Resin Acid in Tree Moss Extract

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Lichens are symbiotic organisms of fungi and algae. The biological significance of lichens and their metabolites has been reviewed by Huneck, who even stated that "good perfumes require lichen extracts."[1] Indeed, as of 1997, about 3,000 tons of lichen were processed—mainly in the Grasse, France area—for the manufacture of a wide variety of extracts, referred to as resinoids, and derived products (*e.g.* colorless/codistillation products, etc).

For a long time, there has been a misunderstanding on the true nature of the lichen extracts currently used in perfume compounding. Until recently, whatever the lichen species, such extracts were frequently called "oak moss", concretes or resinoids. It is now well established that lichen growing on oak trees is specifically *Evernia prunastri*, whereas lichen growing on trees other than oak trees are called "tree moss", which is predominantly *Pseudevernia furfuracea* (synonym: *Parmelia furfuracea*). Sometimes, the latter happens to be mixed with other minor species, such as *Usnea*.

Unfortunately, misidentification of the industrial extracts has not been totally eradicated. Indeed, while an RIFM monograph (#340) rightly mentions that oak moss resinoids are manufactured from *Evernia prunastri*, another one (#562) wrongly cites *Usnea* species as the sole raw material for manufacturing tree moss concretes.

In early 2000, PRODAROM informed RIFM that current "tree moss" resinoids are manufactured from lichen growing predominantly on conifers in Europe—mainly *Pinus* species, and more precisely *Pinus sylvestris*. Due to the way in which the raw material is manually collected, lichen is unavoidably contaminated with elements of pine tree twigs, bark, and needles. In weight, however, this contamination appears to be quite low. Because the lichen often adheres intimately to the wood part, it would be virtually impossible to collect pure lichen, free of any exogenous material, for both practical and economical reasons

It is important to note that although this feature has been previously mentioned by Tabacchi et al., it seems that it has been constantly overlooked by many users and toxicologists.[2] The realization that the manufacture of oak moss resinoid may involve the use of mixed raw materials—a contamination either fortuitous or intentional—has recently resulted in a reappraisal of previous evidence of alleged sensitizing properties of oak moss resinoid.[3-5] As a consequence, taking into account information from RIFM, IFRA recently issued a new standard for tree moss extracts.[a]

It has been established that pine tree resin (colophony) is responsible for dermal sensitisation. Resin acids are the main constituents of colophony. Although these diterpenoids are not direct sensitizers, some are transformed into very potent sensitizers upon oxidation, as in the cases of 15-hydroperoxydehydroabietic acid and 7-oxodehydroabietic acid.[6,7] The latter, a minor component among resin acids, has recently been shown to be specifically involved in the haptenation with lysine.[8]

A likely biogenetic precursor of these sensitizers is dehydroabietic acid (DHA), which represents approximately 50 ± 10 percent of the total resin acids present in tree moss extracts. Therefore, a selective analytical monitoring of this compound appears to be highly desirable.

The chemical composition of tree moss (*Pseudevernia spp.*) has been thoroughly investigated.[9-11] However, to our knowledge, no data, either qualitative or quantitative, can be found in the literature concerning constituents coming from any exogenous contamination. Whereas methods using HPLC to analyze resin acids have recently been recently proposed, there is no report on the specific analysis of these substances in lichen extracts.[12,13] Hereafter is a description of an efficient chromatographic method that features a high degree of selectivity for DHA, together with an excellent sensitivity within the relevant concentration range.

Method: HPLC determination of dehydroabietic acid in moss extracts.

Principle: Dehydroabietic acid (DHA) is quantitatively determined using the external standard method by reverse phase HPLC and spectrofluorimetric detection.

Reagents and chemicals: DHA from Helix Biotech (Richmond, Canada)—99 percent purity; water (distilled and deionized); acetic acid; acetonitrile (HPLC grade); ethanol or methanol (99 percent purity).

[a]IFRA Information Letter No 633, issued 03/19/2001: "…tree moss extracts shall not contain more than 0.8% of dehydroabietic acid (DHA)."

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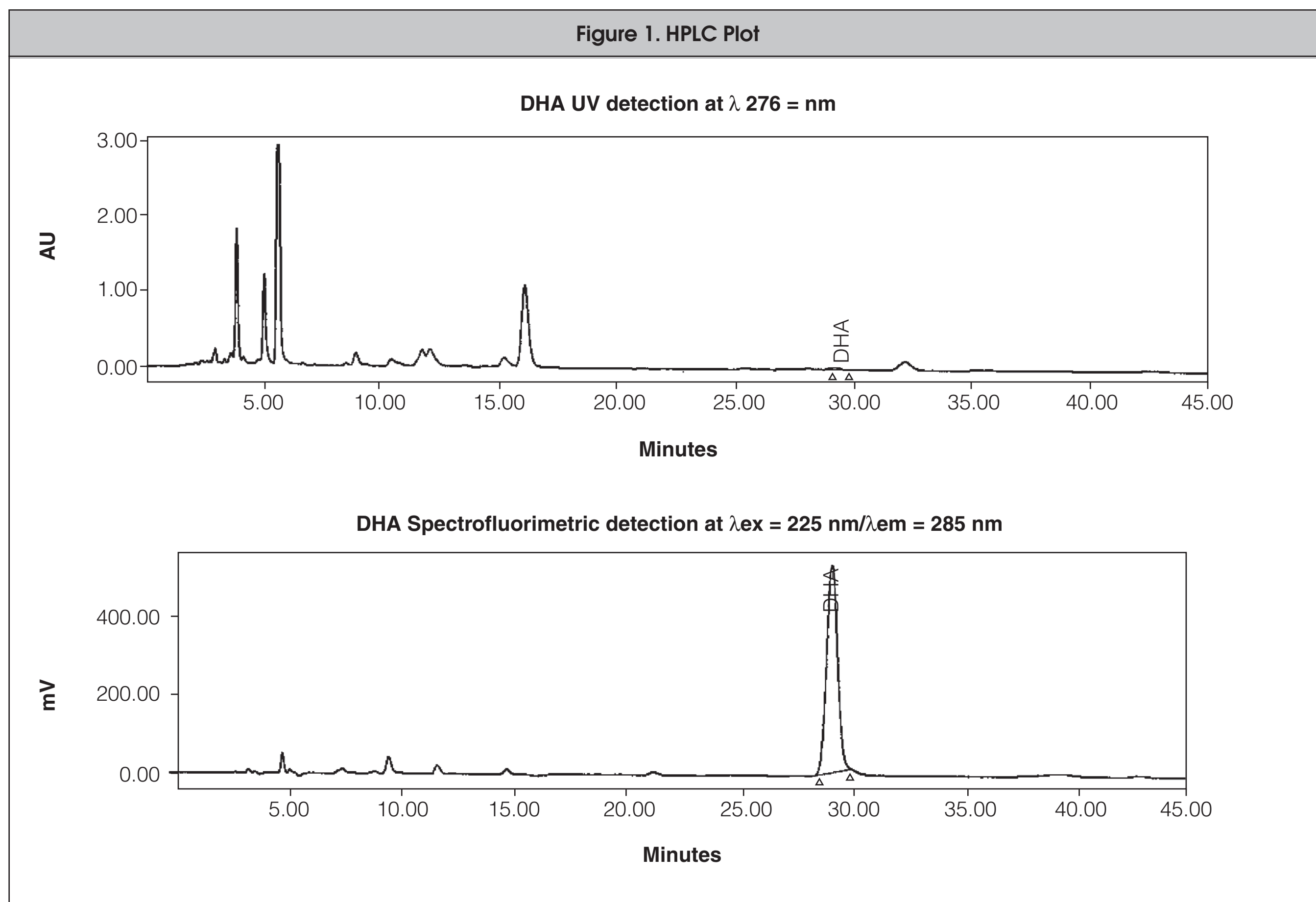
Figure 1. HPLC Plot



Operation conditions: Suitable HPLC system; column: C18 Spherisorb 5 ODS (Chrompack), 250 mm x 4.6 mm (remark: pre-column recommended); solvents: (a) water/acetic acid (98/2), (b) acetonitrile; injected volume: 5 µl; isocratic elution: (a) 50 percent/(b) 50 percent, flow rate: 1.2 ml/min; spectrofluorimetric detection: lex=225 nm/lem=285 nm; approximate retention time for DHA: ~35 min (this retention time can be optimized to 20~25 min by isocratic elution with (a) 38 percent/(b) 62 percent).

Sample Preparation

Standard solutions: External standard solutions are freshly prepared by dissolving 0.015 g of DHA in a 20 ml volumetric flask adjusted with ethanol or methanol. Solution S1 (#750 ppm) is diluted 10 times to produce solution S2 (#75 ppm). Then, solution S2 is diluted two times to produce solution SZ (#37,5 ppm), and solution S3 is diluted 10 times to give solution S4 (#7,5 ppm). The S2, S3, S4 diluted solutions can be made in the elution solvents. 5 ml of each solution (S2, S3, S4) are injected in the HPLC apparatus. Plot the calibration curve: area (DHA)=f (ppm concentration [S2], [S3], [S4]).

Sample preparation—tree moss extract: The sample of tree moss extract is gently heated, mixed and homogenized at 80°C. Solutions of 0.25-0.5 percent of tree moss extract are prepared (for example, weight exactly between 0.5g and 1g of sample in a 20 ml volumetric flask adjusted with ethanol or methanol, then dilute 10 times with the elution solvents in an adequate flask). For tree moss extracts with high DHA content, make an adequate final dilution to be in the standard calibration range. 5 ml of the diluted solution are injected in the HPLC system.

Sample preparation—oak moss extract: The sample of oak moss extract is gently heated, mixed and homogenized at 80°C. Solution of 10-15 percent of oak moss extract is prepared (for example, weigh exactly between 2 g and 3 g of sample in a 20 ml flask adjusted with ethanol or methanol). 5 ml of the diluted solution is injected in the HPLC apparatus.

Table 1. Preliminary round robin tests performed with two representative commercial samples of tree moss and oak moss resinoids

	Tree Moss 1 (%)	Tree Moss 2 (%)	Oak moss 1 (ppm)	Oak Moss 2 (ppm)
Lab. No 1	5.4	5.2	89	45
Lab. No 2	5.7	5.1	83	27
Lab. No 3	5.7	5.2	90	31

Results

Quantitative determinations are made according to the external standard method. The peak area of dehydroabietic acid in the sample is reported on the calibration curve taking into account the dilution factor (Figure 1). Results are an average of minimum two determinations with no more 5 percent difference on DHA percent dosage. The DHA detection threshold in the mosses extracts is below 100 ppm Results (DHA) are shown in Table 1.

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