H_5 , and H_6 positions in CDCl_3 solvent, and their J constants are determined from C_6D_6 solvent.

Table 27. ^{13}C NMR Data of Monoterpenes 2, 7, and 16.
(CDCl_3 , 75 MHz; Chemical shift, ppm)

	C-1	C-2	C-3	C-4	C-5	C-6	C-7	C-8	C-9	C-10
2	110.18	138.55	71.61	67.40	128.93	135.70	71.27	52.61	25.46	26.13
16	110.09	138.5	71.58	67.65	127.31	137.89	66.99	41.50	25.46	27.27
7	110.25	138.44	71.43	69.50	124.50	124.31	135.82	131.18	25.24	68.74

Table 28. The Retention Time and m/z Values of Eight Halogenated Monoterpenes.

Structure	15	14	8
Formula	$C_{10}H_{11}Cl_4Br$	$C_{10}H_{11}Cl_3O$	$C_{10}H_{11}Cl_3$
Molecular Weight	350	252	306
m/z	base peak (89, 91); [$C_{10}H_{11}Cl_4$] ⁺ (271, 273, 275)	base peak (89, 91); [$C_{10}H_{11}Cl_2O$] ⁺ (217, 219)	base peak (89, 91); [$C_{10}H_{11}Cl_4$] ⁺ (271, 273, 275)
Retention Time (min)	16.40 ± 0.06 ^a	14.90 ± 0.06 ^a	17.69 ± 0.06 ^b

Structure	4	9	7
Formula	$C_{10}H_{11}Cl_5$	$C_{10}H_{10}Cl_3Br$	$C_{10}H_{10}Cl_3Br$
Molecular Weight	306	384	384
m/z	base peak (89, 91); [$C_{10}H_{11}Cl_4$] ⁺ (271, 273, 275)	base peak (167, 169, 171); [$C_{10}H_{10}Cl_4Br$] ⁺ (349, 351, 353)	base peak (167, 169, 171); [$C_{10}H_{10}Cl_4Br$] ⁺ (349, 351, 353)
Retention Time (min)	17.82 ± 0.06 ^b	20.19 ± 0.06 ^b	20.72 ± 0.06 ^b

Structure	2	16
Formula	$C_{10}H_{12}Cl_3Br_3$	$C_{10}H_{13}Cl_3Br_2$
Molecular Weight	474	396
m/z	base peak (167, 169, 171); [$C_{10}H_{12}Cl_3Br_2$] ⁺ (395, 397, 399)	base peak (167, 169, 171); [$C_{10}H_{13}Cl_3Br$] ⁺ (317, 319, 321)
Retention Time (min)	21.85 ± 0.06 ^b	19.62 ± 0.06 ^b

^a – Monoterpenes 14 and 15 are introduced into the GC/MS via PTE-5 fused-silica open-tubular column.

^b – Monoterpenes 2, 4, 7, 8, 9, and 16 are introduced into the GC/MS via DB-5 fused-silica open-tubular column.

C. Time-Dependent Extraction of SFE with and without 10% Methanol

Plocamium cartilagineum from Davenport (north of Santa Cruz) was utilized in the study of time-dependent extraction of SFE. The purified halogenated monoterpenes 14 and 15 from Santa Cruz *P. cartilagineum* were used as the external standards to calculate the concentration of the corresponding monoterpenes (14 and 15) found in the SFE extracts. Although the preliminary studies determined the possibility of using SFE for the extraction of *P. cartilagineum*, the extraction time of 3 hours was not reasonable in practical applications. In order to find a more reasonable and practical time when the extraction of *Plocamium cartilagineum* was essentially complete, a time-dependent study of the extraction of *Plocamium cartilagineum* was performed. Supercritical CO₂ at 250-atm of pressure was chosen for the time-dependent experiment in order to reduce the consumption of CO₂ because the process at the high pressure (400 atm) consumed more CO₂ than the extraction at the low pressure (250 atm). Although the solvation strength of supercritical CO₂ decreases at 250 atm of pressure, an increase in extraction time can compensate for a decrease in the solvation strength of CO₂. Hence, the time-dependence study of SFE was used to determine a suitable extraction time for the halogenated monoterpenes.

1. Time-Dependent Extraction with CO₂

Supercritical CO₂ was used at 40°C/250 atm ($\rho = 0.89$ g/mL for CO₂) with a 1.5-mL/min flow rate to extract *Plocamium cartilagineum* for 3 hours. The SFE extracts

were collected in 15 min intervals and analyzed by GC/MS. The average concentrations (3 determinations) of the total extractable materials, and of compounds 14 and 15 obtained by combining each SFE fraction, as a function of the extraction time gives the time-dependent extraction curves as shown in Figure 6. It was found that the total concentrations of the extractable materials, and of the monoterpenes 14 and 15, were 4899 $\mu\text{g/g}$ (RSD = 4.5%), 2456 $\mu\text{g/g}$ (RSD = 26.8%), and 532 $\mu\text{g/g}$ (RSD = 22.4%) respectively after 3 hours of extraction. However, the concentrations of compounds 14 and 15 in a one hour extraction were 2373 $\mu\text{g/g}$ (RSD = 26%) and 337 $\mu\text{g/g}$ (RSD = 22.3%), and in a two hour extraction were 2444 $\mu\text{g/g}$ (RSD = 26.5%) and 458 $\mu\text{g/g}$ (RSD = 22.2%). It can be concluded that one hour of extraction with CO_2 at $40^\circ\text{C}/250\text{ atm}$ using a 1.5 mL/min flow rate was sufficient for the extraction of *Plocamium cartilagineum*. A longer extraction time would not improve the overall extraction efficiency of SFE for the halogenated monoterpenes. Further extraction of the residual algae after SFE with methanol indicated that compound 15 was not fully extracted with supercritical CO_2 in 3 hours. It was found that 1879.8 $\mu\text{g/g}$ (RSD = 13.4%) of compound 15 remained in the residual algae. On the other hand, no compound 14 was detected in the methanol extract. Thus, SFE with pure CO_2 results in the incomplete extraction of some of the halogenated monoterpenes from *P. cartilagineum* in a reasonable time. However, this situation was found to change when a cosolvent was used in the SFE.

Note 1: *Pilocarpum cartilagineum* from Davenport (north of Santa Cruz) was extracted with pure CO₂ at 250 atm/40°C with a 1.5 mL/min flow rate for 3 hours with collection of fractions every 15 min.

Note 2: The total amounts of SFE materials and total concentrations of monoterpenes 14 and 15 (3 determinations) were 4891 µg/g, 2456 µg/g and 533 µg/g respectively in 3 hours.

Note 3: The total concentration of monoterpenes 14 and 15 were 2377 µg/g and 337 µg/g respectively in one hour.

Figure 6. Time-Dependent Extraction Curves with Pure Carbon Dioxide.

2. Time-Dependent Extraction with CO₂ Modified with 10% Methanol

The time-dependent extraction by CO₂ modified with 10% methanol exhibited improved performance for *Plocamium 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 hours. The SFE extracts collected every 15 min were analyzed by GC/MS. The average concentrations (3 determinations) of the total extractable materials, and of monoterpenes 14 and 15 are plotted as a function of the time as shown in Figure 7. 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 in 45 min of extraction were 564.5 µg/g (RSD = 10.4%) and 342.4 µg/g (RSD = 36.8%), respectively, and no monoterpenes of interest were found in the remainder of the SFE fractions. The use of carbon dioxide with 10% cosolvent in SFE enhances the solvation strength of supercritical CO₂, but the low extraction yields of monoterpenes 14 and 15 cannot be readily explained. It is possible that under these conditions many unwanted compounds (most of them were pigments) that interfered with the GC/MS quantitation of the monoterpenes were also extracted.

The time-dependent extraction with pure CO₂ indicates that a time of 60 min is sufficient for *Plocamium 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 if pure CO₂ was used. 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 phenomenon was not observed when 10% methanol was added.

Note 1: *Plocamium cartilagineum* from Danverport (Santa Cruz) was extracted by CO₂ modified with 10% methanol at 250 atm/40°C with a 1.5 mL/min flow rate for 3 hours with collection of fractions every 15 min.

Note 2: The total amounts of SFE materials and total concentrations of monoterpenes 14 and 15 (3 determinations) were 9824 µg/g, 565 µg/g and 343-µg/g respectively in 3 hours.

Note 3: The total concentration of monoterpenes 14 and 15 were 565 µg/g and 343 µg/g respectively in 45 minute.

Figure 7. Time-Dependent Extraction Curves with CO₂ Modified with 10% Methanol.

D. Effect of Pressure on SFE

The purpose of the pressure effect studies is to determine whether the increase of pressure can enhance the solvation strength (extraction efficiency) of supercritical CO₂ for the extraction of halogenated monoterpenes from *Plocamium cartilagineum*. The pressure of the SFE system was tested at 250 atm, 300 atm, 350 atm, and 400 atm with supercritical CO₂ alone to extract the red algae from Santa Cruz under 40°C/60 min with a 1.5 mL/min flow rate. The yields of compounds 14 and 15 as a function of the extraction pressure are shown in Figure 8. It was found that an increase of the pressure enhances the extraction ability of SFE. Above 300 atm, the concentration of compound 14 in the SFE extracts is higher than that at 250 atm. In 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 *Plocamium cartilagineum*.

Note: The SFE processes were performed with pure CO₂ at 40°C/60 min using a 1.5 mL/min flow rate to extract *Plocamium cartilagineum* from Santa Cruz at different pressures, as indicated.

Figure 8. Effect of Pressure on SFE.

E. Comparative Analysis of SFE with Conventional Solvent Extraction

The results from the previous experiments suggested that a "gradient extraction" approach for SFE might be advantageous for the *Plocamium cartilagineum* metabolites. Gradient extraction is commonly used in conventional solvent extraction, but the conditions are significantly different from SFE methods.

1. Santa Cruz *Plocamium cartilagineum*

A gradient extraction of *Plocamium cartilagineum* was performed with pure CO₂ and CO₂ modified successively with 2%, 5% and 10% methanol. Since the solvation strength of supercritical CO₂ is close to that of pure hexane, the gradual addition of methanol increases the solvation strength of the SFE fluid.

SFE was performed by the extraction of the towel-dried algae mixed with the diatomaceous earth successively with pure CO₂ at 250 atm/40°C/60 min, followed by extraction at 250 atm/40°C/30 min with CO₂ 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 hours to check for any compounds not removed by the processing.

The solvents and time used in the conventional extraction were chosen to be as close as possible as those in SFE, except hexane was the primary solvent instead of supercritical CO₂. Figure 9a–9d shows 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 Figure 10a–10d. The compound identified in the first fraction of SFE was compound 14 (t_R 14.90min). The monoterpenes determined in the second and third fractions of SFE were compound 14 (t_R 14.90min) and compound 15 (t_R 16.40 $\pm$ 0.01min). In the final SFE fraction, compound 15 (t_R 16.39min) was found while no compound 14 was observed. The GC/MS chromatograms of the conventional solvent extraction presented 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 11.

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 1625.5 $\mu\text{g/g}$ (RSD = 30%), and is significantly higher than the yield from conventional extraction (560.0 $\mu\text{g/g}$, RSD = 30%) by comparison of both methods in the first hour. The low yield of compound 14 in the conventional solvent extraction is tied to its sensitivity to air oxidation. Since the conditions of SFE are almost oxygen free, the lability of 14 is reduced. However, in conventional solvent extraction, oxidation can occur at multiple steps and is difficult to avoid or minimize.

Table 29 lists the average yields (3 determinations) of monoterpenes 14 and 15 obtained by adding the concentrations of the corresponding monoterpenes found in each fraction of SFE and conventional solvent extraction, respectively. It was found that the

Figure 9. The GC/MS Chromatograms of the SFE Fractions on Santa Cruz Algae. A) the first SFE fraction; B) the second SFE fraction; C) the third SFE fraction; D) the fourth SFE fraction.

Figure 9. The GC/MS Chromatograms of the SFE Fractions on Santa Cruz Algae (Continued).

Figure 10. The GC/MS Chromatograms of the Fractions of Conventional Solvent Extraction on Santa Cruz Algae. A) the first fraction; B) the second fraction; C) the third fraction; D) the fourth fraction.

Figure 10. The GC/MS Chromatograms of the Fractions of Conventional Solvent Extraction on Santa Cruz Algae (Continued).

Figure 11. Comparison of SFE and Conventional Solvent Extraction for the Santa Cruz Algae.

Table 29. Comparison of SFE and Conventional Solvent Extraction for the Santa Cruz Algae.

	14	15
Y_{SFE} ($\mu\text{g/g}$)	1859.6	513.6
$Y_{\text{convention}}$ ($\mu\text{g/g}$)	782.2	235.6
RSD_{SFE} (%)	37.9	41.3
$RSD_{\text{convention}}$ (%)	26	40.6
Recovery (%)	238	218

Note 1: *Plocamium cartilagineum* from Davenport of Santa Cruz was chosen as the extracted subject.

Note 2: 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.

Note 3: Conventional solvent extraction was performed at room temperature with 15 mL hexane for 1 hour, 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 hours.

Note 4: Y_{SFE} ($\mu\text{g/g}$) – Total yield of the monoterpene obtained from the SFE extracts in 2.5 hours extraction time (3 determinations).

Note 5: $Y_{\text{Convention}}$ ($\mu\text{g/g}$) – Total yield of the monoterpene obtained from the conventional solvent extracts in 4.5 hours extraction time (3 determination).

Note 6: RSD – Relative Standard Determination.

Note 7: Recovery – Ratio of Y_{SFE} and $Y_{\text{Convention}}$ ($= Y_{SFE}/Y_{\text{Convention}}$).

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 *Plocamium cartilagineum* than conventional solvent extraction.

2. 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 *Plocamium 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. Figure 12a–12d shows the GC/MS chromatograms of the four SFE fractions. Information about the various fractions of conventional solvent extraction is presented in Figure 13a–13d. The compounds identified in Figure 12 from the SFE fractions include 2 (t_R 21.85min), 7 (t_R 20.72min $\pm$ 0.04), 8 (t_R 17.69min), 4 (t_R 17.82min), and 9 (t_R 20.18min). These five 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 14. It was found that, unlike the result of Santa Cruz *Plocamium cartilagineum*, the extractions of the halogenated monoterpenes with SFE from San Diego algae are not

selective. A further comparison of the recovery in SFE and conventional solvent extraction indicated the different extraction abilities. Table 30 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 hours, compared to 4.5 hours 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.

Figure 12. The GC/MS Chromatograms of the SFE Fractions on San Diego Algae. A) the first SFE fraction; B) the second SFE fraction; C) the third SFE fraction; D) the fourth SFE fraction.

Figure 12. The GC/MS Chromatograms of the SFE Fractions on San Diego Algae (Continued).

Figure 13. The GC/MS Chromatograms of the Fractions of Conventional Solvent Extraction on San Diego Algae. A) the first fraction; B) the second fraction; C) the third fraction; D) the fourth fraction.

Figure 13. The GC/MS Chromatograms of the Fractions of Conventional Solvent Extraction on San Diego Algae (Continued).

Figure 14. Comparison of SFE and Conventional Solvent Extraction for San Diego Algae.

Table 30. Comparison of SFE and Conventional Solvent Extraction for the San Diego Algae.

	2	7	8	4	9
Y_{SFE} ($\mu\text{g/g}$)	62.3	196.1	543	514.3	568.5
$Y_{\text{convention}}$ ($\mu\text{g/g}$)	133.5	534.2	408.4	511	727.7
RSD_{SFE} (%)	89.7	51.4	30.4	29.5	52
$RSD_{\text{convention}}$ (%)	64.4	22.1	20.8	21.8	29
Recovery (%)	47	37	133	100	78

Note 1: *Plocamium cartilagineum* from Casa Beach of San Diego was chosen as the extracted sample.

Note 2: 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.

Note 3: Conventional solvent extraction was performed at room temperature with 15 mL hexane for 1 hour, 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 hours.

Note 4: Y_{SFE} ($\mu\text{g/g}$) – Total yield of the monoterpene obtained from the SFE extracts in 2.5 hours extraction time (3 determinations).

Note 5: $Y_{\text{convention}}$ ($\mu\text{g/g}$) – Total yield of the monoterpene obtained from the conventional solvent extracts in 4.5 hours extraction time (3 determination).

Note 6: RSD – Relative Standard Determination.

Note 7: Recovery – Ratio of Y_{SFE} and $Y_{\text{convention}}$ ($= Y_{SFE}/Y_{\text{convention}}$).

CHAPTER 4

CONCLUSION

Conventional liquid solvent extraction of natural products is time- and labor-intensive work. The high-volume, diluted solvent has to be concentrated before the assay of extracts. The extensive manipulation of the extract can result in the decomposition of the compounds of interest. As an alternative sample preparation method, Supercritical Fluid Extraction (SFE) has been used to extract natural products from the marine red alga *Plocamium cartilagineum*. *Plocamium cartilagineum* contains structurally similar halogenated monoterpenes, which are mainly lipophilic compounds and ideal for SFE study using CO₂.

The time-dependent extraction of SFE with CO₂ indicates that a 60 min is sufficient to extract 4 g of *Plocamium cartilagineum* at a flow rate of 1.5 mL/min, and the extraction time can be reduced to 45 min by the use of CO₂ modified with 10% methanol. However, CO₂ alone does not give quantitative recoveries for the halogenated monoterpenes. CO₂ containing modifier (2% to 5% to 10% methanol) increases the yield of the halogenated monoterpenes, yet the amount of other material (e.g., pigments) in the final extracts is kept to a minimum. Some evidence for selective extraction and high recoveries of specific monoterpenes was observed using a "gradient elution" in SFE from pure CO₂ to CO₂ containing 10% methanol. This was most significantly observed with the Santa Cruz algae, in which one compound, **14**, was obtained in similar purity, but in 238%

higher recovery than using conventional solvent extraction. While the halogenated monoterpenes from San Diego algae were obtained in higher recoveries using SFE, selective extraction of any specific compounds was not observed in this case.

Overall, the SFE extracts were completed in less time than the conventional solvent extracts (several hours vs. several days), and required less labor to yield the completed extraction. The yields of the natural products using SFE were generally higher than conventional solvent extraction, and the interfering compounds (e.g., pigments) in SFE were less evident.

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Appendix A

The GC/MS Data of the SFE and Conventional Solvent Extracts

Table 31. The GC/MS Data of Time-Dependent Extraction with Pure CO₂ for the Sample SC-A.

GC/MS Data of Monoterpene 15 in SC-A

	15	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	A11	A12	A13**
PA ₁	25606254	5113985	8914613	18209803	18329381	14739896	19745639	9415337	26239162	52365231	16782428	25613675	15320608	19745724
PA ₂	28092647	5386826	8849571	18581728	15204066	14901354	19919247	9514003	25631602	52580825	15981241	25944475	14965432	19442592
PA ₃	26726812	5184174	8871172	15398897	19897387	14746132	19308044	9704598	26234870	50991756	11589100	25327393	14997838	26710206
PA	26808571	5228328	8878452	17396809	17810278	14795794	19657643	9544646	26035211	51979270	14784256	25628514	15094626	21966174
C1 (µg/mL)	30.75	6	10.18	19.95	20.43	16.97	22.55	10.95	29.86	59.62	16.96	29.4	17.31	25.2
C2 (µg/mL)		399.96	339.4	399	453.95	169.7	150.18	64.39	149.3	59.62	84.8	58.8	69.24	5040
Y1 (µg/g)		95.23	80.81	95	108.08	40.4	35.76	15.33	35.55	14.19	20.19	14	16.48	1200
Y2 (µg/g)		95.23	176.04	271.04	379.12	419.52	455.28	470.61	506.16	520.35	540.54	554.54	571.02	1771.02

GC/MS Data of Monoterpene 14 in SC-A

	14	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	A11	A12	A13**
PA ₁	27576476	87194271	50249330	61214046	55257710	24160726	17976059	4209049	7896813	17866399	4162818	3606238	3510049	0
PA ₂	28100223	89494668	50303941	62596620	49169085	23870744	17939224	4176382	7492816	17648643	4114940	4297337	3197977	0
PA ₃	28123890	86719055	50339497	53581983	57914214	23659405	17531566	4129477	7693537	17400093	2946053	4283662	3553330	0
PA	27933530	87802664	50297589	59130883	54113670	23896958	17815616	4171636	7694388	17638378	3741270	4062412	3420452	0
C1 (µg/mL)	33.75	106.09	60.77	71.44	65.38	28.87	21.53	5.04	9.3	21.31	4.52	4.91	4.13	0
C2 (µg/mL)		7071.96	2026.07	1428.8	1452.74	288.7	143.39	29.63	46.5	21.31	22.6	4.91	16.52	0
Y1 (µg/g)		1683.8	482.4	340.19	345.89	68.74	34.14	7.05	11.07	5.07	5.38	1.17	3.93	0
Y2 (µg/g)		1683.8	2166.2	2506.39	2852.28	2921.02	2955.16	2962.21	2973.28	2978.35	2983.73	2984.9	2988.83	2988.83

PA₁ – The chromatographic peak area of the first injection; PA₂ – The chromatographic peak area of the second injection;

PA₃ – The chromatographic peak area of the third injection;

PA – The average value of three chromatographic peak areas, $PA = (PA_1 + PA_2 + PA_3)/3$;

C1 – The concentration of the corresponding monoterpene from the GC-MS data, $C1 = C_{standard} \times PA_{analyte} / PA_{standards}$;

C2 – The concentration of the corresponding monoterpene in the SFE extracts, $C2 = C1 \times DF \times V_{extract} / 1mL$;

Y1 – Yield of the corresponding monoterpene in the algae, $Y1 = C2 / W$, (W – Weight of the towel-dried algae for SFE);

Y2 – The total yields of the corresponding monoterpene in the algae, $Y2 = SUM(Y1)$.

** – SC-A13 is the extract which is obtained from the extraction of the SFE residual algae with methanol for 2 hours.

Table 32. The GC/MS Data of Time-Dependent Extraction with Pure CO₂ for the Sample SC-B.

GC/MS Data of Monoterpene 15 in SC-B

	15	B1	B2	B3	B4	B5	B6	B7	B8	B9	B10	B11	B12	B13**
PA ₁	21694729	5179789	5950441	14783001	16214466	12273681	12547909	20123227	16897901	16229284	18861180	23297750	13366976	16763455
PA ₂	21165090	5371871	6110673	17583261	15159572	12652968	12116519	20540226	15435057	16132198	15038717	22146513	13384793	23419725
PA ₃	22763219	5059245	6046219	15081353	15716521	12392609	12269622	20135429	15999796	16376206	15622103	23892188	13438946	23855783
PA	21874346	5203635	6035778	15815871	15696853	12439753	12311350	20266294	16110918	16245896	16507333	23112150	13396905	21346321
C1 (µg/mL)	30.75	7.32	8.48	22.23	22.07	17.49	17.31	28.49	22.65	22.84	23.21	32.49	18.83	30.01
C2 (µg/mL)		487.95	424	370.35	220.7	139.92	150.6	142.45	113.25	114.2	116.05	96.82	75.32	6002
Y1 (µg/g)		125.11	108.72	94.96	56.59	35.88	38.61	36.52	29.04	29.28	29.76	24.82	19.31	1538.97
Y2 (µg/g)		125.11	233.83	328.79	385.38	421.26	459.87	496.39	525.43	554.71	584.47	609.29	628.6	2167.57

GC/MS Data of Monoterpene 14 in SC-B

	14	B1	B2	B3	B4	B5	B6	B7	B8	B9	B10	B11	B12	B13**
PA ₁	23276789	68523740	45022306	27226225	9683096	4262350	2604070	5797703	3693882	2565352	2892094	3147608	1459561	0
PA ₂	25586448	69058002	46184819	26996156	9243270	4329573	2931034	5898719	3685467	2534341	3006207	2715708	2070260	0
PA ₃	24706460	67715611	45645494	26569810	9080646	4164046	3201150	5817704	3997682	2608384	3096765	3233712	1391290	0
PA	24523232	68432451	45617540	26930730	9335671	4251990	2912084	5838042	3792343	2569359	2998355	3032342	1640370	0
C1 (µg/mL)	33.75	94.18	62.78	37.06	12.85	5.85	4.01	8.03	5.22	3.54	4.13	4.17	2.26	0
C2 (µg/mL)		6278.04	3139	617.42	128.5	46.8	34.89	40.15	26.1	17.7	20.65	12.43	9.04	0
Y1 (µg/g)		1609.75	804.87	158.31	32.95	12	8.95	10.29	6.69	4.54	5.29	3.19	2.32	0
Y2 (µg/g)		1609.75	2414.62	2572.93	2605.88	2617.88	2626.83	2637.12	2643.81	2648.35	2653.64	2656.83	2659.15	2659.15

Note: The definitions of PA₁, PA₂, PA₃, PA, C1, C2, Y1, Y2 here are the same as those in Table 31.

** - SC-B13 is the extract which is obtained from the extraction of the SFE residual algae with methanol for 2 hours.

Table 33. The GC/MS Data of Time-Dependent Extraction with Pure CO₂ for the Sample SC-C.

GC/MS Data of Monoterpene 15 in SC-C

	15	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12	C13**
PA ₁	22613954	1035687	2654992	4122395	8612422	9620358	6477219	9579462	3915757	5219862	9169318	10071822	7062645	10663711
PA ₂	23773082	1033436	2806619	4291861	8602128	9338995	6671292	10231492	3865625	5108238	9356149	10057130	6896038	10484384
PA ₃	21704566	1073604	2666971	4671211	8738727	9311026	6741318	9632571	3806947	5185977	9933606	9743500	6924175	10876223
PA	22697201	1047575	2709527	4361822	8657759	9490126	6629943	9821175	3862776	5171359	9486357	9957484	6960959	10674773
C1 (µg/mL)	51.25	2.37	6.12	9.85	19.55	21.43	14.97	22.18	8.72	11.68	21.42	22.48	15.72	24.1
C2 (µg/mL)		157.98	306	218.87	229.91	107.15	74.85	126.87	49.88	46.72	63.83	44.96	46.84	4820
Y1 (µg/g)		42.7	82.7	59.15	62.14	28.96	20.23	34.29	13.48	12.63	17.25	12.15	12.66	1302.7
Y2 (µg/g)		42.7	125.4	184.55	246.69	275.65	295.88	330.17	343.65	356.28	373.53	385.68	398.34	1701.04

GC/MS Data of Monoterpene 14 in SC-C

	14	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12	C13**
PA ₁	51844135	24139431	26481189	8420874	9639158	6949509	3904000	4132796	733434	1050760	874403	1436910	608462	0
PA ₂	53555046	23871995	26836431	8478421	9651174	6553805	4041276	4266633	812528	1037709	825863	1449548	718520	0
PA ₃	53724581	24821009	25979330	8625956	9253194	6515114	4177315	4221544	711593	1038502	878049	1561067	631302	0
PA	53041254	24277478	26432317	8508417	9514509	6672809	4040863	4206991	752518	1042323	859438	1482508	652761	0
C1 (µg/mL)	101.25	46.34	50.46	16.24	18.16	12.74	7.71	8.03	1.44	1.99	1.64	2.83	1.25	0
C2 (µg/mL)		3089.02	2523	360.85	213.56	63.7	38.55	45.93	8.24	7.96	4.89	5.66	3.72	0
Y1 (µg/g)		834.87	681.89	97.53	57.72	17.22	10.42	12.41	2.23	2.15	1.32	1.53	1	0
Y2 (µg/g)		834.87	1516.76	1614.29	1672.01	1689.23	1699.65	1712.06	1714.29	1716.44	1717.76	1719.29	1720.29	1720.29

Note: The definitions of PA₁, PA₂, PA₃, PA, C1, C2, Y1, Y2 here are the same as those in Table 31.

** - SC-C13 is the extract which is obtained from the extraction of the SFE residual algae with methanol for 2 hours.

Table 34. The GC/MS Data of Time-Dependent Extraction with CO₂ Modified with 10% Methanol for the Sample SC-D.

GC/MS Data of Monoterpene 15 in SC-D														
	15	D1	D2	D3	D4	D5	D6	D7	D8	D9	D10	D11	D12	D13**
PA ₁	14092858	2625997	2143445	1287500	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D
PA ₂	14556728	1570992	2114212	1374825	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D
PA ₃	15941689	1751151	2121868	1339627	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D
PA	14863758	1982713	2126508	1333984	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D
C1 (µg/mL)	30.75	4.1	4.4	2.76	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D
C2 (µg/mL)		205	110	92.02	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D
Y1 (µg/g)		102.5	55	46.01	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D
Y2 (µg/g)		102.5	157.5	203.51	203.51	203.51	203.51	203.51	203.51	203.51	203.51	203.51	203.51	203.51

GC/MS Data of Monoterpene 14 in SC-D

	14	D1	D2	D3	D4	D5	D6	D7	D8	D9	D10	D11	D12	D13**
PA ₁	11774428	7841665	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D
PA ₂	11815981	7026938	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D
PA ₃	12035301	6398178	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D
PA	11875237	7088927	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D
C1 (µg/mL)	33.75	20.15	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D
C2 (µg/mL)		1007.5	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D
Y1 (µg/g)		503.75	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D
Y2 (µg/g)		503.75	503.75	503.75	503.75	503.75	503.75	503.75	503.75	503.75	503.75	503.75	503.75	503.75

Note: The definitions of PA₁, PA₂, PA₃, PA, C1, C2, Y1, Y2 here are the same as those in Table 31.

** – SC-D13 is the extract which is obtained from the extraction of the SFE residual algae with methanol for 2 hours.

Table 35. The GC/MS Data of Time-Dependent Extraction with CO₂ Modified with 10% Methanol for the Sample SC-E.

GC/MS Data of Monoterpene 15 in SC-E

	15	E1	E2	E3	E4	E5	E6	E7	E8	E9	E10	E11	E12	E13**
PA ₁	45315288	N/D	6114926	4162898	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D
PA ₂	55800453	N/D	5561403	4401300	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D
PA ₃	63880244	N/D	5807462	4182349	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D
PA	54998662	N/D	5827930	4248849	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D
C1 (µg/mL)	102.5	N/D	10.86	7.92	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D
C2 (µg/mL)		0	543	175.98	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D
Y1 (µg/g)		0	285.79	87.99	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D
Y2 (µg/g)		0	285.79	373.78	373.78	373.78	373.78	373.78	373.78	373.78	373.78	373.78	373.78	373.78

GC/MS Data of Monoterpene 14 in SC-E

	14	E1	E2	E3	E4	E5	E6	E7	E8	E9	E10	E11	E12	E13**
PA ₁	61195255	13223097	5633386	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D
PA ₂	64037502	14393596	5170526	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D
PA ₃	61027460	13252342	5264191	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D
PA	62086739	13623011	5356034	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D
C1 (µg/mL)	101.25	22.22	10.51	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D
C2 (µg/mL)		555.5	525.5	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D
Y1 (µg/g)		292.37	276.58	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D
Y2 (µg/g)		292.37	568.95	568.95	568.95	568.95	568.95	568.95	568.95	568.95	568.95	568.95	568.95	503.75

Note: The definitions of PA₁, PA₂, PA₃, PA, C1, C2, Y1, Y2 here are the same as those in Table 31.

** - SC-E13 is the extract which is obtained from the extraction of the SFE residual algae with methanol for 2 hours.

Table 36. The GC/MS Data of Time-Dependent Extraction with CO₂ Modified with 10% Methanol for the Sample SC-F.

GC/MS Data of Monoterpene 15 in SC-F														
	15	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10	F11	F12	F13**
PA ₁	28896152	4348106	5720555	5604109	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D
PA ₂	27537794	4429103	5700516	5496899	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D
PA ₃	26057361	4785170	5544708	5250937	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D
PA	27497102	4520793	5655260	5450648	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D
C1 (µg/mL)	51.25	8.43	10.54	10.16	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D
C2 (µg/mL)		337.2	263.5	254	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D
Y1 (µg/g)		177.47	138.68	133.68	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D
Y2 (µg/g)		177.47	316.15	449.83	449.83	449.83	449.83	449.83	449.83	449.83	449.83	449.83	449.83	449.83

GC/MS Data of Monoterpene 14 in SC-F

	14	F1	F2'	F3	F4	F5	F6	F7	F8	F9	F10	F11	F12	F13**
PA ₁	37632664	11643746	2178494	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D
PA ₂	45690376	11979992	2197875	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D
PA ₃	52185031	11760528	2137117	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D
·PA	45169357	11794755	2171162	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D
C1 (μg/mL)	101.25	26.44	4.87	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D
C2 (μg/mL)		1057.6	121.75	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D
Y1 (μg/g)		556.63	64.08	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D
Y2 (μg/g)		556.63	620.71	620.71	620.71	620.71	620.71	620.71	620.71	620.71	620.71	620.71	620.71	620.71

Note: The definitions of PA₁, PA₂, PA₃, PA, C1, C2, Y1, Y2 here are the same as those in Table 31.

** -- SC-F13 is the extract which is obtained from the extraction of the SFE residual algae with methanol for 2 hours.

Table 37. The GC/MS Data of the SFE Extracts – Effect of Pressure for the Samples from SC-G to SC-R.

GC/MS Data of Monoterpene 15

	15	SC-G	SC-H	SC-I	SC-J	SC-K	SC-L	SC-M	SC-N	SC-O	SC-P	SC-Q	SC-R
PA ₁	28896152	1663446	1166882	3501942	1669335	566145	1646221	1549175	1569067	882891	972027	1097042	2692564
PA ₂	27537794	1589654	1377577	4087928	2551646	484928	1393667	1235105	1522209	852311	1031972	1020358	2062832
PA ₃	26057361	2108714	1470131	4011324	2582764	560281	1462329	1497439	1003309	888040	1355464	1024128	1897278
PA	27497102	1787271	1338197	3867065	2267915	537118	1500739	1427240	1364862	874414	1119821	1047176	2217558
C1 (µg/mL)	51.25	3.33	2.49	7.21	4.23	1	2.8	2.66	2.54	1.63	2.09	1.95	4.13
C2 (µg/mL)		83.25	62.25	90.12	105.75	25	70	66.5	63.5	40.75	52.25	48.75	103.25
Y (µg/g)		37.84	29.64	42.91	50.36	11.9	33.33	31.67	30.24	19.4	24.88	24.38	49.17

GC/MS Data of Monoterpene 14

	14	SC-G	SC-H	SC-I	SC-J	SC-K	SC-L	SC-M	SC-N	SC-O	SC-P	SC-Q	SC-R
PA ₁	11774428	40461076	25144830	76514265	66657040	28050568	63920578	46314568	38831821	45916962	46236249	29690800	53648508
PA ₂	11815981	42076299	31919094	78812040	70920135	28292701	66351631	48126465	42576027	46327000	44272957	28865793	44048686
PA ₃	12035301	45144710	26995658	79468878	70146825	29231903	67281376	51708097	38154870	42237795	57156458	27096279	43333929
PA	11875237	42560695	28019861	78265061	69241333	28525057	65851195	48716377	39854239	44827252	49221888	28550957	47010374
C1 (µg/mL)	33.75	120.96	79.63	222.43	196.79	81.07	187.15	138.45	113.27	127.4	139.89	81.14	133.61
C2 (µg/mL)		3024	1990.75	2780.37	4919.75	2026.75	4678.75	3461.25	2831.75	3185	3497.25	2028.5	3340.25
Y (µg/g)		1374.54	947.98	1323.98	2342.73	965.12	2227.98	1648.21	1348.45	1516.67	1665.36	1014.25	1590.59

Note: The definitions of PA₁, PA₂, PA₃, PA, C1, C2, Y1, Y2 here are the same as those in Table 31.

Table 38. The GC/MS Data of SFE in Comparison Studies for the Sample SC-S.

GC/MS Data of Monoterpene 15 in SC-S							GC/MS Data of Monoterpene 14 in SC-S						
	15	S1	S2	S3	S4	S5**		14	S1	S2	S3	S4	S5**
PA ₁	23460790	N/D	2146630	2067309	5978884	N/D	PA ₁	12084127	34802146	3853383	1933171	N/D	N/D
PA ₂	19584095	N/D	1824410	2057945	5951533	N/D	PA ₂	11994653	34003448	3478242	2165611	N/D	N/D
PA ₃	19014000	N/D	1908291	2567441	5969655	N/D	PA ₃	12580260	34014437	3393099	2168319	N/D	N/D
PA	20686295	N/D	1959777	2230898	5966691	N/D	PA	12219680	34273343	3574908	2089034	N/D	N/D
C1 (µg/mL)	51.25	N/D	4.86	5.53	14.78	N/D	C1 (µg/mL)	33.75	94.66	9.87	5.77	N/D	N/D
C2 (µg/mL)		0	48.6	50.53	591.2	0	C2 (µg/mL)		2366.5	98.7	57.7	0	0
Y1 (µg/g)		0	24.3	25.26	295.6	0	Y1 (µg/g)		1183.25	49.35	28.85	0	0
Y2 (µg/g)		0	24.3	49.56	345.16	345.16	Y2 (µg/g)		1183.25	1232.6	1261.45	1261.45	1261.45

Note: The definitions of PA₁, PA₂, PA₃, PA, C1, C2, Y1, Y2 here are the same as those in Table 31.

** – SC-S5 is the extract which is obtained from the extraction of the SFE residual algae with methanol for 2 hours.

Table 39. The GC/MS Data of SFE in Comparison Studies for the Sample SC-T.

GC/MS Data of Monoterpene 15 in SC-T							GC/MS Data of Monoterpene 14 in SC-T						
	15	T1	T2	T3	T4	T5**		14	T1	T2	T3	T4	T5**
PA ₁	23460790	N/D	6620492	1965632	6330781	N/D	PA ₁	12084127	43941509	5701315	1941095	N/D	N/D
PA ₂	19384095	N/D	7668394	2049467	6466062	N/D	PA ₂	11994653	45096826	7295104	2197666	N/D	N/D
PA ₃	19014000	N/D	8647810	3266836	6333012	N/D	PA ₃	12580260	45010682	9866435	3074011	N/D	N/D
PA	20686295	N/D	7645365	2427312	6309952	N/D	PA	12219680	44683006	7620951	2404257	N/D	N/D
C1 (µg/mL)	51.25	N/D	18.94	6.01	16.13	N/D	C1 (µg/mL)	33.75	123.41	21.05	6.64	N/D	N/D
C2 (µg/mL)		0	180.94	60.01	645.2	0	C2 (µg/mL)		3085.25	210.5	66.4	0	0
Y1 (µg/g)		0	90.47	30	322.6	0	Y1 (µg/g)		1542.62	105.25	33.2	0	0
Y2 (µg/g)		0	90.47	120.47	443.07	443.07	Y2 (µg/g)		1542.62	1647.87	1681.07	1681.07	1681.07

Note: The definitions of PA₁, PA₂, PA₃, PA, C1, C2, Y1, Y2 here are the same as those in Table 31.

** – SC-T5 is the extract which is obtained from the extraction of the SFE residual algae with methanol for 2 hours.

Table 40. The GC/MS data of SFE in Comparison Studies for the Sample SC-U.

GC/MS Data of Monoterpene 15 in SC-U							GC/MS Data of Monoterpene 14 in SC-U						
	15	U1	U2	U3	U4	U5		14	U1	U2	U3	U4	U5
PA ₁	23460790	2892148	7260847	11930804	16874244	N/D	PA ₁	12084127	65441907	20027745	14697368	N/D	N/D
PA ₂	19584095	2964528	7655639	11489577	16854523	N/D	PA ₂	11994653	60419981	21521931	14277792	N/D	N/D
PA ₃	19014000	3351437	7255636	11949862	17222007	N/D	PA ₃	12580260	61025950	20914805	15012356	N/D	N/D
PA	20686295	3069371	7390707	11790081	16983591	N/D	PA	12219680	62295946	20821494	14662505	N/D	N/D
C1 (µg/mL)	51.25	7.6	18.31	29.21	42.08	0	C1 (µg/mL)	33.75	172.06	57.51	40.5	0	N/D
C2 (µg/mL)		190	180.31	290.21	841.6	0	C2 (µg/mL)		4301.5	570.51	400.5	0	0
Y1 (µg/g)		95	90.16	145.1	420.8	0	Y1 (µg/g)		2150.75	285.25	200.25	0	0
Y2 (µg/g)		95	185.16	330.26	751.06	751.06	Y2 (µg/g)		2150.75	2436	2636.25	2636.25	2636.25

Note: The definitions of PA₁, PA₂, PA₃, PA, C1, C2, Y1, Y2 here are the same as those in Table 31.

** – SC-U5 is the extract which is obtained from the extraction of the SFE residual algae with methanol for 2 hours.

Table 41. The GC/MS Data of Conventional Solvent Extraction in Comparison Studies for the Sample SC-V.

GC/MS Data of Monoterpene 15 in SC-V							GC/MS Data of Monoterpene 14 in SC-V						
	15	V1	V2	V3	V4	V5**		14	V1	V2	V3	V4	V5**
PA ₁	23460790	N/D	1033064	5168801	3336254	1120882	PA ₁	12084127	27042258	10975159	4923449	N/D	N/D
PA ₂	19584095	N/D	829123	5187933	3423759	1136873	PA ₂	11994653	27090250	11755052	5211577	N/D	N/D
PA ₃	19014000	N/D	1030300	5196544	3458469	1378742	PA ₃	12580260	26700681	11377427	5574957	N/D	N/D
PA	20686295	N/D	964162	5184426	3406161	1212165	PA	12219680	26944396	11369213	5236661	N/D	N/D
C1 (µg/mL)	51.25	0	2.39	12.84	8.44	3	C1 (µg/mL)	33.75	74.42	31.4	14.46	N/D	N/D ₁
C2 (µg/mL)		0	23.9	128.4	84.4	199.98	C2 (µg/mL)		1488.4	310.4	144.46	0	0
Y1 (µg/g)		0	11.95	64.2	42.2	99.99	Y1 (µg/g)		744.2	155.2	72.23	0	0
Y2 (µg/g)		0	11.95	76.15	118.35	218.34	Y2 (µg/g)		744.2	899.4	971.63	971.63	971.63

Note: The definitions of PA₁, PA₂, PA₃, PA, C1, C2, Y1, Y2 here are the same as those in Table 31.

** – SC-V5 is the extract which is obtained from the extraction of the SFE residual algae with methanol for 2 hours.

Table 42. The GC/MS Data of Conventional Solvent Extraction in Comparison Studies for the Sample SC-W.

GC/MS Data of Monoterpene 15 in SC-W							GC/MS Data of Monoterpene 14 in SC-W						
	15	W1	W2	W3	W4	W5**		14	W1	W2	W3	W4	W5**
PA ₁	23460790	N/D	832524	2484668	3618447	3363460	PA ₁	12084127	20162378	15460591	4254674	N/D	N/D
PA ₂	19584095	N/D	798881	2733007	3758696	2435180	PA ₂	11994653	20086424	15478643	3954527	N/D	N/D
PA ₃	19014000	N/D	986425	2882586	3651008	3252741	PA ₃	12580260	18487622	15224062	3747560	N/D	N/D
PA	20686295	N/D	872610	2700087	3676050	3017127	PA	12219680	19578808	15387765	3985587	N/D	N/D
C1 (µg/mL)	51.25	N/D	2.16	6.69	9.11	7.47	C1 (µg/mL)	33.75	54.08	42.5	11.01	N/D	N/D
C2 (µg/mL)		0	21.6	66.9	91.1	497.95	C2 (µg/mL)		1081.6	425	110.1	0	0
Y1 (µg/g)		0	10.8	33.45	45.55	248.98	Y1 (µg/g)		540.8	212.5	55.05	0	0
Y2 (µg/g)		0	10.8	44.25	89.8	338.78	Y2 (µg/g)		540.8	753.3	808.35	808.35	808.35

Note: The definitions of PA₁, PA₂, PA₃, PA, C1, C2, Y1, Y2 here are the same as those in Table 31.

** – SC-W5 is the extract which is obtained from the extraction of the SFE residual algae with methanol for 2 hours.

Table 43. The GC/MS Data of Conventional Solvent Extraction in Comparison Studies for the Sample SC-X.

GC/MS Data of Monoterpene 15 in SC-X

	15	X1	X2	X3	X4	X5 ^{**}
PA ₁	23460790	N/D	N/D	2180522	1737588	1729004
PA ₂	19584095	N/D	N/D	1737398	1791974	1946162
PA ₃	19014000	N/D	N/D	1485867	1607501	1474672
PA	20686295	N/D	N/D	1801262	1712354	1716613
C1 (µg/mL)	51.25	N/D	N/D	4.46	4.24	4.25
C2 (µg/mL)		0	0	44.6	42.4	212.5
Y1 (µg/g)		0	0	22.3	21.2	106.25
Y2 (µg/g)		0	0	22.3	43.5	149.75

GC/MS Data of Monoterpene 14 in SC-X

	14	X1	X2	X3	X4	X5 ^{**}
PA ₁	12084127	14943254	10337925	2422729	N/D	N/D
PA ₂	11994653	14255192	9891721	2473277	N/D	N/D
PA ₃	12580260	13843250	9530334	2342345	N/D	N/D
PA	12219680	14347232	9919993	2412784	N/D	N/D
C1 (µg/mL)	33.75	39.63	27.4	6.66	N/D	N/D
C2 (µg/mL)		792.6	274	66.6	0	0
Y1 (µg/g)		396.3	137	33.3	0	0
Y2 (µg/g)		396.3	533.3	566.6	566.6	566.6

Note: The definitions of PA₁, PA₂, PA₃, PA, C1, C2, Y1, Y2 here are the same as those in Table 31.

****** – SC-X5 is the extract which is obtained from the extraction of the SFE residual algae with methanol for 2 hours.

Table 44. The GC/MS Data of SFE in Comparison Studies for the Sample SD-A.

	2	A1	A2	A3	A4	A5**
PA ₁	9106648	2332027	N/D	N/D	N/D	114071
PA ₂	8792756	2009568	N/D	N/D	N/D	128942
PA ₃	9671525	2458902	N/D	N/D	N/D	109002
PA	9190310	2266832	N/D	N/D	N/D	117338
C1 (µg/mL)	63	15.54	N/D	N/D	N/D	0.8
C2 (µg/mL)		194.25	0	0	0	32
Y1 (µg/g)		107.92	0	0	0	17.77
Y2 (µg/g)		107.92	107.92	107.92	107.92	125.69

	4	A1	A2	A3	A4	A5**
PA ₁	8281923	9197209	1848607	1089166	903449	599479
PA ₂	7691865	7737055	1830651	1063016	888280	642512
PA ₃	7986739	7288614	1712867	1095604	957282	485407
PA	7986842	8074292	1797375	1082595	916337	575799
C1 (µg/mL)	50	50.55	11.25	6.78	5.74	3.6
C2 (µg/mL)		631.88	112.5	67.8	114.8	144
Y1 (µg/g)		351.04	62.5	37.67	63.78	80
Y2 (µg/g)		351.04	413.54	451.21	514.99	594.99

	7	A1	A2	A3	A4	A5**
PA ₁	7922284	5577123	477697	393188	549958	423591
PA ₂	8217173	4283875	570996	384010	678740	509790
PA ₃	7598037	4270781	703091	461823	656125	395288
PA	7912498	4710593	583928	413007	628274	442889
C1 (µg/mL)	48	28.58	3.54	2.51	3.81	2.69
C2 (µg/mL)		357.25	35.4	25.1	76.2	107.6
Y1 (µg/g)		198.47	19.67	12.55	38.1	53.8
Y2 (µg/g)		198.47	218.14	230.69	268.79	322.59

	8	A1	A2	A3	A4	A5**
PA ₁	8560136	11985601	1913948	1038239	869198	528341
PA ₂	8663343	10905665	1801689	1015146	874301	551175
PA ₃	8574996	11115065	1850631	1073708	839617	486954
PA	8599492	11335443	1855422	1042364	861039	522156
C1 (µg/mL)	50	65.91	10.79	6.06	5.01	3.04
C2 (µg/mL)		823.87	107.9	60.6	100.2	121.6
Y1 (µg/g)		457.7	59.94	33.67	55.67	67.56
Y2 (µg/g)		457.7	517.64	551.31	606.98	674.54

Table 44. The GC/MS Data of SFE in Comparison Studies for the Sample SD-A (Continued).

GC/MS Data of Monoterpene 9 in SD-A						
	9	A1	A2	A3	A4	A5**
PA ₁	7080066	10351665	1154466	910855	1225504	816124
PA ₂	7296278	8235046	1257799	910994	1344027	985046
PA ₃	6109397	7936375	1377242	1043609	1499894	692173
PA	6828580	8841028	1263169	955152	1356475	831114
C1 (µg/mL)	45	58.26	8.32	6.29	8.94	5.48
C2 (µg/mL)		728.25	83.2	62.9	178.8	219.2
Y1 (µg/g)		404.58	46.22	34.94	99.33	121.78
Y2 (µg/g)		404.58	450.8	485.74	585.07	706.85

Note: The definitions of PA₁, PA₂, PA₃, PA, C1, C2, Y1, Y2 here are the same as those in Table 31.

** – SD-A5 is the extract which is obtained from the extraction of the SFE residual algae with methanol for 2 hours.

Table 45. The GC/MS Data of SFE in Comparison Studies for the Sample SD-B.

GC/MS Data of Monoterpene 2 in SD-B

	2	B1	B2	B3	B4	B5**
PA ₁	9106648	N/D	N/D	N/D	N/D	162115
PA ₂	8792756	N/D	N/D	N/D	N/D	168303
PA ₃	9671525	N/D	N/D	N/D	N/D	120339
PA	9190310	N/D	N/D	N/D	N/D	150252
C1 (µg/mL)	63	N/D	N/D	N/D	N/D	1.03
C2 (µg/mL)		0	0	0	0	51.5
Y1 (µg/g)		0	0	0	0	28.61
Y2 (µg/g)		0	0	0	0	28.61

GC/MS Data of Monoterpene 7 in SD-B

	7	B1	B2	B3	B4	B5**
PA ₁	7922284	4285013	N/D	N/D	1110774	263572
PA ₂	8217173	3496748	N/D	N/D	903150	256266
PA ₃	7598037	4229923	N/D	N/D	1097161	215267
PA	7912498	4003894	N/D	N/D	1037028	245035
C1 (µg/mL)	48	24.29	N/D	N/D	6.29	1.49
C2 (µg/mL)		303.62	0	0	125.8	74.5
Y1 (µg/g)		168.68	0	0	69.89	41.39
Y2 (µg/g)		168.68	168.68	168.68	238.57	279.96

GC/MS Data of Monoterpene 8 in SD-B

	8	B1	B2	B3	B4	B5**
PA ₁	8560136	12778728	2101588	1949693	1908954	285292
PA ₂	8663343	10272693	2115713	1836859	1787178	308861
PA ₃	8574996	12477649	2132706	1839673	1846774	270618
PA	8599492	11843023	2116669	1875408	1847635	288257
C1 (µg/mL)	50	68.86	12.31	10.9	10.74	1.68
C2 (µg/mL)		860.75	123.1	109	107.4	84
Y1 (µg/g)		478.19	68.39	60.56	59.67	46.67
Y2 (µg/g)		478.19	546.58	607.14	666.81	713.48

GC/MS Data of Monoterpene 4 in SD-B

	4	B1	B2	B3	B4	B5**
PA ₁	8281923	9591199	1829234	2320523	2076299	302973
PA ₂	7691865	7184662	1925501	2037118	2129287	253233
PA ₃	7986739	9205173	1986479	2022806	2205778	305208
PA	7986842	8660344	1913738	2126815	2137121	287138
C1 (µg/mL)	50	54.22	11.98	13.31	13.38	1.8
C2 (µg/mL)		677.75	119.8	133.1	267.6	90
Y1 (µg/g)		376.53	66.56	73.94	148.67	50
Y2 (µg/g)		376.53	443.09	517.03	665.7	715.7

Table 45. The GC/MS Data of SFE in Comparison Studies for the Sample SD-B (Continued).

GC/MS Data of Monoterpene 9 in SD-B						
	4	B1	B2	B3	B4	B5**
PA ₁	7080066	10839657	1664911	2208953	3802320	866883
PA ₂	7296278	7378314	1640393	1882328	3783997	865845
PA ₃	6109397	10148822	1805162	2088874	4077588	761075
PA	6828580	9455597	1703488	2060051	3887968	831268
C1 (µg/mL)	45	62.31	11.23	13.58	25.63	5.48
C2 (µg/mL)		778.88	112.3	135.8	512.6	274
Y1 (µg/g)		432.71	62.39	75.44	284.78	152.22
Y2 (µg/g)		432.71	495.1	570.54	855.32	1007.54

Note: The definitions of PA₁, PA₂, PA₃, PA, C1, C2, Y1, Y2 here are the same as those in Table 31.

** – SD-B5 is the extract which is obtained from the extraction of the SFE residual algae with methanol for 2 hours.

Table 46. The GC/MS Data of SFE in Comparison Studies for the Sample SD-C.

GC/MS Data of Monoterpene 2 in SD-C

	2	C1	C2	C3	C4	C5**
PA ₁	9106648	1455563	329692	N/D	N/D	1492389
PA ₂	8792756	1564487	359855	N/D	N/D	1490687
PA ₃	9671525	1356981	398566	N/D	N/D	1503441
PA	9190310	1459010	362704	N/D	N/D	1495506
C1 (µg/mL)	63	10	2.49	N/D	N/D	10.25
C2 (µg/mL)		125	24.9	0	0	410
Y1 (µg/g)		65.79	13.1	0	0	215.79
Y2 (µg/g)		65.79	78.89	78.89	78.89	294.68

GC/MS Data of Monoterpene 7 in SD-C

	7	C1	C2	C3	C4	C5**
PA ₁	7922284	1591151	N/D	N/D	655428	960324
PA ₂	8217173	877581	N/D	N/D	542285	938240
PA ₃	7598037	856005	N/D	N/D	681692	857961
PA	7912498	1108246	N/D	N/D	626468	918842
C1 (µg/mL)	48	6.72	N/D	N/D	3.8	5.57
C2 (µg/mL)		84	0	0	76	222.8
Y1 (µg/g)		44.21	0	0	36.78	117.26
Y2 (µg/g)		44.21	44.21	44.21	80.99	198.25

GC/MS Data of Monoterpene 8 in SD-C

	8	C1	C2	C3	C4	C5**
PA ₁	8560136	6738832	1505703	1214207	401176	3735873
PA ₂	8663343	5765386	1519196	1239436	524309	3711203
PA ₃	8574996	5906463	1468437	1254227	872671	3583020
PA	8599492	6136894	1497779	1235957	599385	3676699
C1 (µg/mL)	50	35.68	8.71	7.19	3.49	21.38
C2 (µg/mL)		446	87.1	71.9	69.8	855.2
Y1 (µg/g)		234.74	45.84	37.84	36.74	450.1
Y2 (µg/g)		234.74	280.58	318.42	355.16	805.26

GC/MS Data of Monoterpene 4 in SD-C

	4	C1	C2	C3	C4	C5**
PA ₁	8281923	4747266	1731271	1347021	2033784	3231764
PA ₂	7691865	4316789	1768735	1241652	2148953	3425096
PA ₃	7986739	4328925	1605622	1371831	2997892	3404278
PA	7986842	4464327	1701876	1320168	2393543	3353713
C1 (µg/mL)	50	27.95	10.65	8.26	14.98	21
C2 (µg/mL)		349.38	106.5	82.6	149.8	840
Y1 (µg/g)		183.88	56.05	43.47	78.84	442.1
Y2 (µg/g)		183.88	239.93	283.4	362.24	804.34

Table 46. The GC/MS Data of SFE in Comparison Studies for the Sample SD-C (Continued).

GC/MS Data of Monoterpene 9 in SD-C						
	9	C1	C2	C3	C4	C5**
PA ₁	7080066	2626457	576346	377444	1273109	3585754
PA ₂	7296278	1635762	528700	242734	1374265	3662221
PA ₃	6109397	1651690	616462	231736	1330134	3656612
PA	6828580	1971303	573836	283971	1325836	3634862
C1 (µg/mL)	45	12.99	3.78	1.87	8.74	23.95
C2 (µg/mL)		162.37	37.8	18.7	174.8	958
Y1 (µg/g)		85.46	19.89	9.84	149.88	504.21
Y2 (µg/g)		85.46	105.35	115.19	265.07	769.28

Note: The definitions of PA₁, PA₂, PA₃, PA, C1, C2, Y1, Y2 here are the same as those in Table 31.

** - SD-C5 is the extract which is obtained from the extraction of the SFE residual algae with methanol for 2 hours.

Table 47. The GC/MS Data of Conventional Solvent Extraction in Comparison Studies for the Sample SD-D.

GC/MS Data of Monoterpene 2 in SD-D

	2	D1	D2	D3	D4	D5**
PA ₁	9106648	N/D	362314	55387	206503	N/D
PA ₂	8792756	N/D	431815	466984	245772	N/D
PA ₃	9671525	N/D	366845	387424	213924	N/D
PA	9190310	N/D	386991	469932	222066	N/D
C1 (µg/mL)	63	N/D	2.65	3.22	1.52	N/D
C2 (µg/mL)		0	26.5	32.2	30.4	0
Y1 (µg/g)		0	13.25	16.1	15.2	0
Y2 (µg/g)		0	13.25	29.35	44.55	44.55

GC/MS Data of Monoterpene 7 in SD-D

	7	D1	D2	D3	D4	D5**
PA ₁	7922284	851679	2507914	6238785	3295995	866783
PA ₂	8217173	964048	2966216	4661233	3559906	913965
PA ₃	7598037	1159828	2507275	4581638	3146979	828061
PA	7912498	991851	2660468	5160552	3334293	869603
C1 (µg/mL)	48	6.02	16.14	31.31	20.23	5.28
C2 (µg/mL)		75.25	161.4	313.1	404.6	351.96
Y1 (µg/g)		37.62	80.7	156.65	202.3	175.98
Y2 (µg/g)		37.62	118.32	274.97	477.27	653.25

GC/MS Data of Monoterpene 8 in SD-D

	8	D1	D2	D3	D4	D5**
PA ₁	8560136	1591531	1792628	3638707	2656377	624673
PA ₂	8663343	1741535	2046326	2358154	2874639	620576
PA ₃	8574996	1952308	1770592	2795717	2534321	577483
PA	8599492	1761791	1869848	2930859	2688446	607577
C1 (µg/mL)	50	10.24	10.87	17.04	15.63	3.33
C2 (µg/mL)		128	108.7	170.4	312.6	235.31
Y1 (µg/g)		64	54.35	85.2	156.3	117.65
Y2 (µg/g)		64	118.35	203.55	359.85	477.5

GC/MS Data of Monoterpene 4 in SD-D

	4	D1	D2	D3	D4	D5**
PA ₁	8281923	2170490	2436158	4027004	2555239	793430
PA ₂	7691865	2306032	2575399	3161083	2832204	857761
PA ₃	7986739	2481627	2333783	3054100	2627993	800416
PA	7986842	2319383	2448446	3414062	2671812	817202
C1 (µg/mL)	50	14.52	15.33	21.37	16.73	5.12
C2 (µg/mL)		181.5	153.3	213.7	334.6	341.3
Y1 (µg/g)		90.75	76.15	106.85	167.3	170.65
Y2 (µg/g)		90.75	166.9	273.75	441.05	611.7

Table 47. The GC/MS Data in Comparison Studies for the Sample SD-D (Continued).

GC/MS Data of Monoterpene 9 in SD-D						
	9	D1	D2	D3	D4	D5**
PA ₁	7080066	909942	2112769	5473531	3337512	1091193
PA ₂	7296278	1051732	2393723	3828268	3754795	1185304
PA ₃	6109397	1170335	2044867	4078407	3335895	1086423
PA	6828580	1044003	2183786	4460068	3476067	1120973
C1 (µg/mL)	45	6.88	14.39	29.39	22.91	7.39
C2 (µg/mL)		86	143.9	293.9	458.2	492.62
Y1 (µg/g)		43	71.95	146.95	229.1	246.31
Y2 (µg/g)		43	114.95	261.9	491	737.31

Note: The definitions of PA₁, PA₂, PA₃, PA, C1, C2, Y1, Y2 here are the same as those in Table 31.

** – SD-D5 is the extract which is obtained from the extraction of the SFE residual algae with methanol for 2 hours.

Table 48. The GC/MS Data of Conventional Solvent Extraction in Comparison Studies for the Sample SD-E.

GC/MS Data of Monoterpene 2 in SD-E

	2	E1	E2	E3	E4	E5 ^{''}
PA ₁	9106648	438022	1004667	1120945	654788	N/D
PA ₂	8792756	446924	1010655	1027411	733068	N/D
PA ₃	9671525	562872	938559	1071821	734129	N/D
PA	9190310	482606	984627	1073392	707328	N/D
C1 (µg/mL)	63	3.31	6.75	7.36	4.85	N/D
C2 (µg/mL)		41.37	67.5	73.6	97	0
Y1 (µg/g)		20.68	33.75	36.8	48.5	0
Y2 (µg/g)		20.68	54.43	91.23	139.73	139.73

GC/MS Data of Monoterpene 7 in SD-E

	7	E1	E2	E3	E4	E5 ^{''}
PA ₁	7922284	566644	2179455	2799131	2634161	474349
PA ₂	8217173	486804	2310430	2662444	2602365	478341
PA ₃	7598037	650656	938559	2558094	2744839	505866
PA	7912498	568034	1809481	2673223	2660455	486185
C1 (µg/mL)	48	3.45	10.98	16.22	16.14	2.95
C2 (µg/mL)		43.12	109.8	162.2	322.8	196.65
Y1 (µg/g)		21.56	54.9	81.1	161.4	98.32
Y2 (µg/g)		21.56	76.46	157.56	318.96	417.28

GC/MS Data of Monoterpene 8 in SD-E

	8	E1	E2	E3	E4	E5 ^{''}
PA ₁	8560136	1310873	1949493	1908615	1781111	229799
PA ₂	8663343	1262989	2131788	1783594	1886060	216107
PA ₃	8574996	1608137	1852880	1827824	1923286	222933
PA	8599492	1393999	1978053	1840011	1863485	222946
C1 (µg/mL)	50	8.11	11.5	10.7	10.83	1.3
C2 (µg/mL)		101.37	115	107	216.6	86.66
Y1 (µg/g)		50.68	57.5	53.5	108.3	43.33
Y2 (µg/g)		50.68	108.18	161.68	269.98	313.31

GC/MS Data of Monoterpene 4 in SD-E

	4	E1	E2	E3	E4	E5 ^{''}
PA ₁	8281923	1680053	2319711	1848185	1829993	338801
PA ₂	7691865	1608093	2503204	1761710	1859933	353202
PA ₃	7986739	1938036	1995401	1801990	2116618	377424
PA	7986842	1742060	2272772	1803961	1935514	356476
C1 (µg/mL)	50	10.91	14.23	11.29	12.12	2.23
C2 (µg/mL)		136.38	142.3	112.9	242.4	148.65
Y1 (µg/g)		68.19	71.15	56.45	121.2	74.32
Y2 (µg/g)		68.19	139.34	195.79	316.99	391.31

Table 48. The GC/MS Data in Comparison Studies for the Sample SD-E (Continued).

GC/MS Data of Monoterpene 9 in SD-E							
	9	E1	E2	E3	E4	E5**	
PA ₁	7080066	850017	2070271	2824502	3243001	626265	
PA ₂	7296278	729338	2188734	2694580	3151864	584888	
PA ₃	6109397	1028892	1919673	2656436	3571163	663777	
PA	6828580	869415	2059559	2725172	3322009	624977	
C1 (µg/mL)	45	5.73	13.57	17.96	21.89	4.12	
C2 (µg/mL)		71.62	135.7	179.6	437.8	274.64	
Y1 (µg/g)		35.81	67.85	89.8	218.9	137.32	
Y2 (µg/g)		35.81	103.66	193.46	412.36	549.68	

Note: The definitions of PA₁, PA₂, PA₃, PA, C1, C2, Y1, Y2 here are the same as those in Table 31.

** – SD-E5 is the extract which is obtained from the extraction of the SFE residual algae with methanol for 2 hours.

Table 49. The GC/MS Data of Conventional Solvent Extraction in Comparison Studies for the Sample SD-F.

GC/MS Data of Monoterpene 2 in SD-F

	2	F1	F2	F3	F4	F5**
PA ₁	9106648	546605	1516621	1143481	1004267	148403
PA ₂	8792756	539938	1590153	1361651	986322	162374
PA ₃	9671525	480777	1356222	1645742	1020315	159261
PA	9190310	522440	1487665	1383625	1003635	156746
C1 (µg/mL)	63	3.58	10.2	9.48	6.88	1.07
C2 (µg/mL)		44.75	102	94.8	137.6	53.5
Y1 (µg/g)		22.37	51	47.4	68.8	26.75
Y2 (µg/g)		22.37	73.37	120.77	189.57	216.32

GC/MS Data of Monoterpene 7 in SD-F

	7	F1	F2	F3	F4	F5**
PA ₁	7922284	1105564	2384322	4284569	3218889	497644
PA ₂	8217173	1251292	2899609	4770161	3341743	518681
PA ₃	7598037	925984	2268616	4375691	3350147	526046
PA	7912498	1094280	2517516	4476807	3303593	514124
C1 (µg/mL)	48	6.64	15.27	27.16	20.04	3.12
C2 (µg/mL)		83	152.7	271.6	400.8	156
Y1 (µg/g)		41.5	76.35	135.8	200.4	78
Y2 (µg/g)		41.5	117.85	253.65	454.05	532.05

GC/MS Data of Monoterpene 8 in SD-F

	8	F1	F2	F3	F4	F5**
PA ₁	8560136	2203429	2518633	3493359	2422276	193572
PA ₂	8663343	2584623	2853062	3777341	2508613	207344
PA ₃	8574996	1954703	2481826	3669546	2370667	197364
PA	8599492	2247585	2617840	3646749	2433852	199427
C1 (µg/mL)	50	13.07	15.22	21.2	14.15	1.16
C2 (µg/mL)		163.37	152.2	212	283	58
Y1 (µg/g)		81.68	76.1	106	141.5	29
Y2 (µg/g)		81.68	157.78	263.78	405.28	434.28

GC/MS Data of Monoterpene 4 in SD-F

	4	F1	F2	F3	F4	F5**
PA ₁	8281923	2512645	2879889	4069545	2567255	372274
PA ₂	7691865	2732205	3332921	3922905	2561467	389460
PA ₃	7986739	2222746	2896775	3690145	2381308	371555
PA	7986842	2489199	3036528	3894198	2503343	377763
C1 (µg/mL)	50	15.58	19.01	24.38	15.67	2.36
C2 (µg/mL)		194.75	190.1	243.8	313.4	118
Y1 (µg/g)		97.37	95.05	121.9	156.7	59
Y2 (µg/g)		97.37	192.42	314.32	471.02	530.02

Table 49. The GC/MS Data in Comparison Studies for the Sample SD-F (Continued).

GC/MS Data of Monoterpene 9 in SD-F						
	9	F1	F2	F3	F4	F5**
PA ₁	7080066	1247839	2892884	5278542	4001361	515926
PA ₂	7296278	1436741	3189828	5588940	3969984	565060
PA ₃	6109397	1118978	2730748	5094351	3816432	541854
PA	6828580	1267853	2937820	5320611	3929259	540947
C1 (µg/mL)	45	8.36	19.36	35.06	25.89	3.56
C2 (µg/mL)		104.5	193.6	350.6	517.8	178
Y1 (µg/g)		52.25	96.8	175.3	258.9	89
Y2 (µg/g)		52.25	149.05	324.35	583.25	672.25

Note: The definitions of PA₁, PA₂, PA₃, PA, C1, C2, Y1, Y2 here are the same as those in Table 31.

** – SD-F5 is the extract which is obtained from the extraction of the SFE residual algae with methanol for 2 hours.

Appendix B

The NMR and Mass Spectra of Eight Isolated Halogenated Monoterpenes

Figure 15. The ^1H NMR Spectrum of Monoterpene 14 (CDCl_3 , 300 MHz).

Figure 16. The ^{13}C NMR Spectrum of Monoterpene 14 (CDCl_3 , 75 MHz).

Figure 17. Mass Spectrum of Monoterpene 14 in GC/MS.

Figure 18. The ^1H NMR Spectrum of Monoterpene **15** (CDCl_3 , 300 MHz).

Figure 19. The ^{13}C NMR Spectrum of Monoterpene **15** (CDCl_3 , 75 MHz).

Figure 20. Mass Spectrum of Monoterpene 15 in GC/MS.

Figure 21. The ^1H NMR Spectrum of Monoterpene 2 (CDCl_3 , 300 MHz).

Figure 22. The ^{13}C NMR Spectrum of Monoterpene 2 (CDCl_3 , 75 MHz).

Figure 23. Mass Spectrum of Monoterpene 2 in GC/MS.

Figure 24. The ^1H NMR Spectrum of Monoterpene 16 (CDCl_3 , 300 MHz).

Figure 25. The ^{13}C NMR Spectrum of Monoterpene 16 (CDCl_3 , 75 MHz).

Figure 26. Mass Spectrum of Monoterpene 16 in GC/MS.

Figure 27. The ^1H NMR Spectrum of Monoterpene 7 (CDCl_3 , 300 MHz).

Figure 28. The ^{13}C NMR Spectrum of Monoterpene 7 (CDCl_3 , 75 MHz).

Figure 30. The ^1H NMR Spectrum of Monoterpene 9 (CDCl_3 , 300 MHz).

Figure 31. The ^1H NMR Spectrum of Monoterpene 9 (C_6D_6 , 300 MHz).

Figure 32. Mass Spectrum of Monoterpene 9 in GC/MS.

Figure 33. The ^1H NMR Spectrum of Monoterpene 8 (CDCl_3 , 300 MHz).

¹H NMR spectrum of compound 8. The chemical structure of 8 is shown above the spectrum. The spectrum displays peaks from 0 to 8 ppm. The following table provides the peak data:

Line	Chemical Shift (ppm)	Integration	Assignment
1	7.166	1.00	H _{1a}
2	7.103	1.00	H _{1b}
3	6.967	1.00	H ₂
4	6.776	1.00	H ₃
5	6.723	1.00	H ₄
6	6.616	1.00	H ₅
7	6.552	1.00	H ₆
8	6.459	1.00	H ₇
9	6.371	1.00	H ₈
10	6.284	1.00	H ₉
11	6.183	1.00	H ₁₀
12	6.085	1.00	H ₁₁
13	6.012	1.00	H ₁₂
14	5.911	1.00	H ₁₃
15	5.805	1.00	H ₁₄
16	5.697	1.00	H ₁₅
17	5.587	1.00	H ₁₆
18	5.475	1.00	H ₁₇
19	5.353	1.00	H ₁₈
20	5.229	1.00	H ₁₉
21	5.103	1.00	H ₂₀
22	4.975	1.00	H ₂₁
23	4.845	1.00	H ₂₂
24	4.713	1.00	H ₂₃
25	4.579	1.00	H ₂₄
26	4.443	1.00	H ₂₅

Figure 35. Mass Spectrum of Monoterpene 8 in GC/MS.

Figure 36. The ^1H NMR Spectrum of Monoterpene 4 (CDCl_3 , 300 MHz).

[illegible]

Year	1967	1968	1969	1970	1971	1972	1973	1974	1975	1976	1977	1978	1979	1980	1981	1982	1983	1984	1985	1986	1987	1988	1989	1990	1991	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013	2014	2015	2016	2017	2018	2019	2020	2021	2022	2023	2024	2025	2026	2027	2028	2029	2030	2031	2032	2033	2034	2035	2036	2037	2038	2039	2040	2041	2042	2043	2044	2045	2046	2047	2048	2049	2050	2051	2052	2053	2054	2055	2056	2057	2058	2059	2060	2061	2062	2063	2064	2065	2066	2067	2068	2069	2070	2071	2072	2073	2074	2075	2076	2077	2078	2079	2080	2081	2082	2083	2084	2085	2086	2087	2088	2089	2090	2091	2092	2093	2094	2095	2096	2097	2098	2099	2100																																																																																																																																																																							
1	1.00	1.01	1.02	1.03	1.04	1.05	1.06	1.07	1.08	1.09	1.10	1.11	1.12	1.13	1.14	1.15	1.16	1.17	1.18	1.19	1.20	1.21	1.22	1.23	1.24	1.25	1.26	1.27	1.28	1.29	1.30	1.31	1.32	1.33	1.34	1.35	1.36	1.37	1.38	1.39	1.40	1.41	1.42	1.43	1.44	1.45	1.46	1.47	1.48	1.49	1.50	1.51	1.52	1.53	1.54	1.55	1.56	1.57	1.58	1.59	1.60	1.61	1.62	1.63	1.64	1.65	1.66	1.67	1.68	1.69	1.70	1.71	1.72	1.73	1.74	1.75	1.76	1.77	1.78	1.79	1.80	1.81	1.82	1.83	1.84	1.85	1.86	1.87	1.88	1.89	1.90	1.91	1.92	1.93	1.94	1.95	1.96	1.97	1.98	1.99	2.00	2.01	2.02	2.03	2.04	2.05	2.06	2.07	2.08	2.09	2.10	2.11	2.12	2.13	2.14	2.15	2.16	2.17	2.18	2.19	2.20	2.21	2.22	2.23	2.24	2.25	2.26	2.27	2.28	2.29	2.30	2.31	2.32	2.33	2.34	2.35	2.36	2.37	2.38	2.39	2.40	2.41	2.42	2.43	2.44	2.45	2.46	2.47	2.48	2.49	2.50	2.51	2.52	2.53	2.54	2.55	2.56	2.57	2.58	2.59	2.60	2.61	2.62	2.63	2.64	2.65	2.66	2.67	2.68	2.69	2.70	2.71	2.72	2.73	2.74	2.75	2.76	2.77	2.78	2.79	2.80	2.81	2.82	2.83	2.84	2.85	2.86	2.87	2.88	2.89	2.90	2.91	2.92	2.93	2.94	2.95	2.96	2.97	2.98	2.99	3.00	3.01	3.02	3.03	3.04	3.05	3.06	3.07	3.08	3.09	3.10	3.11	3.12	3.13	3.14	3.15	3.16	3.17	3.18	3.19	3.20	3.21	3.22	3.23	3.24	3.25	3.26	3.27	3.28	3.29	3.30	3.31	3.32	3.33	3.34	3.35	3.36	3.37	3.38	3.39	3.40	3.41	3.42	3.43	3.44	3.45	3.46	3.47	3.48	3.49	3.50	3.51	3.52	3.53	3.54	3.55	3.56	3.57	3.58	3.59	3.60	3.61	3.62	3.63	3.64	3.65	3.66	3.67	3.68	3.69	3.70	3.71	3.72	3.73	3.74	3.75	3.76	3.77	3.78	3.79	3.80	3.81	3.82	3.83	3.84	3.85	3.86	3.87	3.88	3.89	3.90	3.91	3.92	3.93	3.94	3.95	3.96	3.97	3.98	3.99	4.00

Figure 38. Mass Spectrum of Monoterpene 4 in GC/MS.

IMAGE EVALUATION TEST TARGET (QA-3)

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