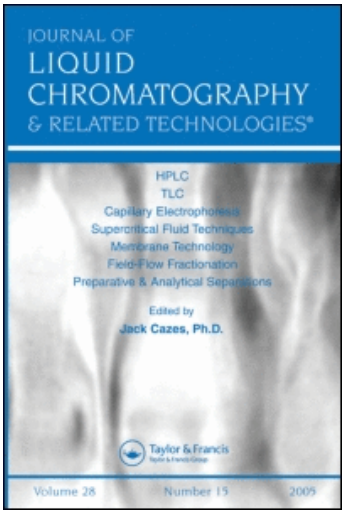

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A REEVALUATION OF ENDOGENOUS LEVELS OF LACINILENE C 7-METHYL ETHER IN BRACTS OF THE GENUS GOSSYPIUM

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ABSTRACT

Lacinilene C 7-methyl ether, lacinilene and their cadalene precursors were extracted from dried green and senescent cotton bracts with methanol-acetone (8:2, v/v). Preliminary separation of the concentrated extract was effected with silica and reverse phase SEP-PAK cartridges. The final eluate, containing the four compounds, was separated on a Supelcosil LC-C_{18} HPLC column using a mobile phase consisting of acetonitrile-methanol-isopropanol-water (45:13:2:40, v/v). The observed amounts of the methyl ether never exceeded 60 ppm, which was sustantially lower than previously reported by others. These determinations were unaffected by the method of tissue preparation and diethyl ether was not as effective as an extraction solvent as methanol-acetone (8:2, v/v). The reported procedure may be applicable to the simultaneous quantification of the two lacinilenes and their cadalene precursors.

INTRODUCTION

The sesquiterpene lacinilene C 7-methyl ether (LCME), a natural product of the cotton plant (Gossypium sps.) has been

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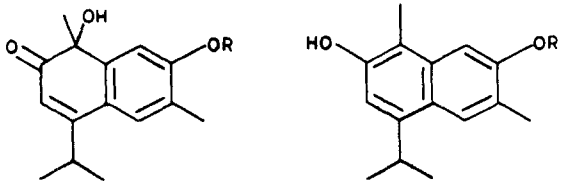
proposed as a possible cause of byssinosis, an occupational respiratory disease affecting some cotton mill workers (1). The unmethylated compound, lacinilene (LC), and its metabolic precursor, 2,7-dihydroxycadalene (DHC), have recently been shown to act as phytoalexins capable of inhibiting bacterial blight of cotton caused by Xanthomonas malvacearum (2). These three compounds plus 2-hydroxy-7-methoxycadalene (HMC), the sesquiterpenoid napthol precursor of LCME (Fig. 1), have been isolated and identified in both green and field dried senescent cotton bracts (3,4).

The lacinilenes and their precursors have been difficult to quantitatively extract and or purify in mg amounts. This is understandable for the two napthol precursors since they rapidly autoxidize on silica gel to form the lacinilenes (4). It is not so simple to rationalize the problem associated with the quantitative extraction of the lacinilenes.

Recent reports indicate that quantitative extraction of LCME could be obtained by using a long term (2-7 days) ether soxhlet technique followed by direct resolution of the concentrated ether extract with normal phase HPLC (5,6). The quantities reported in various bract, gin trash and dust samples varied from 14 to 500 ppm. It was also reported that LCME was



(LC) R = H
(LCME) R = Me

(DHC) R = H
(HMC) R = Me

FIGURE 1. Lacinilene C (LC), 2,7-Dihydroxycadalene (DHC), Lacinilene C 7-methyl ether (LCME), and 2-Hydroxy-7-methoxy-cadalene (HMC).

heat labile *in situ* and that tissue prepared in a Wiley mill contained appreciably less LCME than those prepared by grinding in liquid N_2 or dry ice.

We have previously developed an expedient semi-quantitative method for obtaining all four compounds from aqueous bract extracts (7). The extract was pumped with a peristaltic pump through a reverse phase SEP-PAK. The four compounds were adsorbed by the packing, subsequently eluted and resolved using reverse phase HPLC. During this work, it was apparent that our values were considerably lower than those reported by other workers using the ether extraction technique (5).

We now report on a new extraction technique for LC, LCME, DHC and HMC and include a quantitative measure of endogenous levels of LCME. The final HPLC step remarkably enhances the resolution of the four compounds and considerably shortens the analysis time as compared to our previous work. Our determinations of LCME concentrations are unchanged from our previous qualitative observations. Also, we were unable to observe any detrimental effect of heat on the amount of extractable LCME.

MATERIALS AND INSTRUMENTATION

SEP-PAK silica and reverse phase cartridges were obtained from Waters Associates (8). Chromatographic solvents were either HPLC grade or distilled A.C.S. grade filtered through a 0.2 μm Millipore filter. A Tracor Instruments #950 high pressure pump was used to deliver the solvent through a 4.6 mm x 15 cm Supelcosil LC-18 column (Supelco, Inc.). Samples were introduced via injector model 7120 (Rheodyne, Inc.), fitted with a 20 μl loop. Column effluent was monitored at 250 nm with a Tracor 970A variable-wavelength detector and recorded with a Hewlett-Packard 3380A integrator.

METHODS

All procedures were conducted in subdued light. Dried green cotton bracts (*Gossypium hirsutum* cultivar Stoneville 256) were

supplied by Cotton, Inc. (Raleigh, NC). Field dried senescent bracts (cvs. Deltapine 16, Stoneville 213, Storm Prof 37 and Rogers glandless 6) were collected locally from experimental plots. The standard curve and extraction efficiencies were obtained from triplicate samples.

One gram of bract powder (Wiley mill ground to 40 mesh) was extracted with 100 ml of methanol-acetone (8:2, v/v) at room temperature for varying amounts of time. One-half g samples were also subjected to soxhlet extraction with 150 ml ether for 48 hours. Whole bracts were pulverized in liquid N_2, passed through a 20 mesh screen, and heat treated (100°C/15 min.). One g samples of this material was also extracted with methanol-acetone (8:2, v/v). With all samples, the extraction mixtures were vacuum filtered through Whatman #3 filter paper, concentrated by rotoevaporation (30°C) to approximately 0.5 ml *in vacuo* and quantitatively transferred to a silica SEP-PAK with the aid of acetone. The SEP-PAK was dried with N_2 gas and eluted with 10 ml of 55% acetone. The acetone was removed by rotoevaporation (30°C) and the resulting aqueous solution was placed on a reverse phase SEP-PAK as previously described (7). An aliquot (20 µl) of the final solution (5 ml) was injected onto the columns which was equillibrated with a mobil phase consisting of acetonitrile-methanol-isopropanol-water (45:13:2:40 v/v) at a flow rate of 1 ml/min. A standard curve of LCME versus detector response was obtained using previously prepared LCME. The purity of this material was verified by HPLC and mass spectrometry.

RESULTS

The solvent system of MeCN-MeOH-iso-PrOH-H_2O (45:13:2:40, v/v) was developed using the general concept of the Snyder solvent selectivity triangle (9). The criteria for selecting an appropriate system were 1) to adequately resolve LC from DHC, 2) to increase peak sharpness of HMC, and 3) to reduce analysis time. This approach resulted in the development of an isocratic solvent system capable of resolving the four compounds in under 15 min (Fig. 2).

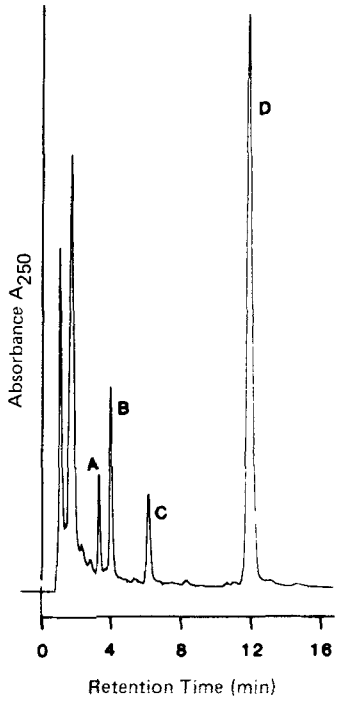


FIGURE 2. A representative HPLC chromatogram of lacinilene C (A), 2,7-dihydroxycadalene (B), lacililene C 7-methyl ether (C) and 2-hydroxy-7-methoxycadalene (D) obtained by methanol-acetone extraction (8:2, v/v).

The calculated line of best fit for the standard curve [y = (x + 1291.3) ÷ 1287.4] had a regression coefficient of 0.99 (Fig. 3). The final concentration (ppm) of the 1 g sample is equal to 1/4 times the calculated LCME from the above curve.

The extraction efficiency for LCME and the other three compounds was determined by extracting 1 g tissue with 100 ml methanol-acetone (8:2 v/v) for an extended time period ($\sim$ 3 days) with intermittent work up to observe when the extractable amounts were less than 1% of total. These amounts were then added together to obtain a total yield (100%) of each compound. At 16 hr the extraction efficiency for LCME was 96%. The overall

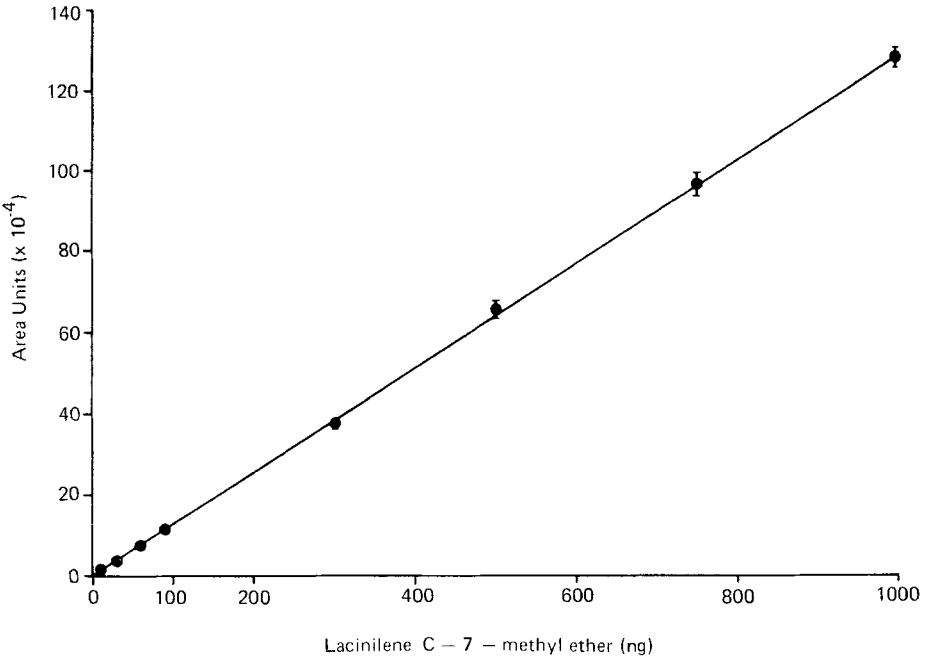


FIGURE 3. Standard curve for lacinilene C 7-methyl ether.

efficiency of the cleanup and analysis technique, determined by a work-up of a known amount of pure LCME from methanol-acetone, was 92 ± 2%. Thus, the combined total efficiency for LCME recovery from bract was estimated at 88%. The extraction efficiency at 16 hr for LC, DHC and HMC was 96%, 99% and 98%, respectively (Fig. 4). The extractability of these four compounds in the methanol-acetone solvent system was very efficient and over 60% extraction of all four were obtained within 15 min. The efficiency of ether extraction as compared to methanol-acetone extraction was 72% (LC), 85% (DHC), 82% (LCME), and 99% (HMC).

Heat lability of the compounds *in situ* was determined using the heated and non-heated powders prepared from whole bracts pulverized in liquid N_2. No substantial differences were detected in these preparations (Table 1).

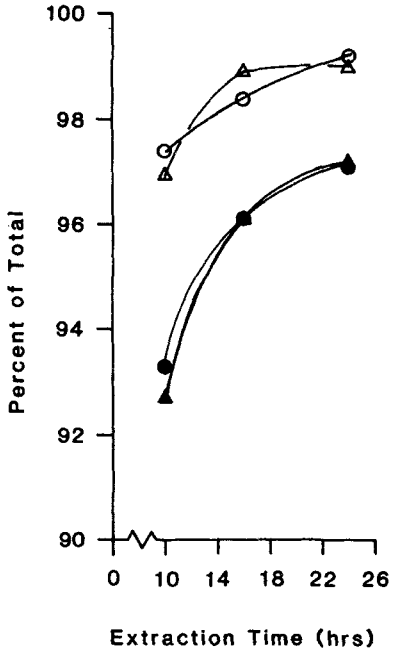


FIGURE 4. Extraction efficiency for lacilinene C (●), 2,7-dihydroxycadalene (△), lacilinene C 7-methyl ether (▲), and 2-hydroxy-7-methoxycadalene (o).

TABLE 1

The Effect of Pre-Heating Tissue on Compound Stability.

	LC	DHC	LCME	HMC
Wiley Mill	64	87	79	97
no heat*	87	91	80	92
100° C for 15 min*	72	89	79	97

Samples were extracted for 2 hrs and values are percent of total.
*Samples were ground to 20 mesh size.

TABLE 2

Lacinilene C 7-Methyl Ether Concentrations in Bract Tissue from Selected Cultivars.

Cultivar	LCME (ppm)
St 256 (green)	55
DP 16 (senescent)	38
St 213 (senescent)	24
SP 37 (senescent)	7
Gl 6 (senescent)	6

Using the technique described above, LCME concentrations were determined in four field dried senescent bract samples and one dried green sample (Table 2). The green bract sample contained 55 ppm of LCME. The field dried senescent bracts from DP 16 and St 213 contained 70 and 43 percent of this amount, respectively. The Texas cv SP 37 contained substantially less; it was only slightly higher than the glandless Rogers Gl 6.

DISCUSSION

Methanol-acetone (8:2, v/v) is an efficient extracting solvent for both the lacinilenes and the cadalenes. Long term ether refluxing offers no real advantage for extracting these compounds.

Our method of sample preparation clearly is not as simple as the direct injection of a concentrated ether extract onto the column as reported by others (5,6). However, our method does offer a number of important practical advantages. The UV transparency of the final preparation allows compound detection at a compromise wavelength close to the extinction maximums of the lacinilenes and cadalenes. This enhances sensitivity for the

lacinilenes by approximately two-fold and for the cadalenes by approximately eight-fold over earlier techniques (5,6). The reverse phase system also exhibits highly consistent retention times for each of the four compounds and does not require any washing between analytical runs.

Our results indicate that LCME occurs in cotton bract tissue in concentrations less than 60 ppm. These data are in agreement with our past large scale extractions using water, ether or methanol-acetone. We could not confirm the report (5) that LCME concentrations in bract tissue approached 500 ppm. We were also unable to demonstrate the detrimental effect of heat on LCME lability in situ as previously reported (5).

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