Extraction of Bioactive Sesquiterpene Lactones from *Magnolia grandiflora* Using Supercritical Carbon Dioxide and Near-Critical Propane

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The bioactive sesquiterpene lactones parthenolide and costunolide, as well as the tricyclic sesquiterpene cyclocolorenone, were extracted from the leaves of *Magnolia grandiflora* by supercritical extraction with carbon dioxide and by near-critical extraction with propane. Parthenolide, costunolide, and cyclocolorenone in the extracts were identified by ^{1}H NMR and quantified by HPLC. Results indicate that supercritical carbon dioxide selectively removed parthenolide, costunolide, and cyclocolorenone when compared to standard liquid dichloromethane or near-critical propane extraction. HPLC traces show that the carbon dioxide extract is virtually clean of any chlorophyll or fatty material peaks which hinder isolation of these chemicals.

Keywords: *Supercritical fluid; natural products; bioactive; Magnolia grandiflora*

INTRODUCTION

Sesquiterpenes. Chemical structures of the sesquiterpenes parthenolide, costunolide, and cyclocolorenone are shown in Figure 1. Parthenolide, a sesquiterpene lactone, is known to exhibit a wide spectrum of biological activities which include cytotoxic, antibacterial, and anti-fungal properties (Picman, 1986; Fischer, 1990). Its anti-inflammatory (Heptinstall *et al.*, 1985) and antirheumatoid arthritis activities (Pattrick *et al.*, 1989) have been reported. Parthenolide is the active principle in European feverfew, *Tanacetum parthenium* (Awang *et al.*, 1991), which has been in popular use for the prophylactic treatment of migraine headaches for several centuries. Recently, two placebo-controlled trials established the potential of feverfew as a prophylactic against migraine headaches with reductions in both frequency and severity of migraine attacks (Johnson *et al.*, 1985; Murphy *et al.*, 1988). Parthenolide is also an inhibitor of human platelet aggregation and degranulation (Groenewegen and Heptinstall, 1990; Marles *et al.*, 1992).

Recent findings in our laboratory have shown that sesquiterpene lactones act as potent germination stimulants of witchweed, *Striga asiatica* (Fischer *et al.*, 1990). This parasite causes severe reductions in the yields of major staple crops (corn, wheat, sorghum, rice, millet, and sugarcane) in Africa and Asia (Ramaiah, 1983). A potential method for witchweed control involves the use of stimulants to promote seed germination in the absence of a suitable host, which results in death of the witchweed seedling. Parthenolide induces germination of witchweed (42–54%) over a broad concentration range from 10^{-5} to 10^{-9} M (Fischer *et al.*, 1990).

The blood fluke is responsible for human schistosomiasis (bilharzia), which affects more than 200 million people in many tropical countries (Marston and Hostettmann, 1985; Hostettmann and Marston, 1987). Costunolide and several biogenetically derived lactones prevent human skin penetration of the cercariae of *Schistosoma trematodes* (Vichnewski *et al.*, 1976; Garcia *et al.*, 1976).

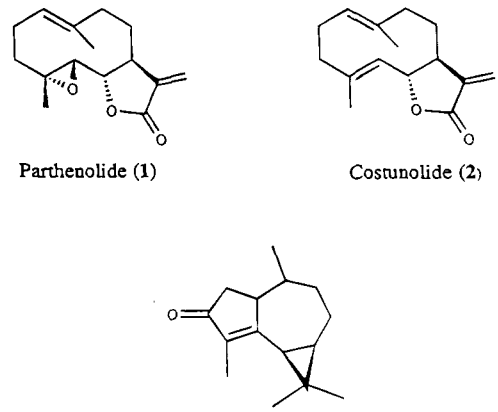

Figure 1. Chemical structures of parthenolide (**1**), costunolide (**2**), and cyclocolorenone (**3**).

Costunolide shows a strong inhibiting effect on the movement of the nematode larvae (Seki *et al.*, 1991), with a minimal lethal concentration of 250 μg/mL, and has a cholagogic and antiulcer effect (induced by restraint in water) in mice (Yamahara *et al.*, 1985). It is also one of the major constituents in costus root oil, which is obtained from the Himalayan *Saussurea lappa* and was used in the cosmetic industry (Fischer, 1991).

The tricyclic sesquiterpene cyclocolorenone (Hellyer and Lassak, 1967; Mikhailova *et al.*, 1978) presents phytotoxic and antimicrobial properties (Jacyno *et al.*, 1991), as well as antifungal activity as the active constituent of *Ledum* L. (Belousova *et al.*, 1989). Its epimer also plays an important role in a dipterocarp as a defensive sesquiterpenoid (Richardson *et al.*, 1989).

Magnolia grandiflora (Magnoliaceae), which is a major tree of the pine–hardwood forests in the southeastern United States, has been shown to be an excellent source of parthenolide (El-Feraly and Chan, 1978) as well as costunolide and cyclocolorenone. However, isolation of these compounds by column chromatography is tedious and time-consuming (Fischer, 1991). Large-

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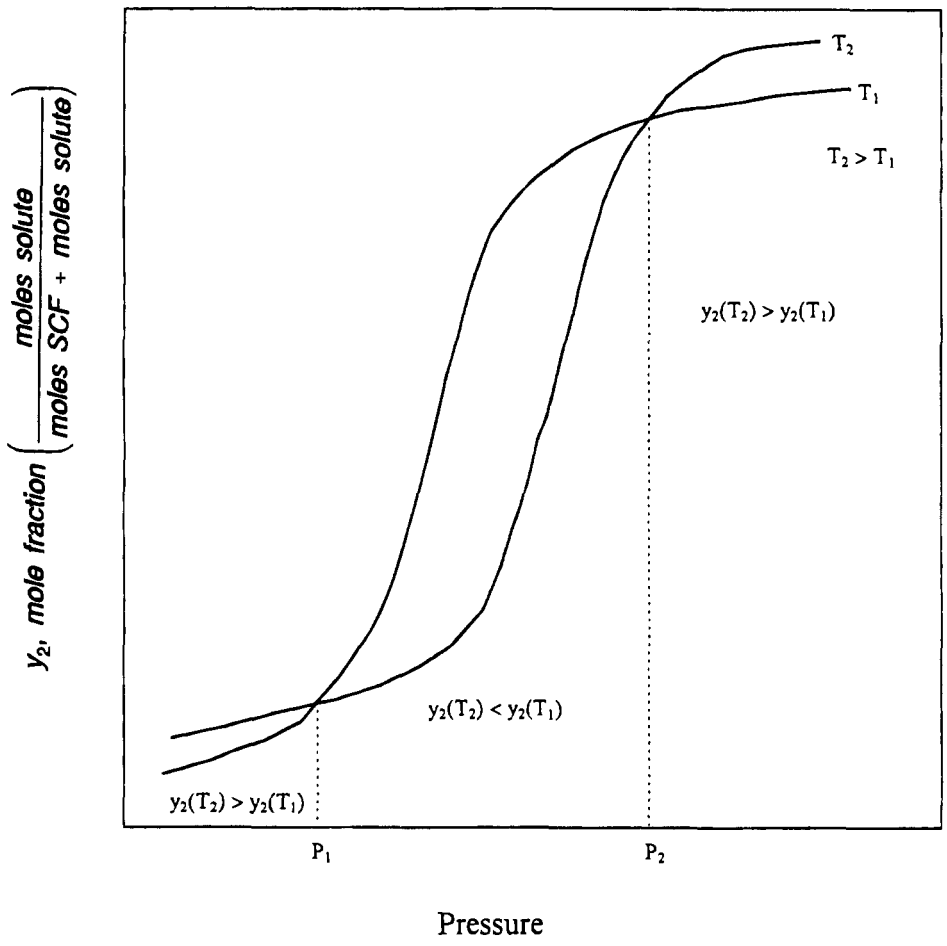

Figure 2. Solubility of a nonvolatile solute.

scale column isolation is not likely because of the volumes of solvents that would be required. It was the objective of this study to test the applicability of supercritical fluid extraction for selective removal of these compounds from leaves of *M. grandiflora* by using carbon dioxide and near-critical propane as solvents.

Supercritical Fluid Extraction. Typically, extraction of a natural product requires recovery of only a small portion of the original matrix. This can be an ideal application for supercritical fluid extraction (SCFE), with the most notable success being the commercial decaffeination of raw coffee beans (McHugh and Krukonis, 1986).

Traditional liquid organic solvents tend to be nonselective, often making additional time-consuming and costly separations necessary. However, the selectivity of a SCF may be manipulated by choice of the solvent, by control of the temperature and pressure, or by the use of modifiers; since most of the SCFs currently being investigated are gases at room temperature, complete separation of solvent and solute is possible with a simple pressure drop. SCFE of natural products has been primarily limited to the use of carbon dioxide as the solvent. This is because carbon dioxide has relatively low critical properties and is nontoxic, environmentally acceptable, and inexpensive.

SCFE of a nonvolatile solute can often be explained in terms of the solubility diagram (solute mole fraction, y_2, in the SCF phase versus pressure) shown in Figure 2. The shapes of the isotherms and locations of the crossovers, P_1 and P_2, will vary with each system, but the concept is the same throughout. The crossovers can be explained in terms of the vapor pressure of the solute and the density of the solvent (Pang and McLaughlin, 1985; Chimowitz and Pennisi, 1986). An increase in temperature at constant pressure causes an exponential increase in the vapor pressure of the solute but a decrease in solvent density. Below P_1 solute concentration in the SCF phase is higher at higher temperature (T_2); here solute vapor pressure effects are more important than density changes in the solvent. Between P_1 and P2 density effects dominate. Above P_2 density changes with pressure are small, so vapor pressure effects again become important.

Wong and Johnston (1986) reported solubility data for two similar sterols, cholesterol and stigmasterol, in SC carbon dioxide. Their data showed a P_2 crossover for both cholesterol and stigmasterol near 170 bar. A P_1 crossover was not observed for the temperature and pressure ranges studied (~100 to ~300 bar and 35, 40, 60 °C). Although the sterols are chemically similar in structure, the solubility of cholesterol was ~3 times that of stigmasterol at all conditions. Li and Hartland (1991) measured the solubility of the xanthine stimulants, theobromine and caffeine, in SC carbon dioxide. Caffeine showed a P_2 crossover near 220 bar, and theobromine showed a P_2 crossover near 135 bar. Caffeine and theobromine are again chemically similar in structure; however, caffeine showed nearly 2 orders magnitude greater solubility. These solubility differences, in structurally similar compounds, are primarily caused by vapor pressure differences in the compounds.

Favati et al. (1991) showed a P_2 crossover near 300 bar in the SC carbon dioxide extraction of evening

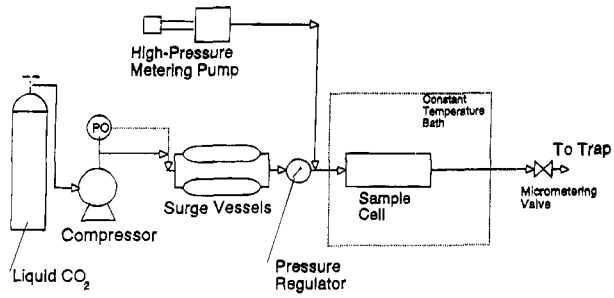

Figure 3. Supercritical fluid extraction apparatus.

primrose oil from evening primrose seeds. Other studies have included extraction of lipids from a filamentous fungus (*Saprolegnia parasitica*) (Cygnarowicz-Provost *et al.*, 1992) and vindoline and catharanthine extraction from Madagascan periwinkle (*Catharanthus roseus*) (Lee *et al.*, 1992).

Cosolvents (or entrainers) can be used to increase extraction efficiencies. Wong and Johnston (1986), Larson and King (1986), and Cygnarowicz-Provost *et al.* (1992) found that using various organic entrainers (methanol, ethanol, or acetone) in SC carbon dioxide increased the solute solubilities in their respective systems. Li and Hartland (1991) found that the cosolvent ethanol increased theobromine solubilities, while having almost no effect on caffeine solubility; on the other hand, when water was used as a cosolvent, caffeine solubility increased, while theobromine solubility was unchanged. Wigfield and Lanouette (1993) found that an entrainer was necessary to recover any of the herbicide fluazifop-P butyl and its metabolite fluazifop-P acid from fortified onions.

These results indicate that biomolecule solubility in a supercritical solvent can be complex and difficult to predict. Often three factors play a key role: the vapor pressure of the solute, the density of the solvent, and site–site interactions, including solvent–solute, solute–solute, and solute–matrix interactions. Cosolvents may be added to take advantage of specific solute–solvent interactions. Experiments are necessary to determine which factors are important for each system of interest. This study was performed to evaluate and compare the effectiveness of supercritical carbon dioxide and near-critical propane as solvents of biomolecules contained within *M. grandiflora*.

MATERIALS AND METHODS

Chemicals. Extractions were performed with industrial grade carbon dioxide (Liquid Carbonic, Chicago, IL), and samples were trapped in reagent grade dichloromethane (EM Science, Gibbstown, NJ). Both were used as received. The HPLC analysis was performed with HPLC grade methanol (Mallinckrodt, Paris, KY). Deionized and doubly distilled water, which was filtered with a 0.20 μm filter, was also used.

Plant Preparation. The leaves of local *M. grandiflora* were air-dried at ambient temperature and then chopped to improve mass transfer.

SCFE Procedure. A schematic of the continuous flow SCF extraction system is given in Figure 3. Liquid carbon dioxide or propane at ambient temperature was fed to a diaphragm compressor (Lewa Model ELM-1) and compressed to a pressure between 200 and 350 bar. The compressed solvent was stored in surge tanks which dampen pressure fluctuations. The solvent then flowed to a horizontal tubular extractor, where the pressure was controlled by a Tescom regulator (Model 44-1124) to within ±0.3 bar. In the extractor, a fixed bed of *M. grandiflora* leaves was contacted by the solvent. Between 10 and 100 g could conveniently be loaded in the tube extractor. The extraction pressure was monitored by a Heise digital pressure gauge (Model 710A, 0–207 bar, ±0.07 bar). Both the feed line and tubular extractor were immersed in a constant-temperature bath (Haake Model 5708) which could be maintained from ambient to 80 ± 1 °C. Downstream of the bed, a micrometering valve was used to control the flow rate and reduce the pressure to atmospheric. Flow rates were measured before pressure letdown using a Micromotion mass flow meter (Model DCR-3). Extractions reported here were performed at flow rates of approximately 1 g/s. The exhaust was passed through a cold trap containing dichloromethane to aid in collecting precipitated biomolecules.

Dichloromethane Extraction. Dried and ground aerial plant parts (80 g) were extracted with dichloromethane (DCM) by soaking for 48 h. The slurry was filtered with a slight vacuum and the green filtrate evaporated *in vacuo*, yielding a dark green crude extract.

Quantitative Analysis by HPLC. The crude extract obtained from the SCFE experiment was dissolved in methanol (MeOH). Part of each extract was not soluble in MeOH; this fraction was dissolved in $CHCl_3$. The MeOH-soluble fraction was filtered through a 0.45 μm LID/X-type syringeless filter. For the HPLC analysis, a Hewlett-Packard (Model 1090) liquid chromatograph with a photodiode array detector was used. The UV spectra of parthenolide, costunolide, and cyclocolorenone have λ_{max} values at 214, 213, and 268 nm, respectively. Two wavelengths, 220 and 230 nm, were used to analyze the samples in the HPLC; 230 nm was used in an attempt to improve the response of cyclocolorenone. The improvement was marginal; therefore, the HPLC results reported are at 220 nm.

A Carbosphere 10 ODS, 250 × 4.6 mm i.d. column (Phenomenex, Palos Verdes, CA), was used to analyze all of the samples. A Spherisorb S5 ODS-1, 5 μm, 250 × 4.6 mm i.d., column (Phase Separations, Deeside, U.K.) was used to verify the results of the carbon dioxide extract samples. The flow rate of the mobile phase was 1 mL/min, and it was continuously degassed with helium. The extracts obtained by SCFE with CO_2 were analyzed using a mobile phase of 70% MeOH/30% H_2O. The extracts obtained with C_3H_8 were analyzed with a mobile phase of 65% MeOH/35% H_2O to aid in the separation of the parthenolide and cyclocolorenone. During the initial HPLC analyses, an attempt was made to use naphthalene as an internal standard; however, better results were obtained from an absolute calibration for each product. Six different concentrations of standards were analyzed to establish the calibration curve of each of the compounds (parthenolide, costunolide, and cyclocolorenone). All peak areas were measured as the arithmetic means from triplicate injections.

Identification by NMR. Parthenolide, costunolide, and cyclocolorenone were isolated from local *M. grandiflora* using the procedures detailed by Fischer (1991) and El-Feraly and Chan (1978). The structure of parthenolide had been previously established by spectral (Govindachari *et al.*, 1965; El-Feraly and Chan, 1978; Ogura *et al.*, 1978) and X-ray analyses (Quick and Rogers, 1976), and its ^1H and ^{13}C NMR spectra were unambiguously assigned using modern 2D NMR methods (Fischer *et al.*, 1991).

The ^1H and ^{13}C NMR spectral data of costunolide were previously reported (Rodrigues *et al.*, 1978; El-Feraly and Chan, 1978; El-Feraly *et al.*, 1979). Costunolide's crystalline structure has been reported (Bovill *et al.*, 1976, 1978) as well as its silver nitrate complex (Linek and Novak, 1978). The ^1H NMR spectral data of cyclocolorenone have also been previously reported (Rao and Davis, 1982; Jacyno *et al.*, 1991) as well as the ^{13}C NMR data (Jacyno *et al.*, 1991).

RESULTS AND DISCUSSION

The extraction of *M. grandiflora* was carried out using both carbon dioxide and propane. Extractions with both solvents were performed at 40 and 50 °C, with reduced pressures (operating pressure/critical pressure) slightly greater than 1. For carbon dioxide, the reduced tem-

Table 1. Composition of *M. grandiflora* Extracts Obtained with Supercritical Carbon Dioxide and Near-Critical Propane

solvent	T (°C)	P (bar)	solvent density (g/mL)	solvent mass (g)	loaded sample mass (g)	extract (mg)	parthenolide (mg/kg of sample)	costunolide (mg/kg of sample)	cyclocolorenone (mg/kg of sample)
CO_2	40	75.8	0.245	785	20.0	36.85	194	37.5	604.5
CO_2	50	75.8	0.199	848	19.4	23.7	65.5	14.4	322.2
C_3H_8	40	44.8	0.479	526	19.0	98.25	59.0	5.3	249.0
C_3H_8	50	44.8	0.457	73	14.9	43.65	17.5	0.7	38.3
DCM	ambient		1.325		80	590	88.5	trace	497.1

peratures (operating temperature/critical temperature) were slightly greater than 1, indicating that the extraction conditions were supercritical. However, for propane, the reduced temperatures were on the order of 0.85, indicating that the extracting conditions were in the near-critical or subcritical region. Operating above the critical temperature of propane (96.7 °C) may cause degradation of the biomolecules. Furthermore, depending on the location of P_1 and P_2 (Figure 2), liquid propane may have a higher solvent capacity for biomolecules than supercritical propane (Coorens *et al.*, 1988).

Table 1 reports the averaged results of at least duplicate extractions. These results show that propane extracts at least twice the total mass that carbon dioxide does at each temperature. However, the amount of each high value component extracted is more important than the total mass extracted. Carbon dioxide extracts 3.5 times more parthenolide, almost 3 times more costunolide, and 1.3 times more cyclocolorenone than propane at 40 °C. These differences are even greater at 50 °C. At 40 °C the carbon dioxide extract is 45% desired biomolecules, and at 50 °C it is 33%. Using propane at these same temperatures the extract is only 6% and 2% desired biomolecules, respectively. Thus, carbon dioxide extracts a greater quantity of the desired products and is more selective than propane.

One possible explanation for carbon dioxide extracting more desired biomolecules is that more carbon dioxide was used during each extraction. However, propane, at the experimental conditions, always extracted more total biomass. Because cold traps were used, it was possible to visually observe when the extraction was complete. Using either carbon dioxide or propane, the extraction appeared to be complete within the first few grams of solvent flow. The reason for exploring SCFE was to see if it can produce a cleaner extract. Propane ultimately may have extracted more of the desired products given additional solvent, but the presence of fatty materials, as shown in the HPLC traces (Figure 4, discussed below), makes propane an inefficient solvent for this system. The results do suggest that SCFE with carbon dioxide produces an extract capable of giving analytical grade chemicals with little or no need for cleanup. In fact, a slight temperature reduction of the carbon dioxide extract caused crystallization of parthenolide.

The standard technique used to recover these compounds is solvent extraction with dichloromethane (DCM). DCM extraction gives a product that contains approximately 1.2% parthenolide, 6.74% cyclocolorenone, and traces of costunolide. These values are of the order of those for propane at 40 °C but are lower than those for the carbon dioxide extraction. Additionally, visual observation showed that the carbon dioxide extract was slightly yellow, indicating that few or no chlorophyll-containing compounds were extracted. The propane extract was dark yellow, while the crude extract from the DCM extraction was green; it contained substantial amounts of chlorophyll.

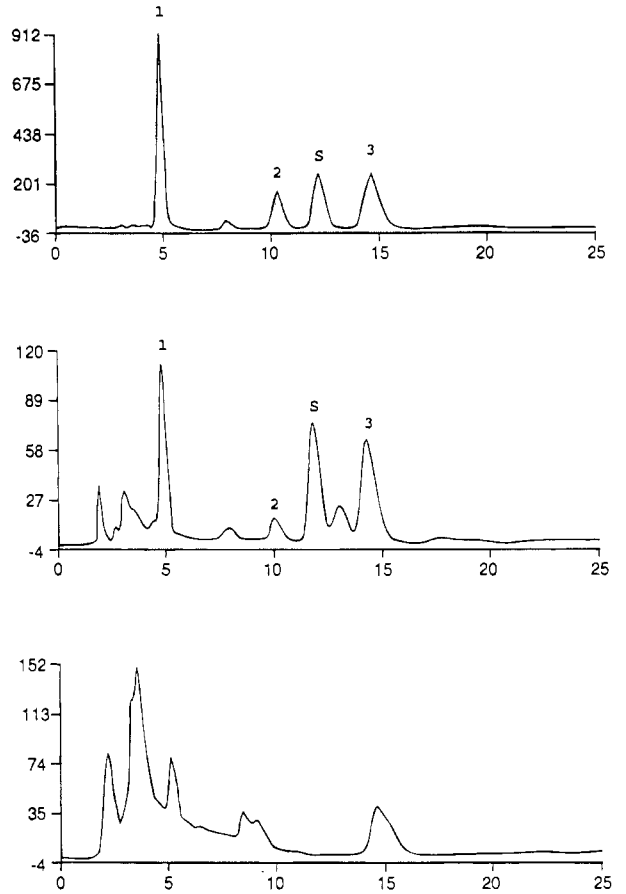

Figure 4. HPLC trace of *M. grandiflora* extraction with (a, top) CO_2 at 40 °C, (b, middle) propane at 40 °C, and (c, bottom) DCM at room temperature. Peaks: parthenolide (**1**), costunolide (**2**), cyclocolorenone (**3**); standard (s) was naphthalene. Detection was at 220 nm.

Figure 4 gives the HPLC chromatograms of the methanol-soluble crude extracts obtained with carbon dioxide and propane at 40 °C and with DCM. ^{1}H NMR spectral and GC–MS data showed that the methanol-insoluble part of each extract contained mainly fatty acids. Figure 4a shows that the carbon dioxide extract contains the three desired products as major compounds. The propane HPLC trace in Figure 4b shows many additional small peaks that were not identified but which surely hinder product recovery. The most noticeable differences between the traces from the carbon dioxide and propane extractions are the large peaks at 3–3.5 min. These peaks are attributable to fatty materials which were favorably extracted by propane. Another difference is the new unidentified peak at 13 min, which is near the cyclocolorenone peak. The DCM extraction (Figure 4c) shows even more undesired material than propane. In this case the peaks at 3–3.5 min are larger than in the propane extract, and the parthenolide peak has many others very close

to it. In both the propane and DCM extracts, baseline resolution is poor, making product quantification difficult.

Carbon dioxide, at the conditions studied, removed more desired biomolecules than either near-critical propane or DCM extraction. Increasing temperature, for carbon dioxide, reduced recovery of desired biomolecules. Therefore, over the experimental conditions studied, operation was between P_1 and P_2 (Figure 2); here the extraction is more sensitive to density changes than solute vapor pressure effects. However, density considerations alone are not sufficient to explain the experimental results. At the experimental conditions, the density of propane is approximately twice that of carbon dioxide at both temperatures. This may explain why propane extracts more overall mass than carbon dioxide, but it offers no explanation for propane extracting fewer of the biomolecules of concern. One explanation may be that the extraction of parthenolide, costunolide, and cyclocolorenone is controlled by the site–site interactions of the solvent and solute, but apparently the greater density or the nonpolar nature of the propane causes it to extract more undesired fatty material than does carbon dioxide, allowing carbon dioxide to produce a cleaner extract.

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