ELSEVIER

Journal of Chromatography A, 710 (1995) 273–285

JOURNAL OF CHROMATOGRAPHY A

Liquid chromatographic–thermospray mass spectrometric analysis of sesquiterpenes of *Armillaria* (Eumycota: Basidiomycotina) species

Peadar Cremin[a], Dervilla M.X. Donnelly[a], Jean-Luc Wolfender[b], Kurt Hostettmann[b,*]

[a]*Department of Chemistry, University College Dublin, Dublin 4, Ireland*

[b]*Institut de Pharmacognosie et Phytochimie, École de Pharmacie, Université de Lausanne, CH-1015 Lausanne, Switzerland*

First received 17 January 1995; revised manuscript received 26 April 1995; accepted 26 April 1995

Abstract

Conventional HPLC analysis of the sesquiterpene aryl esters present in the crude extracts of the pathogenic basidiomycete *Armillaria* is complicated by difficulties in identification solely on the basis of retention time. On-line coupling of liquid chromatography with thermospray mass spectrometry (LC–TSP-MS) provides a means for their direct detection. Crude extracts of seven *Armillaria* species were analysed providing a semi-quantitative survey of sesquiterpene aryl ester production within the genus. The distribution of these metabolites and its significance with regard to species differentiation and pathogenicity are discussed. Unknown components were also detected by on-line LC–TSP-MS screening of extracts, facilitating a targeted isolation route.

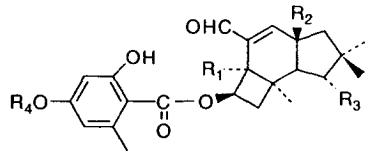
1. Introduction

The pathogenic basidiomycete *Armillaria* is the cause of widespread root disease in deciduous and coniferous trees and can also exist as a saprophyte, particularly on tree stumps [1]. Five European and 20 worldwide species have been classified with virulence varying widely from species to species and within strains of the same species [2,3]. Our previous phytochemical examinations led to the isolation of several biologically active sesquiterpene aryl esters from *Armillaria* species [4]. These metabolites possess varying antibiotic and antifungal activity and comprise three major structural types represented by armillyl orsellinate (J), melleolide (I) and armillane (F) (Fig. 1). The structural similarity of this series of sesquiterpene aryl esters suggests a common biosynthetic pathway. However, the role played by each of these metabolites in this pathway and their relationship to pathogenicity within the genus are of interest.

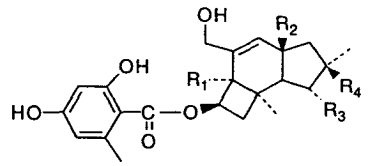
An initial study of the distribution of sesquiterpene aryl esters within the genus involved a high-performance liquid chromatographic (HPLC) analysis of crude extracts of five *Armillaria* species [5]. This led to the tentative linking of higher concentrations of certain sesquiterpenes to virulent strains. However, retention time data alone are insufficient for the identification of the sesquiterpenes of interest because of

* Corresponding author.

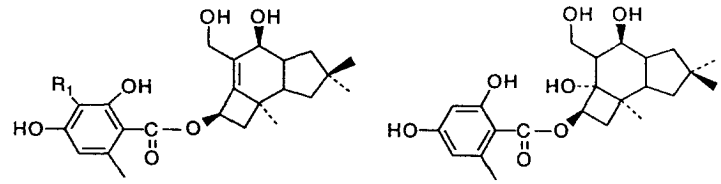
0021-9673/95/$09.50
SSDI 0021-9673(95)00514-5



	R_1	R_2	R_3	R_4
A : melledonal	OH	OH	OH	H
C : 5'-methoxy melledonal	OH	OH	OH	OCH_3
D : 13-hydroxy-4-methoxymelleolide	OCH_3	OH	H	H
I : melleolide	OH	H	H	H
K : 4-methoxy melleolide	OCH_3	H	H	H
M : 4-dehydromelleolide	H	H	H	H



	R_1	R_2	R_3	R_4
B : 13-hydroxhdihydromelleolide	OH	OH	H	H
E : 14-hydroxydihydromelleolide	OH	H	H	OH
G : 10-hydroxydihydromelleolide	OH	H	OH	H
L : dihydromelleolide	OH	H	H	H
H : 4-dehydro-14-hydroxydihydromelleolide	H	H	H	OH
N : 4-dehydrodihydromelleolide	H	H	H	H



	R_1	
J : armillyl orsellinate	H	F : armillane
O : armamiol	Cl	

Fig. 1. Structures of the sesquiterpene aryl esters studied.

interference between the main sesquiterpenes and minor components.

Here we report on the liquid chromatographic–thermospray mass spectrometric (LC–TSP-MS) analysis of a standard mixture of 15 sesquiterpene aryl esters and crude extracts of seven *Armillaria* species (Table 1). This allowed a rapid semi-quantitative survey of sesquiterpene distribution within the genus. On-line coupling of liquid chromatography with ultraviolet spectrophotometry (LC–UV) and with mass spectrometry (LC–MS) in the analysis of crude plant extracts provides important structural information on metabolites directly in their biological matrices [6,7].

2. Experimental

2.1. Chemicals

HPLC-grade water was prepared by distillation on a Büchi (Flawil, Switzerland) Fontavapor 210 distillation instrument and passed through a 0.50-μm filter (Millipore, Bedford, MA, USA). HPLC-grade methanol from Maechler (Reinach,

Table 1
Armillaria genus: species and strains investigated

Species	Pathogenicity[a]	Host range	Strains examined[b]
Armillaria mellea	Pathogenic	Broadleaved, conifers, vines, fruit trees and shrubs	UCD 520, UCD 618, UCD 619, UCD 622, UCD 662, UF IM-CT-91, UF IM-SR-VIR, UF IM-QG-SAP
Armillaria ostoyae	Moderately pathogenic	Conifers (associated with fir species in Europe)	UCD 663, UCD 664, UCD 666, UCD 667, CBS 434.72
Armillaria tabescens (European)	Non-pathogenic	Broadleaved (associated with oak species in Europe)	UF IM-TAB-1, UF IM-TAB-202, UF IM-TAB-303, INRA PT 89.92, INRA PT 83.39, INRA PT 90.5
Armillaria monadelpha (*Armillaria tabescens*, N. American)	Pathogenic	Broadleaved, conifers, fruit trees and shrubs	CBS 132.72, CBS 129.26
Armillaria gallica	Non-pathogenic	Broadleaved (associated with oak species in Europe)	UF 303
Armillaria cepestipes	Non-pathogenic	Broadleaved and conifers	UF 505
Armillaria novae-zelandiae	Moderately pathogenic	Broadleaved (associated with species of southern beech)	CBS 432.72
Unknown	Unknown	Unknown	UF A79

[a] Based on widespread distribution and ability to cause disease in natural forests, plantations and amenity planting [1,3].
[b] Strains examined were from the following collections: UCD = Department of Botany, University College Dublin, Dublin 4, Ireland; UF = Dr. M. Intini, Patologia Vegetale, Università di Firenze, Florence, Italy; CBS = Centraalbureau voor Schimmelcultures, Baarn, Netherlands; INRA = Professor J.J. Guillaumin, INRA, Clermont-Ferrand, France.

Switzerland) was passed through a 0.45-μm filter. Ammonium acetate was obtained from Merck (Darmstadt, Germany). DIFCO potato dextrose broth was obtained from DIFCO Laboratories (Michigan, USA). Merck Kieselgel 60 and Sephadex LH-20 were used as stationary phases for open-column chromatography.

2.2. HPLC conditions

Separations were performed on a Hypersil ODS 5 column (250 × 4.66 mm I.D.) equipped with a Nova-Pak Guard precolumn. A gradient of methanol–water from 75:25 to 90:10 in 30 min (1 ml/min) was used.

2.3. LC–UV analyses

Eluent delivery was provided by an LC-9A HPLC pump (Shimadzu, Tokyo, Japan) equipped with an FCV-9AL low-pressure mixing valve and a Model 7125 injection valve with a 20-μl loop (Rheodyne, Cotati, CA, USA). UV spectra were recorded with an HP-1040A photodiode-array detector and the data were processed on an HP-1090 Chemstation (Hewlett-Packard, Palo Alto, CA, USA).

2.4. LC–TSP-MS analyses

A Finnigan MAT (San Jose, CA, USA) TSQ-700 triple quadrupole instrument equipped with a TSP 2 interface was used for the data acquisition and processing. The temperatures of the TSP were source block 200°C, vaporizer 95°C and aerosol 200–220°C (beginning–end of gradient). The electron multiplier voltage was 1800 V, dynode 15 kV and the filament and discharge were off. Full-scan spectra from m/z 140 to 600 in the positive-ion mode were obtained (scan

time 1.2 s). Concerning the LC part, the eluent delivery was provided by a 600-MS HPLC pump (Waters) equipped with a gradient controller. The UV trace was recorded on-line with a Waters 490-MS programmable multi-wavelength detector. Postcolumn addition of buffer (0.5 *M* ammonium acetate) was achieved with a Waters 590-MS programmable HPLC pump (0.2 ml/min) using a simple tee junction (Waters).

2.5. Samples

Sesquiterpene aryl esters A–O were obtained from previous isolation work and standard solutions prepared by dissolving 0.2 mg of each in 1 ml of methanol. Each strain of the *Armillaria* species listed in Table 1 was grown on DIFCO potato dextrose broth in 12 × 1 l Roux flasks (each containing 200 ml). After incubation in the dark for 35 days at 24°C, these were harvested by filtration. The mycelium was macerated in methanol (2 × 300 ml), evaporated to dryness and then partitioned into chloroform–methanol–water (65:35:20) (2 × 1500 ml). Evaporation of the combined chloroform layers yielded the crude mycelial extract. The culture broth was extracted with hexane (3 × 200 ml) followed by ethyl acetate (4 × 300 ml). Evaporation of the combined ethyl acetate layers yielded the crude culture broth extract. Solutions to be analysed were prepared by dissolving 30 mg of these crude extracts in 1 ml of methanol. The injection volume was 10 μl.

2.6. Armillaria novae-zelandiae samples

Gel filtration on Sephadex LH-20 (methanol) of 4.733 g of the mycelial extract of *Armillaria novae-zelandiae* (CBS 432.72) yielded five fractions. Fraction II (4.034 g) was chromatographed on silica gel with gradient elution from 100:1 to 10:1 chloroform–methanol. This yielded five sub-fractions. Analysis of these sub-fractions was achieved by dissolving 20 mg in 1 ml of methanol with an injection volume of 10 μl.

3. Results

3.1. LC–UV photodiode-array detection

Reversed-phase HPLC utilizing gradient elution with methanol–water was used to separate the 15 sesquiterpene aryl esters A–O (Fig. 1). This separation was monitored by on-line LC–UV photodiode-array detection (Fig. 2), which allows the measurement of the UV spectrum (170–400 nm) of each component eluted. These metabolites possess an identical basic aryl chromophore which renders on-line LC–UV detection ineffective for their differentiation. This structural similarity also leads to similar retention time characteristics and co-elution, render-

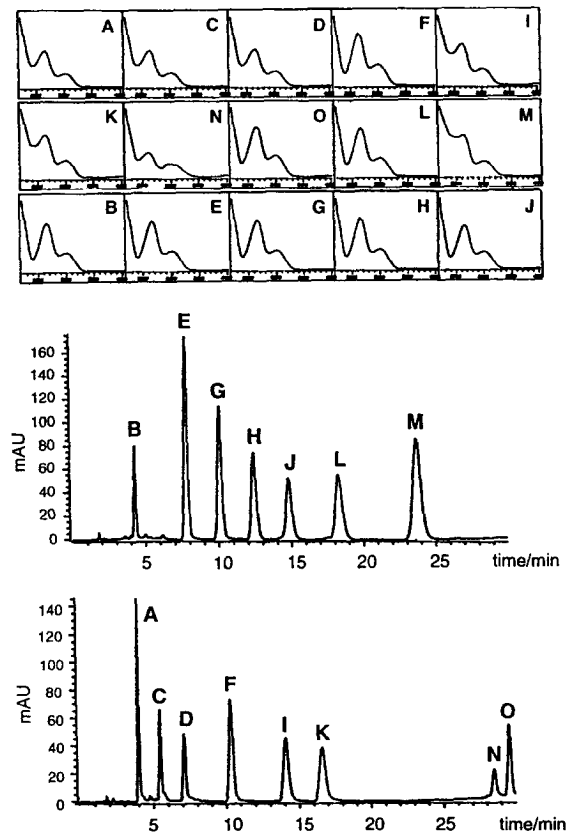


Fig. 2. Gradient HPLC–UV trace for sesquiterpene aryl esters A–O. For conditions, see Experimental. UV traces were recorded at 254 nm with UV spectra recorded from 170 to 400 nm. For peak identities, see Fig. 1.

ing some members of this group indistinguishable by conventional HPLC detection methods.

3.2. LC–MS thermospray tuning

LC–TSP-MS is a soft ionization technique that leads to the formation of adduct ions such as $[M+H]^+$ with molecules. These adduct ions allow the rapid determination of the molecular mass of a component directly after elution from the LC column. Sesquiterpene aryl esters are thermolabile and hence the ability to observe the adduct ions produced is a function of the temperatures set for the thermospray interface. The effect of variation of these parameters was investigated in the positive-ion mode with a solution of armillyl orsellinate (J: M_r 402) in methanol. Total ion current (TIC) and characteristic ion intensities as a function of ion source block temperature and as a function of thermospray vaporizer temperature were measured.

First the mass spectrum of armillyl orsellinate was measured at a source temperature of 200°C and a vaporizer temperature of 90°C for the purpose of determining the characteristic ions of this class of metabolites (Fig. 3). This showed an intense $[M+NH_4]^+$ ion as the base peak. The soft ionization characteristics of TSP-MS lead to a lack of fragmentation and the most intense fragment ions appear at m/z 385 (loss of water) and m/z 217 (loss of aryl group leading to protoilludane fragment ion). These characteristic ions were used to examine the dependence of the mass spectrum of armillyl orsellinate on the TSP-MS parameters. The variation in TIC and characteristic ion intensities as a function of ion source block temperature is illustrated in Fig. 4a. It is observed that although the TIC reaches a maximum at 240°C, at this temperature the base peak is at m/z 217, the protoilludane fragment ion, whereas below 220°C, the base peak is at m/z 420, the ammmoniated adduct of the molecular ion. A temperature of 200°C was then selected to maximize the response for the latter ion. Variation in TIC and characteristic ion intensities as a function of vaporizer temperature are shown in Fig. 4b. This plot indicates that the best ion intensities for the ammoniated ion are to

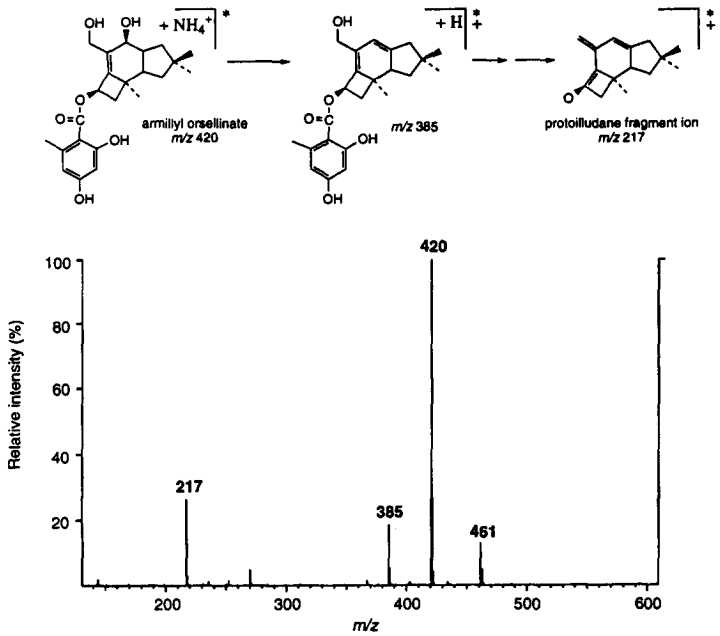


Fig. 3. LC–TSP mass spectrum of armillyl orsellinate (J).

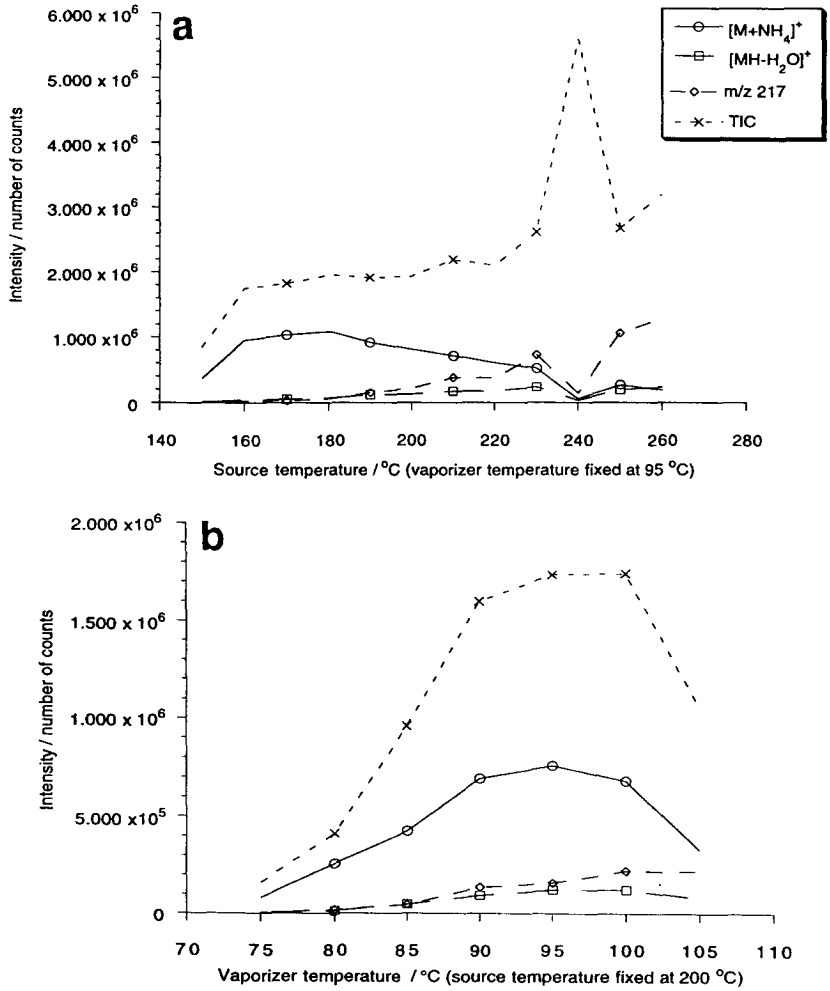


Fig. 4. Influence of (a) ion source temperature and (b) vaporizer temperature on the adduct molecular and fragment ions of armillyl orsellinate.

be obtained at a setting of 95°C. The mass spectrum of armillyl orsellinate was also recorded at different repeller potential values 0–200 V, with only a small variation in intensities observed at extreme values, i.e., less than 20 V or greater than 160 V. The repeller was then set to 100 V. Ammonium acetate was added as postcolumn buffer as this led to an increased intensity for the recorded ions. No improvements were observed using the filament-on or discharge-on modes under these conditions and the analysis was achieved in the filament-off mode.

3.3. LC–MS detection of sesquiterpene aryl esters

Fig. 5 shows the TIC and UV traces for a standard solution of eight of these sesquiterpene aryl esters and also the selected-ion monitoring traces for their adduct ions. A mass of 2 μg of each sesquiterpene was contained in the injection. The chromatographic characteristics and the positive-ion TSP-MS results for the 15 sesquiterpene aryl esters A–O are given in Table 2. The adduct ion $[M + NH_4]^+$ appears as the base peak in the TSP mass spectra for 13 of the

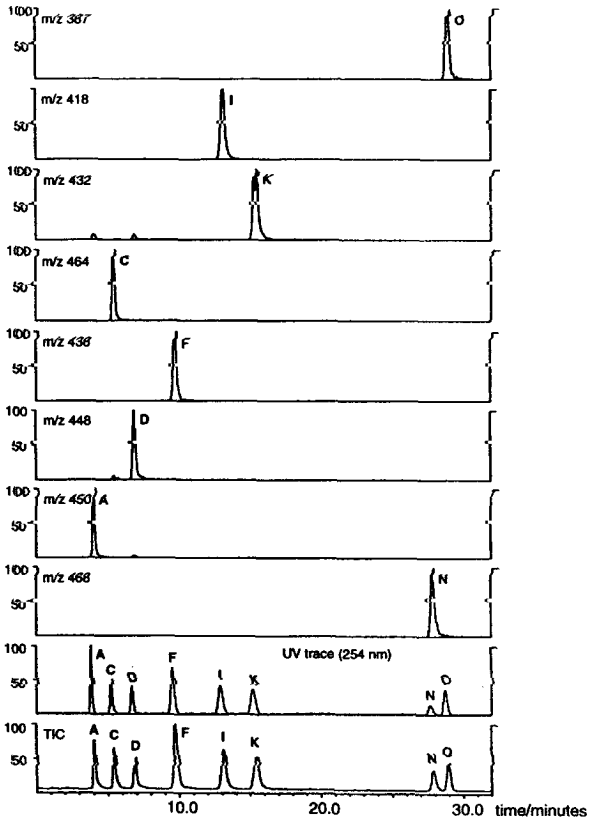


Fig. 5. LC–TSP-MS analysis for eight sesquiterpene aryl esters. For conditions, see Experimental. The UV trace at 254 nm and TIC trace for the standard solution are displayed, in addition to selected-ion traces corresponding to the adduct molecular ions of O, I, K, C, F, D, A and N.

sesquiterpene aryl esters. The abundance of ammoniated adducts in the TSP mass spectra has been explained by stabilization associated with the presence of a double bond and hydroxyl groups [8]. This is supported by the TSP mass spectra of 4-dehydromelleolide (M) and 4-dehydrodihydromelleolide (N), which do not possess a Δ^{2-3} double bond or oxygenation at position 3 and which exhibit the $[M+H]^+$ ion as the most intense peak rather than the ammoniated adduct.

The detection limit for these ions was investigated by injection of various amounts of armillyl orsellinate. This indicated that HPLC–TSP-MS could be used to detect these sesquiterpenes down to levels of 1 pg.

3.4. Extract analysis

Fig. 6 illustrates the analysis of a strain of the pathogenic *A. mellea* species (UCD 619). The LC–UV and TIC trace reveal a complex extract, with many sesquiterpene aryl esters present. However, their close retention times lead to considerable interference with each other and hence much confusion in assigning peaks. This is alleviated, however, by the selected-ion traces, in which each sesquiterpene aryl ester is clearly evident at its expected retention time free from interference. The selectivity of LC–TSP-MS in resolving overlapping components is demonstrated by the determination of armillane (F), *m/z* 420, and 10-hydroxydihydromelleolide (G), *m/z* 436, in this extract (Fig. 6). These two metabolites differ by about 20 s in elution times and can be distinguished by selective ion monitoring. They are indistinguishable, however, by conventional UV detection.

The sensitivity of LC–TSP-MS is useful for analysis of extracts in which these metbolites are present in trace amounts. Initial analysis of the mycelial extracts of INRA PT 90.5, a strain of the non-pathogenic species *A. tabescens*, established the presence of only two sesquiterpene aryl esters, armillyl orsellinate (J) and melleolide (I), which appear as the major components on the LC–UV and TIC traces (Fig. 7). However, selective ion monitoring at *m/z* 432 reveals the presence of an unknown component, P eluting at 26.12 min. Furthermore, selective ion monitoring at *m/z* 468 reveals the presence of armamiol (O). These metabolites are not visible on the UV or TIC traces owing to the predominance of armillyl orsellinate (J) and melleolide (I).

The sesquiterpene aryl esters investigated in this study all exhibit a similar level of MS response. This facilitated a semi-quantitative analysis of their distribution based on observed ion intensities recorded in the analysis of *Armillaria* extracts. Selected-ion monitoring simplifies the process of recording the ion intensity of each sesquiterpene. Extracts of 25 strains were analysed. The intensities of the recorded ions were tabulated and results for some representative individual strains are illustrated in Table 3.

Table 2
Summary of the results of the LC–TSP-MS analysis of sesquiterpene aryl esters A–O

Code	Sesquiterpene	M_r	Retention time (min)	Major ions observed (m/z)[a]	
				$[M + NH_4]^+$	Others
A	Melledonal	432	3.8	450 (100)	433 (5):$[MH]^+$; 397 (1):$[MH - 2H_2O]^+$
B	13-Hydroxydihydromelleolide	418	4.0	436 (100)	419 (22):$[MH]^+$; 401 (80):$[MH - H_2O]^+$; 233 (55)
C	5-Methoxymelledonal	446	5.3	464 (100)	447 (10):$[MH]^+$; 217 (1)
D	13-Hydroxy-4-methoxymelleolide	430	6.8	448 (100)	431 (55):$[MH]^+$
E	14-Hydroxydihydromelleolide	418	7.3	436 (100)	383 (10):$[MH - 2H_2O]^+$; 233 (25)
F	Armillane	420	9.7	438 (100)	421 (25):$[MH]^+$; 251 (15)
G	10-Hydroxydihydromelleolide	418	9.4	436 (100)	419 (40):$[MH]^+$; 233 (50)
H	4-Dehydro-14-hydroxydihydromelleolide	402	11.6	420 (100)	403 (5):$[MH]^+$; 385 (30):$[MH - H_2O]^+$; 217 (80)
I	Melleolide	400	13.25	418 (100)	401 (25):$[MH]^+$; 250 (20):$[MH\text{-aryl}]^+$
J	Armillyl orsellinate	402	13.9	420 (100)	403 (5):$[MH]^+$; 385 (80):$[MH - H_2O]^+$; 217 (60)
K	4-Methoxymelleolide	414	15.7	432 (100)	415 (25):$[MH]^+$; 233 (5)
L	Dihydromelleolide	402	17.2	420 (100)	385 (20):$[MH - H_2O]^+$; 217 (55)
M	4-Dehydromelleolide	384	24.0	402 (80)	385 (100):$[MH]^+$; 217 (5)
N	4-Dehydrodihydromelleolide	386	28.7	404 (20)	387 (100):$[M - H]^+$; 369 (60):$[MH - H_2O]^+$; 343 (30):$[MH - CO_2]^+$
O	Arnamiol	449	29.7	468 (100)	217 (80)

[a] The relative intensity (%) of the ion is given in the parentheses after the m/z value.

These results were also averaged for each species to achieve an overall picture of the distribution of these metabolites within the genus (Table 4). This reveals that sesquiterpene aryl esters are produced by all members of the genus, but not in equal amounts. The species *A. mellea*, *A. tabescens* and *A. ostoyae* were examined in detail with strains from different sources included in the study. The results indicate that the culture broth and mycelial extracts of *A. mellea* possess a higher concentration level of all 15 sesquiterpene aryl esters than those of *A. ostoyae*, which

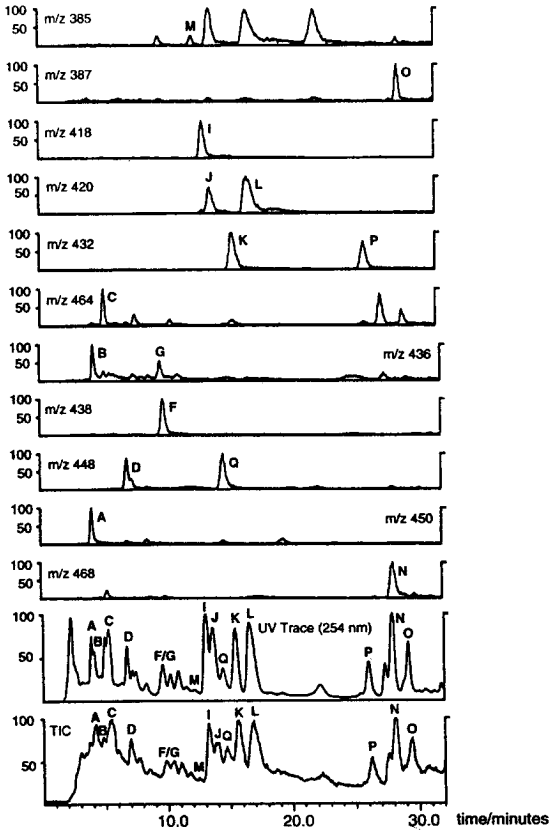


Fig. 6. LC–TSP-MS analysis of mycelial extract of *A. mellea* (strain UCD 619). The UV trace at 254 nm and TIC trace are displayed, in addition to selected-ion traces corresponding to adduct ions of sesquiterpene aryl esters A–O (sesquiterpenes H, *m/z* 420, and E, *m/z* 436, were undetected in this extract) and unknown components P and Q.

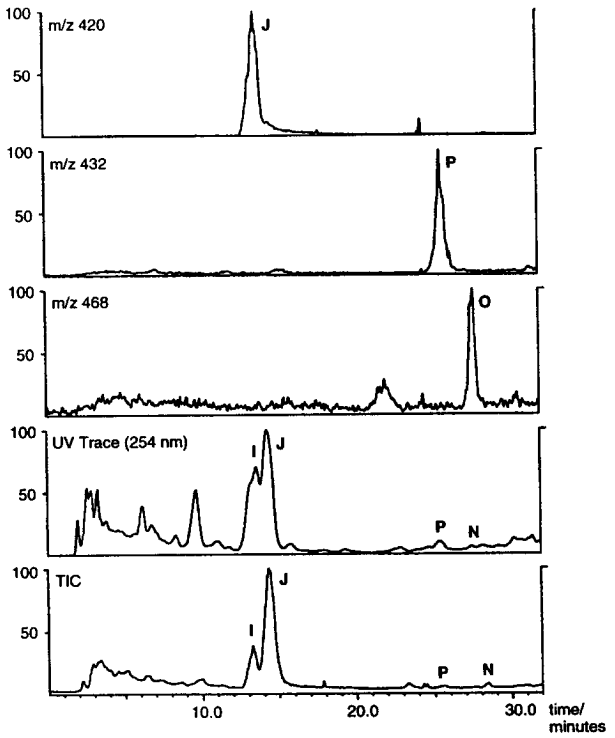


Fig. 7. LC–TSP-MS analysis of the mycelial extract of *A. tabescens* (strain INRA PT 90.5). The UV trace at 254 nm and TIC trace are displayed, in addition to selected-ion traces at *m/z* 420, armillyl orsellinate (J), *m/z* 432, component P, and *m/z* 468, arnamiol (O).

in turn possesses a higher concentration level of sesquiterpenes than extracts of *A. tabescens*. This variation in sesquiterpene aryl ester concentration mirrors the variation in reported pathogenicity of these species (Table 2). Hence the existence of a direct relationship between the level of these metabolites and pathogenicity is supported.

Two strains of *A. tabescens* (N. American), regarded as a serious pathogen in the southern USA, were examined. *A. tabescens* (European) is reported as possessing a low pathogenic ability [3]. Recent mycological studies led to the proposal that *A. tabescens* (N. American) is a distinct and different species from *A. tabescens* (European) and the name *A. monadelpha* is suggested for the former [1]. LC–TSP-MS analysis revealed a considerable difference in their metabolic profiles, with a far higher level of sesquiterpenes present in extracts of *A. monadelpha* in comparison with those of *A. tabescens* (Table 4). Hence our results indicate a possible chemotaxonomic basis for the differentiation of these two species.

One strain each of the species *A. gallica* and *A. cepestipes* were examined. Both of these species are considered as weak pathogens [3]. However, LC–TSP-MS analysis revealed a level of sesquiterpenes present comparable to that found in extracts of *A. mellea*. This suggests that these strains may be members of a pathogenic species based on our previous findings. The pathogenicity of these two strains has not been

Table 3
Occurrence of sesquiterpene aryl esters and components P and Q in extracts of six *Armillaria* strains[a]

Extract	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q
A. mellea UCD 520 culture broth	++	++	++	–	++	+	+	–	++	–	–	++	–	+	+	++	++
A. mellea UCD 520 mycelial	++	–	++	–	+	–	+	–	–	++	–	–	–	+	++	–	++
A. mellea UCD 619 culture broth	+++	+++	+++	++	++	++	+++	–	++	–	++	++	–	+	++	–	++
A. mellea UCD 619 mycelial	++	++	+++	+++	++	++	++	–	+++	+++	+++	+++	++	++	++	+++	+++
A. mellea UF IM-QG-SAP culture broth	+++	++	+++	–	+++	–	++	–	++	–	–	++	++	–	+	–	–
A. mellea UF IM-QG-SAP mycelial	++	++	++	++	++	–	++	–	++	++	++	++	++	++	++	++	++
A. ostoyae UCD 663 culture broth	++	–	++	–	–	–	–	–	++	–	–	–	–	–	–	–	++
A. ostoyae UCD 663 mycelial	++	–	++	–	–	–	–	–	+	–	+	–	–	–	+	–	–
A. monadelpha CBS 137.32 culture broth	++	++	++	++	++	++	++	–	++	++	++	++	++	++	–	–	–
A. monadelpha CBS 137.32 mycelial	++	++	++	+++	++	++	+++	–	+++	++	++	+++	++	++	–	–	–
A. tabescens INRA PT 90.5 culture broth	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	+
A. tabescens INRA PT 90.5 mycelial	+	–	++	++	–	+	–	–	+	++	–	–	–	–	++	++	++

[a] Plus and minus signs indicate the intensity values recorded for these compounds on the TIC trace. – = Intensity values undetectable; + = Intensity values less than $1 \cdot 10^4$ counts; ++ = Intensity values between $1 \cdot 10^4$ and $1 \cdot 10^6$ counts; +++ = Intensity values greater than $1 \cdot 10^6$ counts.

Table 4
Occurrence of sesquiterpene aryl esters and components P and Q in extracts of seven *Armillaria* species[a]

Species	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q
A. mellea culture broth	++	++	+++	+	++	+	+	–	++	–	+	++	+	+	+	+	+
A. mellea mycelial	++	+	++	+	++	+	+	–	++	++	++	++	+	++	++	++	++
A. ostoyae culture broth	++	+	++	+	+	+	+	+	+	+	–	+	–	+	+	+	++
A. ostoyae mycelial	++	+	++	+	–	+	–	–	+	+	–	–	–	+	–	++	+
A. tabescens culture broth	+	–	++	–	–	–	–	–	+	–	–	–	–	–	–	–	+
A. tabescens mycelial	–	–	–	–	–	–	+	+	–	–	–	–	–	+	–	+	–
A. monadelpha culture broth	++	++	++	++	++	++	++	+	+	+	+	+	++	–	+	–	–
A. monadelpha mycelial	++	++	++	+++	+	++	+++	+	+++	++	++	+++	++	+	++	–	–
A. gallica culture broth	+++	+++	++	–	++	++	–	–	++	–	–	++	++	–	–	+	++
A. gallica mycelial	+++	++	++	++	++	++	++	–	++	++	++	++	++	++	++	++	++
A. cepestipes culture broth	+++	++	++	–	++	++	–	–	++	–	–	++	–	++	–	++	++
A. cepestipes mycelial	++	–	+++	++	–	–	–	–	++	–	–	–	–	++	++	++	++
A. novae-zelandiae culture broth	+++	++	++	–	++	++	++	–	++	–	–	–	–	–	–	–	–
A. novae-zelandiae mycelial	+	+	++	+	–	+	+	–	+	+	–	+	–	–	–	–	+
A79 culture broth	++	–	+++	–	–	–	–	–	++	–	–	–	–	–	–	–	++
A79 mycelial	++	–	++	++	–	+	–	–	++	++	++	++	++	++	++	++	++

[a] See footnote to Table 3.

assessed in field tests and it is possible that they are virulent or aggressive members of an otherwise non-pathogenic species. Studies on further strains of *A. gallica* and *A. cepestipes* are necessary before their sesquiterpene metabolite production can be compared with that of other species.

One strain of *A. novae-zelandiae* was examined. The culture broth extract was found to possess a level of sesquiterpene aryl esters comparable to that in extracts of *A. ostoyae* examined (Table 4), with the mycelial extract exhibiting a lower level of concentration. Also examined was an isolate of *Armillaria*, strain A79 of unknown species (Table 1). The mycelial extract of A79 revealed an intermediate concentration of sesquiterpene aryl esters similar to that found in some strains of *A. mellea*. The level of sesquiterpene aryl esters present in the culture broth extract of A79 resembles the levels found in extracts of *A. ostoyae*. This suggests that A79 is a pathogenic rather than non-pathogenic strain and hence a strain of either *A. ostoyae* or *A. mellea* rather than the other species.

This study provides evidence for a direct link between the level of sesquiterpene aryl esters produced and the pathogenicity of the strain. Linking the presence and concentration of a specific metabolite or metabolites to virulence or locating the most important or 'killer toxin' is more problematic. Our previous HPLC–UV quantitative study linked higher concentrations of armillyl orsellinate (J) and arnamiol (O) to extracts of pathogenic species. Of the metabolites examined in this work, melledonal (A) and 5′-methoxymelledonal (C) possess the highest level of concentration in extracts of pathogenic species. Other sesquiterpene aryl esters, namely 13-hydroxydihydromelleolide (B), 14-hydroxydihydromelleolide (E), melleolide (I) and dihydromelleolide (L), possess a slightly lower level of concentration. 13-Hydroxy-4-methoxymelleolide (D), armillane (F), 10-hydroxydihydromelleolide (G), armillyl orsellinate (J), 4-methoxymelleolide (K), 4-dehydromelleolide (M) and arnamiol (O) exhibit the lowest concentration levels of the sesquiterpenes studied in extracts of pathogenic species. Preliminary studies on the biological activity of sesquiterpene aryl esters has indicated armillyl orsellinate (J) to be the most active of these metabolites, with melledonal (A) possessing no biological activity [9]. Biosynthetic studies have demonstrated that these sesquiterpene aryl esters are produced via a common biosynthetic pathway originating from the cyclization of humulene to give the protoilludyl cation [9]. It is possible that the end product of this pathway is the active armillyl orsellinate (J) and that high concentration levels of the inactive melledonal (A) and 5′-methoxymelledonal (C) in extracts of pathogenic species may indicate that these compounds act as stores or key intermediates to armillyl orsellinate.

While the level of these metabolites may show variations, they all appear to follow a similar gradient of higher concentration in pathogenic, lower in moderately pathogenic and lowest in non-pathogenic species. An exception is 4-dehydro-14-hydroxydihydromelleolide (H), which is either undetectable or at low levels in all extracts. This metabolite has been isolated from *A. manadelpha* and is unknown in other species.

3.5. Variations within each species

Considerable variation in sesquiterpene production exists amongst strains of the same species. For example, of the *A. mellea* strains, UCD 619 produces a high level of almost all 15 sesquiterpene aryl esters, whereas UCD 520 and UF IM-QG-SAP exhibit more varied sesquiterpene profiles (Table 3). We believe that this variation in sesquiterpene aryl ester production reflects the variation in virulence within the species, a factor which has confounded foresters in assessing the threat posed by an infestation, since although one strain may be non-pathogenic, another of the same species may be strongly pathogenic. This variation may depend on factors such as host type, its condition and the habitat. *A. tabescens* (European) has long been accepted as non-pathogenic on its natural host, *Quercus* spp., in northern Europe. However, it has been reported as highly pathogenic on *Eucalyptus* spp. in southern France [10] and

Citrus spp. in Corsica [11]. This southward distribution of root disease also appears in reports upon the behaviour of *A monadelpha* [*A. tabescens* (N. American)] [12] and is noteworthy because observations in China [13] associated severe root infection by *A. tabescens* with high soil temperature. Thus environmental factors appear to have a direct relationship on the pathogenic ability of these species. It may be that these factors directly or indirectly affect the metabolic pathways and production of the basidiomycete. A study involving strains of *A. tabescens* (European) and *A. monadelpha* from differing hosts and conditions is under way to assess the impact of these factors on sesquiterpene aryl ester production.

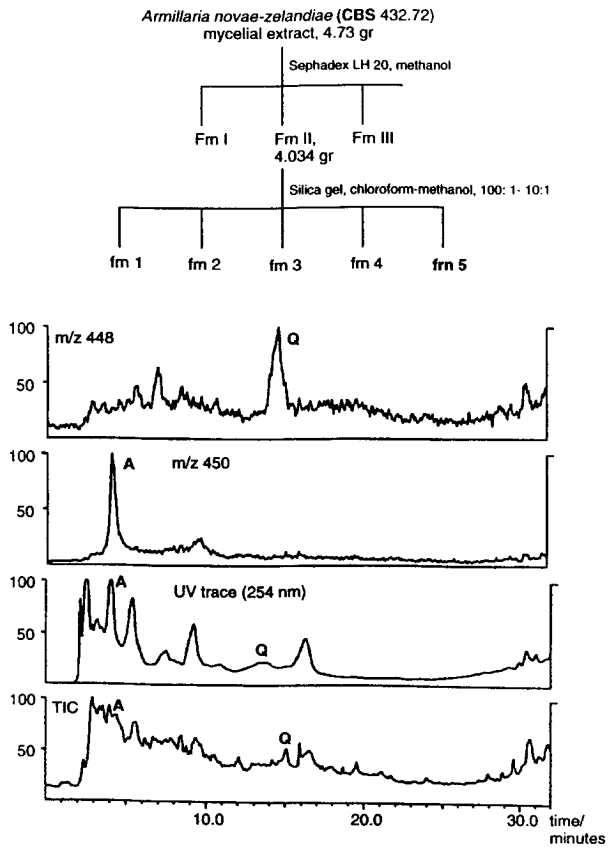


Fig. 8. LC–TSP-MS analysis of subfraction 5 of chromatographic separation of mycelial extract of *A. novae-zelandiae* (strain CBS 432.72) (see inset). The UV trace at 254 nm and TIC trace are displayed, in addition to selected-ion traces corresponding to adduct ions of 13-hydroxy-4-methoxymelleolide (D) and melledonal (A).

3.6. Unknown components

Selected-ion monitoring of extracts for the presence of 4-methoxymelleolide (K) at m/z 432 and for 13-hydroxy-4-methoxymelleolide (D) at m/z 448 revealed the presence of two unknown components, P and Q, respectively (Fig. 6). The clear ionization of P eluting at 26.12 min and Q eluting at 14.6 min under these conditions as well as their distribution within the extracts (Tables 3 and 4) suggest that they may be sesquiterpene components. Given that ammoniated adducts are the norm in this study, this suggests a relative molecular mass of 414 for P and 430 for Q. These molecular masses may correspond to a number of sesquiterpene aryl esters previously isolated from *Armillaria* species. The low fragmentation characteristics of thermospray ionization is limiting in further structural elucidation of such components. However, its usefulness in their isolation is illustrated by the analysis of fractions I–V collected from a successive separation of a mycelial extract of *A. novae-zelandiae* on Sephadex and silica (Fig. 8). Subfraction 5 is clearly targeted as having the highest level of species Q and isolation work on this fraction is continuing.

4. Conclusion

The results indicate that LC–TSP-MS can be successfully applied to the identification of sesquiterpene aryl esters of extracts of *Armillaria* species. Previously developed HPLC procedures for sesquiterpene aryl ester analysis have been successfully coupled with TSP to provide an on-line analysis. The advantages of our conventional HPLC analysis is thus retained, namely speed of analysis, reproducibility and ability to handle small amounts of sample. LC–TSP-MS further allows the identification of components not just on the basis of retention time but also from on-line molecular mass information. Thus

another dimension is provided through which we can observe these metabolites.

Fungal extracts are complicated biological matrices and the isolation of the biologically active components present is often a complex and tedious process. The developed method allows us to determine these components in a semi-quantitative fashion from direct analysis of the extracts. This is particularly useful in conjunction with standard isolation techniques. Components of interest can be determined rapidly and, although the structural information on these components may be limited, extracts and/or fractions which are rich in these components can be readily targeted.

That *Armillaria* species can cause serious disease has been known for over a century, but its propensity to do so has been a matter of controversy. This analysis supports a direct relationship between the level of sesquiterpene aryl ester metabolites produced by a strain and its virulence. LC–TSP-MS analysis may thus provide a direct method of determining the pathogenic ability of any strain of this wood-rotting basidiomycete. This technique is also applicable to the analysis of infected wood tissue. The level and identity of sesquiterpene aryl esters present may be established and hence the threat posed by an infestation of *Armillaria* assessed.

References

[1] G.A. Kile, J.J. Guillaumin, C. Mohammed and R. Watling, in M. Johansson and J. Stenlid (Editors), Proceedings of the Eighth IURFO International Conference on Root and Butt Rots, 1993, pp. 411–436.
[2] J. Rishbeth, Plant Pathol., 31 (1982) 9–17.
[3] C.G. Shaw and G.A. Kile, Agricultural Handbook No. 691, Forest Service, USDA, Washington, DC, 1991.
[4] D.M.X. Donnelly and D.J. Coveney, in K. Hostettmann and P.J. Lea (Editors), Proceedings of the Phytochemical Society of Europe, 1987, pp. 19–32.
[5] O. Dunne, Ph.D. Thesis, National University of Ireland, 1993.
[6] J.L. Wolfender and K. Hostettmann, J. Chromatogr., 647 (1993) 191.
[7] M. Maillard, J.L. Wolfender and K. Hostettmann, J. Chromatogr., 647 (1993) 147.
[8] E.B. Hansen, J. Abian, T.A. Getek, J.S. Choinski and W.A. Korfmacher, J. Chromatogr., 603 (1992) 157.
[9] R.H. Hutchinson, Ph.D. Thesis, National University of Ireland, 1990.
[10] B. Lung-Escarmant, D. Poincot and B. Taris, presented at the 27th Colloquium of the Société Française de Phytopathologie, Agronomie, 5 (1985) 559.
[11] E. Laville and R. Vogel, Arboric. Fruit., 31 (1984) 34.
[12] A.S. Rhoads, Lloydia, 19 (1956) 193.
[13] L.W. Chang, Q.F. Liu and G. Li, Acta Phytopathol. Sin., 12 (1982) 41.