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Lichen extracts as raw materials in perfumery. Part 2: treemoss

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ABSTRACT: This is a comprehensive review of extracts from the lichen *Pseudevernia furfuracea* (treemoss) that are used in the fragrance industry. Qualitative and quantitative analytical aspects are critically reviewed and the results are compared to those of the related oakmoss extracts. It is shown that more than 90 constituents have been identified so far in treemoss extracts, including 42 depsides, depsidones or depside-derived compounds, and 42 triterpenes or steroids. Constituents of certain host trees, mainly *Pinus* species, generate specific analytical and toxicological issues which need to be considered in addition to those related to the known degradation products of lichen compounds. A new classification of lichen extracts used as raw materials in fragrance compounding is proposed

Keywords: *Pseudevernia furfuracea*; treemoss; fragrances; depsides; triterpenes; steroids; resin acids; contact allergy

Introduction

Following on from the previous article on oakmoss (*Evernia prunastri*),[1] the present review deals with treemoss extracts that are used in fragrance compounding. In principle, so-called treemoss extracts are mostly manufactured from *Pseudevernia furfuracea* (L.) Zopf., a lichen which is particularly common on coniferous trees, mainly pine, and cedar trees. Four chemical races of *P. furfuracea* have been defined: one contains mainly physodic acid, a second physodic and olivetoric acids, a third olivetoric acid and the last one physodic, olivetoric and oxyphysodic acids (chemical structures are shown below).[2]

Lichens other than *Evernia prunastri* (true oakmoss) are also processed industrially, such as *Ramalina fraxinea* (L.) Ach., sometimes admixed with *Usnea caucasica* Vain. and harvested mainly from beech trees (*Fagus* spp.) in Macedonia.[3] When these lichens are processed in the presence of *E. prunastri*, whatever the proportions, the resinoids obtained should not be called 'oakmoss' but rather 'treemoss resinoids'.

The Chemical Abstracts Service (CAS) has assigned various Registry Numbers (RN) to treemoss extracts:

- 90028-67-4, defined as: 'extractives and their physically modified derivatives, etc. of *Evernia furfuracea*, Usneaceae'. However, it should be pointed out that the correct botanical and family names are, respectively, *Pseudevernia furfuracea* and Parmeliaceae.
- 92129-88-9, defined as: 'tree moss wax, *Pseudovernia furfuracea.* Extractives and their physically modified derivatives *Evernia furfuracea*'. Although this assignment is somewhat obscure, one can consider that it corresponds to the same type of products defined under RN 90028-67-4.
- 94944-93-1, defined as: '*Evernia furfuracea* extracts, ethanolysed'. The same remark applies regarding the botanical name. As mentioned previously,[1] this RN is likely to correspond to most of the industrially available extracts, provided they are produced from pure *Pseudevernia furfuracea* lichen, free of exogenous products.
- 68648-41-9 and 68917-40-8, both defined as: 'Extractives and their physically modified derivatives, *Evernia furfuracea* and *Usnea barbata*, Usneaceae'. Because another name under RN 68648-41-9 is 'cedarmoss oil', one can admit that the declared lichens species grow specifically on cedar trees.

Considering this confusing situation, however, one can reasonably admit that:

- CAS RN 94944-93-1 should be used specifically for absolute oils prepared from concrete oils manufactured from pure *P. furfuracea*, whatever the host tree.
- CAS RN 68648-41-9 applies to any extractive (concrete or absolute oils) of mixed lichen species, containing mainly *P. furfuracea* and *U. barbata* and growing on cedar trees. As a result, this RN corresponds to resin acid-less treemoss extracts (see below).
- CAS RN 68917-40-8 applies to any other extract, whatever the purity, origin, host tree, etc.

Therefore, extractives of impure *P. furfuracea* growing on pine trees can be assigned this RN. Clearly, no RN has been assigned to any extract obtained from *Ramalina* spp. in either pure form or lichen mixtures.

As of 1997, about 1900 tons of such treemoss and 700 tons of oakmoss were processed each year in France. In 2007, the total quantity of treemoss and oakmoss processed by French producers was only 540 and 550 tons, respectively (source: Prodarom, Grasse, 9 January 2009).

Other related lichens are used for the production of industrial fragrant extracts, such as *Parmelia nepalensis*, *Usnea* spp. and *Ramalina* spp. in Nepal. However, this production did not exceed ca. 60 tons in 1994.

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As we pointed out in the first article,[1] the fragrance industry indiscriminately indicated under the name of 'oakmoss' the lichen collected on oak trees or the lichen collected on other trees. However, distinction has been made traditionally between 'cedarmoss' collected mainly in Morocco on cedar trees (*Cedrus atlantica*), and 'treemoss' gathered from pine trees (*Pinus* spp.), although the lichen species is actually the same (*P. furfuracea*). The difference in fragrance is mainly attributable to the wood, twigs, bark, etc. from pine trees, which contaminate the lichen to various extents.[4]

In using similar solvents and standard procedures, and starting from lichen containing 15–20% humidity, the extraction yields are 5.5–6.0% and 2.5–3.0% for 'pine treemoss' and 'cedar treemoss', respectively.

Several sources mention that extracts of *Lobaria pulmonaria* (L.) Hoffm.—a foliose lichen, whereas *E. prunastri* and *P. furfuracea* are fruticose lichens—have been used in fragrance compounds. Although the reality and importance of this remains unclear, this lichen species is not processed industrially today.

Qualitative Composition of Treemoss Extracts

The chemical composition of treemoss (*P. furfuracea*) has been thoroughly investigated, starting from the lichen itself and typical industrial extracts.[5–14] It was reported that the lichen growing on pine trees (*Pinus sylvestris*) is largely *P. furfuracea*, with minor proportions of other species: *Hypogymnia physodes*, *Usnea* spp., *Alectoria capillaries* and *Parmelia sulcata*.[3,7] This generated mainly qualitative data, which are reported in Huneck and Yoshimura's compilation.[25] As in the case of true oakmoss,[1] whatever the extraction solvent or process, either batch or continuous,[24] the duration and intensity of the hydrolytic pre-treatment can indeed generate a variety of extracts with different compositional characteristics.[3,23] This variability is drastically increased depending on the origin of the lichen (see Introduction) and the host conifer tree, viz. *Pinus* or *Cedrus* spp. When standard treemoss is collected on pine trees in the Massif Central, France (Figure 1a), it is usually made of 40–70% by weight of components (wood, bark, twigs, needles, etc.) harvested from the tree itself. In some cases, this proportion can even reach 80% (Figure 1b).

Apart from these analytical studies, which were focused on the raw material that is used in the fragrance industry, a number of papers have been published on lichen substances identified in *P. furfuracea*. Classical analytical methodologies, including separation and spectroscopic techniques for structure determination, are similar to those involved in similar cases.[1] Mono-aryl compounds (Figure 2) are listed in Table 1, while depsides and depsidones (Figure 4) are listed in Table 2.

Depsides are readily hydrolysed, and the intermediate benzoic acid derivatives can be thermally decarboxylated. For example, in the case of chloroatranorin, increasing the intensity of the hydrolytic process (tepid or hot water or steam) results in its almost complete degradation, with the production of methyl *β*-orcinol carboxylate **9**, chloroatranol **15** and chlorohaematommic acid **16**.[3,23]

Direct analysis by gas chromatography–mass spectrometry (GC–MS) of an extract of *P. furfuracea* growing on cedar trees shows the presence of large amounts of olivetonide **20** and 5-(2-oxoheptyl)-resorcinol **23**, the latter not described,[21] and which is likely to be an artifact generated during the analysis by thermal decarboxylation of olivetonic acid **22** (Figure 3).[23]

Isopropyl haematommate **14** is clearly an artifact that is formed from atranorin during the extraction with isopropanol.[14] Rhizonic acid **19**, already known from lichens, has been tentatively identified in *P. furfuracea* collected in Turkey, together with a lactone which has been assigned the structure $\mathbf{S_1}$ (Figure 5).[26] However, this structure might be corrected to that of vulpinic acid **43**, since the mass spectrum and the carbon nuclear magnetic resonance (CNMR) data are very similar. The chlorinated amino-depside $\mathbf{S_2}$ claimed by the same authors is also unlikely, at least as far as the B-ring of this depside is concerned,[26] which should motivate further studies (e.g. hydrolysis experiments, crystallization, melting point, X-ray analysis). Using an authentic sample, perlatolic acid **31** was unambiguously identified by high performance liquid chromatography–ion trap mass spectrometry (HPLC–ITMS) operating in MS^3.[13] Although it was found for the first time in this lichen species, it had been identified previously in other *Parmelia* spp.[22] Apart from atranorin **25** and chloroatranorin **26**, which represent up to 80% of the depside fraction,[8] olivetonide **20**, physodone **32** and isophysodic acid **34** (which

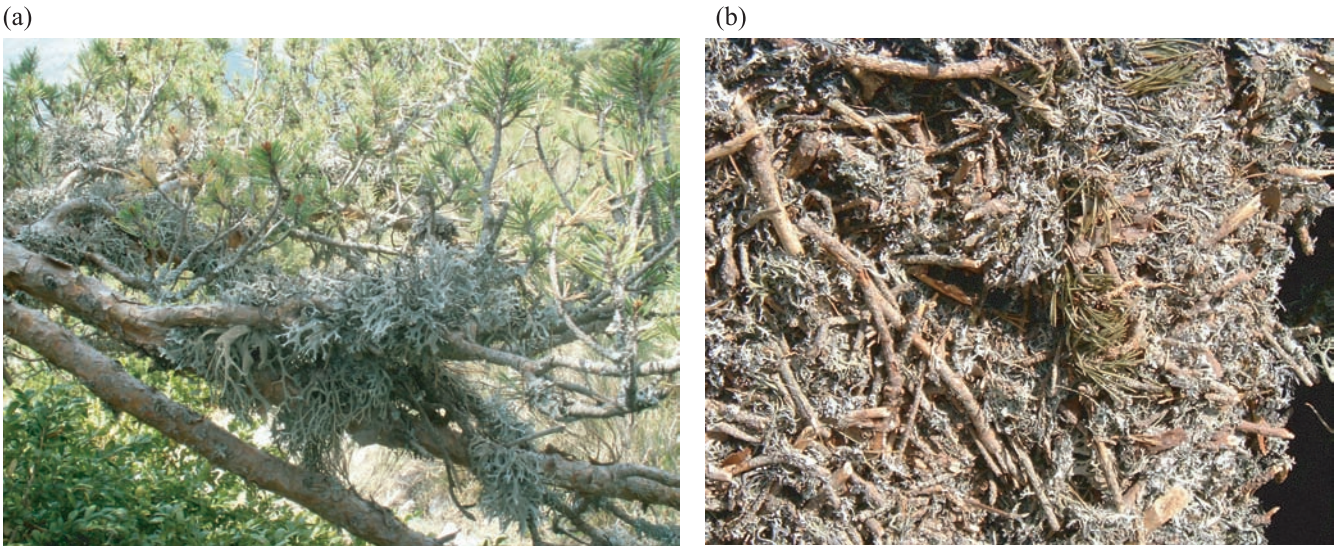

Figure 1. (a) *Pseudevernia furfuracea* on pine tree; (b) raw 'pine treemoss' before processing.

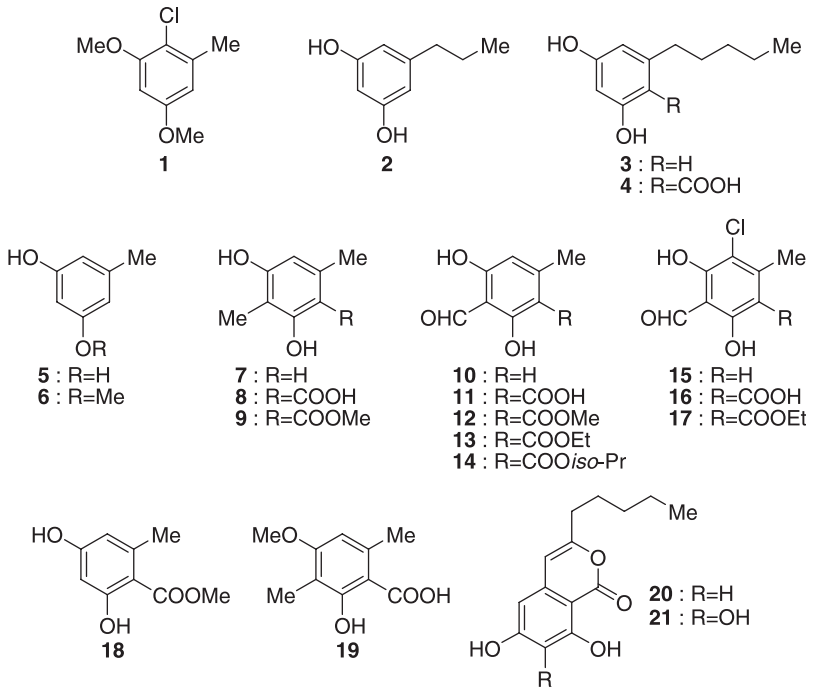

Figure 2. Chemical structures of mono-aryl compounds identified in *P. furfuracea*

Table 1. Monoaryl compounds identified in *P. furfuracea* extracts

Compound no.	Name	CAS RN	MW	References
1	2-Chloro-3,5-dimethoxytoluene	NA	186.6	14
2	Divarinol	500-49-2	151.2	14
3	5-Pentylresorcinol (= olivetol)	500-66-3	180.2	14,21
4	Olivetolcarboxylic acid	491-72-5	224.2	21
5	Orcinol	504-15-4	124.1	21
6	Orcinol monomethylether	3209-13-0	138.1	21
7	β-Orcinol	488-87-9	138.1	21
8	β-Orcinolcarboxylic acid	4707-46-4	182.2	7,13
9	Methyl β-orcinolcarboxylate	4707-47-5	196.2	7,15
10	Atranol	526-37-4	152.1	13
11	Haematommic acid	479-25-4	196.1	7,13
12	Methyl haematommate	34874-90-3	210.1	13
13	Ethyl haematommate	39503-14-5	224.2	13,14,35
14	Isopropyl haematommate	NA	238.2	14
15	Chloroatranol	57074-21-2	186.6	6,13
16	Chlorohaematommic acid	56410-39-0	230.7	21
17	Ethyl chlorohaematommate	57857-81-5	258.7	13,35
18	Methyl orsellinate	3187-58-4	182.1	21
19	Rhizonic acid	479-26-5	196.2	26
20	Olivetonide	3734-54-1	248.3	7
21	Hydroxy-olivetonide	NA	264.3	7

results from the rapid and total isomerization of physodic acid **35**) are considered to be characteristic elements of industrial extracts of *P. furfuracea*.[10,23] 2′-*O*-methylphysodone **33** originates from the decarboxylation of the corresponding acid **36**, previously recorded as a constituent of this species.[18]

It is worth mentioning the possibilities offered by the direct analysis of a neat lichen sample by tandem mass spectrometry (MS/MS), in placing it directly in the ionization chamber of the mass spectrometer.[12] This technique bypasses the tedious extraction and separations steps, which involve relatively large amounts of lichen. In most cases, in addition to compiled data on thin layer chromatography (TLC), negative chemical ionization–MS/MS (NCI–MS/MS) provides decisive information for identifying lichen substances, provided that a comprehensive database has been assembled beforehand. As a result, one would expect HPLC–MS/MS methodologies, which have recently been developed

Table 2. Depsides and depsidones identified in *Pseudevernia furfuracea* extracts from various origins

Compound no.	Name	CAS RN	MW	References
25	Atranorin	479-20-9	374.3	5,6
26	Chloroatranorin	479-16-3	408.8	6,8
27	Lecanoric acid	480-56-8	318.3	19,20
28	Imbricaric acid	491-57-6	402.4	17
29	Microphyllinic acid	491-46-3	528.6	17
30	Olivetoric acid	491-47-4	472.5	8
31	Perlatolic acid	529-47-5	444.5	13,21
32	Physodone	58005-58-6	440.5	7,15
33	2′-*O*-methylphysodone	62806-12-6	454.5	7
34	Isophysodic acid	188347-29-7	470.5	7
35	Physodic acid	84-24-2	470.5	5,7,18
36	2′-*O*-methylphysodic acid	56484-74-3	484.5	7,18
37	3-Hydroxyphysodic acid	53899-46-0	486.5	7,16,18
38	Virensic acid	668-14-4	358.3	16
39	Physodalic acid	90689-60-4	416.3	8
40	Alectoronic acid	54226-87-8	510.6	18
41	Furfuric acid	100508-93-8	552.5	9
42	Fumarprotocetraric acid	489-50-9	456.3	17

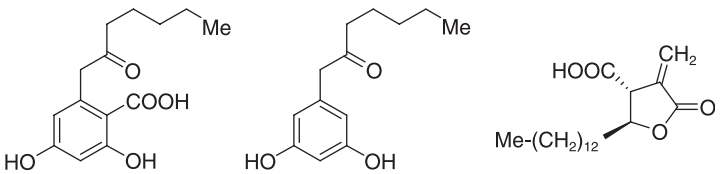

Figure 3. Miscellaneous compounds identified in *P. furfuracea*. Mass spectrum of **23** [*m/z*, (%)], 222 (M^+, 35), 166 (3), 124 (23), 123 (50), 99 (100), 71 (62), 55 (29), 43 (100), 41 (32)

for other families of natural products, to apply efficiently to lichen substances as well.

Similarly, volatile compounds present in fresh lichens (*P. furfuracea* and *Evernia prunastri*) can be analysed directly by solid phase micro-extraction (SPME) sampling and analogous sorptive trapping, followed by GC–MS. In this way, atranol and chloroatranol (and other mono-aryl compounds) can be readily detected in the volatile fraction from the crude lichen. This demonstrates that hydrolysis and decarboxylation of atranorin and chlorotranorin occurs in the 'living' lichen (ca. 15–20% humidity) in the absence of any artificial treatment.[21] It is known that depside hydrolases, present in unactivated form in the lichen, can be activated again in the presence of water (rain water, etc.)

Furfuric acid **41** has been isolated in small amounts (2% of the depside–depsidone fraction) by extraction of 2.4 kg *P. furfuracea* from Massif Central (France) after careful cleaning from other lichen species and elimination of wood and bark residues. The structure of this unique depsidone has been confirmed by synthesis[27] and also, in one step, by the acid-catalysed alkylation of methyl β-orcinolcarboxylate **9** or atranorin **25**, with physodalic acid **39**.[28] This reaction is so facile that it supports the claim that **41** is an artifact of the isolation procedure of *P. furfuracea* contaminated with *Hypogymnia physodes*.[28] However, subsequent experiments confirmed the natural origin of **41**, at least in this specific chemotype of *P. furfuracea*. It appeared that the extraction conditions (hexane) were not sufficiently acidic to catalyse the reaction. Indeed, when a mixture of *Evernia prunastri* (containing methyl β-orsellinate and atranorin) and 10% of *H. physodes* was extracted under the same conditions, only physodalic acid was observed and isolated, whereas furfuric acid **41** was not detected (R. Tabacchi, unpublished information). However, this does not rule out a possible '*in vivo* relationship' between the two species, resulting in some enzyme-mediated reaction between their metabolites.

In addition to the usual depsides and their derivatives present in *Parmelia* species, the main constituent of commercial 'tree-moss' resinoid obtained from *Parmelia nepalensis* (*vide supra*) is protolichesterinic acid **24**.[21] Not surprisingly, since this resinoid is stated to be obtained by extraction with ethanol, it also contains significant amounts of sugars (xylitol, arabitol, etc.) which are easily identified by GC–MS after silylation.[21] Such sugars have been previously identified in *P. furfuracea*.[29]

Starting from lichen collected on conifers (mainly *P. sylvestris*), and after tedious separation of the lichen from any component derived from the host tree, a number of sterols and triterpenes have been identified by GC, UV, IR, NMR and MS.[11,30] They are listed in Table 4, and their structures are shown in Figure 6. It is highly probable that these compounds are metabolites from the host tree (*Pinus* spp.),[13] involving a migration from the host to the parasitic lichen. This hypothesis is well supported by two facts:

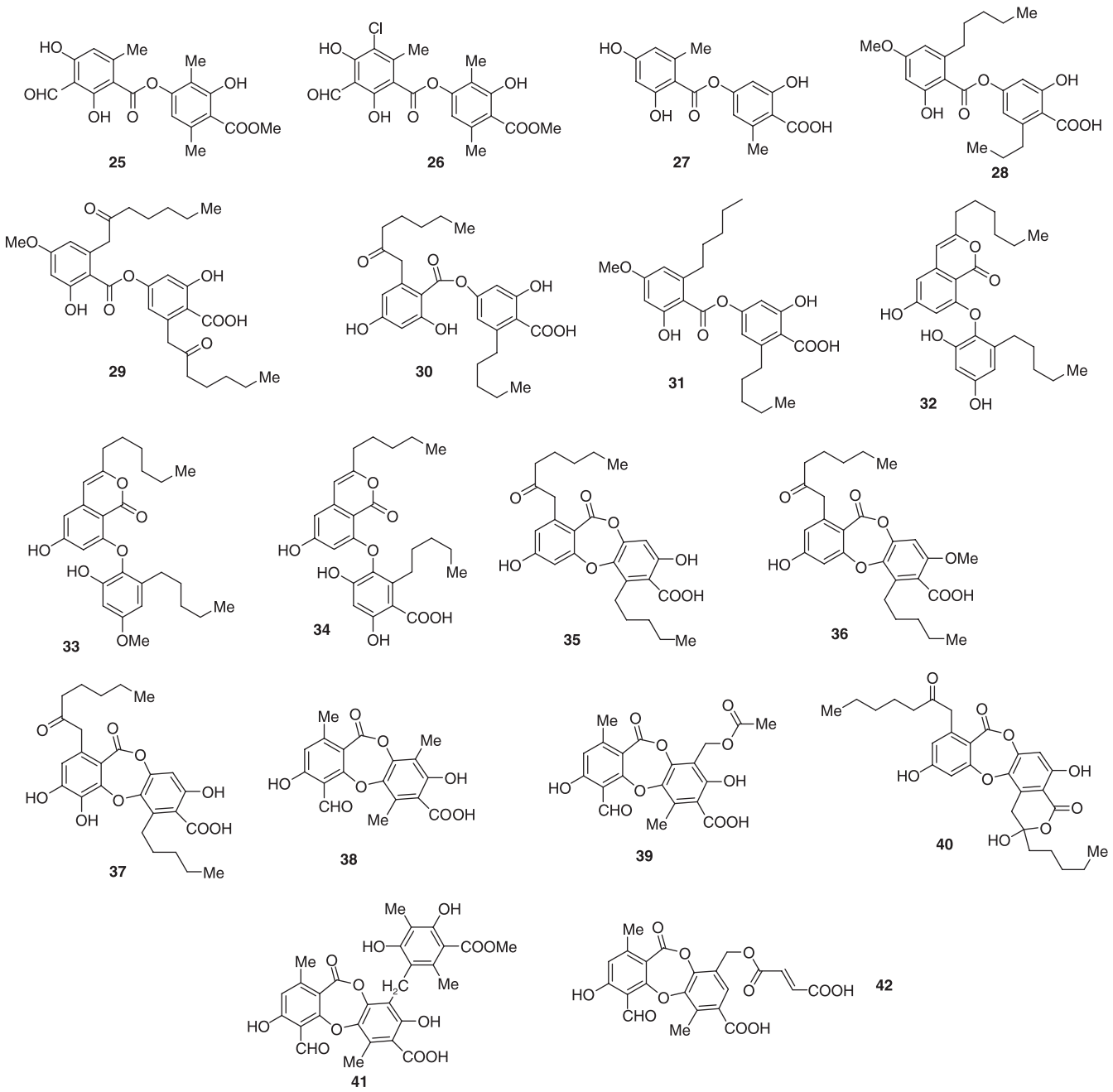

Figure 4. Chemical structures of depsides and depsidones identified in *P. furfuracea*

1. Most of the sterols and triterpenoids listed in Table 4 have been previously identified in Pinaceae,[31] such as *Pinus monticola*,[32] *P. luchuensis*,[33] *Picea jezoensis* var. *hodoensis*.[34]
2. In a typical experiment, a 1 kg sample of 'pine treemoss' was separated into three fractions: one part (A, 36%) was wood devoid of lichen, a second part (B, 29%) comprised mainly lichen (21%) intimately mixed with small pieces of wood, bark, twigs etc. (8%), and a third 'intermediate' part (C, 35%) consisted of large pieces of wood (22%) covered with some lichen (13%). Each fraction was extracted using exactly the same procedure and the concentration of β-sitosterol and stigmasterol (together), chosen as target sterols, was measured by various methods, including HPLC (Table 3).[13] The results confirm that these sterols are components of the host tree and are also present in the thallus of the lichen. A similar phenomenon is observed with resin acids, which are well-known components of *Pinus* (colophony, see below).

All this suggests that terpenoids and sterols biosynthesized in the host tree migrate to the parasitic lichen.

It is well known that lichens can biosynthetize triterpenes and steroids, even when growing in the absence of a host organism, e.g. on rocks. The extent and truly parasitic nature of the relationship between the lichen and its host tree needs to be further investigated with additional lichen species, and host trees that are not conifers. However, it is now clearly established, at least in the case of *P. furfuracea* growing on *Pinus* spp., that di- and triterpenes, as well as steroids, biosynthesized in the host tree do

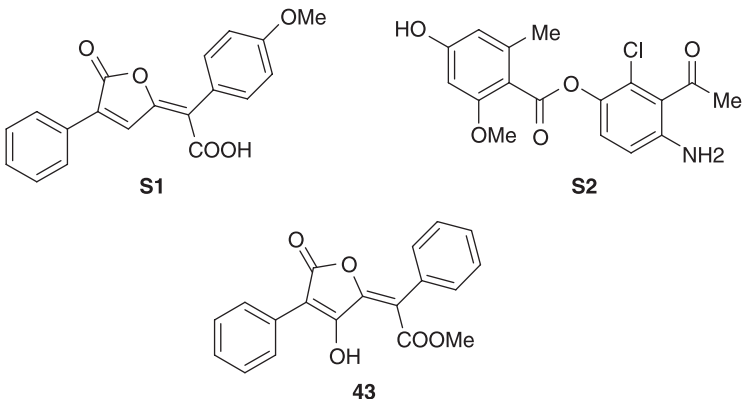

Figure 5. Unconfirmed structures of *P. furfuracea* metabolites

Table 3. Distribution of diterpenes and selected sterols in treemoss fractions[13]

Fraction of biomass (see text)	A	B	C
Lichen content (%, w/w)	0	72	37
Sterols (%, w/w)[a]	10.0	1.9	4.6
Total resin acid (%, w/w)[b]	16.0	2.2	4.8

[a]As β-sitosterol + stigmasterol, by HPLC. [b]By GC–MS with silylation with internal standard.

indeed migrate into the lichen. In the case of resinoids obtained from lichen growing on cedar trees, one should expect to detect terpenoids which are produced by the host tree (*C. atlantica*) and which are also present in the well-known essential oil. However, such studies are currently in progress and have so far not been reported (D. Joulain, unpublished information).

Quantitative Composition of Treemoss Extracts

The content of defined lichen compounds in *P. furfuracea* has only rarely been measured. In most cases, the preferred analytical technique is HPLC.[2,16] For example, atranorin **25**, physodic **35**, hydroxyphysodic **37** and virensic acid **38** are claimed to represent 0.11–0.19%, 1.46–3.78%, 1.69–3.44% and 1.14–1.46%, respectively, in dried lichen from Slovakia.[16] For the same reasons that have been discussed previously in the case of oakmoss,[1] quantitative data on the composition of industrial resinoids and other extracts of *P. furfuracea* are even more scarce. For example, a commercial treemoss absolute oil was found to contain 8.7% methyl β-orcinolcarboxylate **9**, 0.4% ethyl haematommate **13**, 0.3% ethyl chlorohaematommate **17** and 0.3% **25**.[35]

Contrary to depsides, which can undergo various degradations (hydrolysis, decarboxylation, alcoholysis) to mono-aryl compounds, which therefore may be readily available as reference compounds, depsidones (viz. **35**–**42**), present in larger quantities in treemoss than in oakmoss, do not generate such compounds, but rather diphenyl ethers (**32**–**34**). Such ethers can be quite diverse, depending on the extraction conditions, thus drastically complicating the analytical task. Moreover, they are not volatile and their contribution to the odour of the extracts is negligible. However, nowadays, thanks to the availability of efficient columns and instrumentation, GC–MS can be quite effective for the analysis of mono-aryl compounds and even some depsides, since silylated compounds with molecular weights (MW) up to 800 g/M can be eluted below 300 °C. For example, persilylated atranorin (MW 590) is eluted at 230 °C on a 30 m long non-polar column. A typical composition of an industrial treemoss absolute oil is shown in Table 5.[13]

A global quantification method of polyphenols, using the Folin–Ciocalteu method and 96-well plate assays, can be applied with success to the rapid screening of many samples of *P. furfuracea*.[36]

The Resin Acid Issue in Commercial 'Moss' Resinoids

As we mentioned earlier[1] and have discussed again above, treemoss resinoids that are produced from the lichen collected from pine trees contain variable amounts of resin acids (colophony), depending on the proportion of 'wood' components. Colophony is a complex mixture of diterpene acids (resin acids, ca. 90%), including abietic acid **85** as the main constituent.[37] It has been found that, when highly purified, abietic acid is non-allergenic. However, it rapidly autoxidizes to the hydroperoxide **86**, which is identified as a major allergen in colophony.[38] Other oxidized species derived from dehydroabietic acid (DHA) **87**, such as 15-hydroperoxy-dehydroabietic acid **88** and 7-oxodehydroabietic acid (7-ODHA) **89**, are also sensitizers (Figure 7). In particular, a haptenation mechanism involving **89** has been proposed, which may account for the allergic contact dermatitis (ACD) observed from exposure to resin acids.[39] The qualitative monitoring of the oxidation of diterpenoid resins can be achieved by HPLC coupled with atmospheric pressure chemical ionization MS (LC–APCI–MS) or APCI–MS/MS.[40]

In 2000, it was reported that products marketed under the name 'oakmoss absolute' by two patch-tests suppliers, viz. Trolab and Chemotechnique, actually contained resin acids.[41] This meant that these products were mixtures of extracts, including one obtained from *P. furfuracea* growing on pine trees. As a consequence, it was surmised that the use over the years of these products in patch-testing for ACD to oakmoss may have been a source of misdiagnosis. However, it was shown subsequently that the diagnostic value of oakmoss absolute as an indicator of fragrance ACD has been, and is, unaffected by the resin acid contamination.[42] Concomitant observations showed that a strongly statistically significant association between oakmoss absolute and colophony (resin acids) triggers only a small increase in rates of allergic response to colophony in oakmoss-positive patients.[43]

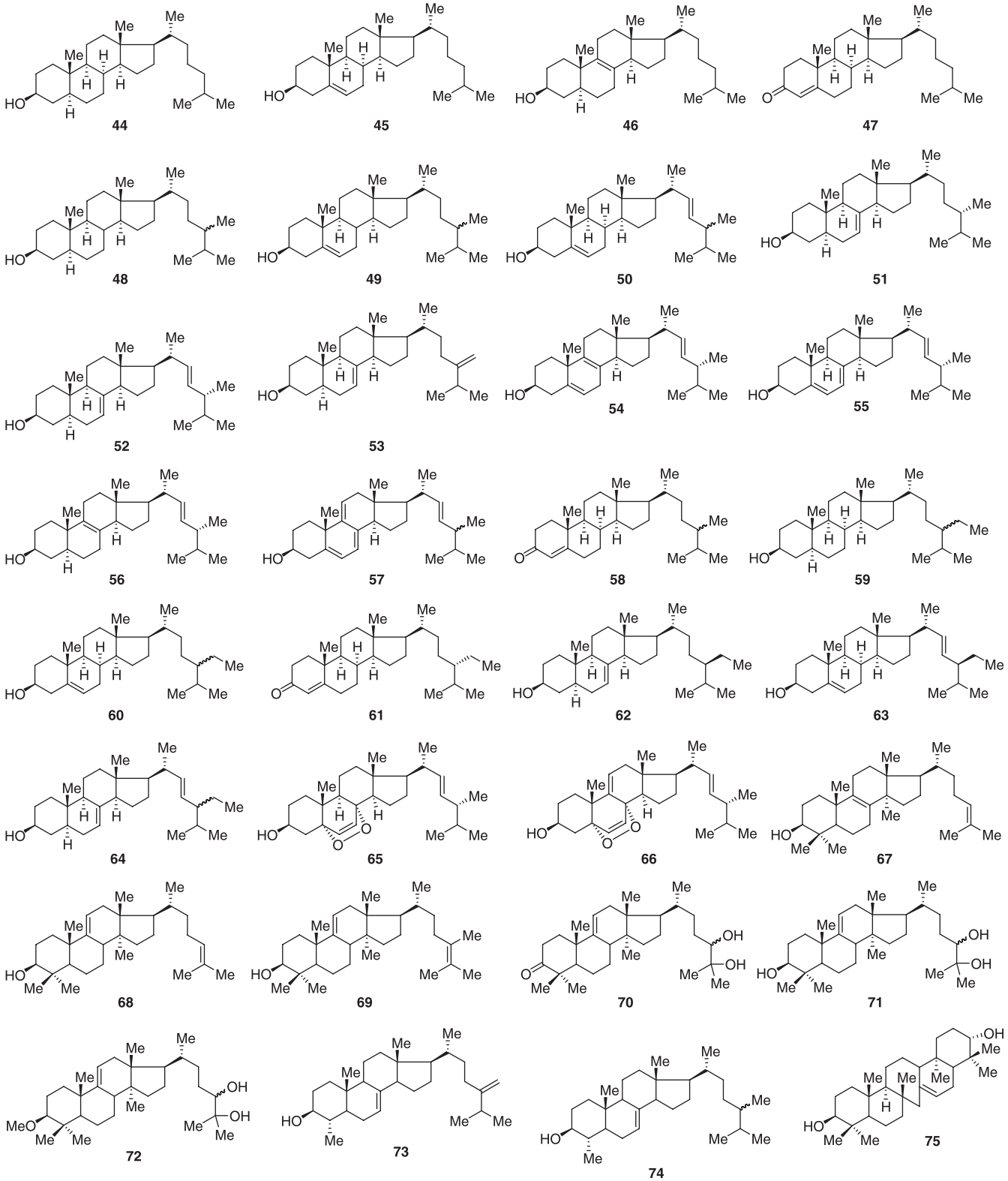

Figure 6. Serrattene derivatives identified in *P. furfuracea* growing on pine trees

In July 2001, the International Fragrance Association (IFRA) issued a recommendation ('standard') on oakmoss, stating:

> 'In the presence of treemoss extracts, the level of oakmoss has to be reduced accordingly such that the total amount of both extracts does not exceed 0.1% in the final product. Oakmoss extracts used in perfume compounds must not contain added treemoss. Treemoss contains resin acids. The presence of resin acids can be detected by using a routine analytical method available from IFRA. However, traces of resin acids are unavoidable in current commercial qualities of oak moss. As an interim standard, these traces must not exceed 0.1% (1000 ppm) dehydroabietic acid (DHA)'.

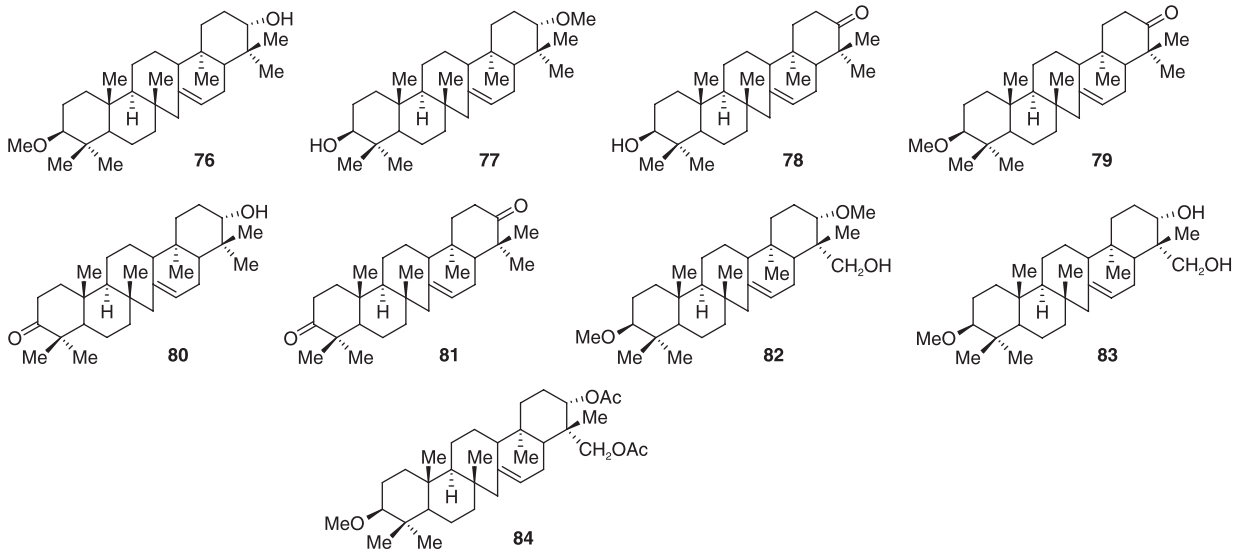

Figure 6. (*Continued*)

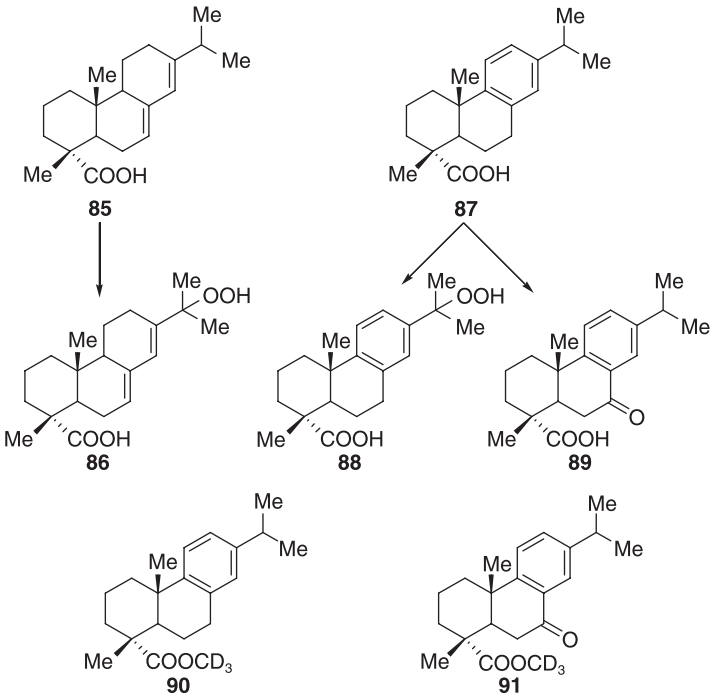

Figure 7. Resin acids and their oxidation products (selection)

A method for quantifying resin acids involves the determination of dehydroabietic acid (DHA) by HPLC with fluorimetric detection, whilst assuming that DHA represents 40% of the total resin acids.[44] This IFRA-recommended method (see below) is efficient in every respect, and shows that typical commercial treemoss absolute oils contain 5–6% w/w DHA. It is also sensitive enough to detect and quantify DHA in a low ppm range (<100 ppm) in contaminated oakmoss extracts. An alternative global method for determining resin acids involves their separation by gel permeation chromatography (GPC), followed by methylation of the acids by diazomethane.[41] Using this method, the contents of resin acids in two commercial samples of treemoss absolute oils were found to be 11.4% and 8.1%. In this mixture, the concentrations of 7-oxo-dehydroabietic acid (7-ODHA) were found to be 1.6% and 1.1%, respectively, but the method of quantification of this single compound by gas chromatography–mass spectrometry (GC–MS) is unknown.[41] In contrast, the selective quantification of DHA and 7-ODHA can be achieved by GC–MS with internal standardization.[13] It requires two steps: the extraction of the acid fraction; followed by *in situ* methylation with diazomethane and the trideuterated esters of DHA (**90**) and 7-ODHA (**91**) as internal standards (IS). Due to the excellent selectivity and sensitivity of this method, quantification of DHA and 7-ODHA at the sub-ppm level is possible in lichen absolute oils, and even in finished cosmetic products.

Table 4. Steroids and triterpenoids identified in *Pseudevernia furfuracea*

Compound no.	Name	CAS RN	MW	References
44	Cholestan-3β-ol	80-97-7	388.7	11,30
45	Cholest-5-en-3β-ol	57-88-5	386.6	11,30
46	Cholest-8-en-3β-ol	7199-91-9	386.6	11,30
47	Cholest-4-en-3-one	601-57-0	384.6	11,30
48	Ergostan-3β-ol	6538-02-9	402.7	11,30
49	Ergost-5-en-3β-ol	290299-12-6	400.7	11,30
50	Ergosta-5,22-dien-3β-ol (=brassicasterol)	474-67-9	398.6	11,30
51	Ergost-7-en-3β-ol (=fungisterol)	507-78-9	400.7	5,11,30
52	Ergosta-7,22-dien-3β-ol	17608-76-3	398.6	11,30
53	Ergosta-7,24(28)-dien-3β-ol	17105-77-0	398.6	11,30
54	Ergosta-5,8,22-trien-3β-ol (=lichesterol)	50657-31-3	396.6	11,30
55	Ergosta-5,7,22-trien-3β-ol (=ergosterol)	57-87-4	396.6	5,11,30
56	Ergosta-8,22-dien-3β-ol	36904-77-5	398.6	11,30
57	Ergosta-5,7,9(11),22-tetraen-3β-ol	26596-35-0	394.6	11,30
58	Ergost-4-en-3-one	51014-22-3	398.6	11,30
59	Stigmastan-3β-ol	83-45-4	416.7	11,30
60	Stigmast-5-en-3β-ol (=β-sitosterol)	5779-62-4	414.7	11,30
61	stigmast-4-en-3-one	1058-61-3	412.7	11,30
62	Stigmast-7-en-3β-ol (=schottenol)	6869-99-4	414.7	11,30
63	Stigmast-5,22-dien-3β-ol (=stigmasterol)	83-48-7	412.7	11,30
64	Stigmast-7,22-dien-3β-ol	18070-03-6	412.7	11,30
65	Ergosterol-5α,8α-peroxide	2061-64-5	428.6	11,30
66	3β-Hydroxy-5,8-*epi*-dioxyergosta-6,9(11),24(28)-triene	78342-37-7	426.6	11,30
67	Lanosterol	79-63-0	426.7	11
68	Lanost-9(11)-en-3β-ol (T)	28032-52-2	426.7	11
69	24-Methyllanost-9(11)-en-3β-ol (T)	NA	440.7	11
70	Lanost-9(11)-en-3β,24,25-triol	NA	460.7	11
71	3β-Methoxylanost-9(11)-en-24,25-diol	NA	474.7	11
72	24,25-Dihydroxylanost-9(11)-en-3-one	385384-28-1	458.7	11
73	4α-Methylergosta-7-24(28)-dien-3β-ol	NA	412.7	11
74	4α-Methylergost-7-en-3β-ol	77122-68-0	414.7	11
75	Serrat-14-en-3β,21α-diol (=serratenediol)	2239-24-9	442.7	11
76	3β-Methoxyserrat-14-en-21α-ol	NA	456.7	11
77	21α-Methoxyserrat-14-en-3β-ol	NA	456.7	11
78	3β-Hydroxyserrat-14-en-21-one	3787-73-3	440.7	11
79	3β-Methoxyserrat-14-en-21-one	NA	454.7	11
80	21α-Hydroxyserrat-14-en-3-one	NA	440.7	11
81	Serrat-14-en-3,21-dione	1449-07-6	438.7	11
82	3β,21α-Dimethoxyserrat-14-en-24-ol	NA	486.8	11
83	3β-Methoxyserrat-14-en-21α-30-diol	94805-72-8	472.7	11
84	3β-Methoxyserrat-14-en-21α-30-diol, diacetate	NA	556.8	11

T, tentative identification; NA, no assignment.

A method has been developed for the selective removal of resin acids from treemoss extracts, when manufactured from lichen growing on pine trees.[45] Table 5 shows a list of concentrations of selected relevant constituents of treemoss before and after such processing.[13] It is noteworthy that, although it still contains the same normal levels of atranol and chloroatranol but undetectable amounts of the atranorins, the resin acid-depleted oil did not induce any reaction in two separate human repeated insult patch tests (HRIPTs), according to the Marzulli–Maibach protocol, on a total of ca. 200 volunteers at 3% concentration (Robertet, unpublished data).[13] As we have demonstrated above, resin acids are not only present in the wood debris from the host pine tree, but they also migrate into the lichen. Since dehydroabietic itself is not a sensitizer, it would be worthwhile to monitor the oxidation rate of this hydrocarbon during its migration in the thallus of the lichen. Would depsides, which are polyphenols, act as anti-oxidants? In order to rule out a possible bias or misinterpretation, the following experiment was carried out: pure intact lichen (free of any wood debris) was soaked briefly in hexane (3 min at 20 °C), yielding extract No. 1. Then, the drained lichen was ground and extracted again with hexane in a Soxhlet apparatus for 5 h, yielding extract No. 2. Quantification of 7-ODHA was performed on extracts Nos 1 and 2 by GC–MS with deuterated ISs, as above. The results demonstrate that it is indeed present mainly in the thallus of the lichen (ca. 10 ppm), whereas ca. 0.7 ppm only is recovered from its outer part.[13]

Table 5. Quantitative analysis of treemoss absolutes prepared from lichen growing on pine trees[13]

Component	Regular absolute (%)	Resin acid—less absolute (%)
β-Orcinol **7**[a]	0.016	0.01
Atranol **10**[a]	0.37	0.31
Methyl β-orcinolcarboxylate **9**[a]	3.70	3.55
Chloroatranol **15**[a]	0.22	0.15
β-Orcinolcarboxylic acid **8**[a]	0.012	0.014
Methyl haematommate **12**[a]	0.20	0.20
Ethyl haematommate **13**[a]	0.22	0.97
Haematommic acid **11**[a]	0.075	0.044
Ethyl chlorohaematommate **17**[a]	0.4	0.4
Olivetonide **20**[a]	0.30	0.30
2′-*O*-methylphysodone **33**[a]	0.79	1.14
Physodone **32**[a]	2.39	4.69
β-Sitosterol **60**/Stigmasterol **63**[a]	4.12	8.0
Atranorin **25**[b]	ND	ND
Chloroatranorin **26**[b]	ND	ND
Dehydroabietic acid[b,c]	4.12	0.29
7-Oxodehydroabietic acid[c]	0.19	0.09

[a]GC–MS after silylation with internal standardization. [b]HPLC with external standardization. [c]GC–MS after methylation (CH_2N_2) with internal standardization. ND, not detected (limit of detection, ca. 30 p.p.m. in a resinoid).

Moreover, the ratio of concentrations of 7-ODHA vs. DHA is higher in the lichen than in the wood bearing the lichens. This suggests that lichen compounds do not protect DHA against oxidation and may even act as pro-oxidants. These results do not contradict recent observations showing that methanol extracts of *P. furfuracea* have low antioxidant activity, in comparison with other lichen species.[46]

Other Toxicological Issues

Whatever the type of lichen that is used for the manufacture of treemoss resinoids, the corresponding absolute oils are expected to contain significant levels of atranol **10** and chlorotranol **15**, since atranorin **25** and chloroatranorin **26** are major constituents of these lichens (Table 5). The latest opinion of the Scientific Committee on Consumer Products (SCCP) concerning **10** and **15** is clearly focused on a use level based on elicitation (previously sensitized individuals).[47] This new paradigm, if it becomes official, overturns the present strategy of the safe use levels of fragrance ingredients based on induction and labeling of allergens.

In a previous opinion, the SCCP stated:

> 'As atranol and chlorotranol are such potent allergens (and chloroatranol particularly so), they should not be present in cosmetic products'.[48]

This opinion is largely based on results obtained from a bioassay-guided fractionation of an oakmoss absolute.[1] From a methodological viewpoint, following a fractionation using an *in vivo* allergy assay is a reductionistic approach, and one should be aware that toxicity could derive from a synergistic interaction of more than one compound, and that there could not be a single 'culprit' in case of a toxic activity. Most importantly, the SCCP indicated that 'oakmoss absolute is a strong sensitizer', whereas 'treemoss is unlikely to be a skin sensitizer under the conditions of the test' (local lymph node assay).[48] Hence, it would be logical to take into account the toxicological tests performed on treemoss still containing high levels of **10** and **15** (see above), and previous observations showing that 'hydrolysed atranorin'—thus likely to contain **10** and/or haematommic acid **11**—did not elicit any reaction on three sensitized individuals out of eight who had tested positive to atranorin, themselves representing 1% of the tested patients.[49] Furthermore, one can question the validity of results obtained from patch-testing sensitized humans with the two synthetic atranols of uncertain purity, and from 'high' to 'low' concentrations.[50] Individuals who give low thresholds of elicitation in sequential dilution patch tests are not always the most responsive in repeated open application test (ROAT) studies, and vice versa.[51]

An IFRA standard has recently been released that specifies that:

> 'tree moss extracts shall not contain more than 0.8% of dehydroabietic acid (DHA) as a marker of 2% of total resin acids. The concentration of DHA (about 40% of the total resin acids) in tree moss can be measured with an HPLC reverse phase-spectrofluorimetry method. Further, levels of atranol and chloroatranol should each be below 100 ppm in tree moss extracts.'

Consequently, when 'pine treemoss' absolute oils with low risk of ACD are desired in order to comply with existing recommendations and regulations, one needs to reduce the levels of both resin acids and atranol/chloroatranol. This task appears to be quite tricky, although it is not technically unattainable. In principle, patented methods would indeed apply to both tasks.[45,52–54] The only limiting factors are cost and odour effectiveness. Is this going to trigger the disappearance of 'pine treemoss' from the market?

A recent publication reported that 'oakmoss exerts prominent photo toxicity in an *in vitro* assay with photohaemolysis occurring upon exposure to both UVB- or UVA-rich sources'. Regretfully, however, the exact nature and origin of the tested 'oakmoss' (true *Evernia prunastri* extract?) were not mentioned.[55]

Lichen Extracts as Fragrance Ingredients

In perfumery, lichen extracts are generally classified in the 'woody' and 'green' notes categories. The top note of true oakmoss absolute provides a unique and characteristic seaweed and marine effect that is highly prized by perfumers. Whether or not it is a contribution of the algal portion of the symbiotic algae:fungus of the lichen, this has sometimes prompted the addition to oakmoss of seaweed extracts, in an attempt to enhance this effect in a number of natural specialties. Phenolic notes are clearly provided by volatile orcinol derivatives, and contribute to the woody complexity of the product. While it is discrete but real in the case of oak and cedar trees, the contribution of the host plant is obvious in 'pine treemoss' extracts, with tar-like and heavy pine needle/terpenic notes. In some cases, the plant material is collected at ground level, together with true mosses or even fungi, which are responsible for the earthy/mouldy character of the extracts, reminiscent of humus and forest undergrowth.[56–58] Undoubtedly these fragrance raw materials are unique and so complex that they cannot be replaced by any synthetic blend. The odour of lichen (so-called 'moss') extracts has not yet revealed all of its secrets. Indeed, when carrying out extensive and detailed fractionation of absolute oils, analytical chemists observe that all fractions, irrespective of their complexity, still tend to smell 'mossy'. This suggests that trace constituents play an important organoleptic role.

Conclusion

We have shown herein and in the previous review article that the understanding of the chemistry of industrially relevant lichen extracts—so-called 'moss extracts'—is considerable. In the case of treemoss, we now report more than 90 constituents. This should put an end to allegations claiming that these complex products are 'partly known'[50] in quoting irrelevant textbooks such as Arctander's.[58]

On the other hand, it appears that so-called 'moss' resinoids are manufactured by solvent extraction of a variety of lichens, and sometimes of poorly defined mixtures of lichens. Whatever the extraction technology, a serious concern persists about the accurate naming of these important fragrance raw materials, depending on the botanical species involved. As we have pointed out several times, confusion has existed and still exists as to what is really 'oakmoss' and 'treemoss'. This confusion was aggravated by the wide-spread allegation, albeit not always founded, that cheaper treemoss was used to 'cut' (dilute) the more noble oakmoss extracts. Undoubtedly, this confusion has been detrimental to the fragrance industry in several respects. It would certainly be desirable to redefine which product is which. Based on what has been demonstrated in these articles, we now propose to define only three categories of lichen (so-called 'moss') extracts:

- 'Oakmoss' extracts and their physically derived products, which are obtained exclusively from *Evernia prunastri*, a lichen that grows mainly, but not only, on oak trees.
- 'Cedarmoss' extracts, obtained exclusively from *Pseudevernia furfuracea* growing on cedar trees (*Cedrus atlantica*).
- 'Treemoss' extracts, obtained from any other lichen species growing on any host plant.

In principle, it would be easy to characterize such oakmoss resinoids or absolute oils by the specific presence of evernic acid and evernin (or what remains of these depsides after processing), along with large amounts of usnic acid, whereas cedarmoss resinoids would contain specifically physodic and/or isophysodic acid but no usnic acid at all (<5 ppm; D. Joulain, unpublished information).

Once these definitions are accepted and enforced, it would be logical to carry out the appropriate toxicological tests, starting from samples representative of industrial production, in contrast with most experiments that have given rise to recent publications dealing with the ACD of these fragrance raw materials.

As we have mentioned above, the quantity of treemoss that is processed today, for the manufacture of extracts for the fragrance industry, is only about one-third of what it was 10 years ago. This is apparently the result of the industry's self-regulation with respect to resin acids. During the same period, the consumption of oakmoss has also been declining, from 700 to 550 tons. Thus, the total consumption of lichens is now only 30% of what it was in 1997. It is foreseeable that the new drastic constraints concerning the atranols will induce a further decrease in the usage of lichen extracts for fragrance compounding.

It would therefore be desirable to undertake a comprehensive survey of the uses of lichens, *other than in fragrances*, to up-date the quantities of lichens that are really harvested and traded in Europe and North America. This would possibly shed light on, and reveal some causes of allergies induced by, otherwise unexpected exposure to these products.

Addendum

After the submission of the previous article, we became aware of a publication reporting new constituents of *Evernia prunastri* collected in the Canary Islands, Spain. Those that are missing in the first article are listed in Table 6, and their structures are shown in Figure 8.[59] It is interesting to note the identification

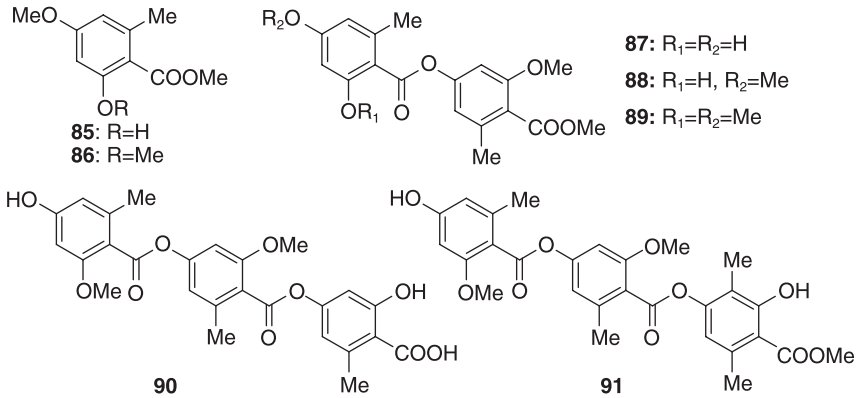

Figure 8. Chemical structures of new compounds identified in *Evernia prunastri*[59]

Table 6. New compounds identified in *Evernia prunastri*[59]

Compound no.	Name	CAS RN	MW
85	Methyl everninate	520-43-4	196.2
86	Methyl di-*O*-methylorsellinate	6110-37-8	210.2
87	Methyl 2′-*O*-methyllecanorate	124521-14-8	346.3
88	Methyl 2′-*O*-methylevernate	NA	360.4
89	Methyl 2,2′-di-*O*-methylevernate	79080-47-0	374.3
90	2,2′-Di-*O*-methylgyrophoric acid	863781-98-0	480.5
91	Prunastrin	863919-47-5	508.5

of two new tridepsides **90** and **91**. However, the identification of several methyl esters raises the question of whether these products exist as such in the lichen, or are formed during their extraction with methanol.

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