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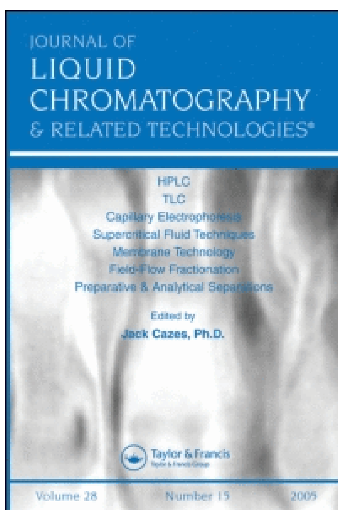

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DETERMINATION OF PULEGONE IN *H. pulegioides* AND PEPPERMINT OIL BY THIN LAYER CHROMATOGRAPHY WITH DENSITOMETRY

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ABSTRACT

A method was developed for determination of the phytotoxic terpene pulegone using silica gel thin layer chromatography with detection by acetic acid-sulfuric acid-anisaldehyde reagent and densitometric scanning. The method was used to monitor levels of pulegone in the plant *Hedeoma pulegioides* and to assay the compound in a commercial peppermint oil. The storage of pulegone was found to be localized in the foliage and stem of the plant and to be highest in the spring.

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INTRODUCTION

Pulegone is a phytotoxic terpene produced by a variety of plants, including the annual *Hedeoma pulegioides* (American Pennyroyal) (1,2). The present study involved the quantification of pulegone in the foliage, stem, roots, and flower of *H. pulegioides* at 30 day intervals during the 1982 growing season. The soil around the plants was also tested for the presence of pulegone at these times. The quantification of pulegone was carried out over a complete growing season in order to assess varying concentration levels for each plant organ during the growth cycle. This information was applied to apparent allelopathy in the field.

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In addition to its interest as a phytotoxic compound, the pulegone content of commercial products is of importance because of the toxicological effects on humans (3). Pulegone is found in many plant oils, such as peppermint oil, which are used as flavoring agents in the pharmaceutical, food, and confectionery industries (4). Because of its biological and commercial interest, methods have been devised for the determination of pulegone in the oils of various plants, most often based on gas-liquid chromatography. Separation and quantification of pulegone by GLC is made difficult by the presence of interfering compounds that overlap the pulegone peak. Therefore, elaborate extraction and derivatization methods and multiple GLC detectors are required for the analyses (4,5).

This paper describes a simple procedure employing thin layer chromatography with densitometry for the direct determination of pulegone in plants and plant oils. Because of the selectivity of the detection reagent, no extensive sample preparation was required. The method is shown to be accurate and sensitive to low ng amounts of pulegone.

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EXPERIMENTAL

Plant and Soil Analysis

Standard solutions of pulegone were prepared in absolute ethanol at concentrations of 50.0-500 ng/µl by dilution of a 1.00 mg/ml ethanolic stock solution. Whole *H. pulegioides* plants and soil from around the plants were collected under similar environmental conditions at 30 day intervals from May 26, 1982, until September 28, 1982, from an area in northeast Pennsylvania. Composite samples were collected from both partial shade and fully sunny locations. The collected materials were dried at ambient temperature for one week prior to extraction. Three samples each of approximately 0.12 g of dried foliage and stem and 1.0 g of dried soil, roots, and flower were accurately weighed into screw-topped vials and extracted by soaking for 24 hours with

5.00 ml of absolute ethanol. At the end of this period the vials were shaken, the sample was allowed to settle, and the clear extract was applied for TLC.

Extracts and standards were applied at 1.0 μl levels with a Drummond Dialamatic microdispenser to origins located 2 cm from the bottom of Baker-Flex IB2 silica gel sheets (20 x 20 cm). The layers were developed for a distance of 10 cm with hexane-ethyl acetate (5:2 v/v) in a paper-lined, rectangular glass N-tank that was pre-equilibrated with the mobile phase for at least 10 minutes. Chromatograms were air dried and pulegone was detected by lightly spraying the layer with acetic acid-sulfuric acid-anisaldehyde (100:2:1 v/v) prior to heating in an oven at 100°C for 5 minutes.

Pulegone zones were scanned with a Kontes Chromaflex fiber optics densitometer equipped with a baseline corrector and strip chart recorder, using the single beam (5 mm head)-transmission mode and the visible wavelengths from the longwave UV source. Peak areas were calculated using the equation: area = height x width at half height, and calibration curves were plotted as peak area vs. ng of pulegone applied. The amount of pulegone in the extract spots was then determined by interpolation. If the peak area from the extract was not in the linear range (50-500 ng) of the pulegone calibration curve, the solution was quantitatively diluted prior to spotting. Mean pulegone concentrations were calculated from the triplicate analyses of each sample. A portion of foliage, stem, root, soil and flower from each collection was dried at 100°C for 24 hours in order to obtain a baseline dry weight to which the level of pulegone was related.

The identity of the chromatographic zone believed to be pulegone was confirmed using a Finnegan 4000 gas chromatograph/mass spectrometer with INCOS data system and 70eV electron impact source. The 1.8 m x 2 mm 3% OV-101 column was programmed from 100 to 300°C at 15°C/minute. Sufficient pulegone for the MS analysis was obtained by preparative TLC of a scaled-up extract from a larger plant sample. The mass spectrum was compared to the library spectrum of pulegone and a spectrum of an injected pulegone standard.

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Peppermint Oil Analysis

Exactly 2 ml of a commercial peppermint oil was diluted with ethanol to 100 ml in a volumetric flask. One µl of sample was spotted along with bracketing standards, and the amount of pulegone determined as described above. Standard addition was used to evaluate the accuracy of the assay. Identical aliquots of oil were measured and diluted, but 20 mg of pulegone standard was added to one of the samples before analysis. The difference between analyses was compared to the spike level to calculate recovery.

RESULTS AND DISCUSSION

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Collections of plants and soil were made in 1982 on May 26 (day 0), June 25, August 7 and 21, and September 28. Flowers did not appear on the plants until late summer, and no pulegone was found in the flower extract from the single collection. Roots and soil from around the plants were analyzed from each collection; pulegone was never detected in these samples, even when 10 µl of extract was spotted. Based on the amount of sample extracted, the extract volume spotted, and the sensitivity of the detection reagent (50 ng/spot), the sensitivity limit of the assay was approximately 60 µg/g. Therefore, amounts of pulegone in flowers, roots, and soil below this value would not have been detected. If necessary, the sensitivity limit of the analysis could be improved by extraction of a larger sample, concentration of the extract to a small volume prior to spotting, and/or spotting a larger volume on the layer.

Figures 1 and 2 show the decreasing pulegone concentrations found in *H. pulegioides* stem and foliage over the period studied. Pulegone was found in greatest concentration in the foliage, which suggests that it is stored and possibly synthesized there, and may be released into the environment from this location. The concentration of pulegone in the foliage was greatest early in the season and decreased steadily until August, at which time the concentration remained at a low and relatively steady level throughout the rest

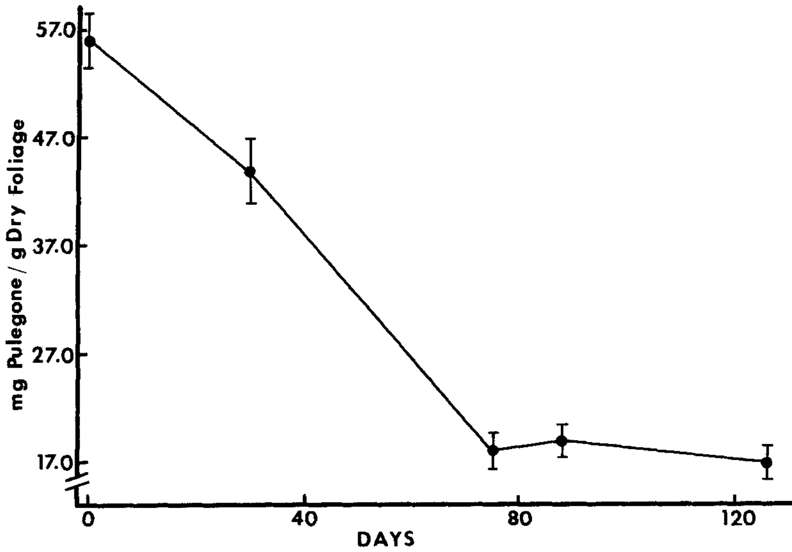

FIGURE 1. Graph of mean pulegone concentration in _H. pulegioides_ foliage as a function of date of collection. The bars indicate the standard deviation of triplicate analyses.

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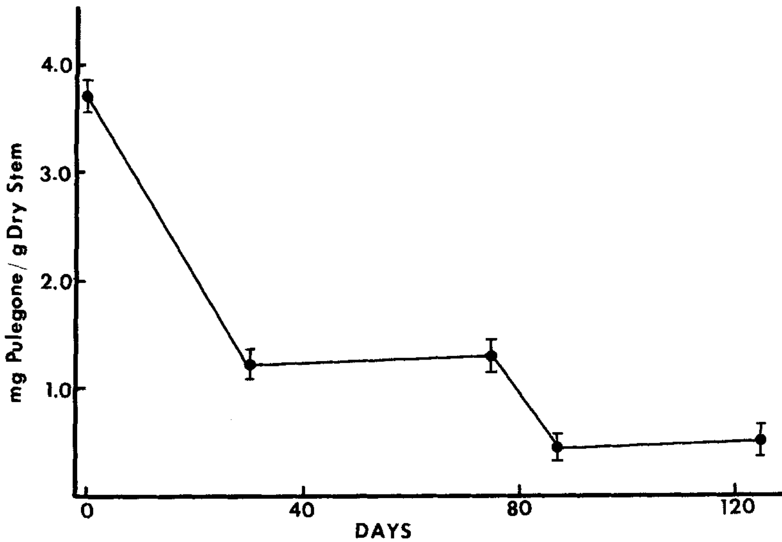

FIGURE 2. Graph of mean pulegone concentration in _H. pulegioides_ stem as a function of date of collection.

of the season. The concentration of pulegone was also highest in the stem at the beginning of the season. This concentration decreased sharply until late June and then gradually decreased through the rest of the growing season, as shown in Figure 2. The time relationship generally corresponds to the study of Farley and Howland (5), which found a decrease of pulegone content from August to September.

To verify complete pulegone extraction, a number of the initial foliage samples was extracted for a second 24 hour period, and this extract was spotted with the first for TLC analysis. Since results showed that at least 95% of the total pulegone was recovered in the first extract, a single extraction was routinely used in all analyses.

The anisaldehyde reagent detected pulegone as a purple zone against a white background at an R_F of 0.43. The reagent is relatively selective for terpenes, and only two faint zones other than pulegone were detected on chromatograms of extracts spotted at the levels described above. Typically, one brown zone was located near the origin and a faint green zone, probably due to chloroplast pigments, close to the solvent front. The densitometric calibration curve [peak area (mm^2) vs. ng pulegone spotted] was fit to the linear equation $Y = A + (B \cdot X)$ with the following typical values: index of determination (r^2) = 0.999, A = -42.0, B = 3.74. Because of slight differences in these values from layer to layer, bracketing standards were always developed in parallel with samples.

To confirm identity of pulegone, foliage extract was streaked onto a preparative TLC plate, the pulegone zone was scraped and eluted with ethanol, and the eluate was analyzed with a directly coupled gas chromatograph/quadrupole mass spectrometer. The mass spectrum had a molecular ion peak of m/z = 152, a base peak of 81, and other prominent peaks were at 137, 109, 95, 91, 67, 53, 41. The close match (r = 0.998) between the extract spectrum, the spectrum of an injected pulegone standard, and the pulegone spectrum in the instrument library, and the correspondence of GC retention times (2.8 minutes) between the sample and standard identified the TLC zone as pulegone.

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The concentration of pulegone in a "concentrated peppermint oil" purchased in a local health food store was found to be 5.8 mg/ml by the densitometric method, which is within the range of pulegone content found for a series of commercial peppermint oils by a much more complex GC method (5). To verify the accuracy of quantification by densitometry, 2.0 ml of this sample was spiked with 20.0 mg of pulegone, and reanalyzed. The resultant value of 14.9 mg/ml represented 91% recovery of the added pulegone, which is adequate for trace analysis at the ng level. Several other purple and brown zones appeared on the chromatogram of the oil (R_F values of 0.28, 0.59, 0.65, 0.70), but none interfered with scanning the pulegone zone.

The quantitative TLC method described provides a simple, accurate means for determination of pulegone during botanical phytotoxicity studies or for assay of this toxic compound in peppermint oil to be added to consumer products. It would also be useful for analysis of finished products, for which the International Organization of the Flavor Industry Committee of Experts (4) has set a pulegone limit of 20-250 ppm, after extraction and concentration steps. Because of the resolving power of TLC and the use of a sensitive and relatively selective terpene detection reagent, pulegone appeared as a separate zone that was not overlapped by interferences, as is often the case when analyzing similar samples by gas chromatography. Samples other than those used in these studies might require cleanup prior to spotting.

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