A rapid method for diterpene resin acid analysis of pine needle oleoresin

THOMAS V. MAGEE AND DUANE F. ZINKEL
United States Department of Agriculture, Forest Service, Forest Products Laboratory. Madison, WI, U.S.A. 53705-2398

Received January 28, 1986
Accepted April 22, 1986


A method for diterpene resin acid analysis has been developed for pine oleoresin samples at the submilligram level. The method utilizes the removal of acidic components by a micro-DEAE-Sephadex ion-exchange column and analysis of the recovered resin acids, as the methyl esters, by capillary gas–liquid chromatography.


Introduction

Analysis of pine needle oleoresin for diterpene resin acid composition has been studied with respect to chemotaxonomic and genetic applications (1). The analysis requires separation of the neutral from the acidic components, for which a quantitative separation method using DEAE-Sephadex has already been developed (2, 3). However, because of the sample size generally used in this method (100–150 mg acids), the time needed for the separation alone is the major constraint in the overall resin acid analysis. Also, a sample of this size is far in excess of that needed for determination of resin acid composition by gas–liquid chromatography (GLC). We have modified the DEAE-Sephadex separation method for samples as small as a single needle, resulting in a decrease in total analysis time, while meeting the requirements for quantitative GLC. This methodology will permit extensive chemotaxonomic surveys using the resin acid composition of foliage similar to the many reports in the literature (e.g., see review by von Rudloff (4)) on foliar monoterpenes composition.

Methods

Oleoresin sampling

The choice of method for obtaining a needle oleoresin will depend on the requirements of the research and the characteristics of the species. See Results and discussion for a detailed treatment of this question.

Column preparation

A standard disposable glass Pasteur pipette (23 cm length) was used as a column. The tip was fire polished, forming an inside lip to retain a small wad of glass wool. The tapered portion of the pipette held the column packing; the cylindrical portion served as a solvent reservoir. The pipette was filled to the reservoir with diethyl ether (Et2O) - methanol (MeOH) - water (79:20:1) and a dilute suspension of DEAE-Sephadex ion-exchange material (in the basic form (3)) in the same solvent was carefully introduced in portions onto the glass wool. Following the addition of each DEAE-Sephadex portion, nitrogen pressure was applied to the pipette to improve the homogeneity and speed of packing and to purge vapor pockets from the bed. About 10 cm (<100 mg) of dry column packing was used for a 0.5-mg sample (0.0002 mequiv. acid; the resin acid capacity of the column packing was approximately 1 mg). Slightly less packing was used for a 0.05-mg (0.0002-mequiv.) acid aliquot.

DEAE-Sephadex separation

Et2O-MeOH-H2O (79:20:1) was used as both solvent and eluent. The oleoresin, dissolved in 1 mL, was introduced onto the column packing. Neutrals were washed through with 5 mL eluent under N2 pressure (14 kPag = 2 psig). Eluent saturated with CO2 was used to recover the acids. The saturation was carried out by shaking the eluent under CO2 pressure (4 psig, compressed gas cylinder) in a long-neck, 100-mL round-bottom flask with 3 outer joint; CO2 was introduced into the flask through a 90° elbow adapter with a small outlet hole for fingertip pressure control. The acids were eluted with 5 mL of CO2-saturated solvent under slight CO2 pressure (2-5 psig, generated from dry ice (5)).

Gas–liquid Chromatography

GLC was done with a Hewlett-Packard model 5840 or 5880 gas chromatograph equipped with a flame ionization detector. An inlet splitter system with a split ratio of 100:1 was used. A combination of BDS (1,4 butanediol succinate) and methyl silicone (e.g., DB-1 of J & W Scientific, Inc., Rancho Cordova, CA) capillary columns (5, 6) were used in this work.

In preparation for GLC, the acid fraction was methylated in Et3O-MeOH (9:1) with a slight excess of ethereal diazomethane (7). The esterified sample was taken to dryness under a stream of nitrogen and redissolved in 100 µL methyl tert-butyl ether. A 3-µL aliquot injection resulted in the application of 100 ng per major resin acid component (methyl ester) to the BDS column.

Results and discussion

Oleoresin extraction methods

Oleoresin can be obtained in several ways from single as well as from several needles. One method is to cut the needle into ca. 3-cm segments and apply slight pressure along the edges...
between the thumb and forefinger to force oleoresin exudation at one end of the segment: the exudate is then drawn up into a fine glass capillary by capillary action, or obtained by surface contact with solvent. Another method is to cut the needle into ca. 0.5-cm segments and extract the oleoresin. A nonpolar solvent such as n-pentane in either approach will minimize extraction of extraneous substances (e.g., chlorophylls). The extraction method was found to give the highest yields, but is more prone to extraction of nonoleoresin substances than is the exudation method. The extraction of needles ground after freezing with liquid nitrogen (8, 9) will provide high recovery of oleoresin, but with a sizable contamination by unwanted needle components. Needle oleoresin yields are highly dependent upon species, maturity, and freshness. For example, we obtained adequate amounts of exuded oleoresin from *Pinus strobus* and *Pinus ponderosa* needles, but solvent extractions was necessary with *Pinus virginiana* and *Pinus taeda*.

Single needle extractions of fresh *Pinus resina* yielded ca. 0.05 mg diterpene resin acids per needle; the recovery of resin acids from the DEAE-Sephadex columns was quantitative at this level.

**Diterpene resin acids**

In our experience, the diterpene resin acid content of fresh mature pine needles ranges from 5 to 15 mg/g needles. In previous studies, extracts from approximately 10-g samples of fresh needles were used for resin acid analysis (10–12). The separation of neutrals from acids was accomplished by the DEAE-Sephadex method (2, 3). In this method, the acids exchange onto the DEAE-Sephadex and the neutrals pass through the column; quantitative recovery of acids is subsequently achieved by elution with CO₂-saturated Et₂O–MeOH–H₂O (89:10:1) eluent. Our modification of this method reduces sample size by over two orders of magnitude. This corresponds to a sample containing on the order of 0.5–1 mg diterpene resin acids, a typical yield from three to five fresh, mature needles. The procedural modifications include the use of a Pasteur pipette as a column and elution with CO₂-saturated Et₂O–MeOH–H₂O (79:20:1) (5) under slight CO₂ pressure.

Pine needles have a greater variety of diterpene resin acids than the usual resin acids of xylem. This includes resin acids with the carboxyl groups in several different chemical environments and, hence, of potentially different acidities. The possibility of selectivity among these resin acids in the ion-exchange process, particularly at submilligram levels, was of concern and, thus, was examined. Compounds were chosen to represent those carboxyl positions encountered in pine resin acids: the C-18 carboxyl (equatorial), the C-19 carboxyl (axial), and the C-15 carboxyl (terminal and primary as in certain labdane acids) (for systematic nomenclature of diterpenes, see 13). Dehydroabietic acid (8, 11, 13-abietatrien-18-0ic acid) was selected as representative of C-18 carboxylic acids. Manoyl oxide acid (8, 13β-epoxy-14-labdien-19-0ic acid) and comminic acid (8(17), E-12, 14-labdatrien-19-0ic acid), obtained from *P. resina* needle oleoresin, were selected as representative of C-19 carboxylic acids. Antionic acid (8(17), E-13-labdadien-15-0ic acid) was selected for the C-15 carboxylic acid. All of these resin acids were reproducibly and quantitatively recovered from the ion-exchange column.

The micro-DEAE-Sephadex methodology is not limited to the pine resin acids, but can also be applied to the resin acids in other conifers and to the study of epicuticular wax acids (14, 15).

**Time efficiency**

The time span required for separation of neutrals from acids is less than 30 min with the modified method. If four or five simultaneous separations are performed, the preparation of 40–60 samples per person per 8-h day is feasible. This is better than a five-fold decrease in total analysis time from the original DEAE-Sephadex method. With the new method, the total time for determination of resin acid composition is no longer dependent upon the DEAE-Sephadex separation; rather, it is dependent upon the time needed for GLC analysis.

**GLC analysis**

Quantitative determination of diterpene resin acid composition was done gas chromatographically. The choice of column or columns will depend upon the nature of the resin acid constituents. Generally, GLC on a nonpolar column (methyl silicone such as DB-1) at 170°C allows for the detection and quantitation of oxygenated resin acids often found in needle oleoresin and gives adequate resolution of most of the other components. However, supplementary GLC on a BDS column may be needed to obtain data for components not resolved by the nonpolar column.

Samples of the oleoresins at the 0.5-mg scale (ca. three to five needles used) were diluted with 100 µL of solvent such that 3-µL injections (split 100:1) applied sufficient material to the column for quantitative analysis, i.e., well in excess of the 20 ng needed for each compound (6). Samples of ca. 0.05 mg resin acids (from single needles) require 10–15µL solvent and manual injection (3 µL) of the solution for quantitative analysis. Smaller sample sizes (from parts of needles, tissue cultures, etc.) would require special injection techniques, as well as possible further refinement of the DEAE-Sephadex procedure.

**Other oleoresin components**

The methodology reported herein for resin acids, with respect to both isolation and separation, also can be adapted to the nonacidic terpenes. Sufficient oleoresin can be obtained usually from three to five needles for rapid determination of mono- and sesqui-terpene composition by direct GLC injection of oleoresin (dissolved in the appropriate volume of a solvent). For example, with both *P. sylvestris* and *P. ponderosa*, the exudation from only two needles in a small volume (20 µL) of n-pentane gave sufficient levels of the major mono- and sesqui-terpenes for GLC analysis. The simplicity of this approach (cf. 16) should have advantages over direct injection techniques for a single conifer needle, as developed previously (see 17), or micro-distillation methods (17, 18). For compositional analysis of neutral diterpenes (such as the diterpene alcohol isohabenol (8, 19, 20)), with the more usual nonpolar capillary GLC columns, the DEAE-Sephadex separation will remove potentially interfering resin acids.

5. **Han, J. S.**, and D. F. Zinkel. 1986. GLC determination of the


