

Rapid Analysis of Abietanes in Conifers

P. J. Kersten · B. J. Kopper · K. F. Raffa · B. L. Illman

Received: 27 February 2006 / Revised: 14 August 2006 / Accepted: 24 August 2006 /

Published online: 3 November 2006

© Springer Science + Business Media, Inc. 2006

Abstract Diterpene resin acids are major constituents of conifer oleoresin and play important roles in tree defense against insects and microbial pathogens. The tricyclic C-20 carboxylic acids are generally classified into two groups, the abietanes and the pimaranes. The abietanes have conjugated double bonds and exhibit characteristic UV spectra. Here, we report the analysis of abietanes by reversed-phase high-performance liquid chromatography using multiwavelength detection to optimize quantification of underivatized abietic, neoabietic, palustric, levopimaric, and dehydroabietic acids. The utility of the method is demonstrated with methanol extracts of white spruce (*Picea glauca*) phloem, and representative concentrations are reported.

Keywords Abietic · Neoabietic · Palustric · Levopimaric · Dehydroabietic · White spruce · Diterpenes · HPLC

Introduction

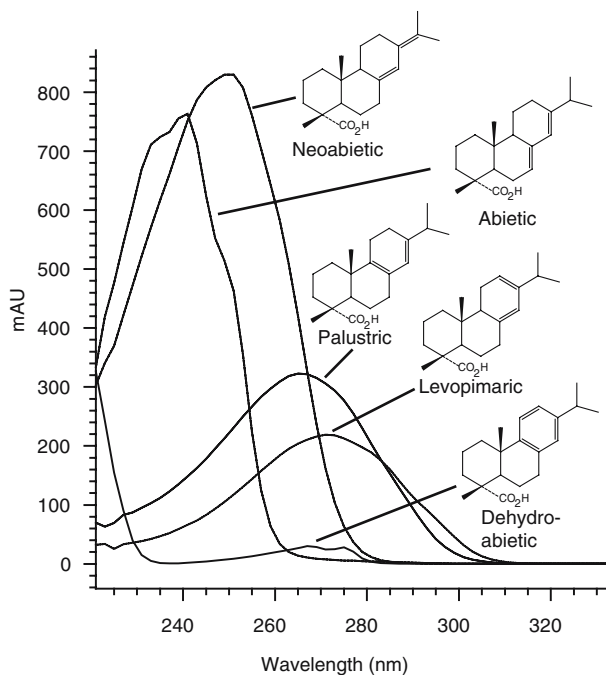
Resin acids have been shown to have broad and highly active biological properties, being involved, for example, in both constitutive and induced plant defenses against numerous insects and microorganisms (Björkman and Gref, 1993; Franich and Gadgil, 1983; Gref 1987; Kopper et al., 2005). The high concentrations, patterns of storage, and elicitation by both herbivore feeding and pathogen infection further suggest important defensive roles (Gref and Ericsson, 1985; Tomlin et al., 2000). This appears to be particularly true of

P. J. Kersten (✉) · B. L. Illman
Forest Products Laboratory, USDA Forest Service, Madison, WI 53726, USA
e-mail: pkersten@wisc.edu

B. J. Kopper · K. F. Raffa
Department of Entomology, University of Wisconsin, Madison, WI 53706, USA

B. J. Kopper
USDA Animal Plant Health Inspection Service, Raleigh, NC 27606, USA

Fig. 1 Structures and spectra of abietanes. A 1-mM mixture of five common abietanes (20 μ l injections) was analyzed by HPLC (see “Methods and Materials”) and spectra obtained at the apex of each peak. Retention times for the abietanes are given in Fig. 2



conifers (Wagner et al., 1983; Larsson et al., 1986). The activity of individual plant defense compounds commonly varies with the presence of other compounds, with interactive effects such as synergism frequently yielding results not predictable from analysis of individual components (Berenbaum, 1985). Moreover, concentrations of phytochemicals are typically highly variable in nature, due to both environmental and genetic differences. Thus, it is commonly necessary to analyze large numbers of samples to determine ecological significance. Based on these considerations, it is desirable to have an easy, quantitatively reliable method for separating resin acids from plant tissues. A significant analytical challenge arises from the similarity of their structures (Fig. 1).

The most common method used for resin acid analysis is gas chromatography (GC) of the methyl esters (Zinkel and Engler, 1977) with detection by flame ionization or mass spectrometry. However, this method has disadvantages, including instability of the derivatized samples (Latorre et al., 2003) and hazards of methylating reagents (potentially explosive and carcinogenic). Furthermore, considerable sample preparation is typically required with crude biological samples, in addition to derivatization. This may include extraction, partitioning of acid components into liquid or solid phase, solvent evaporation, redissolving sample in GC-compatible solvent, and removing moisture from the sample prior to injection.

There have been recent attempts to provide simple and robust analytical methods for resin acid analysis by high-performance liquid chromatography (HPLC). Reversed-phase HPLC of underivatized resin acids in river water does not resolve the structural isomers studied (abietic, isopimaric, and pimaric), and detection by negative ion electrospray mass spectrometry does not provide isomeric speciation (McMartin et al., 2002). Determination of dehydroabietic and abietic acids in Chinese medications (Lee et al., 1997) and adhesive (Lee et al., 1994) with UV detection is reported but without analysis for other abietanes.

Likewise, coumarin ester derivatives of nonaromatic resin acids are not resolved (Volkman et al., 1993).

Here, we report a simple method for the analysis of abietanes by reversed-phase HPLC. Advantages include: (1) no sample derivatization is required and therefore related expenditures and hazards are avoided; (2) extraction and chromatographic conditions are mild, and therefore, the sample components, and consequently their biological activities, should be unchanged; and (3) all components of the HPLC mobile (methanol, acetic acid, and water) phase are volatile and therefore recovery of compounds from fractionated sample is simplified. These benefits are particularly advantageous in biological studies that require rapid analysis of plant materials and screenings for biological activities.

Methods and Materials

Chemicals Abietic (90–95%), neoabietic (99+%), palustric (90–95%), levopimaric (95+%), and dehydroabietic (99+%) acids were purchased from Helix Biotech (British Columbia, Canada).

Sample Preparation Stock solutions of standard resin acids were prepared in 95% ethanol at approximately 2 mg/ml. These solutions were diluted using positive displacement syringes to prepare solutions of defined concentrations according to absorbance at λ_{\max} and literature (Joye and Lawrence, 1967) molar absorption coefficients: abietic at 241 nm ($24,150 \text{ M}^{-1} \text{ cm}^{-1}$), neoabietic at 252 nm ($24,540 \text{ M}^{-1} \text{ cm}^{-1}$), palustric at 266 nm ($9,060 \text{ M}^{-1} \text{ cm}^{-1}$), levopimaric at 272 nm ($5,800 \text{ M}^{-1} \text{ cm}^{-1}$), and dehydroabietic at 268 nm ($698 \text{ M}^{-1} \text{ cm}^{-1}$) and 276 nm ($774 \text{ M}^{-1} \text{ cm}^{-1}$).

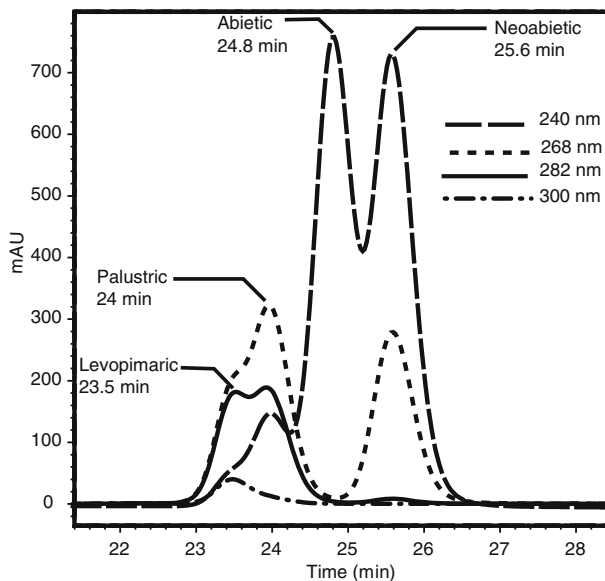
White spruce (*Picea glauca*) samples were collected from 10 mature trees in a planted stand in Dane County, WI, USA, in August. For the preparation of extracts, approximately 500 mg of phloem were cut into 2-mm pieces, extracted with 4 ml methanol, filtered through glass wool, and the extracts stored at -20°C . Extracts were filtered through 0.45- μm Teflon syringe filters prior to HPLC.

Instrumentation and Software A Hewlett-Packard (Palo Alto, CA, USA) series 1050 HPLC, fitted with an Alltech (Deerfield, IL, USA) Alltima C18 column (5 μm , 250 \times 4.6 mm) and a Hewlett-Packard diode array detector was used. An isocratic mobile phase with a ternary solvent system (85%, 5%, and 10%; methanol, 5% acetic acid, water, respectively) was run at a flow rate of 1 ml/min. Data were analyzed with Agilent ChemStation (Santa Clara, CA, USA) for LC 3D software.

Results and Discussion

Method Development Abietanes have distinctive spectra that we used here, together with chromatographic separation, to distinguish and quantify the resin acids by HPLC. The spectra of the individual components (Fig. 1), chromatographically separated from a 1-mM mixture of standards, show λ_{\max} values of 240 nm (abietic), 250 nm (neoabietic), 266 nm (palustric), 272 nm (levopimaric), and 266 nm (dehydroabietic). This is in excellent agreement with spectra obtained with the standards run separately, indicating little or no

Fig. 2 Multiwavelength analysis. HPLC chromatographs obtained from a 1-mM mixture of abietanes (Fig. 1) are shown at wavelengths to optimize detection and differentiation between components. Dehydroabietic acid ($rt=15.5$ min) is well resolved from the other four abietanes and detected at 268 nm

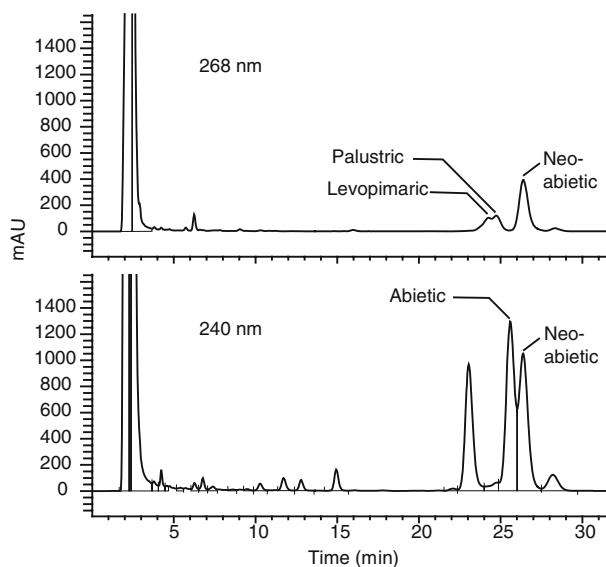


interference in the mixture. Neoabietic and abietic acids have the greatest response factors in the diagnostic wavelength region, followed by palustric, levopimaric, and dehydroabietic acids. These response factors are also in reasonable agreement with literature values of the relative molar absorption coefficients at the corresponding λ_{\max} values (see “Methods and Materials”).

Although detection of analytes at λ_{\max} provides the greatest sensitivity (absorbance units), it may not allow the determination of component concentrations if peaks are not chromatographically resolved. However, the overlay spectra of the abietanes (Fig. 1) indicated that it might be possible to distinguish the abietanes in a mixture and minimize interferences by careful choice of detection wavelengths. Also, detection at an isosbestic point provides summation of component concentrations at a wavelength of equal response. The purpose is to allow quantification in terms of absolute concentration, instead of arbitrary absorbance units, even in cases where chromatographic separation is not complete. Specifically, at 282 nm, palustric and levopimaric acid give equal response; at 240 nm, abietic and neoabietic acid give equal response; at 268 nm, neoabietic acid is detected without interference from abietic acid; and at 300 nm, levopimaric acid is preferentially detected over palustric acid.

Chromatograms of a 1-mM mixture at these four wavelengths are presented in Fig. 2 and provide a test to determine whether a multiwavelength approach can provide quantitative results. Detection at 268 nm allows baseline resolution for neoabietic acid ($rt=25.6$ m) without interference from other components, even its nearest neighbor abietic acid ($rt=24.8$ m), because it does not absorb at this wavelength. Both abietic and neoabietic acids are detected with essentially the same response at 240 nm with little interference from levopimaric and palustric acids. Similarly, palustric and levopimaric acid give the same response at 282 nm with no interference from neoabietic acid (chromatographically resolved) or abietic acid (no absorbance at 282 nm). Levopimaric and pimaric acids can be further distinguished at 300 nm, and concentrations determined without interference from the other abietanes. Dehydroabietic acid ($rt=15.5$ min) is well resolved from the other four abietanes and detected at 268 nm. For

Fig. 3 Abietanes in *P. glauca*. HPLC analysis of methanol extract (20 μ l injection) from *P. glauca* is shown with detection at 240 and 268 nm



convenience, response factors for the abietanes can be normalized to that of abietic acid at 240 nm by using standards determined individually: 240 nm: abietic (100%), neoabietic (102%), levopimaric (7.3%), palustric (19%); 268 nm: abietic (0%), neoabietic (40%), levopimaric (26%), palustric (40%); 282 nm: abietic (0%), neoabietic (1.1%), levopimaric (24%), palustric (22%); 300 nm: abietic (0%), neoabietic (0%), levopimaric (5.5%), palustric (1%).

Accordingly, the concentrations of abietanes in the 1-mM mixture were in close agreement with predicted levels (from simple dilution of stocks to prepare the mixture). For illustration, Fig. 2 chromatograms results are: abietic (1.08 mM at 240 nm), neoabietic (1.0 mM at 268 nm; 1.1 mM at 240 nm), levopimaric (1.08 mM at 282 nm; 1.02 mM at 300 nm), palustric (1.21 mM at 282 nm, 1.14 mM at 268 nm), and levopimaric–palustric sum (2.06 mM at 282 nm peak area). Peak heights are used for response factors to minimize the contribution of neighboring analytes, except in the case of levopimaric–palustric acid summation where peak areas can be used without interferences. This may allow in principle a more accurate determination of palustric by subtracting levopimaric acid concentration (1.02 mM at 300 nm) from levopimaric–palustric sum (2.06 mM at 282 nm).

The basis for the analytical technique described here relies on both the optimal combination of wavelengths for detection and the chromatographic resolution of the peaks. The degree of resolution we observe (Fig. 2) is similar to that reported previously with a Hypersil C8 column and a mobile phase of methanol–water–propanol (Rigol et al., 2003). However, the order of elution of the abietanes in their case is different (levopimaric–neoabietic < abietic < palustric). It is noteworthy that the coelution of levopimaric and neoabietic was problematic with detection by negative ionization/mass spectrometry because no fragmentation was observed (Rigol et al., 2003). The analytes of identical m/z 301 were not distinguished from each other, in contrast to the multiwavelength UV detection method presented here. We have included acetic acid in the mobile phase to stabilize pH conditions for the acid components, thus improving reproducibility and peak shape.

Detection of Abietanes in *P. glauca* The HPLC of methanol extract from *P. glauca* phloem is shown in Fig. 3. Spectral analyses of the analytes at peak apices correspond to standards using automated spectral library search. As illustrated before with standards, the following is observed: abietic (1.83 mM at 240 nm), neoabietic (1.39 mM at 268 nm and 1.44 mM at 240 nm), levopimaric (0.54 mM at 282 nm, 0.49 mM at 300 nm, and 0.56 mM at 268 nm), palustric (0.46 mM at 282 nm and 0.42 mM at 268 nm), and levopimaric–palustric sum (1.04 mM at 282 nm peak area). This corresponds to 4.0 mg abietic, 3.1 mg neoabietic, 1.2 mg levopimaric, and 0.95 mg palustric acids per gram of dry extracted phloem. The averages \pm standard deviations for 10 samples are 3.1 ± 1.6 mg abietic, 3.1 ± 2.2 mg neoabietic, 2.4 ± 2.6 mg levopimaric, and 2.2 ± 2.2 mg palustric acids per gram dry extracted phloem. The concentration of dehydroabietic acid was too low, or the interferences in the oleoresin too high, for reliable detection. A major peak at 23 min (observed at 240 nm) has a spectrum similar to that of abietic acid. This unknown was observed in all *P. glauca* samples tested.

In summary, the analytical method presented here for common abietanes has advantages particularly useful in ecological studies where large numbers of samples are required for statistical validation, and where it is important to retain the biological activities of the plant constituents. This is demonstrated with complete methanol extracts of conifer phloem with no prior fractionation. Therefore, all components are available from the chromatographic separation for subsequent chemical or biological characterizations. Samples are not derivatized (in contrast to typical GC methods), and therefore biological activities of fractionated components are retained. Components include not only the diterpenes but also monoterpenes, sesquiterpenes, and phenolics in oleoresin. The method is simple and rapid, employing robust reversed-phase HPLC. Detection at four wavelengths and spectral analysis provides confirmation and/or refinement for quantitative determination of abietic, neoabietic, levopimaric, and palustric acids.

Acknowledgements This work was supported in part by USDA-NRI WIS04746, NSF DEB-0314215, McIntire-Stennis, and the University of Wisconsin College of Agricultural & Life Sciences.

References

- BERENBAUM, M. 1985. Brentown revisited: Interactions among allelochemicals in plants. *Rec. Adv. Phytochem.* 19:139–169.
- BJÖRKMANN, C. and GREF, R. 1993. Survival of pine sawflies in cocoon stage in relation to resin acid content of larval food. *J. Chem. Ecol.* 19:2881–2890.
- FRANICH, R. A. and GADGIL, P. D. 1983. Fungistatic effects of *Pinus radiata* needle epicuticular fatty and resin acids on *Dothistroma pini*. *Physiol. Plant Pathol.* 23:183–195.
- GREF, R. 1987. Resin acids and resistance of *Pinus sylvestris* to *Melampsora piniotrqua*. *Eur. J. For. Pathol.* 17:227–230.
- GREF, R. and ERICSSON, A. 1985. Wound-induced changes of resin acid concentrations in living bark of Scots pine seedlings. *Can. J. For. Res.* 15:92–96.
- JOYE, N. M. and LAWRENCE, R. V. 1967. Resin acid composition of pine oleoresins. *J. Chem. Eng. Data* 12:279–282.
- KOPPER, B. J., ILLMAN, B. L., KERSTEN, P. J., KLEPZIG, K. D., and RAFFA, K. F. 2005. Effects of diterpene acids on components of a conifer bark beetle–fungal interaction: Tolerance by *Ips pini* and sensitivity by its associate *Ophiostoma ips*. *Environ. Entomol.* 34:486–493.
- LARSSON, S., BJÖRKMANN, C., and GREF, R. 1986. Responses of *Neodiprion sertifer* (Hym., Diprionidae) larvae to variation in needle resin acid concentration in Scots pine. *Oecologia* 70:77–84.

- LATORRE, A., RIGOL, A., LACORTE, S., and BARCELO, D. 2003. Comparison of gas chromatography-mass spectrometry and liquid chromatography-mass spectrometry for the determination of fatty and resin acids in paper mill process waters. *J. Chromatogr. A* 991:205–215.
- LEE, B. L., ONG, H. Y., KOH, D., and ONG, C. N. 1994. High-performance liquid chromatographic method for determination of dehydroabietic and abietic acids, the skin sensitizers in bindi adhesive. *J. Chromatogr. A* 685:263–269.
- LEE, B. L., KOH, D., ONG, H. Y., and ONG, C. N. 1997. High-performance liquid chromatographic determination of dehydroabietic and abietic acids in traditional Chinese medications. *J. Chromatogr. A* 763:221–226.
- MCMARTIN, D. W., PERU, K. M., HEADLEY, J. V., WINKLER, M., and GILLIES J. A. 2002. Evaluation of liquid chromatography-negative ion electrospray mass spectrometry for the determination of selected resin acids in river water. *J. Chromatogr. A* 952:289–293.
- RIGOL, A., LATORRE, A., LACORTE, S., and BARCELO, D. 2003. Direct determination of resin and fatty acids in process waters of paper industries by liquid chromatography/mass spectrometry. *J. Mass Spectrom.* 38:417–426.
- TOMLIN, E. S., ANTONEJEVIC, E., ALFARO, R. I., and BORDEN, J. H. 2000. Changes in volatile terpene and diterpene resin acid composition of resistant and susceptible white spruce leaders exposed to simulated white pine weevil damage. *Tree Physiol.* 20:1087–1095.
- VOLKMAN, J. K., HOLDSWORTH, D. G., and RICHARDSON, D. E. 1993. Determination of resin acids by gas chromatography and high-performance liquid chromatography in paper mill effluent, river waters and sediments from the upper Derwent Estuary, Tasmania. *J. Chromatogr.* 643:209–219.
- WAGNER, M. R., BENJAMIN, D. M., CLANCY, K. M., and SCHUH, B. A. 1983. Influence of diterpene resin acids on feeding and growth of larch sawfly, *Pristiphora erichsonii* (Hartig). *J. Chem. Ecol.* 9:119–127.
- ZINKEL, D. F. and ENGLER, C. C. 1977. Gas-liquid chromatography of resin acid esters. *J. Chromatogr.* 136:245–252.