Symbiosis, 11 (1991) 193–206
Balaban, Philadelphia/Rehovot

Direct Analysis of Lichens by Tandem Mass Spectrometry

R. TABACCHI, P. ALLEMAND and G. TSOUPRAS
Institut de Chimie de L'Université, Av. de Bellevaux 51, CH-2000 Neuchâtel, Switzerland
Tel. 004138252815, Fax 004138242695

Received April 24, 1990; Accepted November 21, 1990

Abstract

Until now all chemical analysis of lichens has been carried out using classical techniques: extraction, separation and identification.

Tandem mass spectrometry renders the first two phases unnecessary. Using this method one analyses the ions produced when the lichen is placed directly in the source. The advantages of this technique are obvious, the use of a very small amount of plant material and rapid analysis. The technical aspect of the technique poses certain problems. The presence in the lichen of a small number of substances in large quantity and a number of others in small amounts means that a direct analysis is difficult. The facile fragmentation of typical lichen components (depsidones and depsides) leads to a large number of low mass fragments and very weak parent ions. This is especially true in EI. In the positive CI mode, ions which can be used in MS-MS experiments are rare. In negative CI the results are more promising but no systematic study exists of the application of this soft ionisation technique to even the best known lichen products. Experimental conditions are also of great importance.

The comparison of the results obtained on direct analysis by MS-MS and by classic extraction methods of the lichens: *Pseudevernia furfuracea* (L.) Ach. and *Lobaria pulmonaria* (L.) Hoffm. shows the advantages, the disadvantages and the limits of this technique.

Keywords: MS-MS analysis, chemical ionisation, depsides, depsidones, *Pseudevernia furfuracea*, *Lobaria pulmonaria*

Abbreviations: EI = electron impact, CI = chemical ionisation, NCI = negative chemical ionisation, CID = collision induced decomposition, MS-MS = tandem mass spectrometry, TLC = thin layer chromatography

1. Introduction

Lichens are organisms of very slow growth. In spite of being widely distributed, for certain species it is difficult, if not impossible, to obtain sufficient quantities of material to carry out an investigation on their composition by classical methods (i.e. extraction, isolation and identification). We have undertaken a study to assess whether direct analysis of lichens by MS-MS offers a rapid and sure alternative.

The perfection of this technique posed several problems. The presence in lichens of a small number of compounds in large quantities and numerous others in small or trace amounts renders direct analysis difficult. The facile fragmentation which some characteristic lichen components (depsides) undergo leads to a number of small abundant fragments and very weak parent ions which are lost in the background spectral noise. This is especially true in the EI mode. We observed for example in the case of *Cladonia* sp. the presence of fumarprotocetraric acid, a thermally unstable compound which fragments rapidly, which meant that we were unable to observe the other components. Better results are obtained in the positive CI mode (Harrison, 1983), but ions which can be used in MS-MS studies are rare. Quasimolecular ions were observed but the CID spectra gave no useful fragmentation information. On the other hand, negative CI gave more promising results but unfortunately no systematic study has been made on the application of this soft ionisation technique to the more common lichen components (depsides, depsidones, depsones, triterpenes). This represents a considerable amount of work because the number of spectra to be measured is very large. Such a study needs to precede an MS-MS study of lichens. We thought that MS-MS could be used as a method of direct analysis of lichens using the different ionisation techniques in the same manner solvent systems that are used in TLC. The advantage of this method over TLC, the main method used today in chemo-taxonomy of lichens, would be the obtainment of clear reproducible and complete spectral data. It is obvious that the investment required for the two methods does not bear comparison. In spite of the difficulties due to lack of literature and data of negative CI spectra for the compounds to be studied, we carried out some experiments.

2. Materials and Methods

Organisms

The lichens examined were *Pseudevernia furfuracea* (L.) Ach. and *Lobaria pulmonaria* (L.) Hoffm.

Experimental

EI spectra were measured on Nermag R30-10 triple quadrupole mass spectrometer. Spectra were acquired under computer control (PDP-11/73). The pressure of the source was 7×10^{-6} Torr. The source temperature varied between 150° and 280°C. NCI spectra were measured on the same instrument using methane or ammonia as reagent gas. The collision energy for the CID spectra varied between 20 and 40 eV and the pressure of the collision gas (argon) was 0.5–1.1 10^{-1} atm.

3. Results

For the perfection of the method we first chose to study the MS-MS spectra of a series of typical compounds: atranorin, chloratranorin and methylbarbatate.

When a depside is analysed by NCI/MS, two main ions are formed: $M^{-\cdot}$ and $(M\text{-}H)^{-}$. An MS-MS study of these two ions showed that their fragments gave ions containing either the A or S ring. In the case of atranorin (Fig. 1), m/z 373 gave two fragments (m/z 195 and 178) from S and A rings respectively (Fig. 3), m/z 374 has two possible origins, $M^{-\cdot}$ or $(M\text{-}H)^{-}(^{13}C)$ (Fig. 2). Four fragments were observed from this peak, m/z 177, 195 and m/z 178, 196 which corresponded either to the same fragments containing ^{13}C, or to $S^{-\cdot}$ and A^{-}, (Fig. 2 and scheme Fig. 4). NCI/MS with thermalysed electrons (CH_4) gave only $M^{-\cdot}$ (Fig. 5) and leads mainly to m/z 178. A new ion (m/z 552) was formed by combination of an atranorin molecule with an S unit. This spectrum confirms that the other peaks in NCI-NH_3/CID-spectra were due mainly to the fragmentation of $(M\text{-}H)^{-}(^{13}C)$.

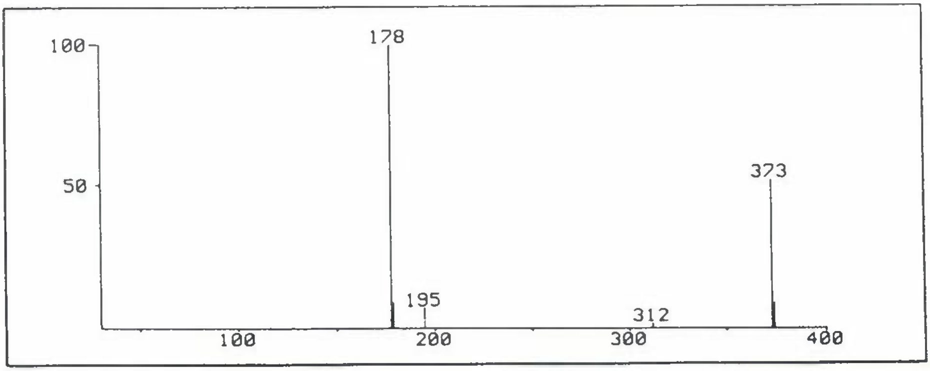


Figure 1. NCI (NH_3) spectrum of atranorin

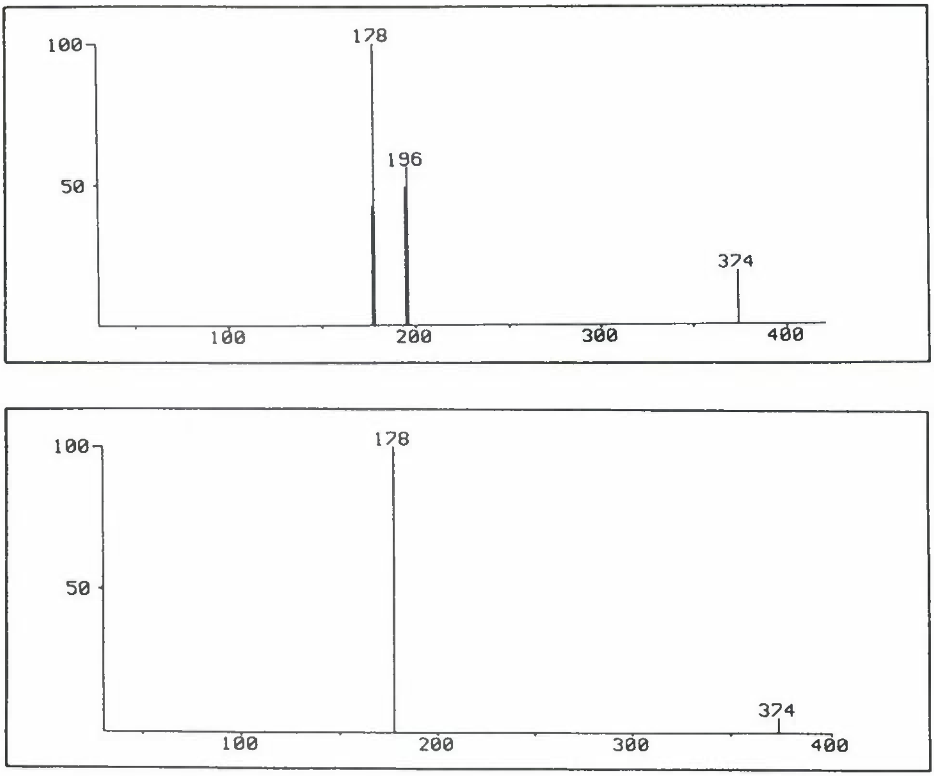


Figure 2. Daughter ions of m/z 374 and parent ion of m/z 178

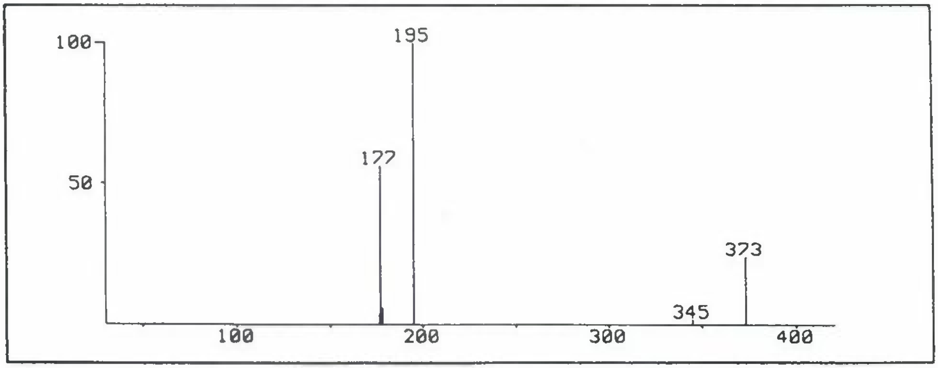


Figure 3. Daughter ions of m/z 373

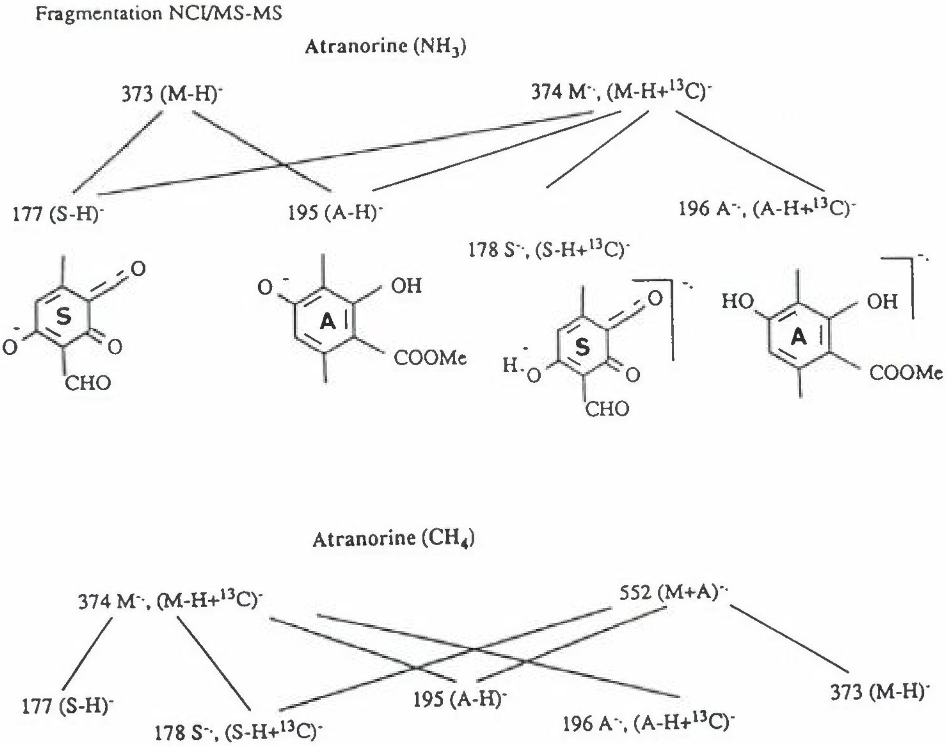


Figure 4. Fragmentation scheme of atranorin (NCI, MS-MS)

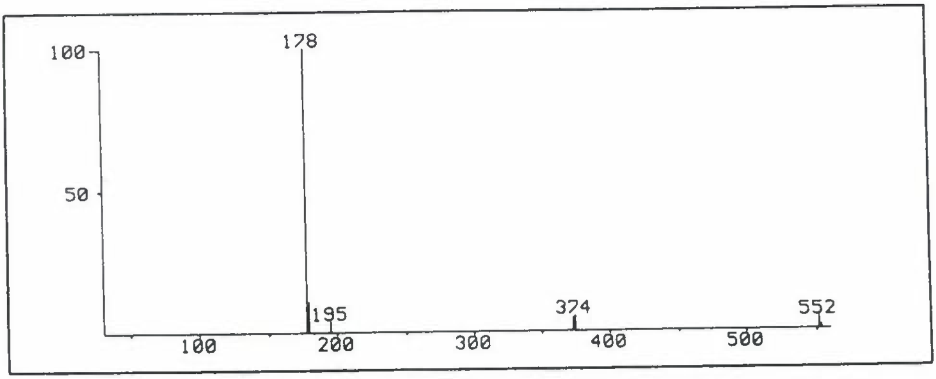


Figure 5. NCl (CH_4) spectrum of atranorin

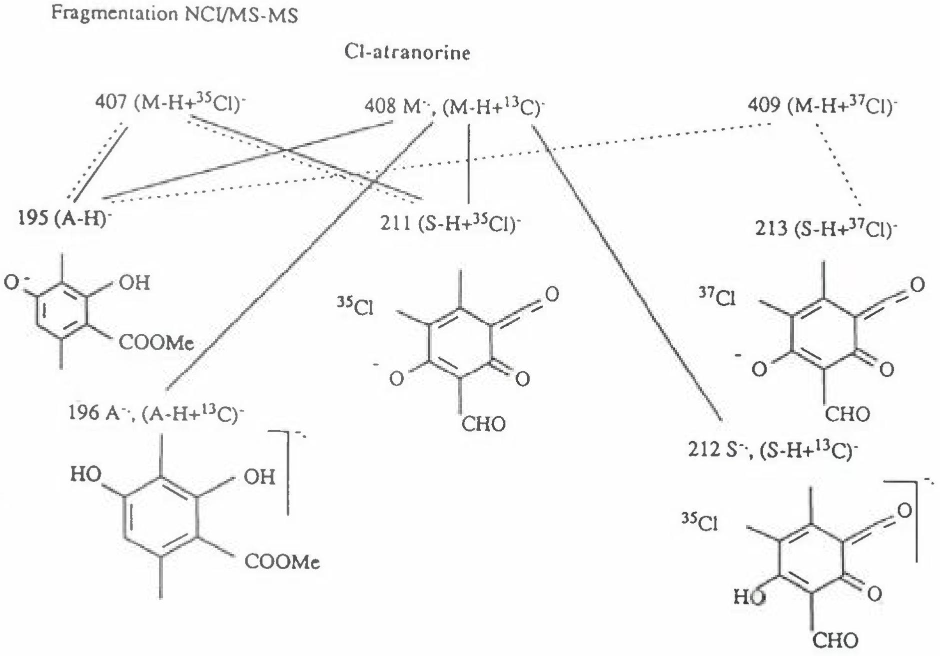


Figure 6. Fragmentation scheme of chloratranorin (NCI, NH$_3$ MS-MS)

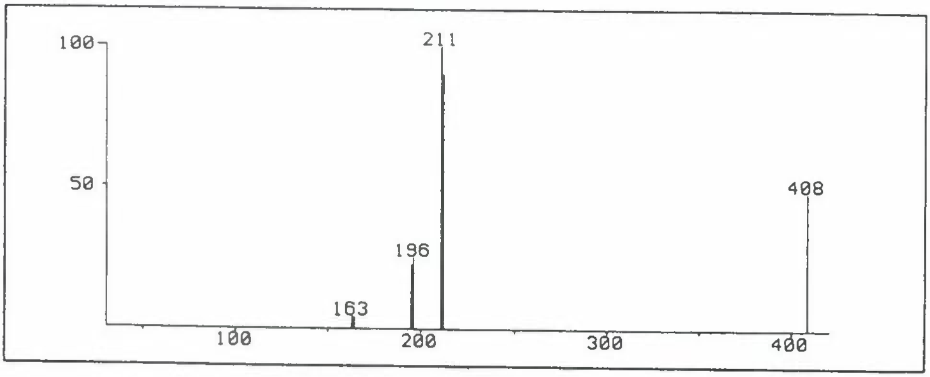


Figure 7. NCI (NH$_3$) spectrum of chloratranorin

The fragmentation pathways of chloratranorin (Fig. 6) were similar to those of atranorin. However, ionization by loss of a hydroxyl proton is favoured by their greater lability. Consequently, in this case m/z 408 was principally due to (M-H) $^{-}$(^{13}C) (Fig. 7). The spectrum of the daughter ions confirmed this through the relative intensities of the ions compared with the spectrum of daughter ions on m/z 374 from atranorin. Daughter ions of m/z 407 (^{35}chloratranorin) (Fig. 8) compared to daughter ions of m/z 409 (^{37}chloratranorin) (Fig. 9) confirmed that m/z 195 corresponds to cycle A and m/z 211 or m/z 213 to cycle S. Comparison of the MS-MS spectra of atranorin (Fig. 10) and methyl-barbatate (Fig. 11) gave useful information. Both compounds have the same molecular weight, but substitution of ring S is different. The substituents in methyl-barbatate were less electrophilic than those of atranorin. Thus, fragmentation of M$^{-\cdot}$ (m/z 374(CH$_4$))leads mainly to m/z 195

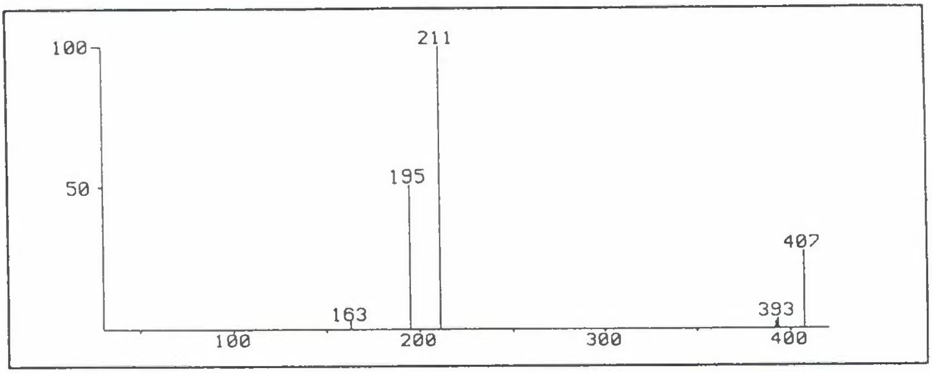


Figure 8. Daughter ions of m/z 407

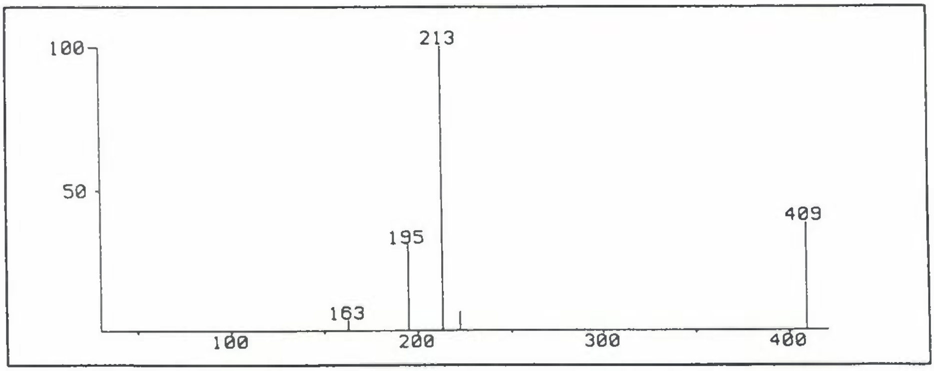


Figure 9. Daughter ions of m/z 409

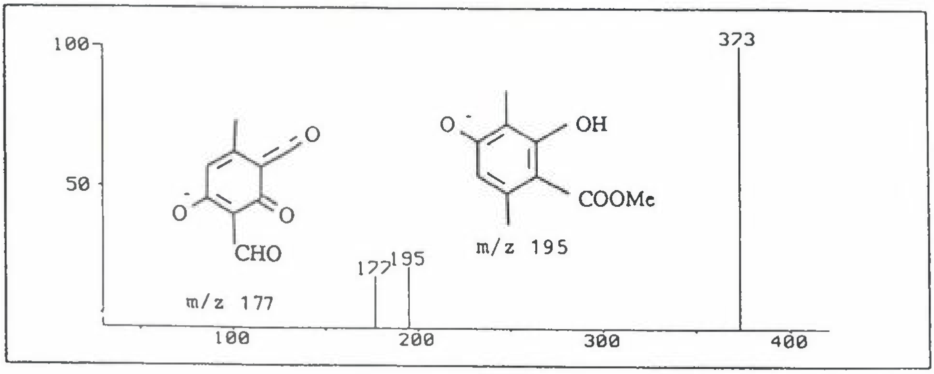


Figure 10. Daughter ions of m/z 373 of atranorin (NCI, CH_4)

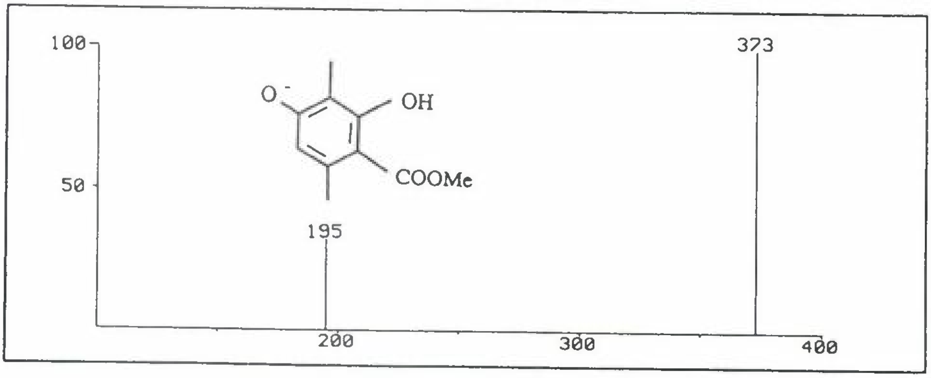


Figure 11. Daughter ions of m/z 373 of methyl barbatate (NCI, CH_4)

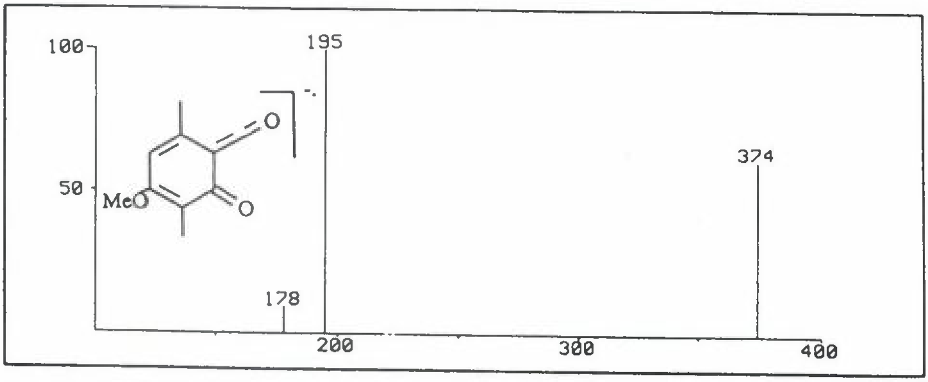


Figure 12. Daughter ions of m/z 374 of methyl barbatate (NCI, CH_4)

(Fig. 12). In the collision spectrum of m/z 373 ((M-H) $^-$/CH_4), no fragment from ring S was observed, because there is only one hydroxyl on this ring. For fragmentation, two are necessary, one for ionization $(M\text{-}H)^-$, and the other for the fragmentation transfer mechanism.

To illustrate the study of a lichen by MS-MS we chose as an example *P. furfuracea* whose chemical composition was already well known (Tabacchi et al., 1983; Gunzinger, 1985). This lichen had the advantage of containing several depsides and depsidones. In the NCI-MS (NH_3) of a small fragment (2 mg dry sample) of the lichen, directly introduced in the ionisation chamber of the MS-instrument, we recognized the following (M^--H) ions (Fig. 13):

We did not observe olivetoric acid (MW 472), also present in the lichen. The fragment of m/z 247 could be assigned to the S cycle of this depside, but also to the main daughter ion of physodic acid (1) (Fig. 14). Molecular, quasimolecular and M-H ions of this depsidone were absent. The loss of the C_5H_{11} chain led to m/z 354.

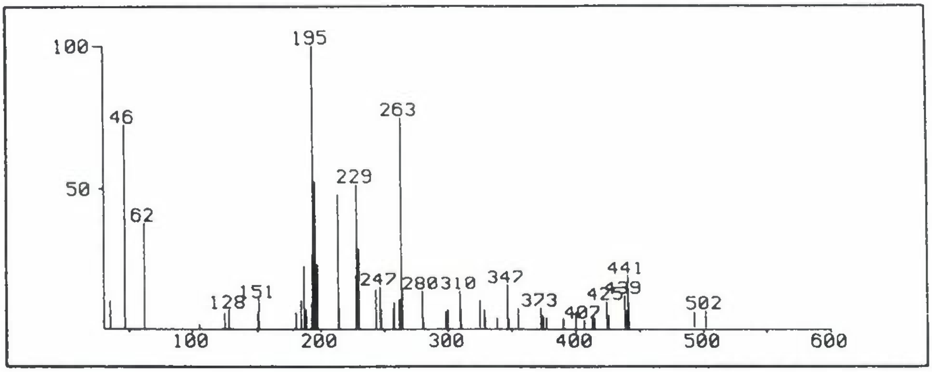
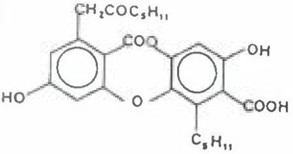


Figure 13. Total NCI-MS (NH_3) of *Pseudevernia furfuracea*

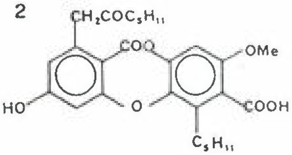
m/z	Substance
373	Atranorine
407	Chloratranorine
425	Physodic acid
439	2′-Methyl-O-physodic acid Isophysodic acid 2′-O-Methyl-physodone
441	3-Hydroxy-physodic acid

Isophysodic (4), 2′-O-methyl physodic acid (2) and 2′-O-methyl physodone (5) gave a common ion at m/z 439 (with a daughter ion at m/z 246) (Fig. 15). m/z 263 observed in the CID spectrum of m/z 241 of 3-hydroxy-physodic acid (3) (Fig. 16) corresponds to the hydroxylated m/z 247 ion of physodic acid.

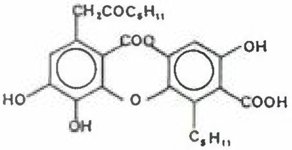
1



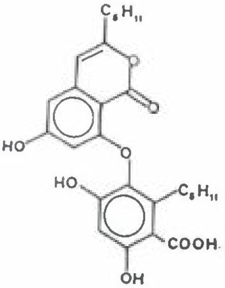
2



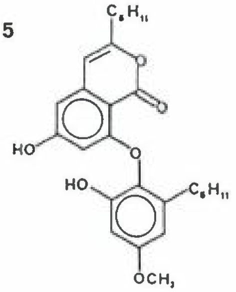
3



4



5



In studying *L. pulmonaria* we wanted to test our method of identification of a lichen (2 mg dry sample), by MS-MS, for which we had no data. As no library exists of the NCI spectra of lichen compounds we did not try to identify the constituent compounds of the lichen by this technique alone but rather compared the results thus obtained with those obtained by a rapid extraction and simple purification of the same material. The lichen (ca. 15 g, dry) was collected near Bern (Switzerland), powdered and then extracted with a MeOH-$CHCl_3$ (1:1) mixture. The extract was separated from either soluble and ether non-soluble fractions. The ether soluble fraction was column chromatographed (SiO_2) and the various fractions further purified by HPLC. Four products were thus obtained:

stictic acid
8′-O-methyl-stictic acid
2′-O-methyl-connorstictic acid
ergosterol peroxyde

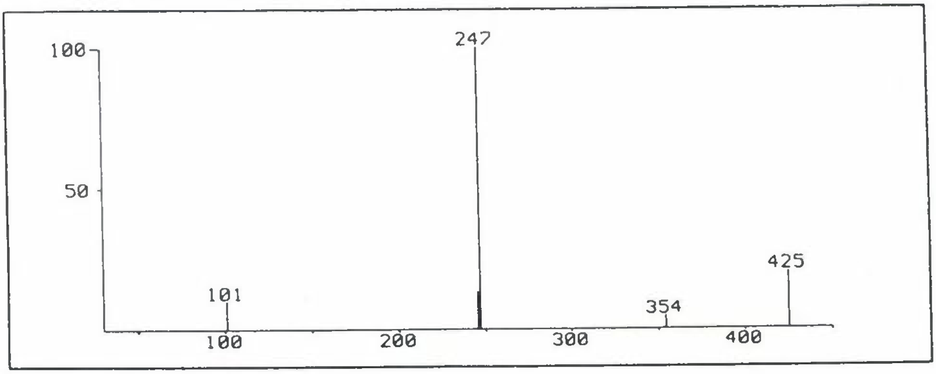


Figure 14. Daughter ions of m/z 425

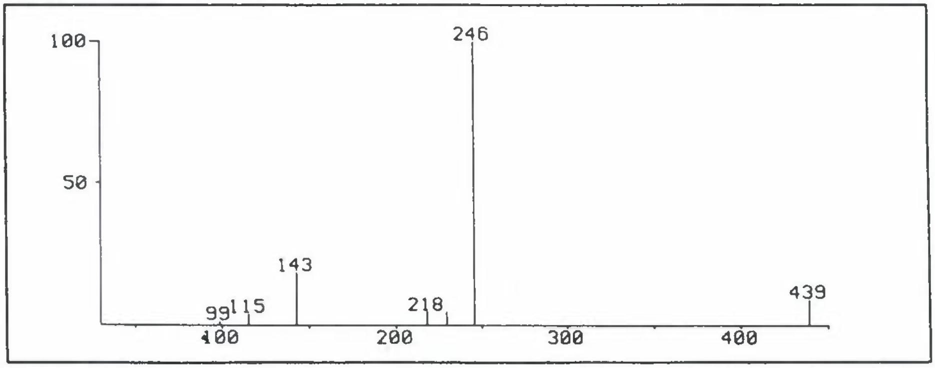


Figure 15. Daughter ions of m/z 439

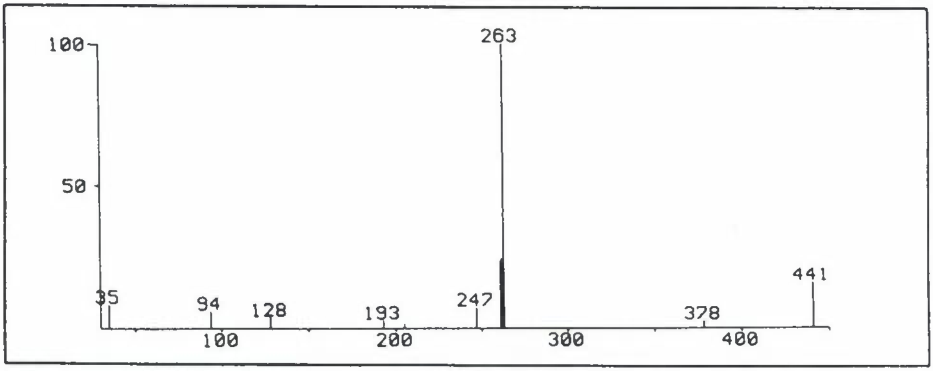


Figure 16. Daughter ions of m/z 441

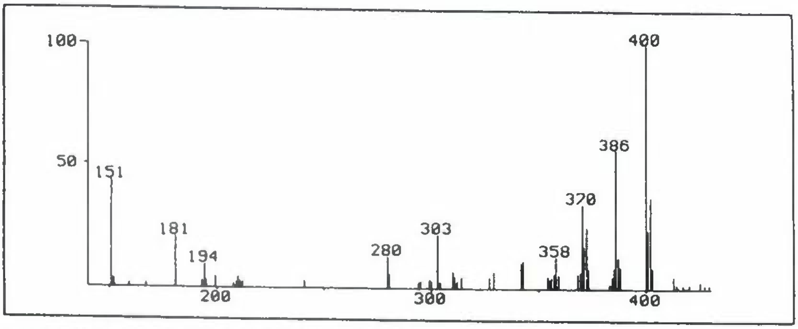


Figure 17. Total NCI-MS (NH_3) of *Lobaria pulmonaria*

The NCI-MS-MS study was relatively difficult to carry out (Fig. 17). We noticed small variations in the chemical composition depending on which part of the lichen (thallus, reproductive organs) was studied. This result could be of great interest as it opens a new field of research, i.e. the study of the variation of components in different parts of the lichen. To obtain meaningful results in this type of study it is more important that experimental conditions must be completely reproducible.

Without discussing the details of the M^- ion fragmentation, the spectra we obtained were very similar to those obtained under the same conditions (ionisation and collision energy, ammonia pressure) with the pure samples (Figs. 18, 19, 20).

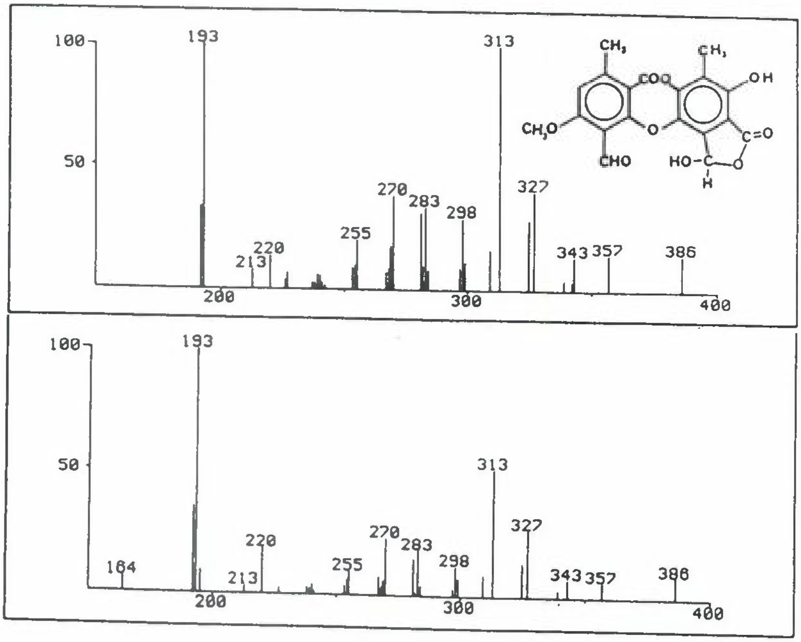


Figure 18. Daughter ions of m/z 386 from pure stictic acid and in the untreated lichen

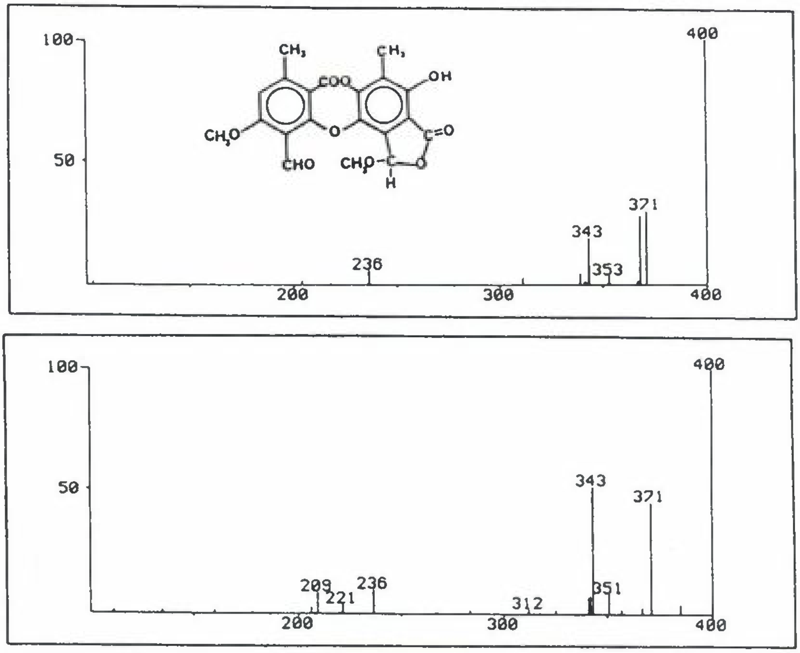


Figure 19. Daughter ions of m/z 400 from pure 8′-O-methyl-stictic acid in the untreated lichen

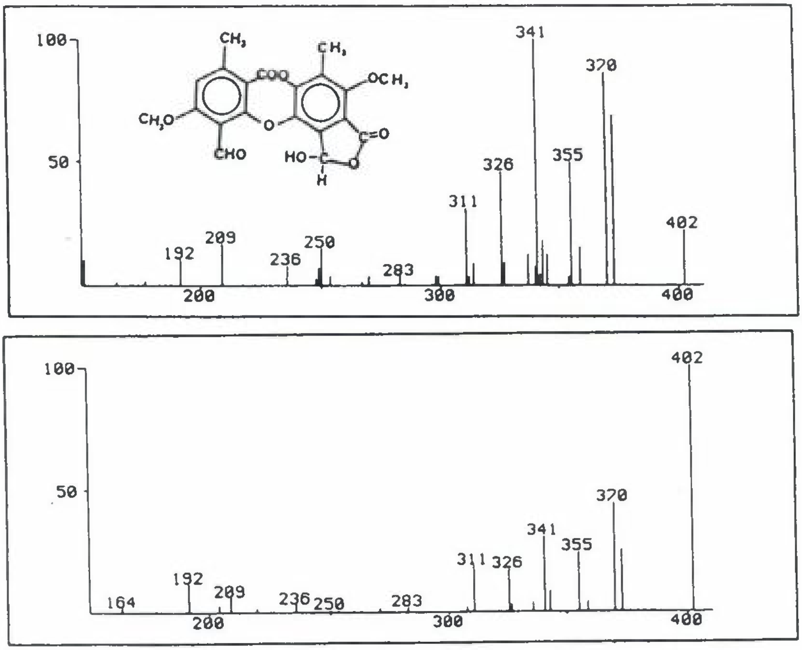


Figure 20. Daughter ions of m/z 402 from pure 2′-O-methyl-connorstictic acid in the untreated lichen

4. Conclusion

The classical analysis technique extraction, separation and identification is slower than direct analysis. Larger quantities of material and more manipulations are required. One of its great advantages is that pure compounds can be isolated which can then be fully characterised using spectroscopical techniques (UV, IR, NMR, MS). The use of GC-MS would no doubt reduce the length of analysis, but the mixture of products must be amenable to this technique and again proper spectroscopic analysis is precluded. Our results show that direct analysis by negative CI-MS-MS is rapid and can be carried out on very small quantities of material. For meaningful results several criteria must be fulfilled. These are linked to the nature of the compounds in the mixture or difficulties in finding optimal experimental conditions. The thermal instability and low volatility of the depsides and depsidones are major problems. Our results show that lichens can be analysed by direct NCI-MS-MS studies but in some cases extreme caution must be exercised in the interpretation of results. Use of chromatographic methods (TLC and HPLC) in parallel with MS-MS can be a very powerful tool for investigations on lichens.

Acknowledgements

We would like to thank the Swiss National Science Foundation (project 2.043-0.86) for the financial support and Dr. J. Gordon for stimulating discussion and reviewing the English text.

REFERENCES

Gunzinger, J. 1985. Etude de la composition chimique de *Pseudevernia furfuracea* (L.) Ach. Synthèse d'un depside et d'une depsidone. Thèse, Université de Neuchâtel.

Harrison, A.G. 1983. *Chemical Ionisation Mass Spectrometry.* CRC Press, Boca Raton, FL.

Tabacchi, R., Gunzinger, J., Clause, S.G. 1983 Contribution to the knowledges of *Pseudevernia furfuracea.* In: *Proceedings of the IXth International Congress of Essential Oils*, Singapore, 1983, Book 4, p. 65.