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# Investigation by solid-phase microextraction and gas chromatography/mass spectrometry of secondary metabolites in lichens deposited on stone monuments

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**Lichens are ubiquitous organisms formed by symbiotic associations of fungal hyphas and algae that also grow under often extreme environmental conditions. They produce secondary metabolites, the so-called lichen substances, whose structural characterization can give an important contribution to lichen taxonomy. Lichens are also widely employed as biomonitors of atmospheric pollution; being epiphyte organisms they tend, in fact, to accumulate exogenous compounds. Moreover, it could be questioned if the environmental stress alters their secondary metabolites production. Therefore, a new strategy for the analysis of the organic substances absorbed or metabolized by lichens has been developed. This method exploits the dry solid-phase microextraction (SPME) headspace technique coupled with gas chromatography/mass spectrometry (GC/MS). Lichens coating the stone surfaces of monuments, located in small towns between high mountains and far away from urban environments, have been investigated. In the field of cultural heritage, this study can contribute to the knowledge of the state of conservation of outdoor exposed historical monuments.**


Lichens are symbiotic organisms formed by the association of the mycobiont (the fungal partner) and the photobiont (an algal photosynthetic component).[1] They contain a great number of secondary metabolites some of which, like amino acid derivatives, aliphatic acids, terpenoids, steroids, and carotenoids, are ubiquitously present in plants as well as in microorganisms. Many other compounds, belonging to the classes of quinones, chromones, xanthones, dibenzofurans, depsides, depsidones and depsones, are characteristic of this type of cryptogames (i.e. a division of the kingdom *Plantae*).[2] The role of these metabolites in the economy of their life is still a debated question,[3] but certainly their structural features, chemical-physical properties and biological activity could give some indications. Some metabolites absorb UV light, thus protecting the algae against too intensive irradiation;[4] other are defensive agents[3] (antibiotic, antiviral and metal-chelating compounds) and must play an important role for their survival fitness.[5]

The metal-chelating ability of some of these metabolites,[6] as well as the localization of many lichens on lithic surfaces, constitutes also a different facet of the research in this field. They are the first organisms to colonize stone monuments;[7] with time, the surface tends to release cations under the chemical pressure determined by the electron-donating moieties of phenols, carbonylic and carboxylic compounds, and a morphological alteration of the surface is thus produced.

Since lichens are difficult to differentiate morphologically, studies of the natural organic compounds present in these organisms also play an important role in their taxonomy.[8] Finally, it is also worth emphasizing that lichens are not only epilithic, at least those we have studied, but also epiphytic organisms which derive part of their nutrients from the air by continuously filtering it through the entire plant outer layer. In this respect they behave as very sensitive indicators for monitoring air pollution.[9] It is our opinion that it could also be questioned whether environmental stress can cause modifications in their secondary metabolism.

Based on the above propositions, it follows that the identification of lichen substances, whether they are secondary metabolites or organics absorbed from the atmosphere, must be considered an important activity for botanists working on lichen classification and chemists dealing with organic natural compounds, as well as for those involved in the control of environmental pollution and for scientists dealing with the conservation of our cultural heritage.

In this communication we present results that were obtained from the analysis of the organic compounds present in lichens which cover, to a fairly large extent, the stone surfaces of old monuments of the Abruzzi region in central Italy that have never been restored. For this purpose we have used the dry solid-phase microextraction (SPME) headspace GC/MS technique,[10] which offers the advantage of using

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small quantities of material,[11] thus allowing a respectful treatment of the work of art.

## EXPERIMENTAL

Five isolated old villages (Crognaleto, S. Stefano, Cervaro, Barisciano, Castel del Monte) were selected in a wide inland area of the Abruzzi region, in central Italy. They are located between high mountains, higher than 2000 m, and are distant by more than 100 km from large cities and industrial estates. In these towns many historical buildings date back to between the 13th and 17th centuries and now appear to be in a state of neglect. Lichens cover the stone surfaces to a considerable extent, and were thus selected and morphologically identified as *Porpidia cinereoatra* ('Crognaleto'), *Lecanora gangaloides* ('S. Stefano'), *Xanthoria parietina* ('Cervaro'), *Lecanora albescens* ('Barisciano') and *Lepraria incana* ('Castel del Monte').

Sampling was performed in compliance with NORMAL recommendations,[12] by gently scratching the lichen material from the monument surface with a surgical lancet. The finely ground material (20 mg), mainly lichens contaminated with inorganic material, was heated in a vial closed with a screw cap with a septum, under slow magnetic stirring, at 210°C for 30 min. A 100 μm polydimethylsyloxane (PDMS) fiber was then exposed to the headspace keeping the sample at 210°C for a further 30 min, without stirring. The vapors adsorbed on the fiber were then thermally desorbed in the injection port of a Varian Saturn 2000 GC/MS instrument, at a temperature of 270°C for 4 min (splitless mode). The analysis was performed using a capillary column (5% phenylmethylsiloxane, 30 m × 0.25 mm i.d., 0.25 μm film). The temperature program was raised from 35°C (4 min) to 270°C (30 min), at a rate of 10°C/min. The mass spectrometer was operated under the following conditions: ionization energy, 70 eV; *m/z* range, 45–600; scan cycle time, 1 s; transfer line, at 170°C; manifold, at 110°C; and ion trap, at 150°C.

Peak identification was based on mass spectral interpretation and on standard libraries (Wiley, NIST, R.P. Adams—Identification of Essential Oils by Ion Trap Mass Spectroscopy, 3rd edition).

## RESULTS AND DISCUSSION

The stone monuments were selected in an inland area at 1100 m elevation above sea level, far away from industrial estates and heavy vehicular traffic, located between high mountains in the Abruzzi region in central Italy. They all date back to the 16–17th centuries and do not appear to have undergone any level of restoration. Lichens are ubiquitous, heavily affecting the monuments' surfaces which, in many cases, appear covered by a pale yellow-green, hard material distributed in a spotted fashion. The lichens were morphologically identified, and the sampled materials were then analyzed by dry SPME headspace GC/MS (see Experimental section for sampling and chemical analysis procedures). Using this procedure less than 20 mg of material are necessary for the complete analysis, thus leaving only a minor level of injury done to the work of art.

A sample total ion current (TIC) chromatogram, relevant to the lichen species *Porpidia cinereoatra* ('Crognaleto' sample), is presented in Fig. 1. Four more lichen samples were collected, and the corresponding TIC chromatograms are reported in Fig. 2. For each chromatogram, the site and the monument where sampling occurred are also reported, as well as the lichen species. Peak assignments, based on library search identification of the mass spectra (see Experimental section) confirmed by individual mass spectral interpretation and comparison of the spectra with those reported for lichens in the specialized literature,[2] are reported in Table 1 for the

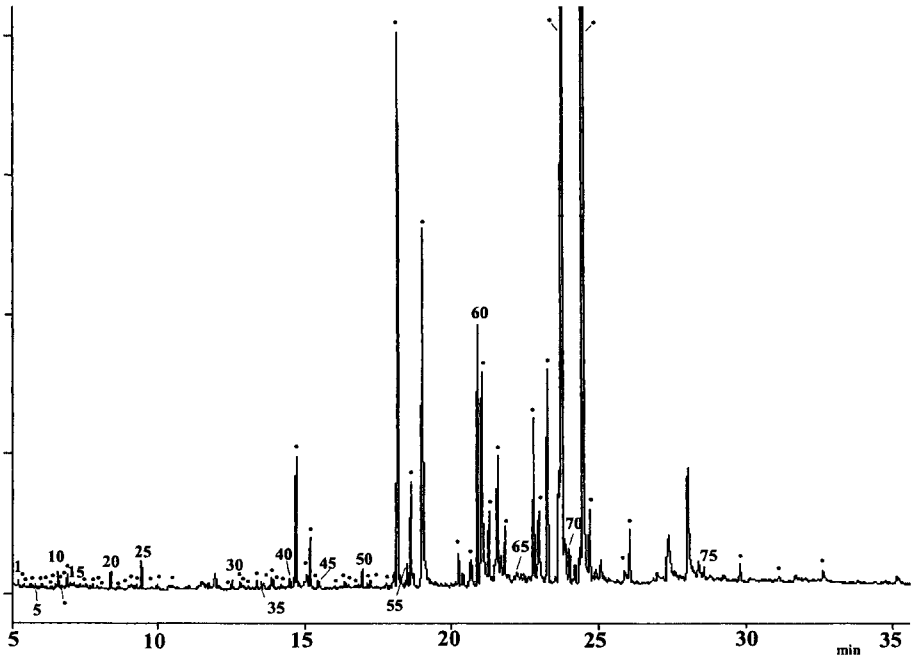


**Figure 1.** Total ion current chromatogram for the sample 'Crognaleto', *Porpidia cinereoatra*. For the sake of clarity only one peak in every five is labeled, the others are marked by dots; see Table 1 for peak assignment.

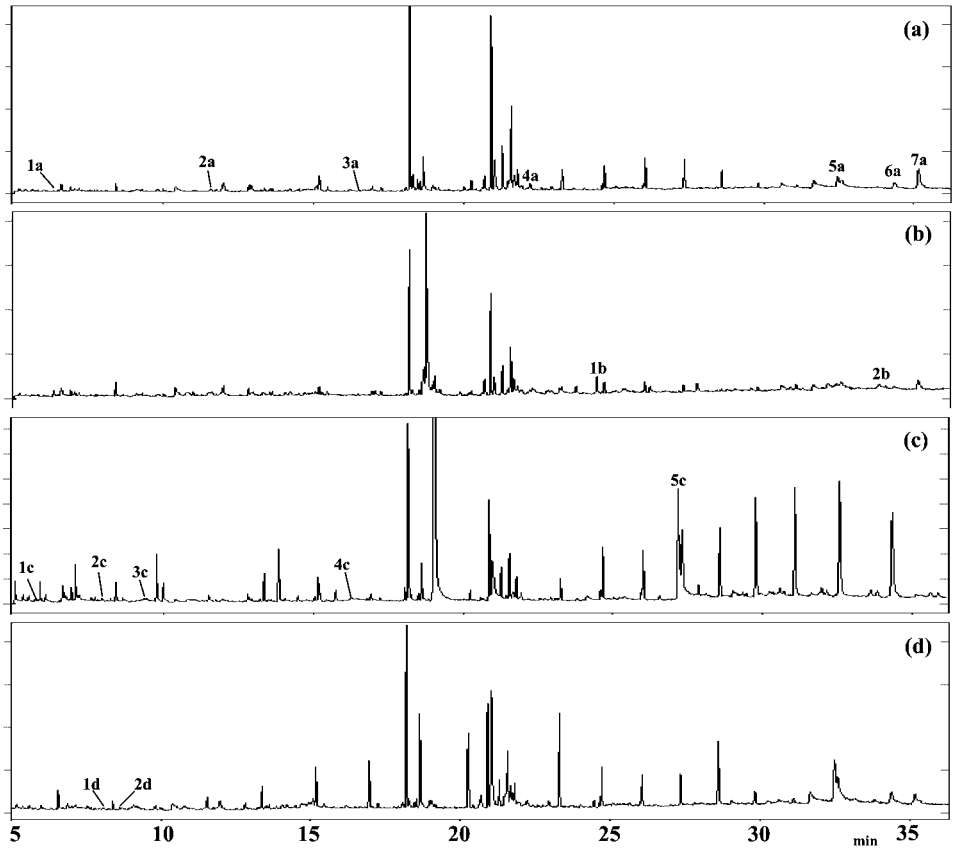


**Figure 2.** Total ion current chromatograms for (a) sample 'S. Stefano', *Lecanora gangaloides*; (b) sample 'Cervaro', *Xanthoria parietina*; (c) sample 'Barisciano', *Lecanora albescens*; and (d) sample 'Castel del Monte', *Lepraria incana*. See Table 2 for peak assignments.

'Crognaleto' sample, and in Table 2 for the other lichens analyzed. In this second table only the identified compounds other than those already reported in Table 1 are listed. Only one chromatogram (Fig. 2(c)) is characterized in its second part by a large envelope of peaks clearly due to hydrocarbons of environmental pollution origin; corresponding mass spectra give evidence supporting this attribution.

It is worth noting that numerous aromatic compounds are clearly present in all the samples analyzed; in particular, as expected, phenols are present in considerable quantities (e.g. orcinol, $\beta$-orcinol, $\beta$-orcinolcarboxylic acid methyl ester). Their function in the algae corpus is to absorb the UV light in order to protect the organism from too intense radiation. Most interestingly, a large number of diterpenoids such as beyerene, rimuene, manool, isophyllocladene, manoyl oxide and phyllocladene itself, were present in only one sample that had collected on the walls of an ancient arch in the village of Crognaleto. These compounds have been reported as typical of many lichen species but, in our opinion, on the basis of the lichen's ability to absorb exogenous substances, they could also reflect the peculiar environment where the organism is located. The village of Crognaleto, in fact, is on high mountains, close to woods that constitute a continuous source of terpenes. Phytol is also always present.

The class of steroids is generally represented by ergosta-5,7,9(11),22-tetraen-3$\beta$-ol and by cholesta-3,5-diene. In addition, it is worth emphasizing the presence of parietin, usnic acid and norlichexanthone. Parietin is a bright yellow-orange to red anthraquinone pigment; anthraquinone derivatives are of great interest because many of them possess significant antibiotic, anti-protozoal and cytotoxic activities.[13] Usnic acid, a yellow dibenzofuran derivative, is a very important biologically active compound occurring in lichens; it possesses antibiotic properties,[14] acting as a strong inhibitor of Gram-positive bacteria,[15] and usually occurs in lichen cortices. Moreover, usnic acid, as well as some peculiar aliphatic and aromatic lichen acids,[2] are strong chelating agents that play an essential role for supplying lichens with minerals from the substrates. Norlichexanthone, a trioxygenated xanthone, also behaves as an antibiotic compound;[16] lichens which contain this compound are *Lecanora reuteri* and *Lecanora straminea*.[17] Xanthones are widely distributed in some higher plant families, as well as in fungi and in lichens; they are of great taxonomic importance and pharmacological interest. Figure 3 shows the mass spectra of two representative lichen substances, parietin (**1**) and usnic acid (**2**).

Historically, mass spectrometry has been a technique widely used for the identification and structural determination of lichen compounds; the results of these studies, as well as a treatment of the mass fragmentation patterns of some typical compounds, were extensively reviewed in 1996 by Huneck and Yoshimura.[2] An original technique, which preceded our study, was developed in the late 1960s by Santesson;[18] it consisted of introducing a small lichen sample directly into the MS source. In this method the identification of the lichen substances was obviously greatly complicated

**Table 1.** Sample 'Crognaleto'; assignment of peaks in TIC chromatogram (Fig. 1)

| Figure ref. | Peak assignment | CAS number | Figure ref. | Peak assignment | CAS number |
|---|---|---|---|---|---|
| **1** | Furfuryl alcohol | 98-00-0 | **40** | Branched alkane (C16) | |
| **2** | Xylene[a] | | **41** | 2,5,6-Trimethyl-3-methoxyphenol | 34883-03-9 |
| **3** | 2-Cyclopentene-1,4-dione | 930-60-9 | **42** | Linear alkene (C16) | |
| **4** | 2-Methyl-3-hexanone | 7379-12-6 | **43** | Linear alkane (C16) | |
| **5** | *n*-Heptanal | 111-71-7 | **44** | Saturated alcohol ($C \geq 14$) | |
| **6** | 2-Acetylfuran | 1192-62-7 | **45** | 3,5-Dimethoxy-4-hydroxyacetophenone | 2478-38-8 |
| **7** | Cyclopentanone | 120-92-3 | **46** | 3,4-Diethoxybenzaldehyde | 2029-94-9 |
| **8** | 1,1,3-Trimethylcyclopentane | 4516-69-2 | **47** | 5,6,7,7a-Tetrahydro-4,4,7a-trimethyl-2(4*H*)-benzofuranone | 15356-74-8 |
| **9** | Benzaldehyde | 100-52-7 | **48** | 2,5-Dimethoxy-4-methylbenzaldehyde | 4925-88-6 |
| **10** | 5-Methylfurfural | 620-02-0 | **49** | Linear alkene (C17) | |
| **11** | Phenol | 108-95-2 | **50** | Linear alkane (C17) | |
| **12** | 1-Acetyl-1*H*-imidazole | 2466-76-4 | **51** | 2,4-Dimethoxy-6-methylbenzaldehyde | 7149-90-8 |
| **13** | 2-Pentylfuran | 3777-69-3 | **52** | Saturated aldehyde ($C \geq 14$) | |
| **14** | Linear alcohol | | **53** | Branched alkane (C18) | |
| **15** | 2-Formylpyrrole | 1003-29-8 | **54** | 6,8-Heptadecadiene | |
| **16** | Cresol[a] | | **55** | Linear alkene (C18) | |
| **17** | 2,6-Dimethyl-3-octene | 6874-28-8 | **56** | Linear alkane (C18) | |
| **18** | 3-Methylbenzaldehyde | 620-23-5 | **57** | Methyl ester of ß-orcinolcarboxylic acid | 4707-47-5 |
| **19** | Orcinol | 504-15-4 | **58** | Linear alkane (C19) | |
| **20** | 7-Methyl-4-octanone | 20809-46-5 | **59** | Isopropyl myristate | 110-27-0 |
| **21** | Long-chain alcohol ($C \geq 8$) | | **60** | Neophytadiene | 504-96-1 |
| **22** | 4,4-Dimethyl-3-methylenedihydrofuran-2-one | 65371-43-9 | **61** | 6,10,14-Trimethyl-2-pentadecanone | 502-69-2 |
| **23** | 2-Acetyl-2-hydroxy-$\gamma$-butyrolactone | 135366-64-3 | **62** | Acetylphytol | 1713612[b] |
| **24** | Linear alkene (C11) | | **63** | Phytol | 150-86-7 |
| **25** | ß-Orcinol | 488-87-9 | **64** | Linear alkane (C20) | |
| **26** | *N*-Furfurylpyrrole | 1438-94-4 | **65** | Norlichexanthone | 20716-98-7 |
| **27** | Unsaturated linear alcohol | | **66** | Beyerene | 3564-54-3 |
| **28** | 5-Hydroxymethyl-2-furaldehyde | 67-47-0 | **67** | Rimuene | 1686-67-5 |
| **29** | Linear alkene (C14) | | **68** | Manool | 596-85-0 |
| **30** | 4-Hydroxybenzaldehyde | 123-08-0 | **69** | Isophyllocladene (kauren-15-ene) | 511-85-3 |
| **31** | 1,1,6-Trimethyl-1,2-dihydronaphthalene | 30364-38-6 | **70** | Manoyl oxide | 596-84-9 |
| **32** | 2,2,3-Trimethylchromene | 38177-45-6 | **71** | Phyllocladene (kauren-16-ene) | 20070-61-5 |
| **33** | Linear alkane (C14) | | **72** | Linear alkane (C22) | |
| **34** | 2,3-Dimethyl-5-methoxyphenol | 34883-01-7 | **73** | Linear alkene (C24) | |
| **35** | Linear alkane (C15) | | **74** | Linear alkane (C24) | |
| **36** | Unsaturated linear alcohol | | **75** | Linear alkane (C26) | |
| **37** | 1,2-Dimethylnaphthalene | 573-98-8 | **76** | Linear alkane (C27) | |
| **38** | Ethylhydroquinone | 2349-70-4 | **77** | Linear alkane (C28) | |
| **39** | 2-tert-Butyl-4-methoxyphenol | 921-00-6 | **78** | Linear alkane (C29) | |

[a]Isomers.
[b]Beilstein Registry Number.

by the complexity of the matrix and by the absence of any previous separation. In our study of the analysis of lichen substances, the SPME technique coupled with GC/MS appears greatly advantageous in terms of sample handling, analysis procedures and ease of mass spectral interpretation. Lengthy and tedious sample preparations are avoided, thus allowing a rapid identification of the compounds of interest and also eventually lichen classification. The relatively large

**Table 2.** Cumulative list of compounds present in the samples 'S. Stefano', 'Cervaro', 'Barisciano', and 'Castel del Monte', other than those already listed for the sample 'Crognaleto' in Table 1

| Figure ref. | Peak assignment | CAS number | Figure ref. | Peak assignment | CAS number |
|---|---|---|---|---|---|
| **1a** | 2,3,4-Trithiapentane (dimethyltrisulfide) | 3658-80-8 | **2b** | Usnic acid | 7562-61-0 |
| **2a** | 2-Octylfuran | 4179-38-8 | **1c** | Methylheptane | |
| **3a** | 3-Hydroxy-1,1,6-trimethyl-1,2,3,4-tetrahydro-naphthalene | | **2c** | 2-(3*H*)-Furanone-5-ethyldihydro-$\gamma$-caprolactone | 695-06-7 |
| **4a** | 3-Methyl-2-(3,7,11-trimethyldodecyl)furan | | **3c** | 3-Decanone | 928-80-3 |
| **5a** | Parietin | 521-61-9 | **4c** | Athranol | 487-69-4 |
| **6a** | Cholesta-3,5-diene | 747-90-0 | **5c** | 5-Hydroxy-7-methoxy-2-pentylchromone | 6972486[a] |
| **7a** | Ergosta-5,7,9(11),22-tetraen-3$\beta$-ol | 85798-12-5 | **1d** | 3,4,5-Trimethylheptane | 20278-89-1 |
| **1b** | Usnetol | 21987-07-5 | **2d** | 2-Formyl-5-methylpyrrole | 1192-79-6 |

[a]Beilstein Registry Number.

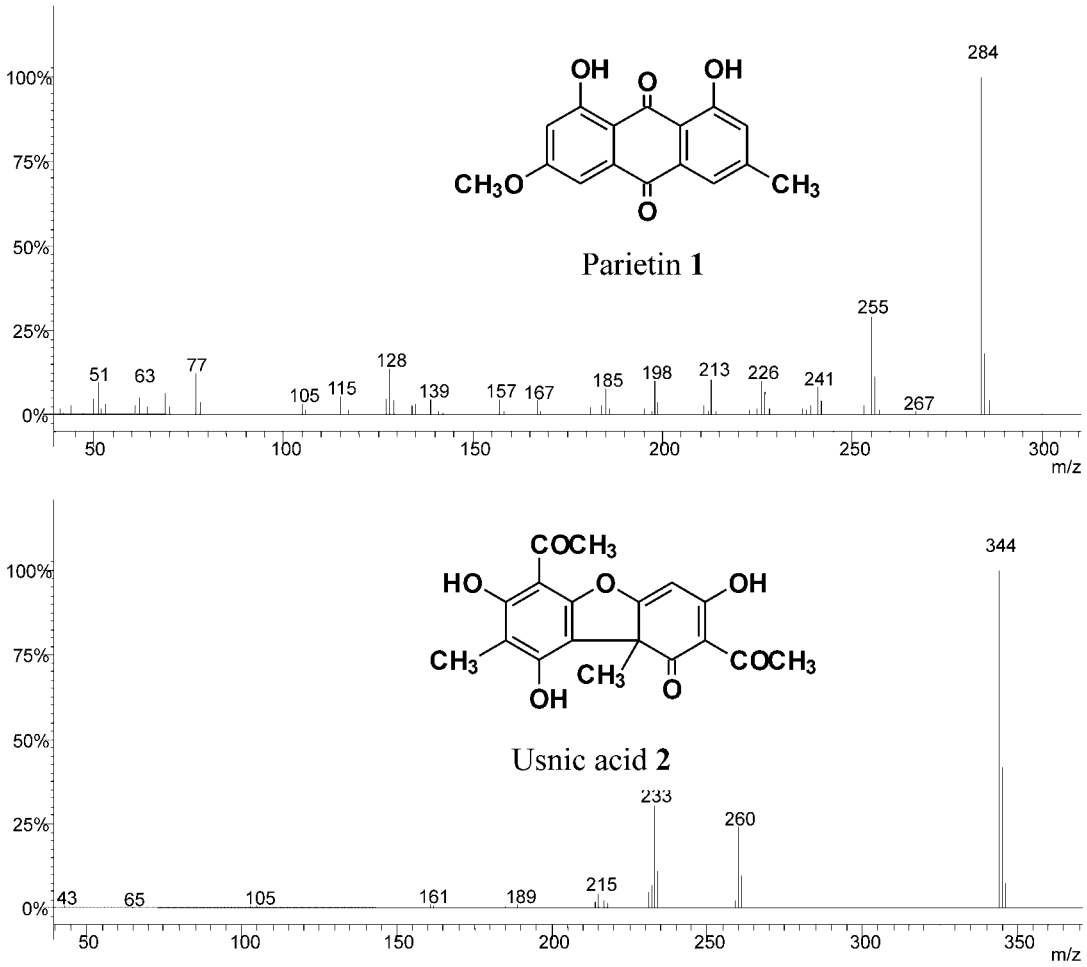


**Figure 3.** EI mass spectra of (a) parietin (**1**) and (b) usnic acid (**2**).

quantities of solvent needed for the classical extraction procedure are no longer necessary. This reflects the absence of possible extraneous compounds derived from the solvents, and is also beneficial with respect to the signal-to-noise ratios in the GC chromatograms and mass spectra. In general, in fact, large unresolved humps appear in the gas chromatograms of the solvent extracts.

## CONCLUSIONS

Our methodology, compared with the solvent extraction procedures, has the advantage of high rapidity of sample preparation, lack of any possible presence of contaminants, and the use of very small amounts of material. The main disadvantage, with respect to the complete identification of the sampled organic material, appears to be the limited sampling of the less-volatile compounds; nevertheless, it is worth emphasizing that metabolites with molecular weights higher than 400 Da were identified without problems in the present work. Also, as to the quantitative aspects of the analyses carried out by the SPME sampling technique, it should be pointed out that the areas of the chromatographic peaks are only partially related to the concentrations of the compounds in the lichen sample. Volatility of the analytes, as well as their relative affinities for the fiber coating, affect to a certain extent the quantitative response.

The analyses can be routinely performed on lichens present on precious substrates like works of art. In such cases, demonstration of the presence of aggressive substances deposited on these objects can give indications on the appropriate restoration procedures. Also, following our methodology, chemical criteria for the taxonomy of lichens can be exploited.[2,19] Finally, the development of a routine procedure for monitoring air pollution in cities and industrialized areas, which makes use of lichens as bioindicators and SPME-GC/MS as the analytical tool, is under study in our laboratories.

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