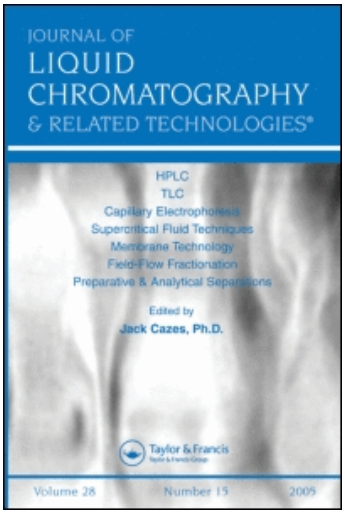


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# HPLC Determination and MS Identification of Dehydroabietic Acid and Abietic Acid in Propolis

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**Abstract:** In the present study, the HPLC method on a $C_{18}$ column with on-line spectrophotometric and fluorimetric detection was used for separation and determination of dehydroabietic acid and abietic acid in propolis. The samples of propolis tincture were prepared prior to the HPLC analysis. The mobile phase for isocratic elution was methanol-water 87:13 containing 0.05% formic acid. Abietic acid was detected with spectrophotometric detection at 238 nm, and dehydroabietic acid was detected with fluorimetric detection (excitation 225 nm, emission 285 nm). The limits of determination (signal/noise ratio 10) were 100 ng/mL for dehydroabietic acid and 200 ng/mL abietic acid. The calibration graphs were linear over a wide interval from the limit of determination to 1 mg/mL. Analytical recovery and reproducibility exceeded more than 89%. The developed method was used for analysis of propolis from Slovakia. Mass spectrometry was used for identification of the studied acids. The results demonstrated that dehydroabietic acid was present in all tested samples of propolis. Its content was different (3.7 μg/g–44.7 μg/g of propolis) depending on the source of propolis.

**Keywords:** Propolis, HPLC, MS, Resin acids

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## INTRODUCTION

Propolis, a natural substance collected by honeybees from buds and exudates of certain trees and plants, is thought to be used in the beehive as a protective barrier against their enemies. Propolis typically consists of waxes, resins, water, inorganics, phenolics, and essential oils, the exact composition of which is dependent upon the source plants.[1,2] Propolis balsam (tincture) is an ethanolic extract of raw (natural) propolis containing the bulk of the organic constituents. This organic fraction has been used in folk medicines in many regions of the world, and has been shown to have various biological activities such as antibacterial, antiviral, anti-inflammatory, anticancer properties.[3] Diterpene resin acids are major constituents of rosin and occur naturally in many softwood species, such as spruce and pine. Rosin is a complex mixture of many compounds. It contains about 90% resin acids and 10% neutral mater. Resin acids found in rosin can be divided into two main types: the abietic-types (abietic, neoabietic, levopimaric, palustric, and dehydroabietic acids) with conjugated double bonds, and the pimaric types (isopimaric and pimaric acids) with non-conjugated double bonds. Of these, the abietic-type acids are easily oxidized when exposed to air and light, and have been reported to isomerise to abietic acid either thermally or by treatment with dilute mineral acids. Dehydroabietic acid and abietic acid are the major acids of the abietic-type found in different types of rosin.[4,5]

The analysis of resin acids is usually carried out by gas chromatography coupled (GC) to flame-ionization (FID), electron-capture (ECD) or mass spectrometry (MS) detection after converting to their methyl esters.[6,7] Other derivatives of resin acids are the pentafluorobenzyl esters, which significantly improved sensitivity and selectivity by ECD and MS versus FID.[8,9] Luong et al.[10,11] presented the capillary electrophoresis method with laser induced fluorescence detection (LIF) for determination of resin acids as their metoxycoumarin esters. The modification of the capillary was performed with a mixture of negatively charged sulfobutylether-$\beta$-cyclodextrin and neutral methyl-$\beta$-cyclodextrin. The HPLC technique was chosen in preference to GC for several reasons, including: HPLC separation is carried out at room temperature, thus avoiding isomerisation of resin acids and no metylation step is needed. The two barriers to the determination of resin acids using HPLC have been the difficulty in separating the various resin acids isomers with a RP system because all the resin acids have similar analyte-column hydrophobic interactions, and there is a lack of a suitable chromophore for the detection, particularly for the non-conjugated resin acids. The metoxycoumarin esters could be detected by UV absorption, while acetoxycoumarin esters were detected by fluorescence spectrophotometry, after post-column alkaline hydrolysis.[6,11,12] Sadhra et al.[13] developed a gradient elution RP-HPLC method with UV detection for the analysis of unmodified, predominantly abietic-type resin acid. A selective and sensitive method for simultaneous determination of dehydroabietic and abietic acids with fluorimetric

and UV detection was presented by Lee et al.[14] McMartin et al.[15] developed a method for analysis of the four resin acids based on LC-electrospray (ESI)-MS. A $C_8$ column was used in this case, and the best separation was obtained by the isocratic mode using 10 mmol/L ammonium acetate in water-acetonitrile as mobile phase. Comparable results were obtained by the LC-atmospheric pressurized chemical ionization (APCI)-MS gradient elution method, published by Rigol et al.[16] using $C_{18}$ column.

In the present study, the isocratic method was used for separation and determination of abietic acid and dehydroabietic acid in propolis samples from Slovakia. Mass spectrometry was used for the identification of studied acids.

## EXPERIMENTAL

### Chemicals



The standards of abietic acid and dehydroabietic acid were purchased in pure grade from ICN Biochemicals, and purified by passing it through a silica gel column (70–230 mesh) with benzene as a mobile phase. TLC on silica gel plates (Merck) with chloroform-methanol (20:1 v/v) mobile phase was used for checking the purity. Abietic and dehydroabieric acid were detected at 254 nm.

Acetonitrile, chloroform, and methanol, were HPLC grade obtained from Merck. Benzene and formic acid were analytical grade obtained from Lachema.

The work was carried out on 4 samples of propolis tincture obtained from propolis collected in East Slovakia. (Preparation of propolis tincture: 150 g of propolis was extracted with 500 mL of pure ethanol for 3 days. The resulting solution was centrifuged at 1000 g for 10 min). The samples of propolis tincture (A, B, C, D) were harvested in the years 2000–2004, and stored in darkness at room temperature until the analysis.

### Instruments

The HPLC system used consisted of a Knauer Model 64 isocratic pump, a 7125 Rheodyne injector with a 20 μL injection loop, a thermostat Model LCT 5100, a Knauer variable wavelength detector (set at 238 nm, for the detection of abietic acid), Shimadzu Model RF-551 fluorescence detector (the excitation and emmision wavelengths were set at 225 nm and 285 nm, for the detection of dehydroabietic acid), and a CSW32 software for peak identification and integration. The analytical columns, Separon SGX $C_{18}$ (125 × 3.9 mm I.D., 5 μm) and Lichrosorb RP-8 (250 × 4 mm I.D., 7 μm), were tested for the separations of the studied acids. The mobile phase consisted of methanol or acetonitrile and water (methanol-water from 75:25

to 90:10 v/v and acetonitrile-water from 60:40 to 90:10 v/v) to which 0.05% of formic acid was added. The column temperature was 30°C and the flow rate of the mobile phase was 0.5 mL/min.

Mass spectrometric analysis was conducted using a MS902S mass spectrometer. MS conditions were as follows: emission 100 μA, electron energy 70 eV, temperature of ionisation chamber 160°C (for dehydroabietic acid) and 110°C (for abietic acid).

### Preparation of Propolis Tincture Samples for HPLC Analysis

The sample of propolis tincture was prepared prior to HPLC analysis as follows: Propolis tincture (2 mL) or spiked propolis tincture was dried with a stream of compressed air at room temperature. Then 0.5 mL of methanol was added and the mixture was centrifuged at 3000 g for 2 min. The solution was injected for HPLC analysis either directly or diluted with methanol, depending on the content of analytes.

The identification of dehydroabietic acid and abietic acid was made by mass spectrometry of fractions of abietic acid and dehydroabietic acid obtained after preparative HPLC analysis (about 150 times injection of prepared sample of propolis tincture or prepared propolis tincture spiked with 100 μg/mL of the studied acids). The fractions of acids under study, after preconcentration under the stream of compressed air (resulting concentrations were 1 mg/mL), were injected into the MS equipment.

## RESULTS AND DISCUSSION

Dehydroabietic acid and abietic acid (Figure 1) are good dissolved in methanol and acetonitrile, and are very poor in water, and for this reason

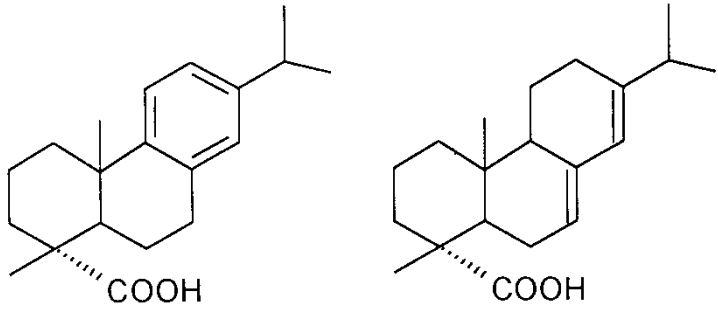


***Figure 1.*** Chemical structures of dehydroabietic acid and abietic acid.

the reversed-phase system was used for HPLC analysis. Several mobile (methanol-water from 75:25 to 90:10 v/v and acetonitrile-water from 60:40 to 90:10 v/v) and stationary phase (Separon SGX $C_{18}$, Lichrosorb RP-8) conditions were evaluated to obtain suitable conditions and analysis time for separation of dehydroabietic acid, abietic acid, and other unknown compounds in propolis. The best results evaluated on the base symmetry of peak and the number of theoretical plates, were obtained on a Separon SGX $C_{18}$. It was observed that the analytes were retained longer in a reversed-phase $C_{18}$ column with methanol than with the same concentration of acetonitrile (Figure 2). Chromatographic performance of dehydroabietic acid and abietic acid was poor if eluted with mobile phase containing less than 70% (v/v) acetonitrile or 80% (v/v) methanol. The addition of formic acid in mobile phase improved the chromatographic efficiency.

The suitable mobile phase for isocratic elution of dehydroabietic acid and abietic acid was methanol-water 87:13 containing 0.05% formic acid. The flow rate was set at 0.5 mL/min. Abietic acid was detected at 14.6 min with spectrophotometric detection at 238 nm and dehydroabietic acid was detected with fluorimetric detection (excitation 225 nm, emission 285 nm) at 9.5 min. The identification of acids in standard solution was based on their chromatographic and fluorimetric properties, and was confirmed by mass



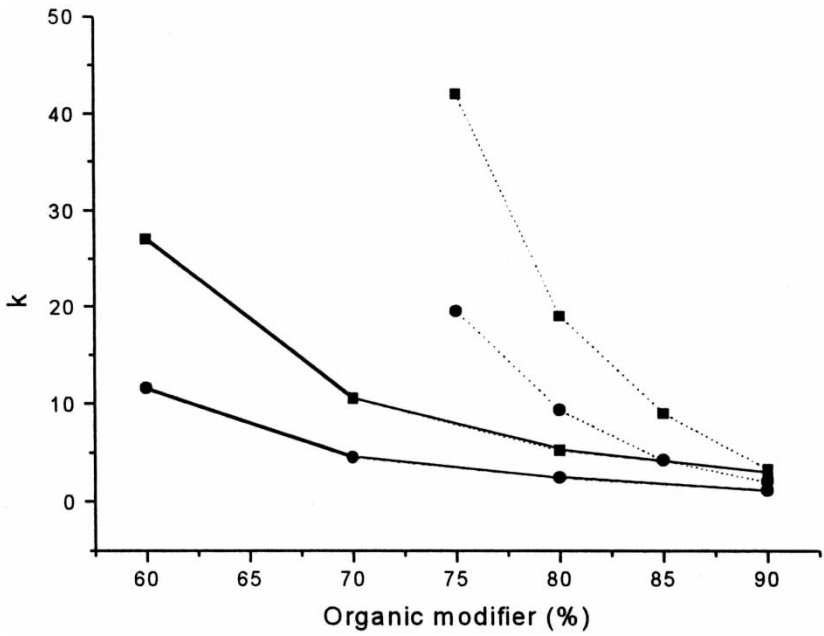


***Figure 2.*** Reversed-phase retention of abietic acid (■) and dehydroabietic acid (●) as a function of mobile phase composition on $C_{18}$ column. Mobile phase: methanol-water (straight line), acetonitrile-water (dot line) containing 0,05% formic acid.

spectra of acid fractions where the characteristics molecular peaks were observed (at m/z 302 for dehydroabietic acid and m/z 300 for abietic acid). The obtained mass spectra were comparable with published spectra of individual resin acids.[13]

Calibration curves were constructed by performing a regression linear analysis of the peak area versus the analytes concentration. Based on a five-point calibration, a linear response was observed from the limit of determination to 1000 μg/mL of studied resin acids:

| | | |
|---|---|---|
| dehydroabietic acid | $y = 13.554 + 13206.713x$ | $r = 0.998$ |
| abietic acid | $y = 15.624 + 3744.108x$ | $r = 0.999$ |

where $x$ was the concentration of dehydroabietic acid or abietic acid and $y$ was the peak area.

The limits of detection, defined as the lowest sample concentration which can be detected, (signal-to-noise ratio of 3:1) were 50 ng/mL for dehydroabietic acid and 100 ng/mL for abietic acid. The limits of determination, defined as the lowest sample concentration, which can be quantitatively determined with suitable precision and accuracy (signal-to-noise ratio of 10:1) were 100 ng/mL for dehydroabietic acid and 200 ng/mL abietic acid.

The intra- and inter-day precisions were determined at two different concentrations of 0.1 and 1 mg/mL for the studied acids. The intra-assay relative standard deviations for every studied concentration were less than 4.1% for both analytes. The inter-assay precision was studied using the spiked samples of propolis tincture that were analysed at least five times within a five-day period. The results show that the concentration values are reproducible with an average inter-assay RSD at the studied concentrations of less than 6.1%. Intra-and inter-day accuracy and precision data are shown in Table 1.

The recoveries of dehydroabietic acid and abietic acid were assessed by comparing the peak areas of prepared samples of propolis tincture or ethanol containing a known amount of studied acids (concentration of 5 and 500 μg/mL of studied acids), with the peak areas obtained from direct

***Table 1.*** Intra- and inter-assay accuracy and precision of the method for determining dehydroabietic and abietic acid in propolis tincture (n = 5)

| Concentration (mg/mL) | Mean (mg/mL) | | RSD (%) | |
|---|---|---|---|---|
| | Intra-assay | Inter-assay | Intra-assay | Inter-assay |
| Dehydroabietic acid | | | | |
| 0.1 | 0.119 | 0.117 | 3.3 | 5.4 |
| 1.0 | 1.057 | 1.069 | 2.7 | 5.7 |
| Abietic acid | | | | |
| 0.1 | 0.121 | 0.119 | 3.9 | 5.8 |
| 1.0 | 0.976 | 0.984 | 4.1 | 6.1 |

***Table 2.*** Concentration of dehydroabietic acid in some Slovak propolis samples determined by HPLC method (n = 3)

| Sample (year) | Concentration (μg/g) |
|---|---|
| Propolis A (2000) | 3.7 ± 1.3 |
| Propolis B (2003) | 9.1 ± 1.3 |
| Propolis C (2004) | 44.7 ± 2.2 |
| Propolis D (2004) | 26.2 ± 1.5 |

injections of a standard solution containing the same concentration of studied compounds. The average recoveries for dehydroabietic acid and abietic acid were more than 95 ± 3% for spiked ethanol and 89 ± 5% for a spiked sample of propolis tincture.

Propolis or "bee glue" is a generic name for the resinous hive product collected by bees from various plant sources. Propolis usually contains a



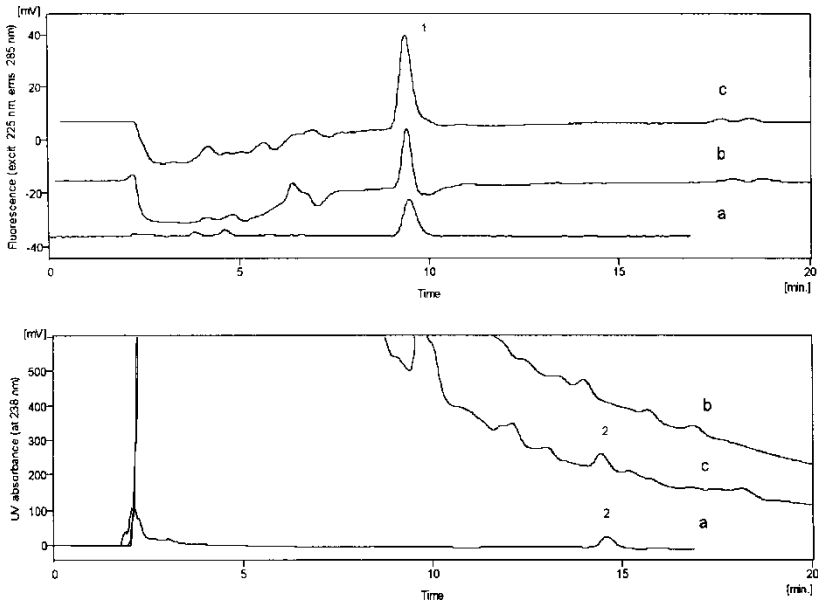


***Figure 3.*** Chromatograms of (a) a standard (50 μg/mL of dehydroabietic acid and abietic acid), (b) a propolis tincture B, and (c) spiked sample of propolis tincture B (spiked with 15 μg/mL of dehydroabietic acid and abietic acid) analysed by isocratic method with on-line spectrophotometric and fluorimetric detection. Legends: (1) dehydroabietic acid, (2) abietic acid. Conditions: stationary phase, Separon SGX $C_{18}$; mobile phase, methanol-water 87:13 containing 0.05% formic acid; other conditions see experimental.

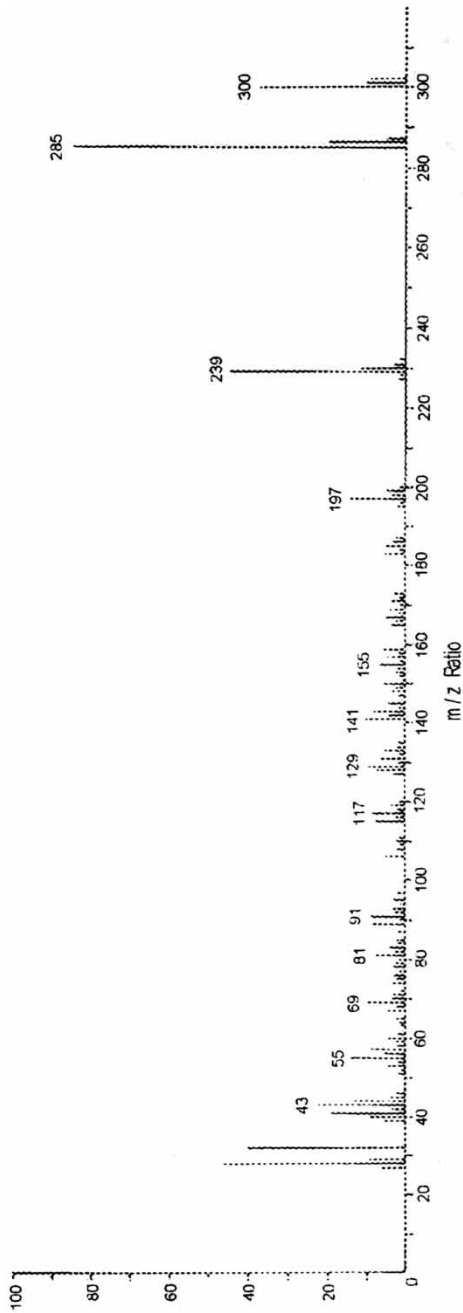


*Figure 4.* Mass spectrum of dehydroabietic acid fraction.

variety of chemical compounds, such as polyphenols (flavonoids, phenolic acids, and their esters), terpenoids, steroids, aromatic alcohols, aliphatic acids and esters, sugars, amino acids,[1–3,17–20] and its composition depends on the vegetation at the site of collection. Diterpene resin acids are constituents of rosin and they occur naturally in many softwood species. Dehydroabietic acid and abietic acid are the major acids of the abietic-type found in different types of resin, or they are final products of isomerization of resin acids with conjugated double bonds.[4,5] The four samples of propolis from Slovakia were analysed by isocratic HPLC with on-line spectrophotometric and fluorimetric detection. The chromatograms of the samples indicate the presence of dehydroabietic acid at concentration level 3.7–44.7 μg/g of propolis (Table 2). Chromatograms of a standard (50 μg/mL of dehydroabietic acid and abietic acid), a propolis tincture, and a spiked sample of propolis tincture (spiked with 15 μg/mL of dehydroabietic acid and abietic acid) are shown in Figure 3. Abietic acid concentration in all tested propolis samples was below the detection limit of this method. The comparison of retentions factors of standard solution peaks with propolis peaks and off-line MS were used for identification of dehydroabietic acid in the fraction of propolis tincture. In the mass spectrum of the dehydroabietic acid fraction (Figure 4) the characteristic molecular ion peak occurred at $m/z$ 300, which can be used for identification of dehydroabietic acid in propolis samples.



## CONCLUSION

The simple, sensitive, and rapid RP-HPLC method for the determination of the dehydroabietic acid and abietic acid in propolis was carried out. The presence of dehydroabietic acid in propolis was demonstrated. The content of dehydroabietic acid in propolis was different (from 3.7 μg/g to 44.7 μg/g of propolis) depending on the source of propolis and on the vegetation at the site of collection.

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