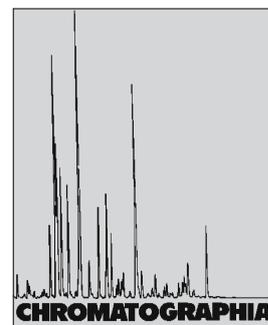


Optimization of a New Extraction Technique for Analysis of Verbenone and *cis*-Verbenol in Pine Seeds



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Abstract

Results from a systematic study of the factors affecting extraction of *cis*-verbenol and verbenone from pine seeds are presented. Five extraction conditions were investigated: extraction solvent, method of extraction, extraction temperature, volume of solvent, and the ratio of the mass of sample to the amount of extraction solvent. The resulting optimized method uses magnetic-stirring-assisted extraction of pine seeds (5 g) with ethyl acetate (75 mL) for 20 min, at room temperature. RSDs were less than 5% for both compounds. GC-FID was used for quantification of *cis*-verbenol and verbenone in the extracts.

Keywords

Gas chromatography
Extraction
Pine seeds
Pinus pinaster
cis-Verbenol
Verbenone

Introduction

There have been many studies of the bioactivity of volatile components, especially monoterpenes, in the essential oil from pines. The antibacterial, antimicrobial, and antifungal activity of natural extracts containing monoterpenes are well known [1–4]. Among the monoterpenes identified in the extracts, two compounds, *cis*-verbenol (C₁₀H₁₆O) and verbenone (C₁₀H₁₄O), have been shown to be highly bioactive [5]. Verbenone has

a repellent effect on the beetles that attack pine trees; the consequent effect is to reduce the number of beetles to below a level that would overcome the natural resistance of attacked trees [6]. Another effect is inhibition of feeding of the pine weevil, both male and female [7]. A strong stimulant effect, on females only, of the old house borer has also been shown [8]. *cis*-Verbenol is also used to control harmful insects and is an intermediate in the synthesis of valuable perfume and medicinal substances [9, 10].

Techniques for extraction of simple monoterpenes from solid material have usually been based on distillation–extraction and hydrodistillation [11–14] using a variety of solvents. These techniques often require long extraction times (between 1 and 3 h), however. Several more rapid extraction techniques have been used in recent years, for example supercritical-fluid extraction using carbon dioxide [15], distillation under reduced pressure, simultaneous purging and solvent extraction [16], solid-phase trapping solvent extraction, headspace solid-phase microextraction, reduced-pressure steam distillation, and simultaneous steam distillation–solvent extraction [17]. In the work discussed in this paper extraction of verbenone and *cis*-verbenol from *P. pinaster* seeds using both ultrasound-assisted extraction (UAE) and magnetic-stirring-assisted extraction (MSAE) has been evaluated.

Improvement of extraction efficiency by use of ultrasound is attributed to the phenomenon of cavitation of the solvent caused by passage of the ultrasonic wave. Cavitation bubbles are produced and compressed during application of the ultrasound. The increase in pressure and temperature caused by the compression leads to the collapse of the bubble. With collapse of bubble a shock wave passes through the solvent, enhancing mixing [18]. Ultrasound also has a mechanical effect, enabling greater penetration of solvent into the sample matrix, increasing

Table 1. Conditions used in the experimental design

Expt.	Solvent	Method ^a	Temp. (°C)	Vol. (mL)	Mass (g)	Verbenone (%) ^b	<i>cis</i> -Verbenol (%) ^b
1	DCM	US	10	25	10	27.4 ± 0.8	25.9 ± 0.5
2	AcOEt	US	10	25	5	31.7 ± 1.0	30.6 ± 0.5
3	DCM	MS	10	25	5	44.7 ± 1.4	40.0 ± 0.4
4	AcOEt	MS	10	25	10	25.9 ± 1.2	24.6 ± 0.5
5	DCM	US	50	25	5	36.5 ± 1.1	36.7 ± 0.5
6	AcOEt	US	50	25	10	29.5 ± 0.9	27.8 ± 0.7
7	DCM	MS	50	25	10	22.3 ± 0.4	19.8 ± 0.5
8	AcOEt	MS	50	25	5	46.2 ± 1.4	44.7 ± 0.9
9	DCM	US	10	50	5	73.7 ± 2.8	67.8 ± 1.3
10	AcOEt	US	10	50	10	42.3 ± 1.9	40.2 ± 1.0
11	DCM	MS	10	50	10	48.6 ± 1.9	45.3 ± 1.0
12	AcOEt	MS	10	50	5	94.7 ± 3.0	92.6 ± 1.8
13	DCM	US	50	50	10	56.0 ± 1.6	51.2 ± 1.0
14	AcOEt	US	50	50	5	97.3 ± 3.1	93.0 ± 1.9
15	DCM	MS	50	50	5	100.0 ± 3.0	100.0 ± 2.0
16	AcOEt	MS	50	50	10	48.2 ± 1.5	45.4 ± 1.5

^a US ultrasound-assisted, MS magnetic-stirring-assisted

^b Relative to the amount obtained in the extraction with the highest recovery (no. 15)

the contact surface area between the solid and liquid phases. This effect, coupled with enhanced mass transfer and substantial disruption of cells, via cavitation bubble collapse, increases the release of intracellular products into the bulk medium. Use of high temperatures in UAE can increase the efficiency of the extraction process by increasing the number of cavitation bubbles formed [18–20]. The high energy involved and the high temperature reached can cause the most volatile compounds to evaporate, however, reducing the efficiency of the extraction.

MSAE is usually found to be a less efficient method of extraction than UAE, because the energy introduced to the system does not overcome the interactions between the compounds to be extracted and the matrix. Because it is a more gentle method of extraction, however, it could be more suitable for volatile compounds.

Although both MSAE and UAE have been applied to the analysis of several terpenes, the literature contains no references to their use in the analysis of *cis*-verbenol and verbenone.

Experimental

Sample

Seeds from *P. pinaster* were obtained from the Station Regionale des Semences de Chefchaouan (Chefchaouan, Morocco). They were collected in the Rif Occidental of Morocco.

Chemicals and Solvents

(1*S*)-(–)-Verbenone and (*S*)-*cis*-verbenol, were obtained from Sigma-Aldrich Chemie (Schnelldorf, Germany). Their purities were 94 and 99%, respectively.

All reagents used were of analytical grade. Ethyl acetate and dichloromethane were purchased from Scharlab (Barcelona, Spain) and octan-2-ol from Merck (Darmstadt, Germany).

Chromatographic Analysis

All extracts were analyzed by use of a Hewlett-Packard (Palo Alto, CA, USA) 5,890 Series II gas chromatograph equipped with flame ionization detection (FID) and split–splitless injection. Compounds were separated on a 60 m × 0.32 mm fused-silica capillary column coated with a 0.25-μm film of DB-Wax (J&W Scientific, Folsom, CA, USA).

The verbenone and *cis*-verbenol peaks obtained from extracts of *P. pinaster* seeds were identified by comparison of their retention times with those of standards, and by GC–MS, performed with a GC 8000 coupled to a MD 800 mass detector (Fisons Instruments, Milan, Italy). Electron-impact mass spectra were acquired at electron energy of 70 eV in the range 40–400 a.m.u. Mass spectra were compared with those in a commercial database of mass spectra (Wiley, Chichester, UK).

For both GC and GC–MS splitless injection was performed with the injector

purge closed for 2 min. The injector temperature was 200 °C and the oven temperature was maintained at 40 °C for 5 min and then programmed at 2 °C min^{−1} to 230 °C, which was maintained for 20 min (isothermal). Helium was used as carrier gas at 40 kPa.

Compounds were quantified by GC–FID. During optimization of the method of extraction recovery was calculated relative to the extraction from which the largest amounts of verbenone and *cis*-verbenol were obtained (largest chromatographic peak area relative to that of the internal standard).

The compounds were extracted by using an ultrasonic water bath of power 400 W (J.P. Selecta, Barcelona, Spain) for UAE. The working frequency was 40 kHz. A magnetic stirring plate (model 1002, Jenway, UK) working at 500 rpm was used for MSAE.

Seeds from *P. pinaster* were ground in a coffee grinder, freeze-dried, and stored at –20 °C until extraction, to guarantee a homogenous sample for all the experiments and that no changes occurred during development of the method of extraction.

Extraction by both UAE and MSAE was performed with dichloromethane (DCM) and ethyl acetate (EtOAc) using high and low temperatures, volumes, and masses. All extractions were performed in triplicate. Sample (5 or 10 g) was placed in a glass beaker with 25 or 50 mL DCM or EtOAc and extracted for 20 min. Internal standard (octan-2-ol, 100 ppm) was then added and the solution was filtered through a 0.45-μm Nylon syringe filter (Millex-HN, Millipore, Ireland).

An experimental design was used for method development. Table 1 shows the experimental conditions evaluated in the experimental design. Minitab software (State College, PA, USA) was used for the experimental design and for treatment of the resulting information.

Results and Discussion

A fractional factorial experimental design was used to optimize the extraction variables organic solvent, extraction method, extraction temperature, volume of solvent, and mass of sample. The starting values for these variables were based on results from previous work with terpenes [21, 22]. A fractional experimental design was used to reduce the

number of experiments. Thirty-two experiments would normally be needed to evaluate the effects of five variables at two levels; a fractional design enables use of 16 experiments instead of 32. Statistical and graphical treatment of the results affords sufficient information to ascertain the best extraction conditions. This type of experimental design has produced good results in previous work with both real and spiked samples [19, 23].

Recovery under each set of extraction conditions was calculated relative to the maximum recovery obtained in the experimental design (denoted 100%; extraction 15). Plots of the main effects for each extraction variable are shown in Fig. 1. The most important factor seems to be the volume of solvent used for extraction, because this results in the largest difference between the two amounts extracted by levels investigated in the experimental design (25 and 50 mL). When 50 mL was used recovery of both verbenone and *cis*-verbenol was almost 40% higher.

The mass of pine seed powder extracted was another important factor. For extraction of 5 g recovery was higher than for extraction of 10 g (30% higher for verbenone and 32% higher for *cis*-verbenol).

Other variables had little or no effect on recovery. Very similar mean recovery was recorded for both verbenone and *cis*-verbenol and use of either DCM or EtOAc as extraction solvent. For the other variables, recovery of verbenone was 8% higher when the higher temperature (50 °C) was used and 5% higher when MSAE was used instead of UAE. For *cis*-verbenol results were similar, 7% higher recovery at the higher temperature (50 °C) and 6% higher when MSAE was used. Because the main-effect plots furnished no definitive information about the best extraction conditions in respect of the method of extraction, the temperature of extraction, and the extracting solvent, plots of interactions between two variables were studied; these are shown in Fig. 2.

The interactions between extraction method (UAE or MSAE) and the most important extraction variables (extraction volume and mass of sample) were checked. It is apparent from Fig. 2 that increasing the extraction volume increased recovery similarly for both UAE and MSAE. This means there were no interactions between these two variables. It was, therefore, not possible to determine the best extractions

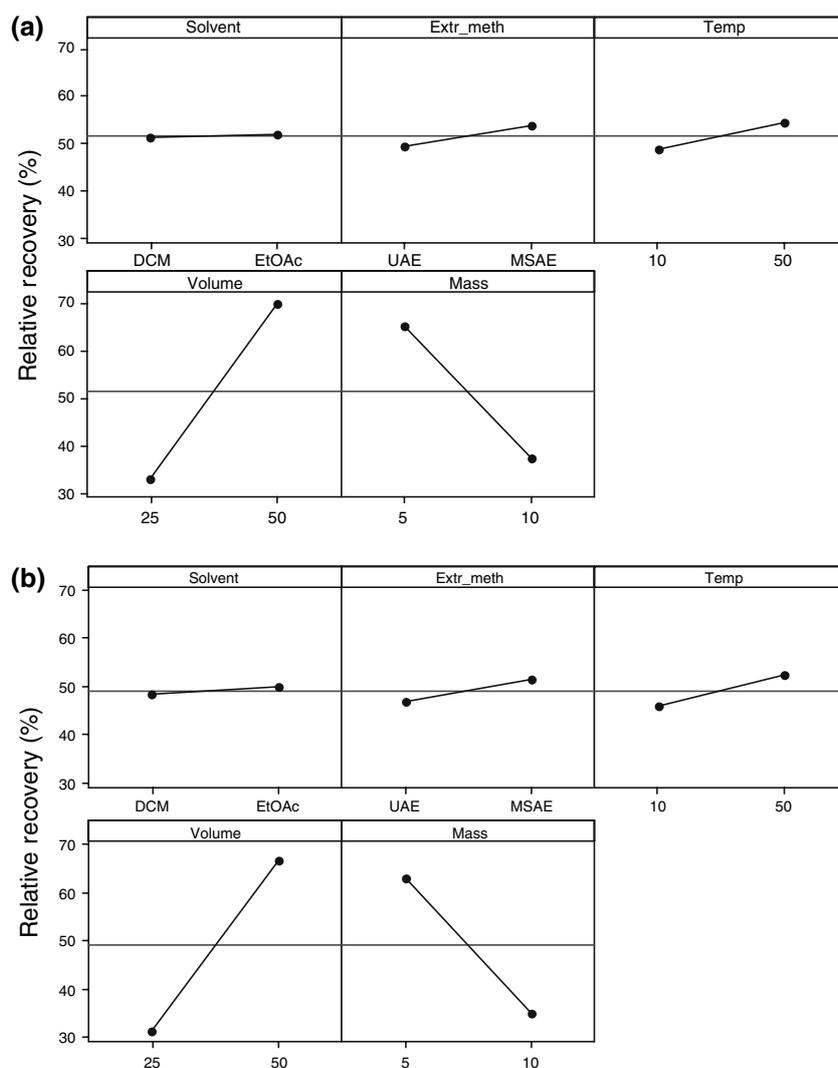


Fig. 1. Main-effects plot for verbenone (a) and *cis*-verbenol (b)

conditions for the extraction method on the basis of this interaction plot. When the interaction between the method of extraction and the amount of sample was evaluated it was found that reducing the mass of sample increased the recovery for both UAE and MSAE, as is also apparent from Fig. 2. For MSAE, average recovery was improved from 38–40 to 75–78% for both *cis*-verbenol and verbenone when the mass of solid sample was reduced; for UAE recovery increased from 38–40 to 58–60% only. Hence, MSAE resulted in higher average recovery than UAE when working with the optimum amount of sample.

There were no clear interaction effects between extraction temperature and extraction solvent, so no additional information could be extracted from the plots.

After evaluation of the main effects and the interaction effects, the values of

the continuous variables (extraction temperature, volume of extracting solvent, and the mass of sample) were optimized. The starting values for this stage of the optimization were chosen on the basis of the results from the experimental design.

Extraction Temperature

Because no clear effect of extraction temperature on recovery was observed, a series of extractions was performed using 5 g sample, 50 mL EtOAc, and temperatures from 10 to 60 °C. Recovery for each set of extraction conditions was calculated relative to the maximum recovery obtained in the temperature study (100%).

It is clearly apparent from Table 2 that increasing the extraction temperature from 10 to 20 °C resulted in increased recovery; there was no difference for

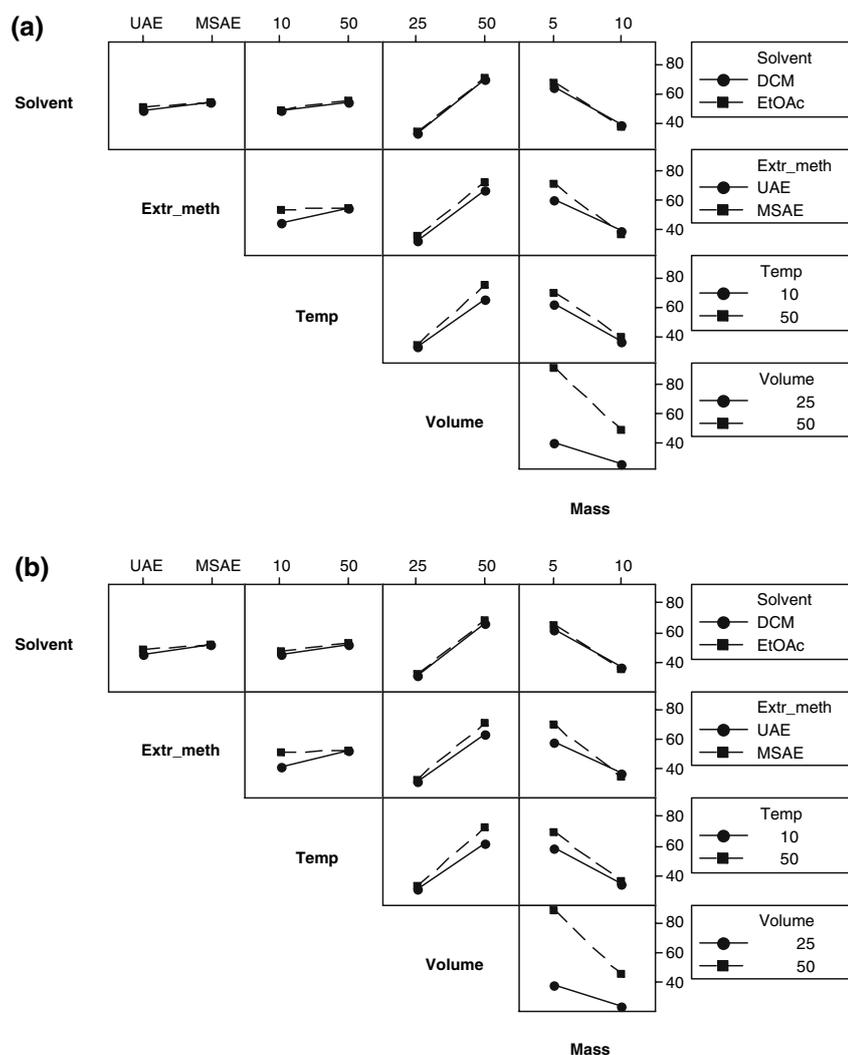


Fig. 2. Interaction plots for verbenone (a) and *cis*-verbenol (b)

Table 2. Recovery^a obtained from extraction at different temperatures

Temp. (°C)	<i>cis</i> -Verbenol (%)	Verbenone (%)
10	86.2 ± 3.5	85.7 ± 2.9
20	100.0 ± 3.8	100.0 ± 1.8
30	95.6 ± 3.8	96.6 ± 1.6
40	97.3 ± 4.5	96.6 ± 2.5
50	86.8 ± 5.6	97.4 ± 4.8
60	94.7 ± 7.5	96.2 ± 6.5

^a Relative to the amount obtained from the extraction with the highest recovery (20 °C)

extractions performed at temperatures from 20 to 40 °C and higher temperatures resulted in a slight decrease in the average recovery for both verbenone and *cis*-verbenol. The higher the temperature the greater the errors in the measurement of recovery, most probably, because evaporation of the solvent during the extraction, resulted in changes in the contact

Table 3. Relative^a recovery obtained by use of different volumes of solvent

Volume (mL)	<i>cis</i> -Verbenol (%)	Verbenone (%)
25	82.5 ± 3.2	80.2 ± 1.9
50	89.9 ± 3.4	89.7 ± 1.9
75	100.0 ± 4.0	100.0 ± 2.3
100	93.8 ± 2.9	85.2 ± 2.1

^a Relative to the amount obtained from the extraction with the highest recovery (75 mL)

surface between the sample and the solvent. Extraction at room temperature was therefore used in all experiments.

Volume of Solvent

Because 50 mL solvent resulted in higher recovery than 25 mL, several experiments were performed using volumes larger than 50 mL. Samples (5 g) were extracted

with 50, 75, or 100 mL ethyl acetate. The results are shown in Table 3. Recovery for different extraction conditions was calculated relative to the maximum recovery obtained in the solvent volume study (100%). It is apparent the highest recovery was obtained by use of 75 mL. This result must be related to the beaker in which the extractions were performed, because higher volumes result in a much lower contact area between the samples and the solvent.

Sample Mass

The amount of sample was the last condition studied during optimization. Because, in the experimental design, it was found that 5 g sample resulted in much greater recovery than 10 g, a series of extractions was performed using between 2 and 5 g sample and 75 mL ethyl acetate at room temperature for 20 min. The results obtained are given in Table 4. Recovery for each extraction condition was calculated relative to the maximum recovery obtained in the sample mass study (100%).

It is apparent recovery of the compounds increased with the increasing mass up to 4 g. For 4 and 5 g sample differences were small. It was therefore, concluded that any amount between 4 and 5 g could be used in the final method.

Repeatability and Quantification

Six extractions were performed under the optimized conditions. Table 5 shows the recovery obtained for both verbenone and *cis*-verbenol. RSDs were below 5% for both compounds. The amount of verbenone was almost twice that of *cis*-verbenol.

To check the method chosen resulted in quantitative extraction, a sample re-extraction study was conducted on the six samples used in the repeatability study. The samples were extracted a second time using the same method. Neither verbenone nor *cis*-verbenol was detected in the second extracts.

The optimized method could therefore be used to determine both verbenone and *cis*-verbenol in pine seeds. The method could be useful in taxonomic studies and in studies of the bioactivity of different varieties of pine.

Table 4. Relative^a recovery obtained by use of different masses of sample

Mass (g)	<i>cis</i> -Verbenol (%)	Verbenone (%)
2	53.0 ± 2.4	44.7 ± 1.6
3	73.5 ± 4.8	64.8 ± 1.7
4	90.4 ± 4.9	90.8 ± 3.6
5	100.0 ± 3.9	100.0 ± 3.2

^a Relative to the amount obtained from the extraction with the highest recovery (5 g)

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Table 5. Repeatability of the method for *cis*-verbenol and verbenone

No.	<i>cis</i> -Verbenol (mg kg ⁻¹)	Verbenone (mg kg ⁻¹)
1	0.397	0.790
2	0.413	0.815
3	0.411	0.808
4	0.420	0.825
5	0.435	0.838
6	0.423	0.818
RSD (%)	3.08	1.97