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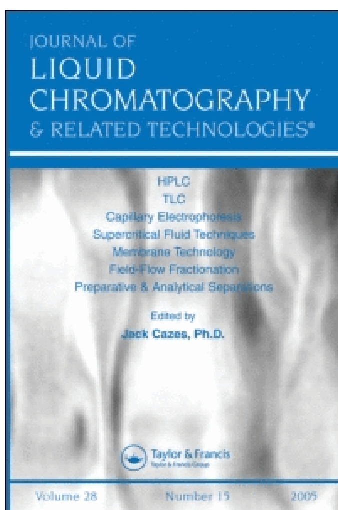

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Determination of Lacinilene C 7-Methyl Ether by High Performance Liquid Chromatography

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DETERMINATION OF LACINILENE C 7-METHYL ETHER BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

An HPLC method is presented for the quantization of lacinilene C 7-methyl ether in cotton dusts. A standard of lacinilene C 7-methyl ether was isolated from extracts of gin trash and a new extraction procedure was developed that effected complete removal of lacinilene C 7-methyl ether from cotton dust. Total dust contains an average of 48.64 μg/g of lacinilene C 7-methyl ether, and the below 20 μm fraction contains 35.9 μg/g of lacinilene C 7-methyl ether.

INTRODUCTION

Lacinilene C 7-methyl ether has been suggested as a causative of byssinosis (1), a respiratory disorder first described in 1705 (2) and characterized by chest tightness and shortness of breath (3). Byssinosis, thought to be caused by a component(s) in the dust generated during processing, occurs in susceptible textile workers in the cotton, flax, jute, and hemp industries. The causative agent(s) is presumed to be removed from cotton dust by washing with water and

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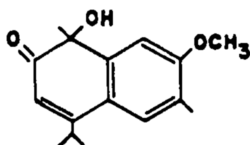

Figure 1. Lacinilene C 7-methyl ether.

can be partially deactivated by steaming (4). Lacinilene C 7-methyl ether is not water soluble but is water extractable, and it has been found in the condensate of a steam distillation of cotton dust.

Lacinilene C, a sesquiterpene ketone, was first isolated from the heartwood of Ohyonire, Ulmus laciniate Mayr (5). A revised structure was established, and its 7-methyl ether (Fig. 1) was isolated from Gossypium hirsutum L. bracts (6). The compound is chemotactic and induces histamine release (7). Quantitative determination of this compound is imperative for orderly biological testing of specific dust samples and synthetically enriched mixtures. There is no known method to date.

Like other compounds of this class, it is unstable, and we feel that high performance liquid chromatography (HPLC) will be the analytical method of choice. We have developed and report here an easy, reliable method for the quantitative determination of lacinilene C 7-methyl ether. We also present accurate extraction procedures.

MATERIALS AND INSTRUMENTATION

Solvents for the HPLC analysis were Waters (8) Liquid Chromatography grade. Diethyl ether and hexane were reagent grade; trace impurities were of no consequence. Lacinilene C 7-methyl ether used as a standard was isolated from "gin trash" in our laboratory.

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Analysis was done on a Waters model 204 liquid chromatograph equipped with a U6K septumless injector, two model 6000-A pumps, a 660 solvent system programmer, and a 100 radial compression module. Detection was accomplished with a Varichrom variable wavelength UV absorption detector. Intergration of peak area by a Hewlett-Packard lab data acquisition system series 3350 was used to quantitate samples.

METHODS

Isolation of enough lacinilene C 7-methyl ether to prepare a standard was achieved by preparative and thin layer chromatography of an extract of gin trash. Forty grams of gin trash was passed through a 20-mesh Wiley mill and extracted with 480 ml water; then the nonpolar compounds were partitioned from the water with three 400-ml volumes of diethyl ether. A dark green oil, which we refer to as gin trash extract, remained after the ether was removed using a roto-evaporator. Our extraction methods are similar to those used for cotton bracts and dust (6,9).

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The extract was loaded onto 2-mm Brinkmann Sil G-200 preparative layer plates and chromatographed with chloroform/acetone/ formic acid (80/19/1) (6). The band fluorescing yellow at about 0.8 R_f value was removed from the plate immediately and eluted from the silica gel with acetone. Under long wave UV light the thin-layer chromatography (TLC) plate revealed that we had separated the yellow fluorescing band into two compounds, lacinilene C 7-methyl ether and a blue-violet compound with nearly the same R_f value. Two successive separations on 0.25-mm silica gel 60 (Merck) TLC plates yielded pure lacinilene C 7-methyl ether.

We were supplied a small amount of authentic lacinilene C 7-methyl ether for TLC comparison (10). Cochromatography of the authentic compound and our isolated compound indicated a pure substance. Two-dimensional TLC analysis in several solvent systems further substantiated this conclusion. We used chloroform/acetone/formic acid (80/19/1) as the first solvent system and either diethyl ether, benzene, hexane, or methanol as the second solvent system. The mass spectrum of our standard matched that of the authentic sample and the spectrum previously reported (6).

Extraction of dust samples for quantitative determinations of lacinilene C 7-methyl ether was done by three methods. Traditionally, water extractions have been done to remove the byssinotic agent from the dust, then the water partitioned with ether to remove the lacinilene C 7-methyl ether. Even though lacinilene C 7-methyl ether is water insoluble, it was first isolated using this technique (6). Cotton dust is a complex matrix, and we wondered if water washing would remove lacinilene C 7-methyl ether completely. We have labeled the traditional extractions A. For comparison, we Soxhlet extracted the dust with ether, labeling these B, or hexane, labeling these C.

Water extractions were done by blending 2 g of dust in an Omni mixer with 18 ml of water for 6 min in the dark to avoid any possible photodegradation; the filtrate was then partitioned three times with 10 ml of ether. The ether portions were combined, reduced to a known volume, and analyzed immediately.

Dust samples for extraction methods B and C weighed from 1.9 to 6 g. The samples were folded in filter paper before being placed

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in the extraction thimbles to prevent loss of any fine dust. A Soxhlet-extraction apparatus was used with a 43 x 123 mm single thickness paper thimble. Extractions were performed in the dark for 48 hr, then an aliquot of the solvent was taken before siphoning back into the boiling flask for TLC and HPLC analyses. The exhaustively extracted dusts were allowed to stand 1 week and re-extracted. The extracts were reduced to a known volume, and HPLC analysis was done immediately.

A dust sample, exhaustively extracted for 1 week and then allowed to stand for 1 week and re-extracted, was reduced to dryness and recovered. Two-tenths milligram of lacinilene C 7-methyl ether, used as a standard, was added to this dust and subsequently Soxhlet extracted with diethyl ether for 48 hr and then HPLC analysis performed on the concentrated extract.

Sample handling after extraction from the dust and before injection into the HPLC is critical. Samples should be refrigerated and stored under dry nitrogen immediately. The ether or hexane used for extractions must not be completely removed during sample concentration. If this should happen, precipitation will occur in the mobile phase when injected into the HPLC instrument even though redissolved first. In some cases we found it necessary to inject 50 µl of sample extract.

Quantitative separations were achieved with a Waters Radial-Pak B cartridge (normal phase activated silica) in conjunction with the radial compression module (RCM-100). This column is packed with a 10 µm spherical silica gel. We flushed the column with ethyl

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acetate, acetone, and 2,2,4-trimethylpentane, respectively, between sample determinations to prevent column contamination. A solvent gradient system worked best, with hexane/acetic acid (99/1) as solvent A and tetrahydrofuran as solvent B varying from 5% to 50% over 10 min. The flow rate was 6 ml/min. Detection was achieved by monitoring UV absorption at 340 nm (6).

A standard curve of instrument response vs. amount of lacinilene C 7-methyl ether was generated. Multiple injections [12] of 1.23 μg of lacinilene C 7-methyl ether dissolved in 2.0 μl of diethyl ether were used to determine the standard deviation of injection and area intergration.

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RESULTS AND DISCUSSION

Dust examined was limited to one that we know to be byssinotically active (11). Total dust was first collected with a high-volume filter and had a wide particle—size range. This dust was then separated into a less than 20-μm fraction (12). The ≤ 20-μm fraction was active to human challenge. Both dust fractions induced histamine release (11).

Table I is a summary of quantitative results. Water extraction in both cases removed only about 30% of the total content of lacinilene C 7-methyl ether. Whether it is difficult to extract from the matrix completely or it is the end product of some decomposition reaction with its equilibrium upset upon Soxhlet extraction is unknown. Complete removal of a natural product from its matrix is a tenuous question. We Soxhlet extracted until we saw no more lacinilene C 7-methyl ether removed from the dust by TLC or HPLC analysis of an aliquot of solvent before it returned to the boiling

TABLE I

Lacinilene C 7-Methyl Ether Content in a Known Active Cotton Dust

Extraction	Total Dust (μg/g)	≤ 20 Micrometers (μg/g)
A. Water Extraction, Ether Partition	16.65	13.80
B. Ether Soxhlet	54.05	35.93
C. Hexane Soxhlet	43.22	35.94

flask. Both hexane and ether Soxhlet extractions totally removed lacinilene C 7-methyl ether from the dust after 48 hr. Concern for the thermal stability of lacinilene C 7-methyl ether prompted us to use ether extractions. From the limited data in this study, lacinilene C 7-methyl ether seems stable at 75°C necessary for hexane extraction.

The possibility that lacinilene C 7-methyl ether may be bound to another compound not soluble in ether or hexane and left behind after extraction must be considered. After complete removal of lacinilene C 7-methyl ether, the dust was allowed to stand at atmospheric conditions for 1 week and then re-extracted. We found that no additional lacinilene C 7-methyl ether was generated. We then added 0.2 mg of pure lacinilene C 7-methyl ether to the dust exhaustively extracted. Upon extracting again with ether, we recovered 96% of the added lacinilene C 7-methyl ether, indicating that lacinilene C 7-methyl ether is not easily bound to compounds left in the dust.

The ≤ 20-μm fraction of the dust contained approximately 77% of the lacinilene C 7-methyl ether content. Total dust would contain fiber that would contribute no lacinilene C 7-methyl ether to

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the sample. More friable plant parts are the major contributors to the ≤ 20-μm fraction of dust. This simple relationship between lacinilene C 7-methyl ether and total or ≤ 20-μm dust should be checked further for statistical relationships. Perhaps total dust content could be estimated from lacinilene C 7-methyl ether measurements.

The consideration of other compounds extracted along with lacinilene C 7-methyl ether is complex. We can account for all fluorescent compounds on the TLC plates in our HPLC chromatogram. However, the peculiar behavior of the sample upon concentration to dryness is indicative of lipids and lignins. Although lacinilene C 7-methyl ether is probably completely taken up in a solvent and this might be an excellent method for eliminating many irrelevant compounds, we chose to concentrate the extract to a known volume (about half). We experienced no problems with this procedure even when we injected 50 μl of extract for analysis. This undoubtly led to some band spreading but it was of no consequence. In fact, some very concentrated trial samples' response peaks were so spike-like that peak height could be used for quantization.

Column contamination using this sample handling method is unavoidable. During our work on this compound we experienced no "ghosting" and no dramatic drop in column efficiency. However, this can be attributed to the regular purging of the column along the recommended lines of a general silica column clean-up procedure.

We found that in some cases an isocratic solvent system of hexane, tetrahydrofuran (THF), and acetic acid (85/14/1) achieved

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adequate separation. Poor resolution sometimes resulted in the hexane extracts because chlorophyll is extremely soluble in hexane. This problem was easily overcome by using a linear gradient with THF varied from 5% to 50% over a period of 10 min with a flow rate of 6 ml/min on the Waters column.

When we first began trying to separate lacinilene C 7-methyl ether from cotton dust, we attempted reverse phase HPLC techniques. We discovered that its residence time on a C_{18} column (6 hr) was too long and allowed decomposition. Normal phase worked quite well. Column selection for use with the RCM-100 is limited, but we did achieve some good separations with a conventional Alltech column (2.5 cm x 4.6 mm I.D.) prepacked with spherisorb (5 µm). Both the Waters and Alltech columns use a spherical silica support phase. In all cases, we achieved near base-line resolution with the Waters column. Fig. 2 is a typical chromatogram with the lacinilene C 7-methyl ether peak shaded.

A conventional column displayed varying solvent synergism effects. Each sample should be spiked with the known to determine which peak in the complex is lacinilene C 7-methyl ether. Using the RCM-100 allows operation at high flow rates that overcomes this effect. In all cases the retention time for lacinilene C 7-methyl ether on the Waters column was 2.5 min; with the conventional column, retention varied from 7.0 to 7.8 min at the 0.5 ml/min flow rate for maximum separation and an isocratic elution.

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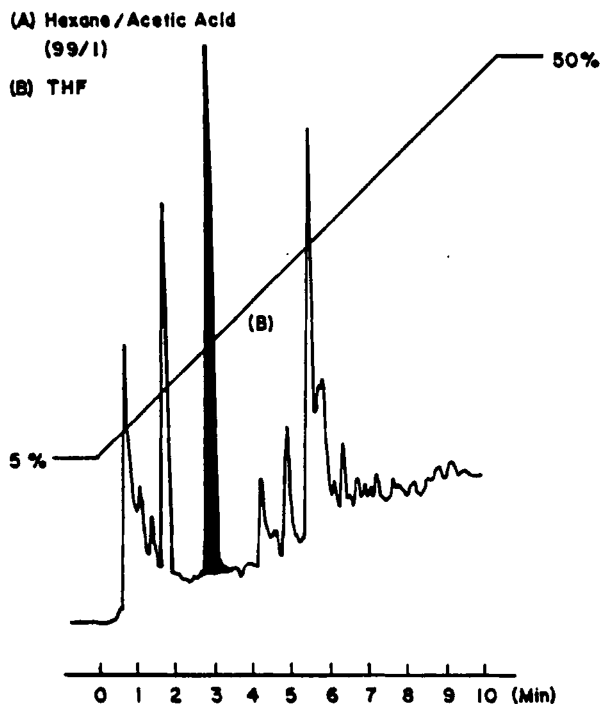

Figure 2. HPLC chromatogram of cotton dust extracts.

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A simple linear relationship (Fig. 3) between peak area and amount of compound injected over a fairly wide range was established. Peak height vs weight of lacinilene C 7-methyl ether was also a linear relationship, and so if peaks are so spiked that area integration is not possible, quantization is still practical.

Multiple injections of the standard were used to calculate the standard deviation of the peak area. The error is probably a combination of determinate and indeterminate. With this information it was calculated that sample replicates of four were necessary to measure a sample of approximately 1.2 μg with 95% confidence level. All samples reported are an average of four trials.

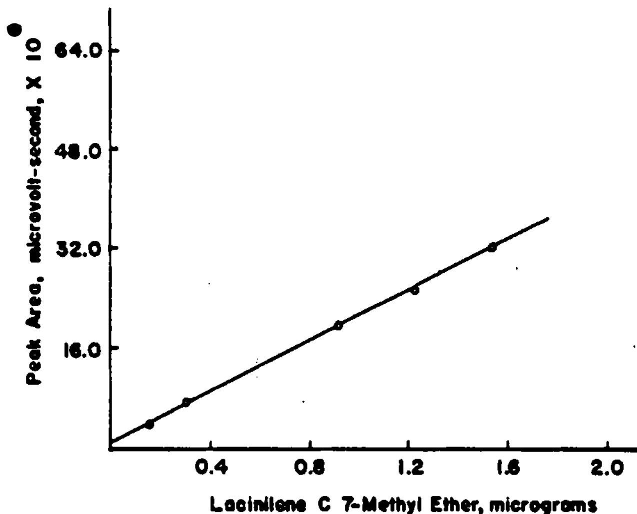

Figure 3. Standard curve depicting the ratio of peak area of lacinilene C 7-methyl ether vs. the weight of lacinilene C 7-methyl ether. The correlation coefficient is r = 0.9996, and the slope of the curve is 20.7356.

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Normal phase HPLC is a reliable method for determining lacinilene C 7-methyl ether in extracts of cotton dusts. We are continuing to quantify other known compounds in this mixture, to identify unknowns, and to investigate the application of preparative HPLC techniques.

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