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CHARACTERIZATION OF OAKMOSS PRODUCTS USED IN PERFUMERY BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

An high-performance liquid chromatographic (HPLC) method was developed to identify and to quantify characteristic substances in commercially available oakmoss products. The procedure offers a rapid and reliable method for routine process and/or quality control. The identity of the registered peaks was confirmed using HPLC coupled on-line with ultraviolet–visible spectroscopy as well as by spectral analysis (^{1}H and ^{13}C nuclear magnetic resonance and mass spectrometry) of the isolated substances. Whereas a freshly prepared laboratory extract of *Evernia prunastri* contains mainly evernic acid, heating at 118°C results in decomposition products such as evernyl, orsellic acid, everninic acid and other phenol derivatives. The results indicate that the often used gas chromatographic method is not readily applicable to the study of lichen compounds, because these are not sufficiently volatile or too unstable at elevated temperatures.

INTRODUCTION

Oakmoss [*Evernia prunastri* (L.) *Ach.*] is a lichen belonging to the family *Usneaceae* which grows primarily on oak trees. It is collected particularly in Yugoslavia, France, Morocco and Algeria. Solvent extracts of oakmoss are extensively used in perfumery. Extracts and absolues are also prepared from the so-called "tree-moss" furnished by the two related lichens *Evernia furfuracea* and *Usnea barbata* which grow predominantly on conifer bark.

Whereas the volatile part of the moss products is well analyzed by gas chromatography–mass spectrometric (GC–MS) methods[1–4], little information exists concerning the amount of high boiling constituents which cannot be vaporized without decomposition.

While odour quality in most cases is placed above other considerations in the processing of natural raw materials for perfumes, there should be other "objective" criteria in order to guarantee the reproducibility of the industrial processing, the use of the right botanical species and the absence of solvents or other natural or synthetic perfume materials. Existing GC methods to characterize oakmoss products have limited applicability for quality control purposes. Therefore, a new high-performance

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liquid chromatography (HPLC) method is proposed for the rapid quantitative and non-destructive characterization of the most relevant oakmoss constituents.

EXPERIMENTAL

Materials

Usnic acid and atranorin were obtained from C. Roth (Karlsruhe, F.R.G.), evernic acid and orcinol from Sigma (Deisenhofen, F.R.G.) and evernyl from Roure Bertrand Dupont (France). Methyl and ethyl evernate were prepared by esterification of evernic acid. 3-Methoxy-5-hydroxy- and 3,5-dimethoxytoluene were synthesized by reaction of orcinol with dimethyl sulphate.

The identity of the synthesized reference substances was confirmed by the individual GC–MS as well as the ^{1}H and ^{13}C NMR data.

Acetonitrile and water used for the mobile phase were of HPLC grade (Merck, Darmstadt, F.R.G.).

Extracts from *Evernia prunastri* were obtained by refluxing the commercially available plant material (Dalmacijabilje, Yugoslavia) for 2 h in a soxhlet apparatus with methanol. After evaporation to dryness, the residue was reconstituted in a few millilitres of the solvent, filtered through a 0.8-μm Millipore filter and applied to HPLC without further treatment. HPLC determination of the industrial extraction batches could be performed directly after dilution and removal of insoluble matter by filtration.

Chromatographic conditions

The HPLC system consisted of an Hewlett-Packard HP 1090 liquid chromatograph with a DR 5 solvent delivery system, variable-volume auto-injector, auto-sampler, thermostatically controlled column compartment and an HP 1040 A diode-array detector which measures absorbance at all wavelengths in the range from 190 to 600 nm simultaneously. The mobile phase was phosphoric acid, adjusted to pH 2.8 with distilled water (A) and acetonitrile (B). The gradient of these two solutions was controlled by the following time programme: 0.0 min, 80.0% A; 0.1–5.0 min, 80.0–70.0% A (linear gradient); 5.1–8.0 min, 70.0% A; 8.1–16.0 min, 70.0–5.0% A (linear gradient). The mobile phase was sparged with helium prior and throughout the analysis to prevent bubble formation. The flow-rate was 0.5 ml/min. For analytical separations an Hypersil ODS 5-μm microbore column, 100 mm × 2.1 mm I.D., obtained from Hewlett-Packard, was used.

RESULTS AND DISCUSSION

A typical chromatogram obtained from commercially available extracts of oakmoss is illustrated in Fig. 1. Using a microbore reversed-phase column in combination with a binary gradient solvent system of dipotassium hydrogenphosphate–phosphoric acid solution and acetonitrile, a very good resolution of most relevant peaks can be achieved within 15 min.

Table I lists the retention times and absorption maxima of all compounds recognized in the lichen extracts. The identity of the registered peaks was confirmed by comparing both the observed retention times and the UV–VIS spectra of the individual peaks with the individual data of the standard compounds.

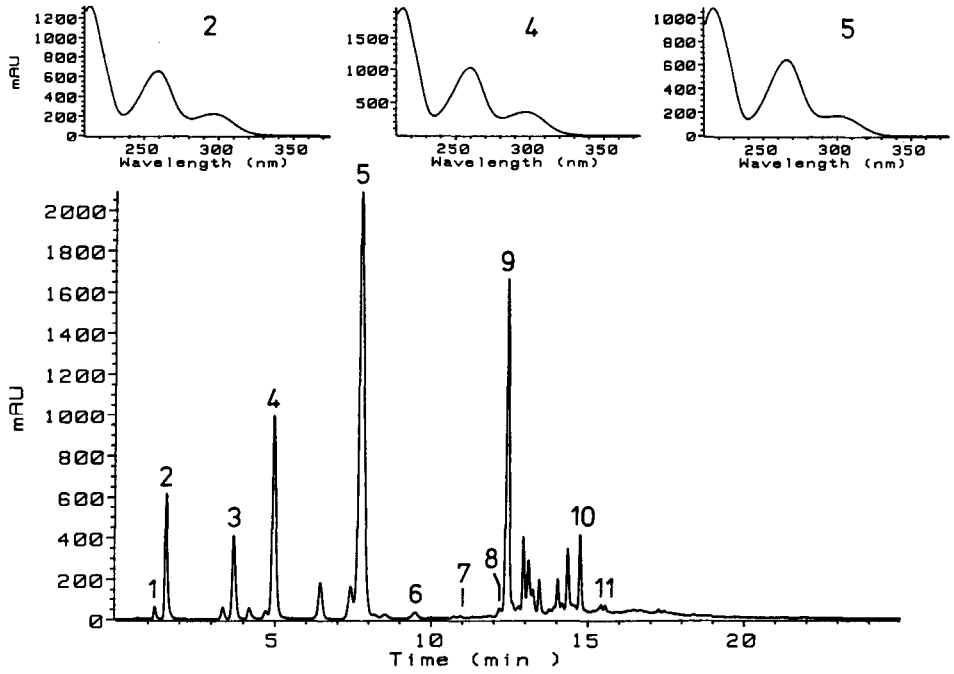

Fig. 1. Reversed-phase HPLC of a commercially available oakmoss extract, detected at λ = 260 nm. UV–VIS spectra presented for peak numbers 2, 4 and 5. Peak identification according to Table I.

TABLE I

ABSORPTION MAXIMA AND RETENTION TIMES OF THE COMPOUNDS DETECTED

Peak No.	*Compound*	*Absorption maxima (nm)*	*Retention time (min)*
1	Orcinol (3-Hydroxy-5-methylphenol)	272	1.1
2	Orsellic acid (2,4-Dihydroxy-6-methylbenzoic acid)	266, 300	1.6
3	Orcinol monomethyl ether (3-Methoxy-5-hydroxytoluene)	272	3.4
4	Everninic acid (2-Hydroxy-4-methoxy-6-methylbenzoic acid)	266, 300	5.0
5	Evernyl (Methyl 2,4-dihydroxy-3,6-dimethylbenzoate)	266, 300	7.7
6	3,5-Dimethoxytoluene	272	9.6
7	Methyl evernate	258, 300	11.0
8	Ethyl evernate	260, 298	12.1
9	Evernic acid	266, 302	12.5
10	Usnic acid	232, 280	14.3
11	Atranorin	248	15.5

In order to identify the compounds corresponding to peaks 2 and 4, it was necessary to isolate the two substances from the extract, because no standards existed. By freezing out the oakmoss extract from chloroform, a crude mixture of both substances was obtained. This fraction was thorougly washed with cold chloroform, dried and subsequently separated by preparative HPLC (C_{18} column, gradient elution with methanol–water). Based on spectral analysis (^1H and ^{13}C NMR, MS) the two pure substances obtained were identified as orsellic acid and everninic acid, respectively[5,6].

Evernyl, which is regarded as having the most important contribution to the typical odour of oakmoss extract, was found to be the main ingredient beside evernic acid in commercially available products (content almost >15%). Due to the similarities in chemical structure, the UV–VIS spectra of orsellic acid, everninic acid and evernyl (Fig. 1) are very similar. Ethyl and methyl evernate were detected only in very small amounts. According to earlier GC studies[4], the presence of orcinol, orcinol monoethyl ether and orcinol dimethyl ether was confirmed. Usnic acid, which to our knowledge has not been found in oakmoss extracts before, is eluted as a well resolved, narrow peak at a retention time of about 15 min. Although it was not possible to get

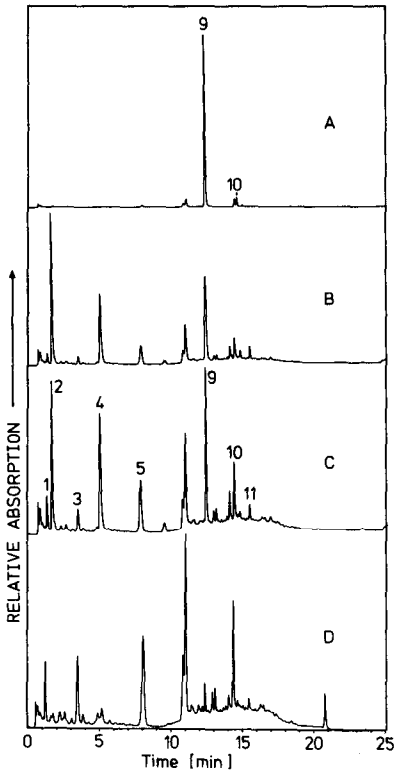

Fig. 2. Chromatograms of a laboratory prepared methanolic extract of *Evernia prunastri* (A) and of the decomposition compounds obtained during a tempering process at 118°C after 1 (B), 1.5 (C) and 3 h (D). Peak identification according to Table I.

a UV–VIS spectrum of the two very small peaks at about 15.5 min, one of them was characterized as the depsid atranorin by its retention time. Furthermore, the assignment is confirmed by the results of earlier studies[7].

Strack *et al.*[7] first performed HPLC separations of lichen substances and found that the main part of a methanolic extract from *Evernia prunastri* consists of evernic acid. Atranorin and chloratranorin were detected only in very small amounts. The existing discrepancy when compared to the corresponding GC results described in the literature[1,3,4] is not discussed by the authors.

In order to clarify the cause of the different results, a laboratory extract from *E. prunastri* was analysed both by an HPLC and a GC method. The HPLC separation was found to be in good agreement to the corresponding results of Strack *et al.* (Fig. 2A), whereas the gas chromatogram shows a composition comparable to that of commercially available products. This fact leads to the conclusion that the characteristic ingredients of *E. prunastri* cannot be vaporized without decomposition.

It is of interest in this context that the laboratory lichen extract does not show any odour characteristics of the corresponding industrially produced materials.

In order to clarify the mechanism of formation of the substances which are responsible for the typical "oakmoss note", the residue of a freshly prepared methanolic extract was heated at 118°C for some hours. The composition of the residue at various stages of this tempering process was monitored by HPLC as illustrated in Fig. 2.

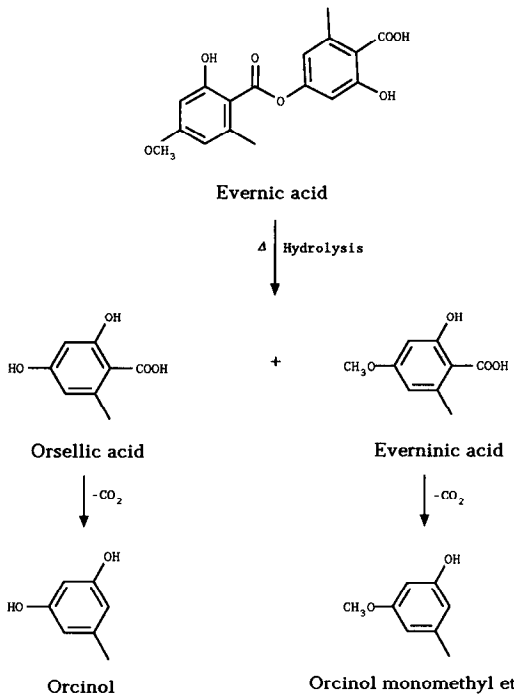

Fig. 3. Degradation reactions of thermally processed evernic acid.

After 1 h, the content of evernic acid decreases and numerous decomposition products result. The degradation of the depsid in a first step mainly yields orsellic acid and everninic acid (Fig. 2B), and after decarboxylation orcinol and orcinol monomethyl ether (Fig. 2D) are formed. The supposed degradation principle is outlined in Fig. 3. A similar scheme was postulated by Pfau[8] in 1937. The same results can be obtained by performing the thermal process with pure evernic acid. On heating the oakmoss extract for 1.5 h (Fig. 2C), the distribution pattern of the detected compounds is very similar to that of a commercially available extract, presented in Fig. 1. On tempering for 3 h, only very small amounts of evernyl and evernic acid are present in the product.

The results obtained lead to the conclusion that GC is of little use in lichen studies, owing to the thermal lability and low volatility of most well known ingredients. As reported[9], especially evernic acid was found to be very unstable to GC analysis even when it was first converted into its trimethylsilyl derivative.

The advantages of the HPLC method presented are:

(i) rapid characterization both of the typical lichen compounds and of the artefacts resulting from the extraction procedure,

(ii) the possibility of quantifying characteristic oakmoss substances in commercially available products for quality control purposes,

(iii) The possibilities of establishing a process control correlating the individual concentrations of the detected ingredients with a desired odour quality.

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