Vinegar Weed Volatile Constituents

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The essential oil from vinegar weed (*Trichostema lanceolatum*) was prepared from the leaves and stems by steam distillation and solvent extraction (yield 0.6%). The oil was analyzed by combined gas chromatography–mass spectrometry and GC retention time. Identified constituents (with rough quantitative indications) were terpinen-4-ol (55%), γ-terpinene (15%), α-terpinene (7.7%), terpinolene (3.2%), *p*-cymene (1.9%), β-phellandrene (1.4%), α-pinene (1.3%), α-terpineol (1.3%), and caryophyllene (1.2%). Twelve other constituents also were identified, at concentrations less than 1%. Acetic acid was identified in the aqueous phase of the distillate, and its abundance in the leaves and stems estimated to be about 0.2%.

This is the second paper from this laboratory on the composition of plant material that is usually shunned by browsing deer, the first paper being on constituents of the leaf oil from California bay (*Umbellularia californica*) (Buttery et al., 1974).

Vinegar weed (*Trichostema lanceolatum* Benth. (Munz, 1959)) is an annual herbaceous plant, up to about 24 in. tall, that thrives through late summer in generally dry areas of the Pacific states, west of the Sierra Nevada and Cascade ranges.

Essential oils from both of these species are reported to be quite effective as inhibitors of activity of rumen microbes from deer and sheep (Oh et al., 1968). Whether or not these ruminants, and perhaps others, are repelled by one or more volatile constituents of these plants is an important question. In the present study, the volatile oil of vinegar weed was separated by steam distillation and solvent extraction and then analyzed by gas chromatography combined with mass spectrometry (GC–MS).

MATERIALS AND METHODS

Source Material. Vinegar weed stems with leaves, a minor amount of flower buds, and very few open flowers were collected from range land at the Hopland Field Station, University of California, Hopland, Calif. in July, 1973. This material was stored in plastic bags at −34 °C. Before each distillation the desired amount of plant material was removed from storage and the stems were cut into 2-in. lengths. Discolored or dry parts and traces of weeds of other species were discarded.

Distillation and Sample Preparation. Two methods were used for preparation of the essential oil from the vinegar weed. The first employed simultaneous steam distillation and solvent extraction with a 12-l. flask and an extraction head of the Nickerson and Likens (1966) type. Quantities of materials used were 1.00 kg of the select weed pieces, 4.1 l. of water, and 250 ml of pentane with 0.03 g of Antioxidant 330 (Ethyl Corporation, Baton Rouge, La.). Distillation was continued for 2.5 h. The pentane extract was washed with 20% of its volume of 10% sodium bicarbonate solution and dried over sodium sulfate. Most of the pentane was removed by distillation with a 40-cm Fenske column packed with glass helices and a water bath at 56 °C (maximum), with a reflux ratio of 2.5/1. The yield of concentrated extract was 18.4 g (65% solvent, estimated).

The second method of preparation started with a simple steam distillation from a 12-l. flask and yielded both the essential oil and sodium salts of volatile acids. Again 1.00 kg of select weed pieces was used, with 5.0 l. of water. Distillation was continued for 2 h. The two layers of the distillate were separated and the oil was washed with 5 ml of 10% sodium bicarbonate solution and dried over sodium sulfate. The yield of oil was 6.0 g.

The aqueous distillate (920 ml from a total of 1040 ml) was titrated with 41.0 ml of 0.100 N sodium hydroxide to pH 8.0. The neutralized solution was washed twice with pentane. Water was removed and the sodium salts were dried at 160 °C to constant weight (0.337 g).

For GC–MS examination of the acids, a sample of the sodium salts was converted directly to trimethylsilyl esters just prior to each analysis by a method adapted from the reaction described by Schuyten et al. (1947). About 3 mg of sodium salts, 200 μl of tetrahydrofuran, and 40 μl of trimethylchlorosilane in a 0.3-ml Reacti-vial (Pierce Chemical Co., Rockford, Ill.) were magnetically stirred a few hours, starting at room temperature, then warming to 60 °C. After the sodium chloride formed was centrifuged down, the supernatant reaction mixture was ready for direct analysis.

GC–MS Analysis. The GC columns used for analysis of the essential oil are 500 ft, 0.02 in. i.d., open-tubular, stainless steel columns. Three different columns were used, the stationary phases being: (1) methyl silicone oil, SF-96 (50) (General Electric, Waterford, N.Y.) mixed with 5% Igepal CO-880 (General Aniline and Film Corp., New York, N.Y.); (2) OV-225 (Ohio Valley Specialty Chemical Co., Marietta, Ohio) with 5% Igepal CO-880; (3) Tween 20 (Atlas Chemical Industries, Inc., Wilmington, Del.) with 5% Igepal CO-880. The SF-96 (50) column was the principal one used. The OV-225 and Tween 20 columns were used for only a few runs, to separate compounds which came together in the same peak with SF-96 (50).

The column used for the silylated acids is similar to the SF-96 (50) column described above but the inside diameter is 0.03 in., and the column was treated with Silyl 8 (Pierce Chemical Co.) at 175 °C before each group of runs. The columns (Mon et al., 1967) and hydrogen flame detectors (FID) (Teranishi et al., 1962) were made in the authors' laboratory. The oven assemblies included commercial temperature programmers and injectors with glass vaporizor tubes (Hamilton Company, Whittier, Calif.).

The mass spectrometer is a Bendix time-of-flight instrument, Model 12-101. The bombarding electron energy was 70 eV. Interfacing with the GC column was through a membrane-type molecular separator (Black et al., 1969)

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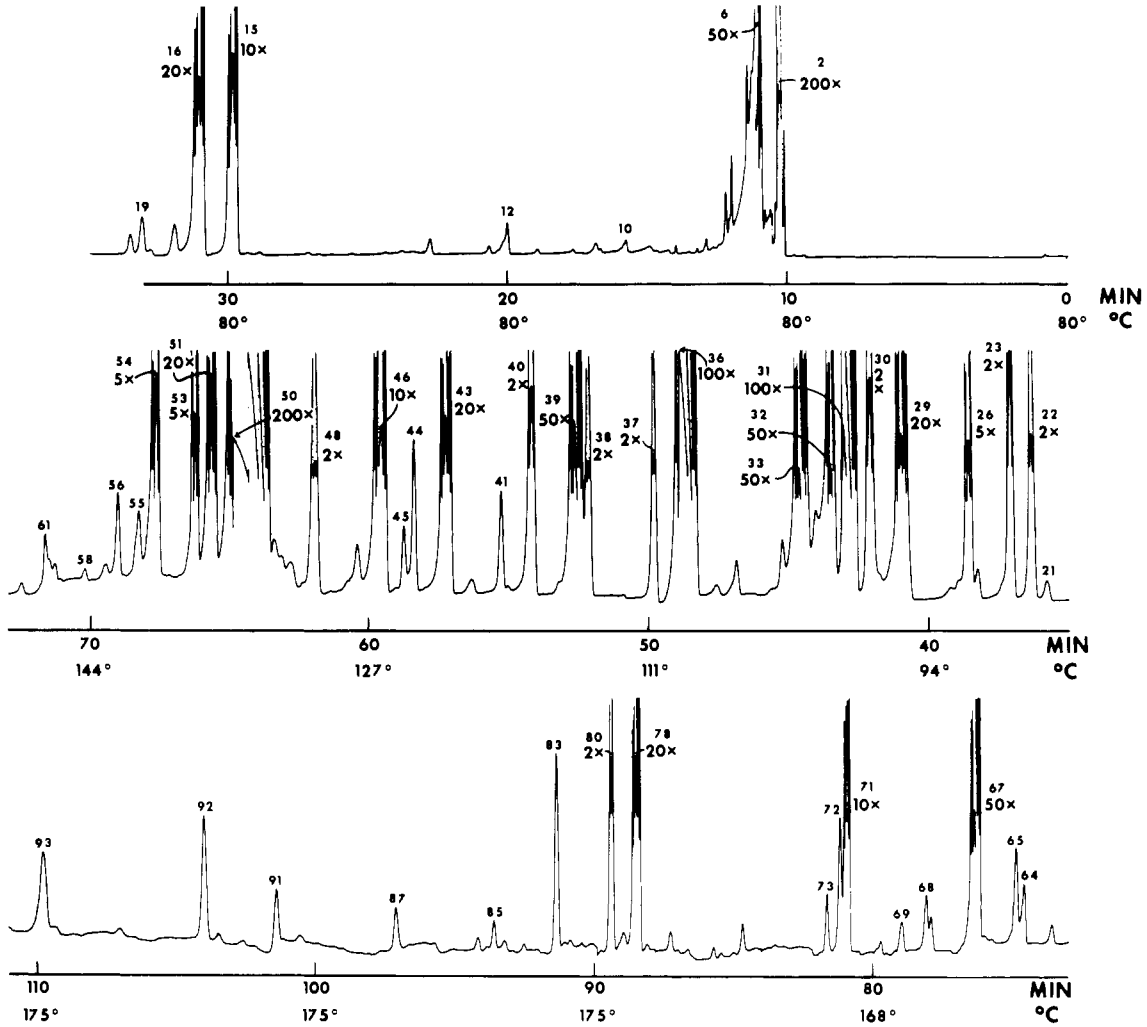

Figure 1. FID chromatogram of vinegar weed oil (pentane extract of steam distillate): column, 500 ft, 0.02 in. i.d., stainless steel, coated with methyl silicone and 5% Igepal; injector temperature, 202 °C; sample size 0.10 μl.

with 0.001-in. methyl silicone rubber sheeting. The separator was operated with the exit from the high-pressure side connected to a mechanical vacuum pump in order to minimize chromatographic peak broadening. Spectra were scanned at 1 decade/s with a Minneapolis-Honeywell Visacorder.

GC–MS runs on the vinegar weed oil (first method) were made in most cases with sample injections of 0.10 μl but in one case with 0.20 μl in order to obtain stronger spectra of the smaller peaks. The helium head pressure was 10 psig, which gave an average linear velocity of about 27 cm/s. The injector temperature was either about 170 or 210 °C. For most of the runs, column temperature was 80 °C for the first 30 min and then was programmed at 1.67 deg/min to 175 °C. Whenever available, reference spectra taken under similar conditions at this laboratory were used preferentially in making the identifications. For the monoterpene hydrocarbons which have quite similar patterns, new reference spectra were obtained on knowns by GC–MS with the present instrument.

Supporting retention time evidence was obtained for all of the peaks identified, by the peak enhancement (enrichment) technique. The same columns were used as for the GC–MS runs, respectively. Solutions (0.02 μl) of the known compounds were coinjected with 0.05-μl samples of the straight vinegar weed extract when examining small peaks, but with 0.05-μl samples of a 5% solution of the extract in hydrocarbon solvent for the larger peaks, in order to make more precise comparisons of retention times. The helium head pressure for these runs and others with the FID was 19 psig, which gave the same average linear velocity as did 10 psig with the mass spectrometer.

For analysis of the silylated acids, the sample size was 0.10 μl, the helium pressure was 2 psig with the MS and 12 psig with the FID, the injector temperature was 140 °C, and the column was isothermal at 80 °C.

Quantitative Estimations. A rough estimation of the concentration of the identified constituents in the oil (first method) was made by multiplying peak height by width at half-height and relating to the sum of the areas of all peaks found in this manner excepting the solvent peaks. Only chromatograms obtained in runs started with the injector at 170 °C were used for this purpose. Chromatograms from both the straight extract and diluted extract were used in order to include the small peaks and yet avoid large errors from overloaded peaks.

RESULTS AND DISCUSSION

Figure 1 shows a GC chromatogram (FID) of vinegar weed oil prepared by simultaneous steam distillation and pentane extraction. Chromatograms of the essential oil prepared by simple steam distillation were quite similar, except for the absence of a few of the small peaks including those of the first 24 min and, of course, the solvent peaks.

Identified constituents are listed in Table I. The values in the last column are only rough semiquantitative indications of the relative concentrations since FID response factors were not determined. Most of the constituents are

Table I. Constituents of Vinegar Weed Oil Identified by GC–MS and Retention Time Matching[a]

Peak no.	Compound	Approx concn % of the oil
2	Pentane	
10	Toluene	0.003
15	α-Thujene	0.74
16	α-Pinene	1.3
19	Camphene	0.012
22	Sabinene	0.13
23	β-Pinene[b]	0.19
26	Myrcene	0.26
29	α-Phellandrene	0.70
31	α-Terpinene[b]	7.7
32	*p*-Cymene	1.9
33a	Limonene	0.20
33b	β-Phellandrene	1.4
36	γ-Terpinene[b]	15
39	Terpinolene	3.2
48	Ethyl benzoate[b]	0.10
50	Terpinen-4-ol[b]	55
51	α-Terpineol	1.3
58	*p*-Cuminic aldehyde	0.011
78	Caryophyllene	1.2
80	α-Bergamotene	0.11
83	α-Humulene	0.06
		90.5

[a] Peak numbers refer to chromatogram in Figure 1. [b] The peak as obtained with the methyl silicone column (Figure 1) represents also a minor proportion of another constituent, detectable by MS but not identified (two minor constituents in peak 50). The minor constituent is estimated to account for about one-eighth of peak 31, less than 20% of peak 48, and less than 10% each of peaks 23, 36, and 50.

terpenoid compounds. Terpinen-4-ol (peak 50) is by far the most abundant of the constituents, amounting to over half of the oil. To our knowledge this compound has not been reported at this high a concentration in the essential oil of any other plant, although it was found at 45% in the oil from the leaves of *Melaleuca alternifolia* Cheel (one of the Australian "tea trees") (Guenther, 1968). α-Terpineol (peak 51) is the only other oxygenated terpene identified. The mass spectral patterns of several other chromatographic peaks indicate that they are probably oxygenated monoterpenes but the patterns do not suggest any compounds for which published spectra are available, excepting a few cases in which retention times did not match.

The second and third most abundant constituents are γ- and α-terpinene (peaks 36 and 31), respectively. This is not surprising since these terpenes and terpinen-4-ol are thought either to have a common progenitor or that the dienes are formed by dehydration of the alcohol (Banthorpe and Charlwood, 1972). Ten other monoterpene hydrocarbons were found, but of these, α-pinene (peak 16), terpinolene (peak 39), and β-phellandrene (peak 33b) are the only ones at about 1% or higher concentration. Limonene (peak 33a), which is widely distributed in nature, is present in very low concentration in the vinegar weed extract. β-Phellandrene was eluted free of limonene from the OV-225 column, while limonene and α-terpinene appeared as single-component peaks with the Tween 20 column.

The mass spectra indicated that eight of the peaks probably represent sesquiterpene hydrocarbons, but only three of these were identified: caryophyllene (peak 78), α-bergamotene (peak 80), and α-humulene (peak 83). Ethyl benzoate was the only ester found, cuminic aldehyde the only aldehyde, and no ketones were found.

The injector temperature for the principal GC–MS runs was about 210 °C. It was subsequently found, both in GC–MS runs and with the FID, that when the injector temperature was 170 °C, peaks 21, 30, and 38 were very much smaller than at the higher temperatures. Thus, it is likely that certain detectable chemical changes occur in the injector at the higher temperatures and that these peaks are due to artifacts. Otherwise only minor differences in peak height were observed between runs made with different temperatures of the injector.

GC–MS analysis of the silylated acids (via their sodium salts) from the aqueous layer of distillate from the simple steam distillation showed a sizable peak shortly after the solvent and excess silylating agent were eluted. The peak in question agreed both in retention time and mass spectrum with the corresponding peak from silylation of authentic sodium acetate by the same procedure. Four very small peaks with longer retention times were observed but none of these could be attributed to acids from vinegar weed. Further evidence that the acidic material in the aqueous distillate was mainly acetic acid is that its equivalent weight, calculated from the amount of alkali used in the titration and the yield of sodium salt, was found to be 60.3 (theory for acetic acid, 60.05).

The yield of volatile oil from vinegar weed leaves and stems was found to be 0.65% (wet weight basis) with the simultaneous steam distillation and pentane extraction, and 0.60% by simple steam distillation. That only a slight increase was found by the former method would be expected from low water solubility of most of the constituents.

The yield of acid (actually prepared only as sodium salt) was 0.025%, based on the weight of starting material. Since most of this appears to be acetic acid, which boils at 118 °C and does not show azeotropic behavior with water, the acid concentration in the still pot residue was probably somewhat greater than in the distillate. On this basis the acetic acid content of the vinegar weed leaves and stems is estimated to be about 0.2%. Since the pH of the solution in the still pot was about 4.8, the total acetate content of the leaves and stems is estimated to be about 0.4%.

All of these quantitative data are from plant material of a single collection. Samples taken at different seasons or sites may vary considerably.

Terpinen-4-ol was found by Oh et al. (1967) to be one of the more effective inhibitors of rumen microbial activity among the terpenoid compounds which they tested. This inhibition and the high concentration of terpinen-4-ol in vinegar weed oil may explain the unpalatability of the plant to deer.

From a comparison of Table I with Figure 1 it is evident that this work is far from a complete analysis of vinegar weed volatiles. Further work is planned to attempt to isolate and identify some of the still unknown constituents, at least those present at moderate and relatively high concentrations.

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Analysis of Blue Cheese for Roquefortine and Other Alkaloids from *Penicillium roqueforti*

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A method has been developed for the analysis of blue cheese containing internal mold for the neurotoxin roquefortine and isofumigaclavines A and B, three alkaloids produced by *Penicillium roqueforti*. Detection was by thin-layer chromatography and semiquantitative estimations were made for roquefortine and isofumigaclavine A. Recoveries of roquefortine added to cheese were 54–66%. Roquefortine was detected in 16 out of 16 samples of blue cheese originating from 7 countries, in estimated concentrations of up to 6.8 ppm. It was usually accompanied by isofumigaclavine A (up to an estimated 4.7 ppm) and traces of isofumigaclavine B were also detected in a few samples. Little or no migration of roquefortine into visibly nonmoldy areas of blue cheese was observed.

Penicillium roqueforti is a fungal species of particular interest to the agricultural and food scientist. Not only is it one of the commonly occurring microorganisms in fermenting silage (Le Bars and Escoula, 1974; Raper and Thom, 1968), including samples associated with mycotoxicoses (Kanota, 1970; Still et al., 1972), but it is also the essential fungus used in the production of Roquefort cheese and other varieties of blue cheese containing internal mold.

Compounds isolated from *P. roqueforti* culture media include "PR toxin", a sesquiterpenoid metabolite (Wei et al., 1973, 1975), and three incompletely characterized substances of unknown structure designated toxins-1, -2, and -3 (Kanota, 1970). The ability of *P. roqueforti* to produce alkaloids was shown by Taber and Vining (1958), Abe et al. (1967), and Bekmakhanova (1974). Only recently, however, have crystalline alkaloids been isolated and characterized. Ohmomo et al. (1975) isolated the known compound festuclavine and two other clavine alkaloids named roquefortine A (the major alkaloid) and roquefortine B, for which the unusual structures 7-acetoxy-6,9-dimethylergoline and 6,9-dimethylergolin-7-ol, respectively, were proposed. A fourth alkaloid, roquefortine C, was not structurally characterized. Scott et al. (1976) obtained two alkaloids from the mycelium of a strain of *P. roqueforti* used in cheese processing. The major alkaloid, designated roquefortine, was assigned the structure 10b-(1,1-dimethyl-2-propenyl)-3-(imidazol-4-ylmethylene)-5a,10b,11,11a-tetrahydro-2*H*-pyrazino-[1′,2′:1,5]pyrrolo[2,3-*b*]indole-1,4-(3*H*,6*H*)-dione and had physical properties similar to those published for roquefortine C. The minor alkaloid, isofumigaclavine A (9-acetoxy-6,8-dimethylergoline), was a stereoisomer of fumigaclavine A (Spilsbury and Wilkinson, 1961). Most of the physical properties reported for roquefortine A (Ohmomo et al., 1975) are similar to those of isofumigaclavine A, and they may in fact be the same compound. However, isofumigaclavine B (6,8-dimethylergolin-9-ol), obtained by hydrolysis of isofumigaclavine A (Scott et al., 1976), had a higher melting point than that reported for roquefortine B. Roquefortine is a neurotoxin, provoking convulsive seizures in mice (Frayssinet and Lafarge-Frayssinet, 1975; Scott et al., 1976). It was therefore important to determine whether it occurred in blue cheese and the approximate amounts that could be found in commercial samples. A semiquantitative thin-layer chromatographic method for the analysis of roquefortine in cheese was developed that also allowed detection of isofumigaclavines A and B.

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EXPERIMENTAL SECTION

Extraction and Cleanup. Fifty grams of cheese was blended in a Waring blender with 50 ml of chloroform, 100 ml of methanol, and 20 ml of water for 2 min at high speed and then for two further 1-min periods after successive additions of 50 ml of chloroform and 50 ml of water, following the procedure of Shih and Marth (1971) for extraction of aflatoxins from cheese. The mixture was filtered under reduced pressure through a pad of Celite filter aid (AW/545) together with ca. 10 ml of chloroform used to rinse the blender jar. The filter cake was rinsed with a further 10–15 ml of chloroform. The chloroform layer was separated and evaporated on a steam bath under a gentle stream of nitrogen to an oily residue, which was dissolved in 50 ml of ethyl acetate and extracted with two 50-ml portions of 0.5 N hydrochloric acid. The combined acid layers were washed with 50 ml of *n*-hexane, made alkaline with ca. 10 ml of 28% ammonium hydroxide solution, and reextracted with 50 ml of chloroform. The