ISOLATION AND CHARACTERIZATION OF SIX HIGHLY INTRINSIC FLUORESCENT METABOLITES IN *ARMILLARIA MELLEA* (VAHL. EX. FR.) KARST

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Abstract

Six fluorescent compounds were isolated and characterized in rhizomorph of *A. mellea* and were presented first as: F_1, F_2, F_3, MF_1, MF_2 and MF_3. These compounds differed by Rf on chromatoplaque, λmax in UV spectra and λex, λem and λmax in fluorescence spectra. Their GC-MS analysis indicated that F_1 has a phenolic moiety and the other five are the benzenic derivatives. F_1 compound was more fluorescent resulting from its hydroxyl group. The difference between the fluorescent compounds of rhizomorph and hyphae suggests the participation of some of these compounds in differentiation. Moreover, these compounds may have antibiotic activity against their hosts or enemies.

Introduction

Armillaria mellea (Vahl ex. Fr) Karst, the honey fungus, is so called because of the honey-coloured carpophore or rhizomorph. This basidiomycete is the cause of widespread root disease in deciduous plants such as Betula sp., *Citrus domestica, Vitis vinifera* , and evergreen trees such as *Juniperus sp.* [1]. Many biologically active sesquiterpene esters have been isolated from different strains of the fungus. The crystalline metabolite, armillane, was isolated from the extracts of the mycelium of *A. mellea* obtained from cultures grown for 30 days [2].

The molecular formula $C_{23}H_{32}O_7$ of the armillane was established by elemental analysis and FARMS (cMH) m/z 421 [2]. *A. mellea* is known to produce chemical defense metabolites possessing antibacterial activity [1]. These compounds, which have an unusual hydroxylation at C-10 and C-13, are new members of the protoilludane sesquiterpenoids, a group of fungal metabolites first isolated from *Clitocybe illudans* [3]. It has been suggested that they are derived from a protoilludane intermediate arising from farnesol via humulene. The first isolated compound of this type from *A. mellea* was armillol arsellinate [4, 5].

In China, tablets containing artificially cultured mycelium of *A. mellea,* are used for the treatment of dizziness, headaches, neurasthenia, insomnia, numbness in limbs and infantile convulsions [6].

We observed that, in the Knop-Heller media, serine promotes differentiation from hyphae to rhizomorph [7]. In this report, some fluorescent compounds are isolated and characterized in rhizomorph; the attacking organ of *A. mellea.*

Keywords: *Armillaria mellea;* Fluorescent compounds

Materials and Methods

A. mellea was obtained from *Quercus sativus* from the forest of Karasang in Amol, Iran. The growth of

the fungal stalk (5 mm long disk) was initiated on malt-agar culture medium at 25°C, which was transferred to other media later. Knop-Heller and ammoniacal Knop-Heller (NO^-_3 replaced by NH_4^+) medium with or without pepton 5 g/l were used to produce mycelia with or without differentiation to rhizomorph [7].

For more rhizomorph production, we used prune juice (20 g prune drupe was filtered through a layer of cheesecloth in boiling distilled water). About 5-10 segments of fungi on malt-agar were transferred to sixty Erlenmeyer flasks and grown for 45 days.

Fungus cultures of various organs were filtered through a layer of cheesecloth and the filtrates were extracted five succesive times with 0.1 volume (V/V) of chloroform in a separatory funnel. The chloroform extracts were evaporated at 60°C.

Vegetative structures from the above cultures were dried for 24-48 h at 60°C, ground and extracted in a Soxholet apparatus for two days with chloroform.

The chloroform extracts were filtered and evaporated. The extracts were then chromatographed on a column of silica gel (0.45 mm) using hexane-ethylacetate (2:1, V/V). Further purification was achieved by preparative TLC (PLC) using the same solvent. Then each band on the plate was chromatographed on a column of silica gel (0.45 mm) using hexane-ethylacetate (2:1, V/V).

Absorption spectra were recorded on a Shimadzu-160 UV-VS spectrophotometer. Corrected fluorescence excitation and emission spectra were obtained by use of a Shimadzu RF-5000 spectrophotometer. Fluorescence spectra of TLC were recorded by a Shimadzu CS-9000 densitometer. Gas chromatography-mass spectrometry (GC-MS) was carried out with the following general conditions:

a) Gas chromatography: apparatus NIST; column DB-5, 30 m; temperature 215°C.

b) Mass spectrometry: apparatus NIST; ionization voltage 70 ev; source temperature 150-215°C.

Results and Discussion

Successive purification of the chloroform extracts of the fungal culture, undifferentiated hyphae, differentiated hyphae and rhizomorph led to the isolation of six intrinsic fluorescent metabolites: F_1, F_2, F_3, MF_1, MF_2, and MF_3 which had vivid blue fluorescence in TLC at 360 nm in the UV-cabinet. The TLC of the last purification of these compounds is shown in Figure 1. The Rf and λmax in UV spectra of these compounds are presented in Table 1.

Their UV and fluorescence spectra are shown in Figures 2 and 3, respectively, and their λex, λem and λmax of fluorescence spectra are presented in Table 2.

These fluorescent compounds also exist in culture

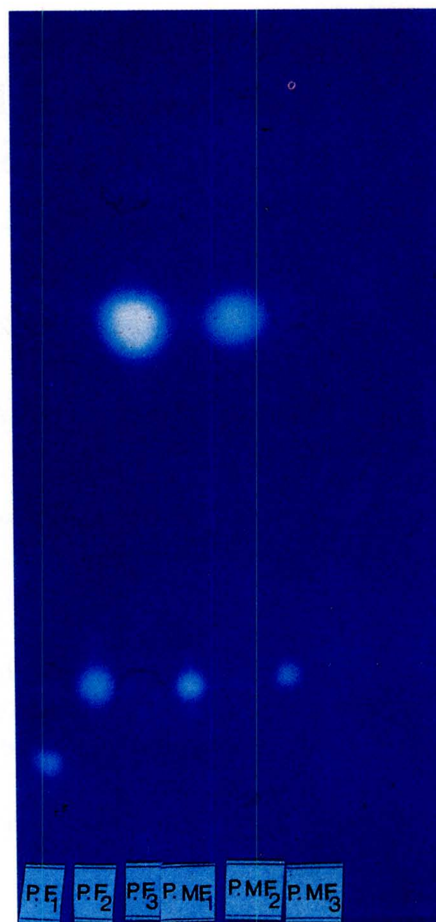

Figure 1. TLC of six purified fluorescent compounds isolated from rhizomorph

Table 1. Characterization of Rf on chromatoplaque and λmax in UV spectra of fluorescent compounds in methanol

Fluorescent Compounds	Rf	Characterization of MeOH λmax (nm) in UV spectra
F_1	0.105	213.4, 231, 259, 298.2
F_2	0.205	213.2, 231.2, 272.8, 314
F_3	0.629	211.8, 268.4, 283.4, 346.8 355.2
MF_1	0.205	214, 230.4, 254.6, 236.4
MF_2	0.629	212, 270.6, 346.4, 355.8
MF_3	0.211	212.8, 270.2, 279.2, 293

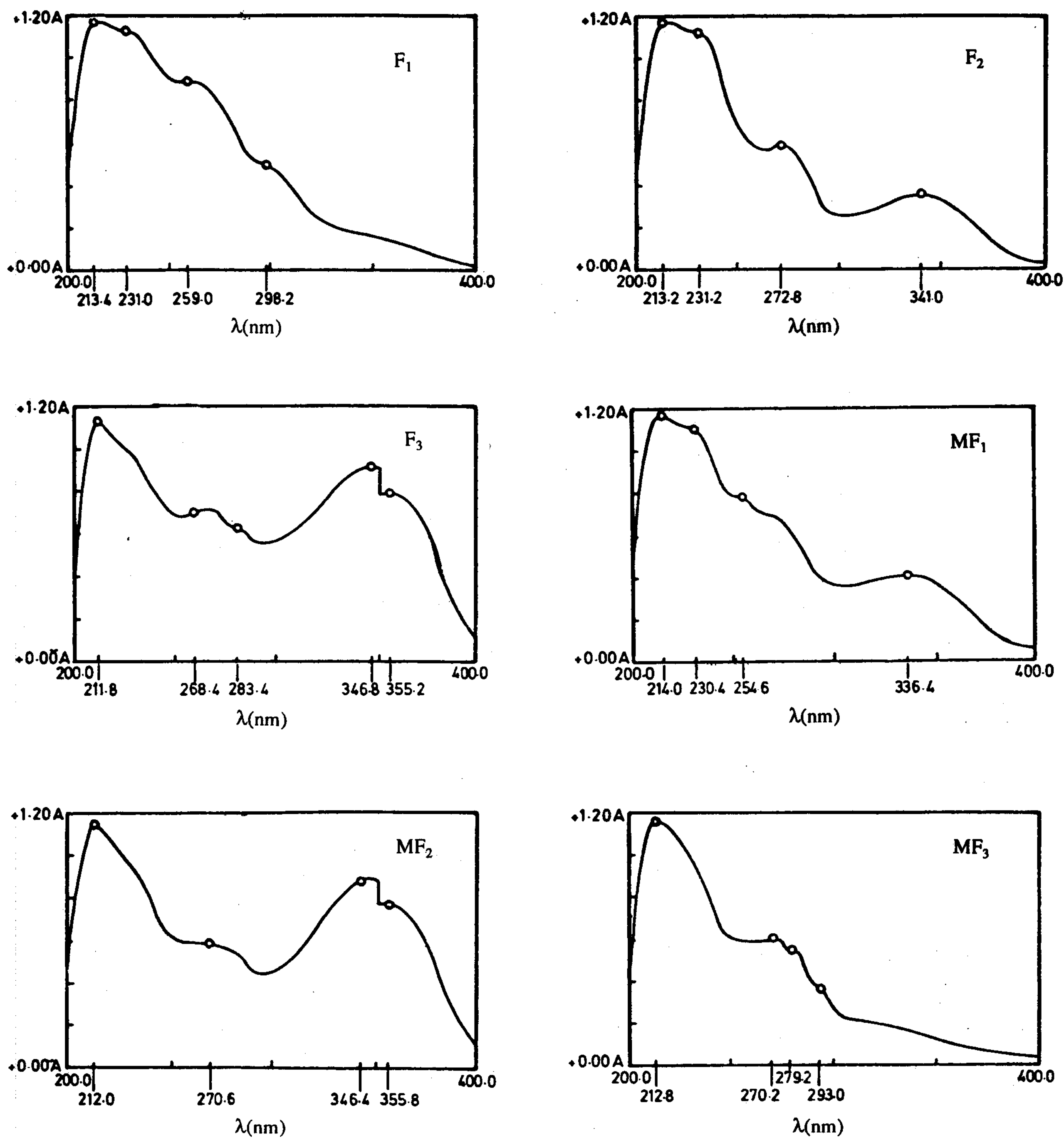

Figure 2. UV spectra of fluorescent metabolites

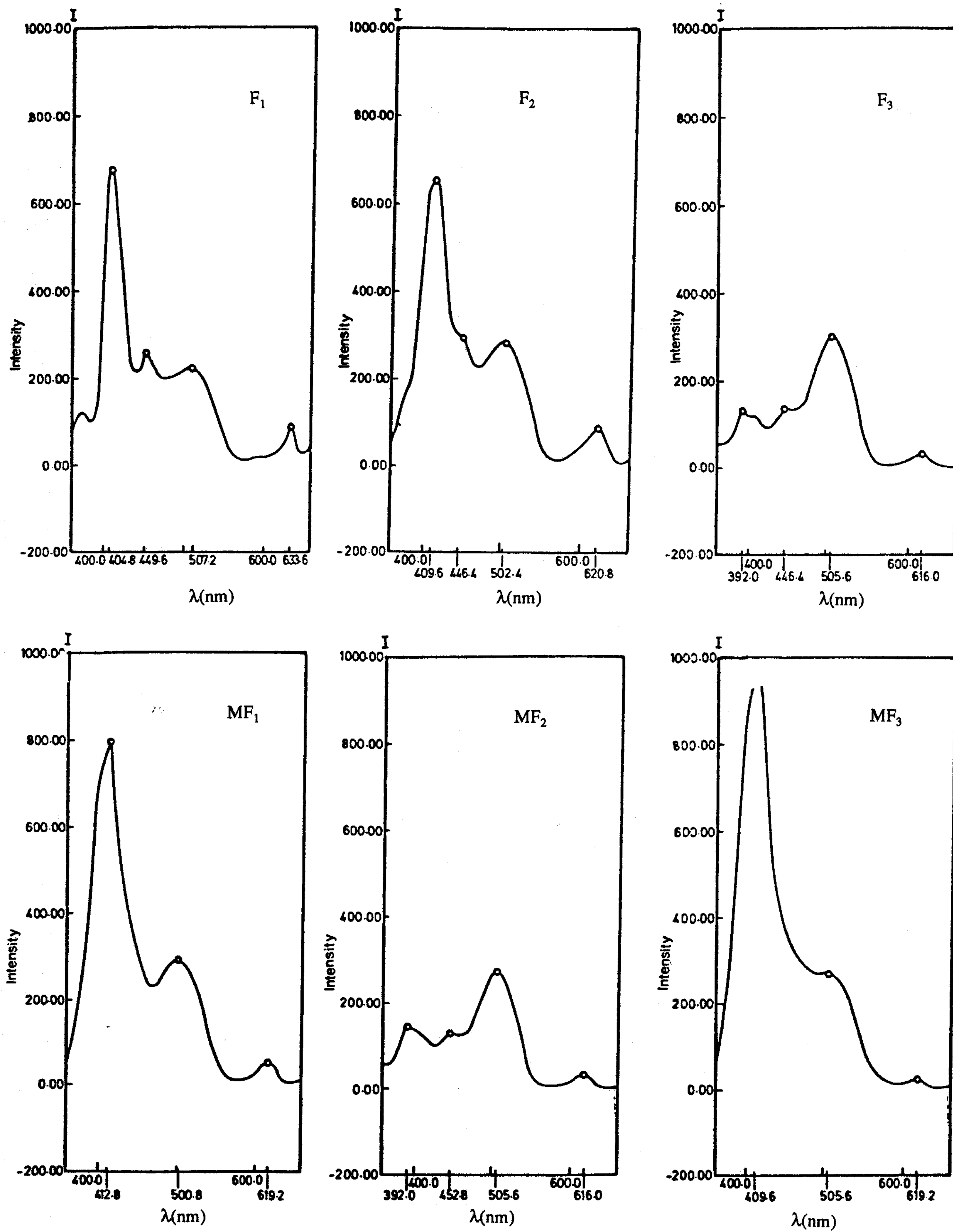

Figure 3. Fluorospectra of fluorescent metabolites

Table 2. λex, λem and λmax of fluorescence spectra in methanol

Fluorescent Compounds	λex (nm)	λem (nm)	λmax (nm)
F_1	360	467.0	440.8, 449.6, 507.2, 633.6
F_2	360	459.2	409.6, 446.4, 502.4, 620.4, 620.8
F_3	365	465.6	392.0, 446.4, 505.6, 616.0
MF_1	360	462.4	412, 8, 500.8, 619.2
MF_2	365	465.6	392.0, 452.8, 505.6, 616.0
MF_3	365	465.6	409.6, 505.6, 619.0

medium of rhizomorph. Fluorodensitometric spectra of the chloroform extract TLC of rhizomorph and its culture medium indicated the similarity (Fig. 4). Fluorescence intensity of the compounds in the methanol and the aqueous media such as Tris-HCl buffer, pH 7.4 is different (Tables 3, 4).

F_1 compound in the Tris-HCl pH 7.4 has remarkable fluorescence intensity because of the hydroxyl group which exists in this compound, but which is not found in others. F_1 with a high

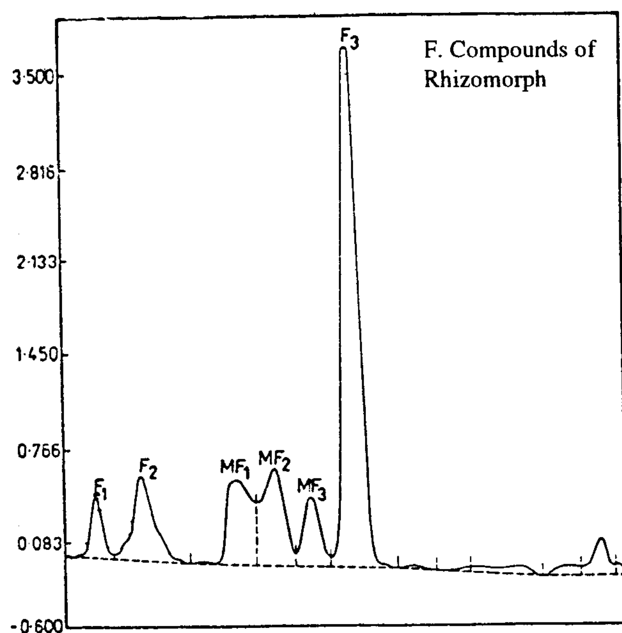

Figure 4. Densitometry of fluorescent compounds in rhizomorph

Table 3. Fluorescence intensity of F compounds in methanol

Fluorescent Compounds	λex (nm)	λem (nm)	Fluorescence intensity in mg ml^{-1}
F_1	360	464.0	18.07
F_2	360	459.2	17.99
F_3	365	465.6	25.02
MF_1	360	462.4	17.25
MF_2	365	465.6	16.75
MF_3	365	465.6	16

Table 4. Fluorescence intensity of F compounds in Tris-HCl, pH 7.4

Fluorescent Compounds	λex (nm)	λem (nm)	Fluorescence in mg ml^{-1}
F_1	356	478.8	119.65
F_2	365	473.6	81.41
F_3	355	470.4	1.73
MF_1	350	467.2	9.03
MF_2	350	456	3.63
MF_3	350	465.6	8.8

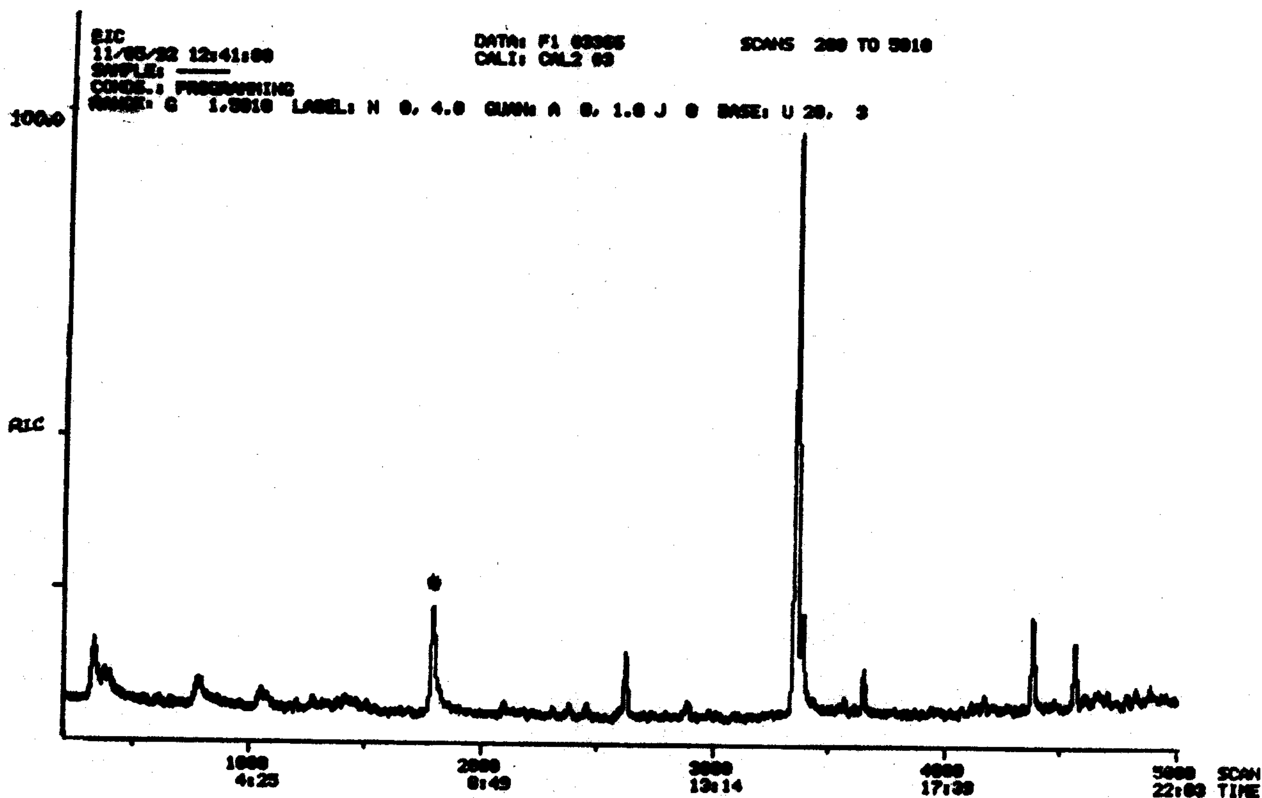

Figure 5a. Total ion profile of F_1 group

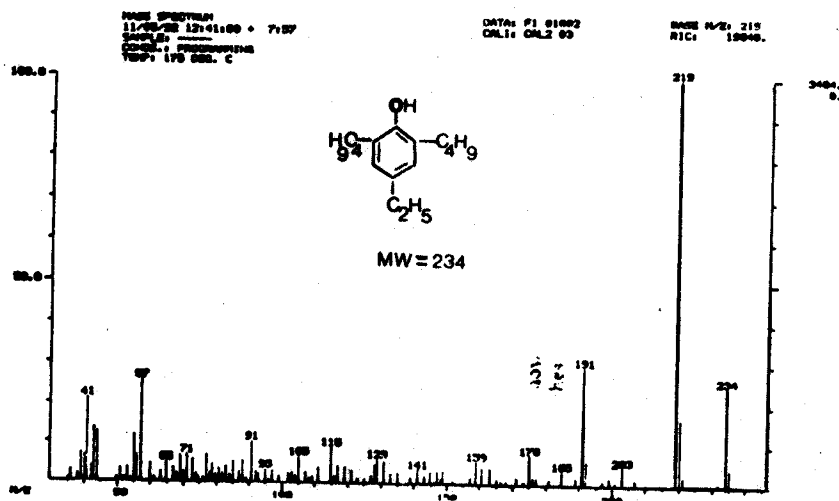

Figure 5b. Mass spectrum of F_1

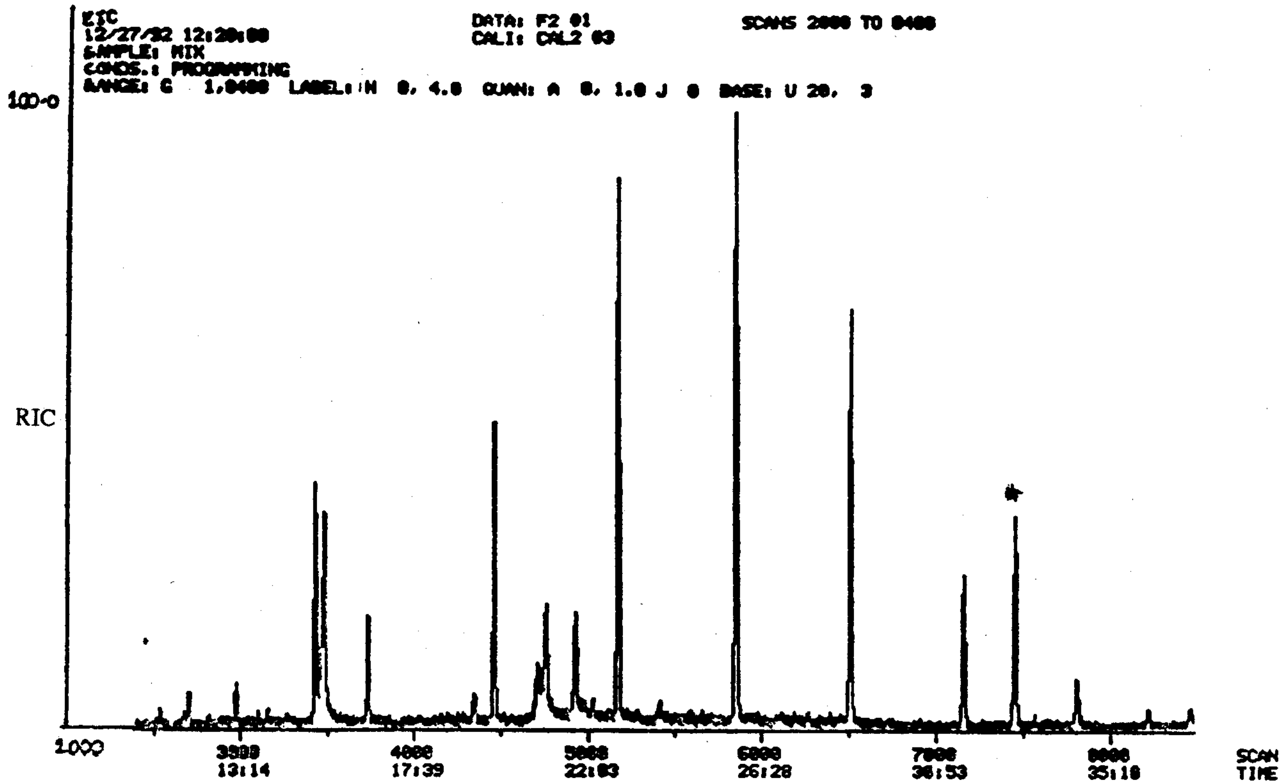

Figure 6a. Total ion profile of F_1 group

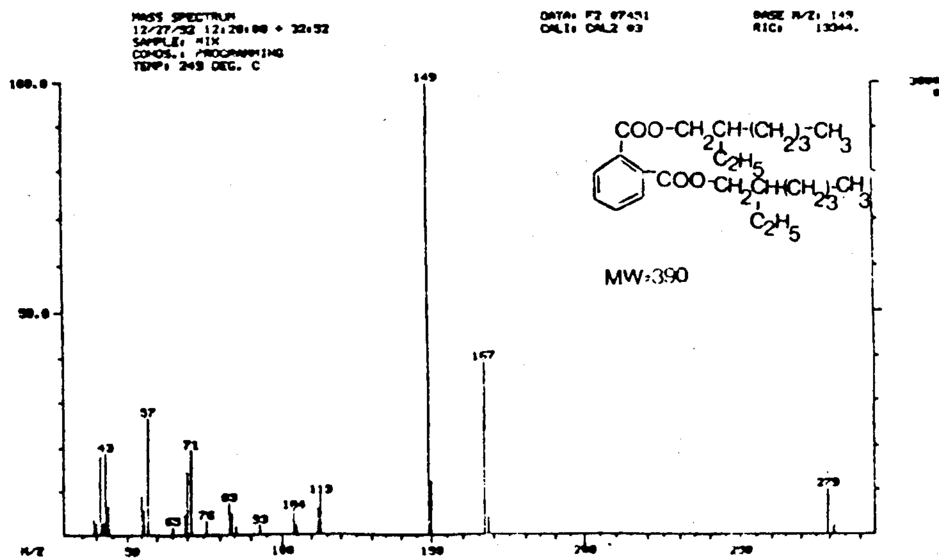

Figure 6b. Mass spectrum of F_2

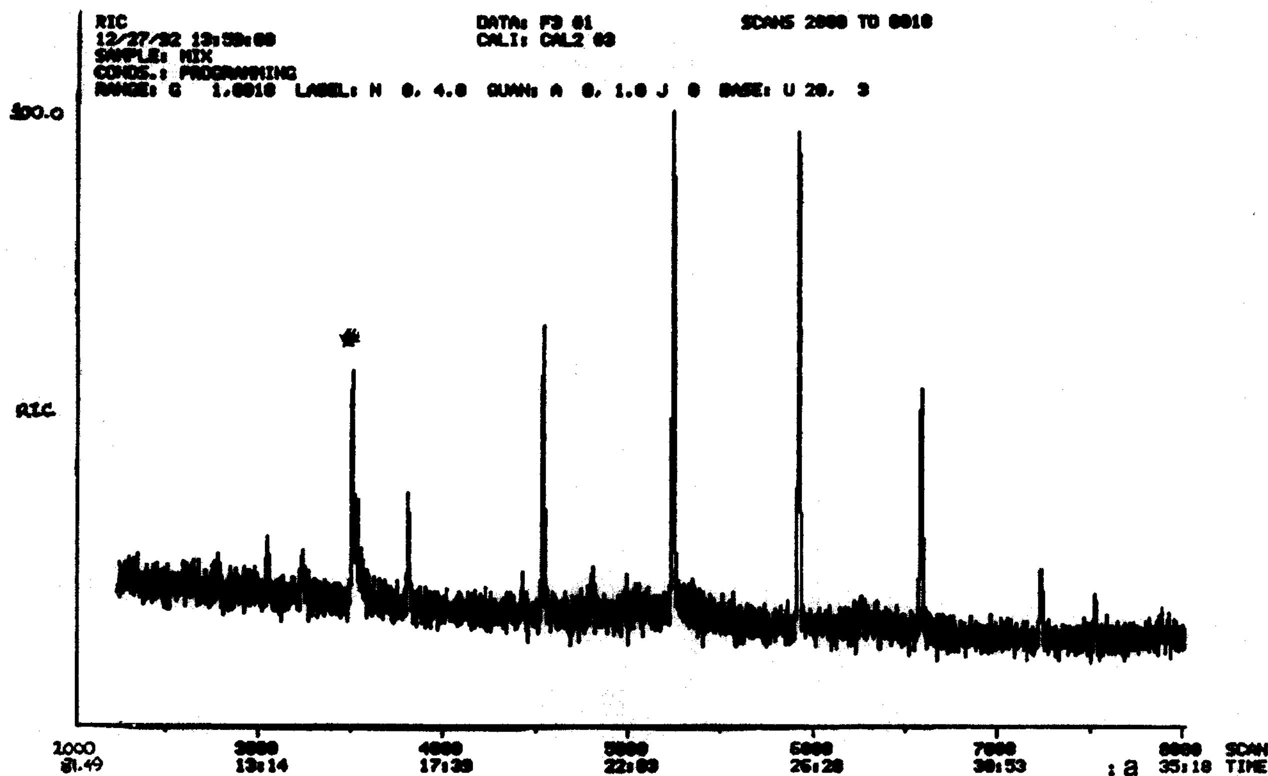

Figure 7a. Total ion profile of F_1 group

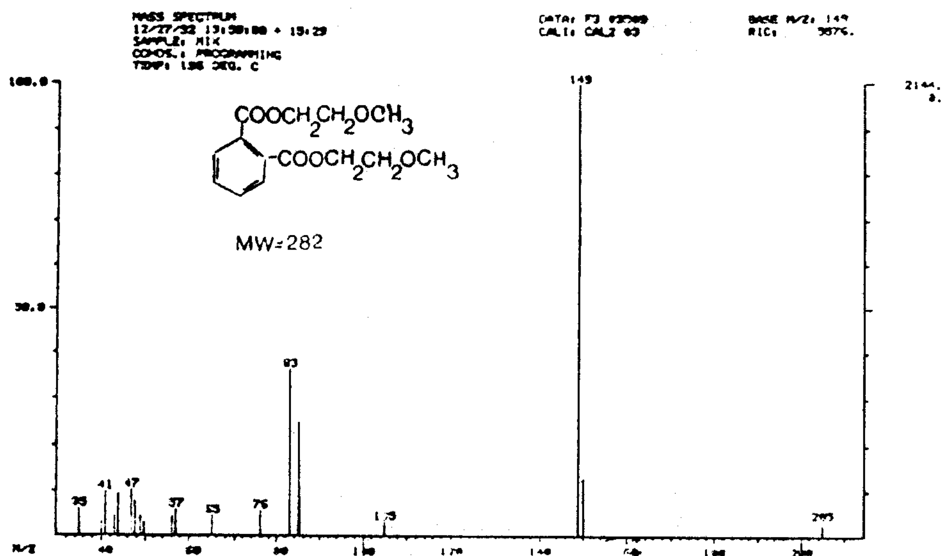

Figure 7b. Mass spectrum of F_3

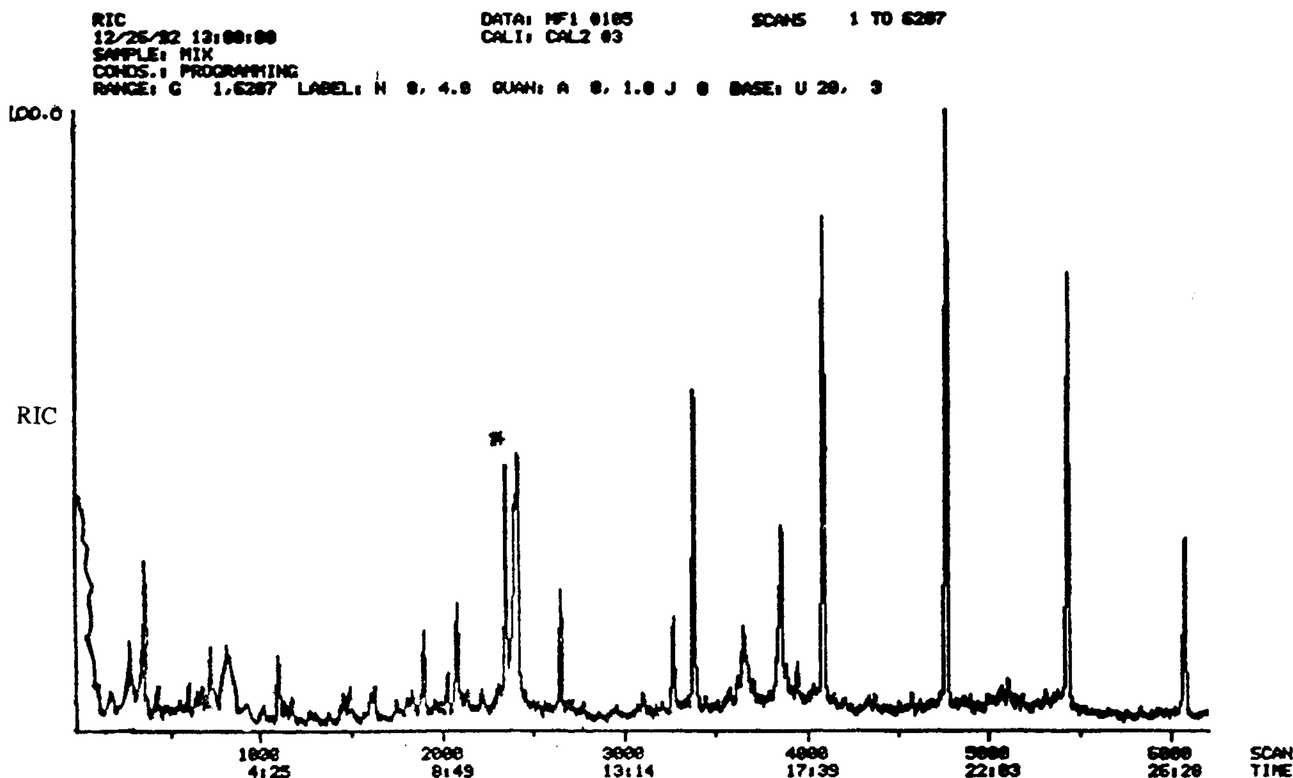

Figure 8a. Total ion profile of MF_1 group

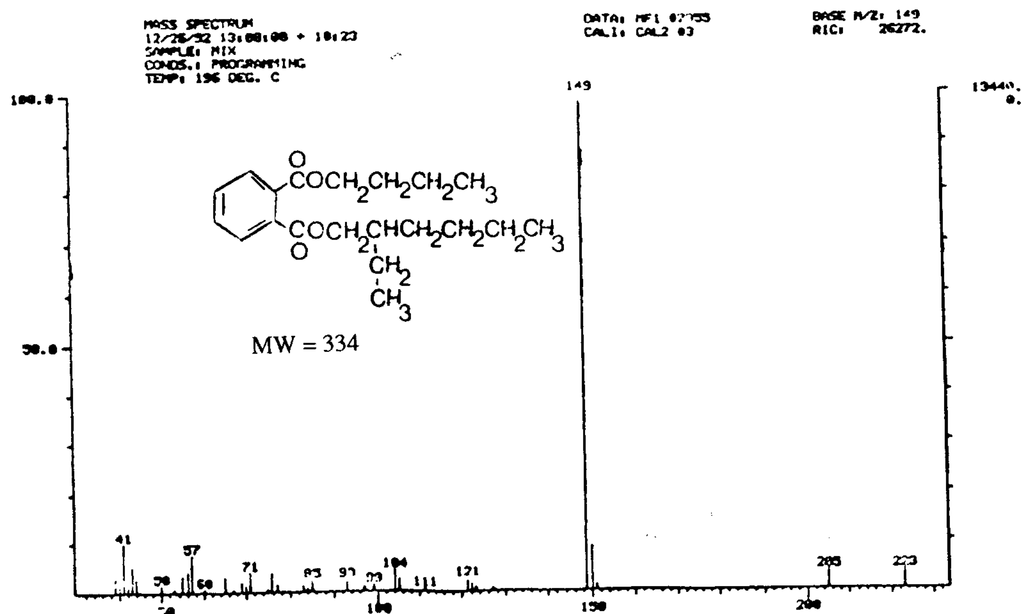

Figure 8b. Mass spectrum of MF_1

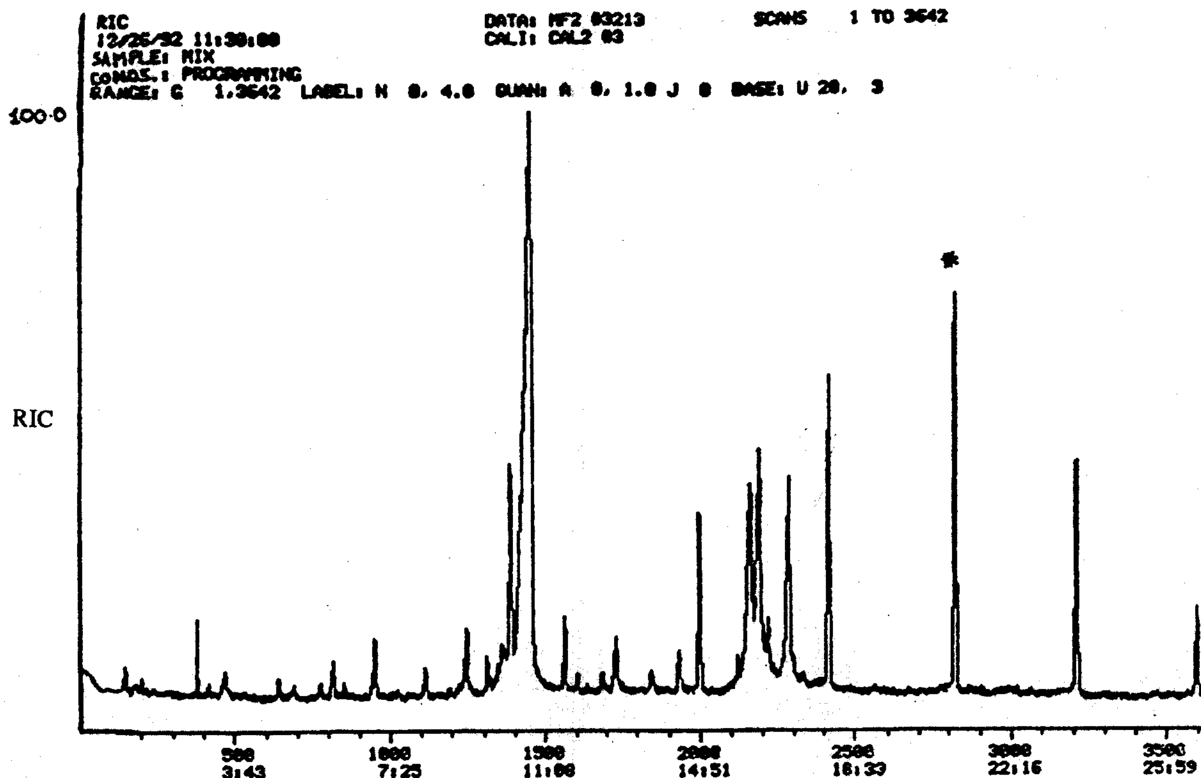

Figure 9a. Total ion profile of MF_2 group

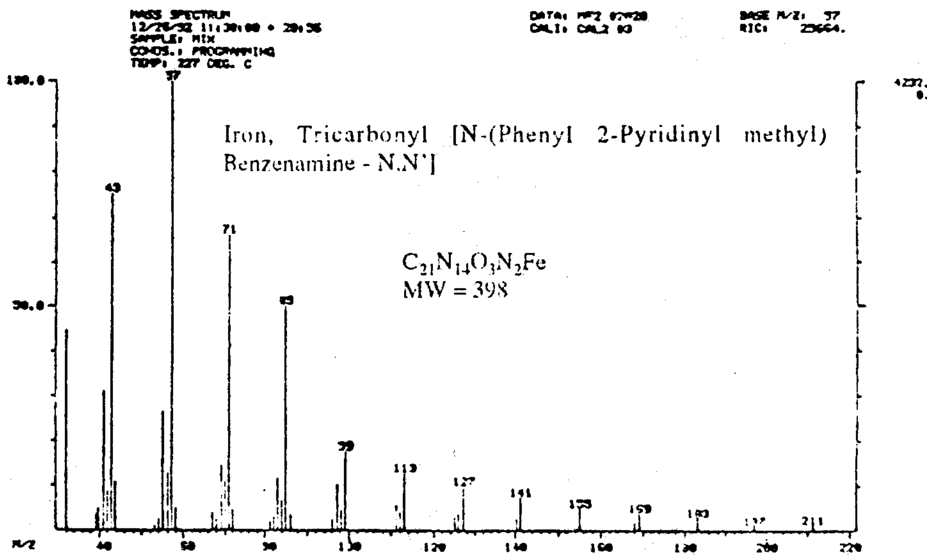

Figure 9b. Mass spectrum of MF_2

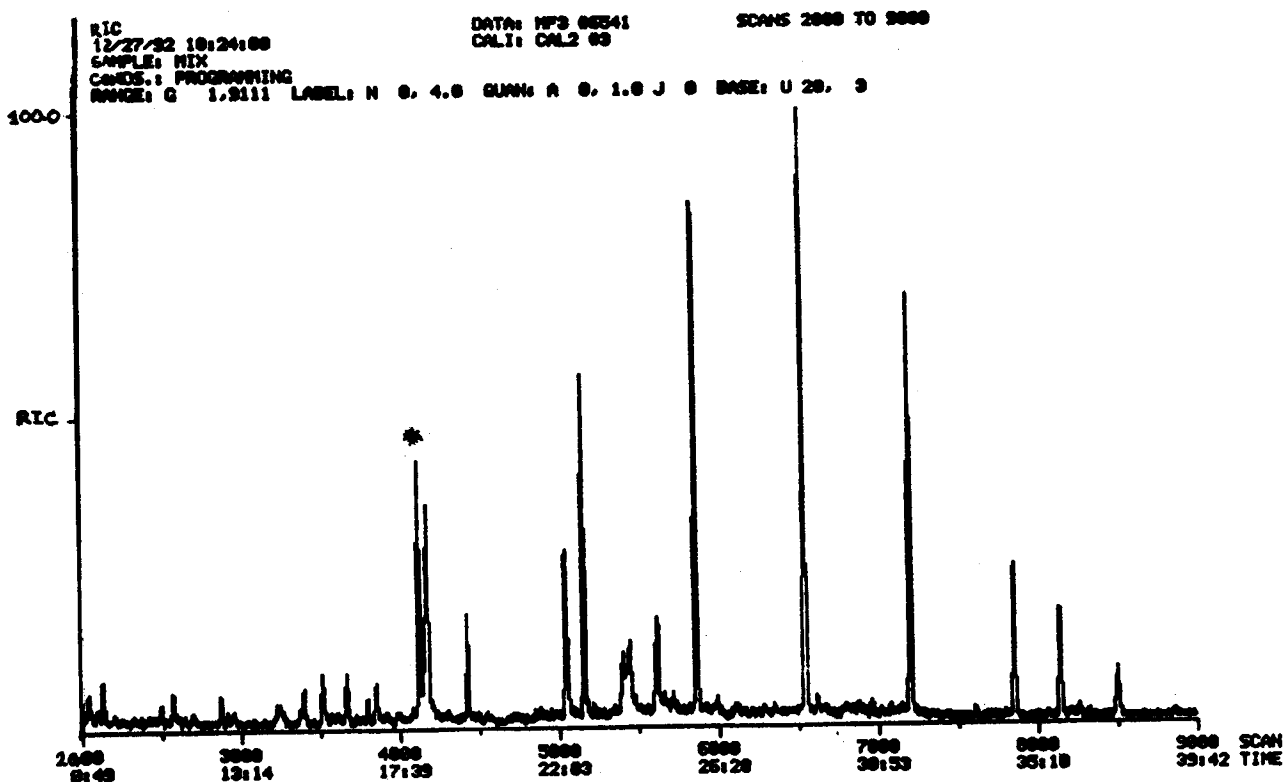

Figure 10 a. Total ion profile of MF_3 group

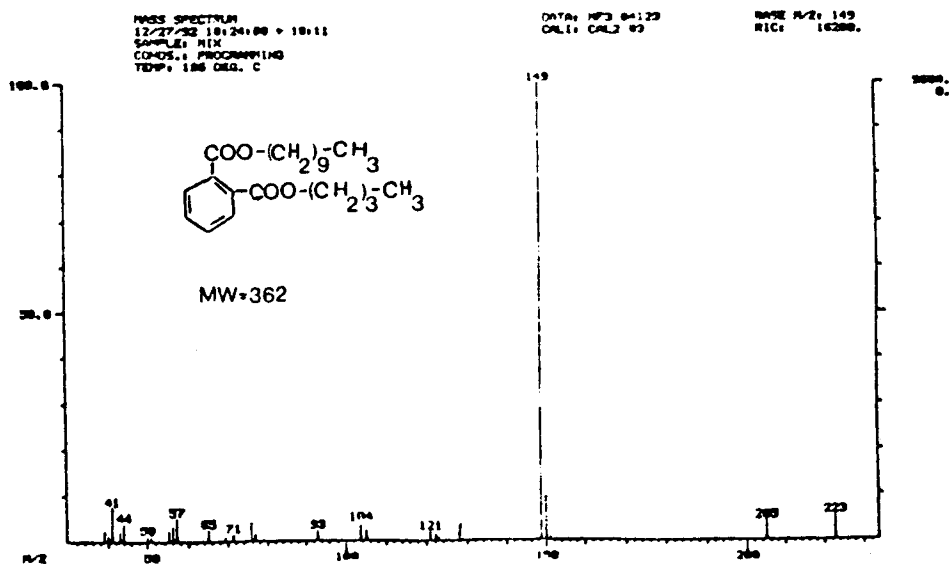

Figure 10 b. Mass spectrum of MF_3

Table 5. Names, formula and molecular weights of the fluorescent compounds

Fluorescent Compounds	Name	Formula	Molecular Weight
F_1	Phenol, 2,6-bis (1,1 dimethyl ethyl) 4-ethyl	$C_{16}H_{26}O$	234
F_2	1,2-Benzendicarboxylic acid diisooctyl - yl ester	$C_{24}H_{38}O_4$	390
F_3	1,2-Benzendicarboxylic acid (2-methoxy ethyl) ester	$C_{14}H_{18}O_6$	282
MF_1	1,2-Benzendicarboxylic acid buty 1-2-ethyl hexyl ester	$C_{20}H_{30}O_4$	334
MF_2	Iron, tricarbonyl (N-pheny 1-2-pyridinyl methyl benzenamine - N, N')	$C_{21}H_{14}O_3$ N.Fe	398
MF_3	1,2-Benzendicarboxylic acid butyl decyl ester	$C_{22}H_{34}O_4$	362

Table 6. Names, formula and molecular weights of the non-fluorescent compounds

Name	Formula	Molecular Weight
Eicosane, 10-methyl	$C_{21}H_{44}$	296
1-Hexadecanoic	$C_{16}H_{34}O$	242
Docosane	$C_{22}H_{40}$	310
Heptadecane, 2,6-dimethyl	$C_{19}H_{40}$	268
Undecane, 3,8-dimethyl	$C_{13}H_{28}$	184
Hexadecanoic acid	$C_{16}H_{32}O_2$	250
1-Heptanol, 2,4-dimethyl-(R, R)-(+)-	$C_9H_{20}O$	144
1-Octanol, 2-butyl	$C_{12}H_{26}O$	180
Decane, 2,4-dimethyl	$C_{12}H_{26}$	170
Octacosane	$C_{28}H_{58}$	394
1-Hexanol, 2-ethyl-2-propyl-	$C_{11}H_{24}O$	172
Hexanoic acid, 2-methyl-3-oxo-ethyl ester	$C_9H_{16}O_3$	172
Octadecanoic acid	$C_{18}H_{36}O_2$	284
Dodecane, 2, 6, 10-trimethyl	$C_{15}H_{32}$	212
Cyclopenta decanone-2-hydroxy	$C_{15}H_{28}O_2$	240

fluorescence intensity may be used in biochemical and biophysical methods for tracing and interaction studies, such as interaction of a fluorescent ligand like actinomycin and DNA [11]. Many of these fluorescent compounds secreted into medium may have antibacterial and fungicidal activity [12]. F_2 compound or 1,2-benzendicarboxylic acid diisooctyl - yl ester (Table 5) may be one of the resistant agents against fungal and bacterial attacks [13].

GC-MS Computer Analysis of Fluorescent Compounds

The study described herein falls within the outline of a comprehensive programme dealing with the construction of a data bank in NIST computer analysis.

Total ion current profiles of the six fluorescent compounds before last purification (F_1, F_2, F_3, MF_1, MF_2, and MF_3 groups) are shown in Figures 5-10. In each profile, the peak with its scan number represents the fully purified fluorescent compounds (F_1, F_2, ... etc) in the last purification. The peaks in each profile, except for the asterisk peak, represent the nonfluorescent compounds.

The name, formula and molecular weight of the fluorescent compounds are presented in Table 5, and of non-fluorescent compounds in Table 6. The structural formula of these fluorescent compounds are identified by some references [8, 9, 10].

None of the fluorescent compounds is from known sesquiterpenoids or sesquiterpene esters group, and therefore differ from armillane [1] and armillol arsellinate [4, 5] of *A. mellea*. However, they exhibit antibacterial and antifungal activity similar to that of armillane and armillol arsellinate [1]. Moreover, the fluorescent metabolites that exist in hyphae are different from those of rhizomorph [14] and the appearance of some of these in explants may be related to the differentiation of rhizomorph on these fragments.

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