Journal of Chromatographic Science 2012;50:358–367
doi:10.1093/chromsci/bms012

Article

Separation and Identification of Volatile Compounds from Liquid Cultures of *Trichoderma harzianum* by GC-MS using Three Different Capillary Columns

Shafiquzzaman Siddiquee[1]*, Bo Eng Cheong[1], Khanam Taslima[2], Hossain Kausar[3] and Md Mainul Hasan[4]

[1]Biotechnology Research Institute, Universiti Malaysia Sabah, JLN UMS, 88400 Kota Kinabalu, Sabah, Malaysia, [2]Fisheries Biology and Genetics Department, Bangladesh Agricultural University, Mymensingh, Bangladesh, [3]Institute of Tropical Agriculture, Universiti Putra Malaysia, JLN UPM Serdang, Selangor Malaysia, and [4]Department of Agricultural Botany, Faculty of Agriculture, Patuakhali Science and Technology University (PSTU), Bangladesh

* Author to whom correspondence should be addressed. Email: shafiqpab@yahoo.com/shafiqpab@ums.edu.my

Received 11 December 2010; revised 6 May 2011

A simple, fast, repeatable and less laborious sample preparation protocol was developed and applied for the analysis of biocontrol fungus *Trichoderma harzianum* strain FA1132 by using gas chromatography–mass spectrometry. The match factors for sample spectra with respect to the mass spectra library of fungal volatile compounds were determined and used to study the complex hydrocarbons and other volatile compounds, which were separated by using different capillary columns with nonpolar, medium polar and high polar stationary phases. To date, more than 278 volatile compounds (with spectral match factor at least 90%) such as normal saturated hydrocarbons (C7–C30), cyclohexane, cyclopentane, fatty acids, alcohols, esters, sulfur-containing compounds, simple pyrane and benzene derivatives have been identified. Most of these compounds have not previously been reported. The method described in this paper is a more convenient research tool for the detection of volatile compounds from the cultures of *T. harzianum*.

Introduction

Many types of live organisms have been sourced from different substances that are utilized for various applications. Recent researchers have estimated that the worldwide number of fungal species is 1.5 million. Of these, approximately 10% have been discovered and described and approximately1% have been examined for their spectra of secondary metabolites (1). *Trichoderma* species have many characteristics that render them of great interest to the research community. Among these characteristics is the production of natural products, or secondary metabolites. These compounds often have obscure or unknown functions in the organism that are of remarkable importance to humankind in medical, industrial or agricultural applications. Some volatile compounds are widely used for antibiotic and immunosuppressant activities as well as less desirable phyto- and mycotoxic activities.

Volatile compounds are appear as intermediate and end products of diverse metabolic pathways and belong to various structure classes such as mono- and sesquiterpenes, alcohols, ketones, lactones, esters or C8 compounds (2, 3). These metabolites have been involved in different biological processes such as biocontrol between microorganisms and their living environments. They can mediate resistance against predators, parasites and diseases, and may be produced for competition between species and to facilitate reproductive processes (4). Volatile compounds of the filamentous biocontrol fungus *Trichoderma*, for example, act antibiotically against plant pathogenic molds and can present plant growth promoting effects (5) as well as systemic resistance to plants, thus rendering plants less susceptible to fungal pathogens (6).

Determination of volatile fungal metabolites usually is done by gas chromatographic (GC) methods and has been described for different fungi such as *Aspergillus*, *Fusarium*, *Mucor*, *Penicillium* and *Trichoderma*. After cultivation of the fungi in liquid (7) or solid growth media (8), volatiles can be extracted in different ways, e.g., with organic solvents (9, 10), solid phase extraction using C18 or silica gel columns (11), online gas enrichment on adsorption tubes (12) or various headspace techniques such as closed loop stripping analysis (13), dynamic headspace (purge and trap) (14) and solid phase microextraction (15, 16).

After extraction and GC separation on nonpolar, medium-polar or high polar stationary phases, the constituents of complex mixtures of volatile compounds can be detected by flame ionization detection (FID) (17) mass spectrometry (MS), which is widely used (18). Mass spectrometric detection offers the possibility of identifying individual volatiles from complex mixtures. Structure characterization and confirmation of identity is usually achieved by comparing mass spectra with library spectra and determining chromatographic retention indices (19, 20).

Filamentous soil fungi of this genus frequently live in association with plant roots. *T. harzianum* has developed effective biocontrol agents to confer increased growth and systemic resistance to plants and against *Ganoderma boninense* phytopathogenic microorganisms of root rot disease of oil palm plantations (21). Due to these beneficial effects, some strains of *T. harzianum*, *T. atroviride* and *T. asperellum* are applied as plant protection agents for the biocontrol of molds and as plant growth promoters in agriculture, fruit growing and vegetable gardening (6, 22, 23). The potential of *Trichoderma spp.* to produce many volatile (e.g., pyrones, sesquiterpenes) and non-volatile secondary metabolites (e.g., peptaibols) has been reviewed by Reino *et al.* (24). Volatile secondary metabolites have been demonstrated to play a key role in the mycoparasitism of *Trichoderma* and its interaction with plants (5). *Trichoderma* species are recognized to produce over 40 different metabolites that contribute to their mycoparasitic and

antibiotic action and produce many important secondary metabolites such as mycotoxins (4).

Various volatiles are physiologically active and play important signaling roles in the microbial kingdom. The 6-pentyl-α-pyrone (6-PAP) is a well-described volatile product of secondary metabolism in *Trichoderma*, with herbicide and antimicrobial activities (25). In addition, eight carbon volatiles 1-octen-3-ol, 3-octanone, 3-octanol and 1-octen-3-one, which are typical mushroom components (26) function as insect attractants and exhibit fungistatic and fungicidal effects (27–29).

Faull *et al.* (30) reported the separation and quantitative determination of a wide variety of organic and inorganic components in microorganisms detected via chromatographic methods. A more profound knowledge of the character and quantity of molecules present in microorganisms facilitates an understanding of the biochemical procedures that are exploited in various biotechnological processes. Advances in the development of molecular biology support the identification of unknown or obscure secondary metabolites. At present, analysis by GC–MS is essential for the identification of natural organic compounds cultured from *T. harzianum*. The different compounds have been identified by using GC–MS techniques. Usually, volatile compounds are identified, such as aromatic compounds, fatty acid, general hydrocarbons and hydroxy or amino compound metabolites. The uses of GC separation approaches have investigated, for instance, the separation of natural compounds from T. *harzianum*. In this paper, the authors aim to detect a wide range of metabolites from the cultures of *T. harzianum* FA1132 during the application of three different capillary columns with high polar, medium polar and nonpolar stationary phases.

Experimental

Cultivation of T. harzianum

T. harzianum strain FA1132 (IMI: 375050) was used in this study (collected from the Mycology Laboratory, Department of Biology, Universiti Putra Malaysia) and grown on potato dextrose agar (PDA) (Difco, USA) at 30 ± 2 OC for 5 days. The isolate was obtained as mycelia in liquid culture of potato dextrose broth (PDB) (Difco, USA) prepared as per the manufacturer's instructions. Aliquots of 250 mL media were decanted into individual 1000 mL Erlehnmeyer flasks with cotton wool stoppers placed over the flask mouths and then autoclaved at 121°C, 1.4 kg cm^{-1} for 15 min. When the flask cooled, the fungal mycelium was placed on culture plates, 5 mm in diameter, and placed to the center of the broth cultured flask. The cotton wool stoppers were then wrapped with aluminum foil, sealed with parafilm and were incubated at 30 ± 2°C (12 h darkness, 12 h light) on a rotary shaker for 14 days at 120 rpm for inoculums cultured of *T. harzianum*.

Extraction procedure

Inoculums cultured of *T. harzianum* were applied according to extraction procedures to collect both extracellular (excreted into the medium) and intracellular metabolites, as shown in Figure 1. A 225 mL aliquot of ethyl acetate was added into inoculums cultured in an Erlenmeyer flask and kept

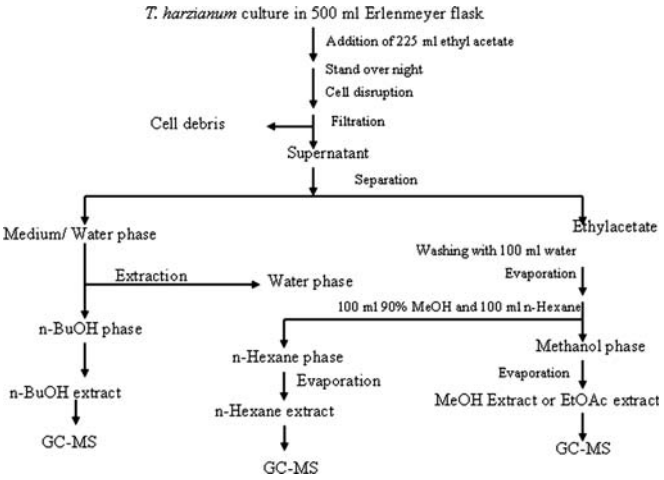

Figure 1. Schematic isolation of volatile compounds from *T. harzianum*.

overnight to ensure that the fungal cell died. The mixtures were then applied to Ultraturrax for 10 min (for cell destruction), followed by filtration using a Büchner vacuum funnel. The extracted mycelium (cell debris) was thrown away and the filtrate containing ethyl acetate phase and medium (water phase) was collected for further processes.

The ethyl acetate phase was separated from the water phase (medium) using a separation funnel for removing the remaining salts and other polar constituents. Subsequently, it was washed twice with distilled water . Immediately after evaporation, the ethyl acetate extracts were each diluted in 100 mL of methanol and *n*-hexane to remove fatty acids and other nonpolar elements. The solvent was then separated by a Büchner vacuum separation funnel. The water phase (medium) was extracted with water saturated with *n*-butanol to collect the polar constituents. All solvents were evaporated using at 40°C by rotary evaporated shaker until 5 mL of solvent was reached, then either GC–MS analysis was immediately used or it was kept in the deep freezer at −20°C. In these experiments, each solvent was repeated three times.

Apparatus and chromatography conditions

Separation of hydrocarbons and other volatile compounds were determined with a GC (Shimadzu, Japan) equipped with a QP5050A MSD mass selective detector. GC–MS analyses were done with ionization energy of 70 eV.

Putative identification of volatile metabolites was performed by three different chromatographic runs by using three different capillary columns with different stationary phases. For the non-polar column (BP1), 30 m × 0.25 mm × 0.25 μm, the oven program had an initial temperature of 60°C for 1 min, then a10°C/min run to 300°C with a final hold at 300°C (5 min); injector temperature was kept at 300°C (splitless) and detector temperature was at 320°C. For the medium polar column (BP10), 50 m × 0.33 mm × 0.25 μm, the oven program had an initial temperature of 60°C for 2 min, then a 10°C/min run to 260°C, with a final hold at 260°C (5 min); the injector temperature was kept at 260°C (splitless) and the detector temperature was 280°C. For the high polar column (BPX70), 30 m ×

Table I
Volatile Compounds Identified from *T. harzianum* using n-Hexane Solvent

Peak	Chemical name	Chemical structure	RT (min)	RI (min)	MW	Abundance (%)
1	Heptane (C7)	C7H16	5.133	5.033	100	2.11
2	1,1-dimethoxy-2-methyl-propane	C6H14O2	5.958	5.817	118	0.25
3	Toluene	C7H8	6.758	6.675	92	0.32
4	3-methyl-2-butanol	C5H12O	7.942	7.825	88	0.06
5	4-hydroxy-2-butenoic acid (methyl ester)	C5H8O3	8.200	8.150	116	0.05
6	Octane (C8)	C8H18	8.325	8.275	114	0.07
7	2,3-dimethyl-1-pentanol	C7H16O	8.933	8.850	116	0.15
8	1,1,2-trimethoxy-ethane	C5H12O3	9.150	9.075	120	0.03
9	Ethylbenzene	C8H10	9.892	9.817	106	0.15
10	1,3-dimethyl-benzene	C8H10	10.250	10.175	106	0.11
11	1-ethenyl-3-methylene-cyclopentene	C8H10	11.042	10.975	106	0.03
12	Nonane (C9)	C9H20	11.083	11.050	128	0.13
13	1-methylethyl-benzene	C9H12	12.083	12.000	120	0.04
14	2,2-dimethyl-butane	C6H14	12.225	12.175	86	0.02
15	2,2-dimethyl-3-methylene-camphene	C10H16	12.942	12.858	136	0.04
16	Propyl- benzene	C9H12	13.150	13.067	120	0.11
17	4-methyl-nonane	C10H22	13.175	13.167	142	0.07
18	2-methyl-nonane (methylnonane)	C10H22	13.317	13.267	142	0.04
19	1-ethyl-2-methyl-benzene	C9H12	13.400	13.342	120	0.32
20	1-ethyl-3-methyl-benzene	C9H12	13.525	13.475	120	0.10
21	3-methylnonane	C10H22	13.542	13.542	142	0.10
22	1,2,4-trimethyl-benzene	C9H12	13.708	13.658	120	0.14
23	Unknown		14.008	13.950		0.19
24	1-decene	C10H20	14.292	14.208	140	0.25
25	*n*-decane (C10)	C10H22	14.558	14.492	142	1.13
26	1,2,3-trimethyl-benzene	C9H12	14.575	14.575	120	0.51
27	2,2,3,4-tetramethyl-pentane	C9H20	15.108	15.083	128	0.01
28	2,2,6-trimethyl-octane	C11H24	15.200	15.158	156	0.01
29	3-ethyloctane	C10H22	15.242	15.217	142	0.04
30	1-methyl- 3-(1-methylethyl) benzene	C10H14	15.458	15.358	134	0.02
31	1,3,5-trimethyl-benzene	C9H12	15.508	15.458	120	0.09
32	1-methyl-2-(1-methylethyl)-benzene	C10H14	15.550	15.550	134	0.02
33	2-ethyl-1-hexanol	C8H18O	15.708	15.642	130	0.08
34	2-methyl-1-decanol	C11H24O	15.808	15.767	172	0.04
35	Indane	C9H10	16.000	15.967	118	0.01
36	2,4,6-trimethyl-octane	C11H24	16.058	16.017	156	0.02
37	2,6-dimethyl-octane	C10H22	16.183	16.133	142	0.05
38	5-ethyl-2-methyl-octane	C11H24	16.292	16.242	156	0.10
39	1,2-diethyl-benzene	C10H14	16.308	16.308	134	0.03
40	2,5-dimethyl-nonane	C11H24	16.400	16.350	156	0.08
41	1-methyl-3-propyl-benzene	C10H14	16.458	16.433	134	0.00
42	5,7-dimethyl-undecane	C13H28	16.475	16.458	184	00
43	2,6-dimethylnonane	C11H24	16.525	16.483	156	0.09
44	2,2,6-trimethyl-decane	C13H28	16.608	16.567	184	0.03
45	4-methyl-decane	C11H24	16.675	16.625	156	0.19
46	3-methyl-decane	C11H24	16.883	16.883	156	0.15
47	α-methyl-beneacetaldehyde	C9H10O	16.917	16.917	134	0.00
48	1,2,4,5-tetramethyl-benzene	C10H14	17.267	17.233	134	0.02
49	4-ethyl-1,2-dimethyl- benzene	C10H14	17.367	17.317	134	0.02
50	6-ethyl-2-methyl-octane	C11H24	17.550	17.508	156	0.02
51	2-ethyl-1,4-dimethyl-benzene	C10H14	17.567	17.550	134	0.03
52	2,3,6,7-tetramethyl-octane	C12H26	17.775	17.733	170	0.04
53	Undecane (C11)	C11H24	17.861	17.792	156	1.53
54	5-ethyl-2-methyl-octane	C11H24	17.958	17.917	156	0.03
55	Nonanal	C9H18O	18.250	18.225	142	0.03
56	2,2,5,5-tetramethyl-hexane	C10H22	18.325	18.300	142	0.01
57	1,2,3,5-tetramethyl-benzene	C10H14	18.625	18.575	134	0.02
58	β phenylethyl alcohol	C8H10O	18.683	18.642	122	0.08
59	1,2,3,4-tetramethyl-benzene	C10H14	18.733	18.725	134	0.02
60	5-dimethyl-1-undecene	C12H24	19.642	19.583	168	0.04
61	1,7,7-trimethyl-camphor	C10H16O	19.758	19.742	152	0.01
62	2,5-dimethyl-dodecane	C14H30	19.833	19.800	198	0.02
63	5-methylene-undecane	C12H24	19.933	19.892	168	0.04
64	3-methylene-undecane	C12H24	20.508	20.458	168	0.04
65	5-dodecene	C12H24	20.617	20.575	168	0.03
66	2-dodecene	C12H24	20.751	20.675	168	1.87
67	Dodecane (C12)	C12H26	20.950	20.908	170	0.12
68	3-ethyl-2,5-dimethyl-hexane	C10H22	21.275	21.250	142	0.01
69	3-dodecene	C12H24	21.375	21.333	168	0.04
70	2-propenoic acid (2-ethylhexyl ester)	C11H20O2	21.842	21.792	184	0.23
71	2,9-dimethyldecane	C12H26	22.533	22.517	170	0.01
72	2,3,7-trimethyl-decane	C13H28	22.658	22.633	184	0.02
73	Unknown		22.800	22.758		0.19
74	Nonanoic acid	C9H18O2	22.883	22.842	158	0.01
75	2-methyl-1,3-cyclohexanedione	C7H10O2	22.942	22.917	126	0.02
76	1,5,6,6-tetramethoxy-3-hexanone	C10H20O5	22.958	22.950	220	0.01
77	5-(2methylproyl)-nonane	C13H28	23.00	22.983	184	0.02
78	3,3-dimethyl heptane	C9H20	23.125	23.100	128	0.01
79	2,3,3-trimethyl-octane	C11H24	23.133	23.125	156	0.01
80	1,5-hexadiene-3-carboxylic acid, methyl ester	C10H16O3	23.167	23.158	184	0.01

Continued

Table I Continued

Peak	Chemical name	Chemical structure	RT (min)	RI (min)	MW	Abundance (%)
81	Tetradecane (C14)	C14H30	23.292	23.258	198	0.03
82	Bicycloheptan-2-ol	C12H2OO2	23.308	23.308	196	0.02
83	4,6,8-trimethyl-1-nonene	C12H24	23.417	23.375	168	0.04
84	Tridecane	C13H28	23.467	23.433	184	0.05
85	1-methoxy-4-(1-propyl)- benzene	C10H12O	23.517	23.492	148	0.05
86	8-methyl-1-undecene	C12H24	23.600	23.550	168	0.05
87	3,8-dimethyl-undecane	C13H28	23.717	23.675	184	0.04
88	2-butyl-1-octanol	C12H26O	23.775	23.750	186	0.03
89	3,7-dimethyl-decane	C12H26	23.833	23.800	170	0.16
90	4-methyl-1-undecene	C12H24	24.025	23.983	168	0.04
91	2-methyl-1-undecanol	C12H26O	24.075	24.050	186	0.04
92	2-methyl-naphthalene	C11H10	24.133	24.108	142	0.01
93	2,6-dimethyl-decane	C12H26	24.208	24.168	170	0.04
94	2,3,5,8-tetramethyl-decane	C14H30	24.300	24.275	198	0.02
95	8-methyl-3-undecene	C12H24	24.375	24.342	168	0.02
96	4,6- dimethyl-dodecane	C14H30	24.408	24.392	198	0.01
97	2-ethyl-1-decanol	C12H26O	24.458	24.433	186	0.01
98	3-tetradecene	C14H28	24.517	24.483	196	0.02
99	1,1,2-trimethyl-cycloundecane	C14H28	24.533	24.525	196	0.02
100	4,11-dimethyl-tetradecane	C16H34	24.600	24.575	226	0.02
101	6-ethyl-undecane	C13H28	24.725	24.700	184	0.02
102	2-butyl-1-decene	C14H28	24.767	24.733	196	0.13
103	Butyldiethylene glycol acetate	C10H2OO4	24.833	24.800	204	0.11
104	5-tetradecene	C14H28	24.883	24.867	196	0.05
105	Octanal dimethyl acetal	C10H22O2	24.983	24.967	174	0.01
106	3-methylene-tridecane	C14H28	25.133	25.092	196	0.14
107	4-tetradecene	C14H28	25.175	25.158	196	0.04
108	3-hexadecene	C16H32	25.311	25.233	224	5.79
109	Unknown		25.394	25.358		0.28
110	9-methyl-1-undecene	C12H24	25.483	25.450	168	0.07
111	Trans-,1-methyl- 2-(4-methylpentyl) cyclopentane	C12H24	25.667	25.642	168	0.02
112	2,6-dimethyl-naphthalene	C12H12	25.883	25.850	156	0.01
113	1,8-dimethyl-naphthalene	C12H12	26.108	26.092	156	00
114	3,5-dimethyloctane	C10H22	26.167	26.125	142	0.02
115	1,5-dimethyl-naphthalene	C12H12	26.217	26.167	156	0.02
116	5-methyl-tetradecane	C15H32	26.233	26.217	212	0.01
117	1,6,10-dodecatrien-3-ol	C15H26O	26.333	26.292	222	0.12
118	4,8- dimethyl-tridecane	C15H32	26.425	26.400	212	0.05
119	3-(1,1-dimethylethyl)-4-methoxy-phenol	C11H16O2	26.542	26.492	180	0.12
120	2,5-cyclohexadiene-1,4-dione, 2,6-bis(1,1-dimethylethyl)-	C14H2OO2	26.618	26.575	220	0.57
121	2-hexanoylfuran (2-furyl *n*-pentyl ketone	C10H14O2	26.750	26.642	166	5.08
122	Hexatriacontane	C36H74	26.875	26.850	506	0.05
123	Pentadecane (C15)	C15H32	26.992	26.958	212	0.23
124	6-tridecene	C13H26	27.033	27.017	182	0.02
125	Tricosane (C23)	C23H48	27.092	27.050	324	0.06
126	*n*-octadecyl ester, Heptafluorobutyric acid	C22H37F7O2	27.175	27.142	466	0.06
127	Butylated hydroxytoluene	C15H24O	27.183	27.183	220	0.03
128	2,4-*bis*(1,1-dimethylethyl)-phenol	C14H22O	27.297	27.225	206	5.31
129	2-methyl-hexadecane	C17H36	27.367	27.342	240	0.05
130	Unknown		27.417	27.383		0.34
131	2,9-dimethylundecane	C13H28	27.475	27.450	184	0.07
132	2-methyl-nonadecane	C20H42	27.575	27.542	282	0.10
133	unknown		27.642	27.600		0.21
134	6-methyl-pentadecane	C16H34	27.667	27.658	226	0.06
135	Nonadecane	C19H40	27.717	27.692	268	0.29
136	4-methyl-pentadecane	C16H34	27.808	27.783	226	0.15
137	7-hexyl-eicosane	C26H54	27.900	27.858	366	0.27
138	5-methyl-6-methylene-decane	C12H24	27.933	27.908	168	0.38
139	3-methyl-hexadecane	C17H36	28.005	27.975	240	0.30
140	2-hexyl-1-decanol	C16H34O	28.142	28.133	242	0.03
141	3,3,4-trimethyl-1-decene	C13H26	28.235	28.183	182	0.87
142	1-hexadecene	C16H32	28.387	28.292	224	10.16
143	Hexadecane (C16)	C16H34	28.433	28.408	226	1.32
144	Triacontane	C30H62	28.494	28.458	422	0.59
145	2-hexyl-1-octanol	C14H3OO	28.533	28.517	214	0.08
146	2-propylheptanol	C10H22O	28.583	28.558	158	0.30
147	2-ethyl-1-dodecanol	C14H3OO	28.667	28.625	214	0.15
148	Unknown		28.717	28.692		0.11
149	Tetratetracontane	C44H90	28.758	28.742	618	0.10
150	1-chloro-octadecane	C18H37Cl	28.842	28.808	288	0.27
151	Eicosane (C20)	C20H42	28.987	28.933	282	1.10
152	5-azulenemethanol	C15H26O	29.073	29.042	222	1.11
153	4-methyl-hexadecane	C17H36	29.156	29.108	240	0.40
154	2-methyl-octadecane	C19H40	29.240	29.200	268	0.62
155	4-cyclohexyl-tridecane	C19H38	29.292	29.267	266	0.16
156	3-methyl-tetradecane	C15H32	29.333	29.308	212	0.28
157	5β, 7βH, 10α-eudesm-11-en-1α-ol	C15H26O	29.367	29.350	222	0.18
158	*n*-dodecenylsuccinic anhydride	C16H26O3	29.425	29.400	266	0.68
159	Undeca-3,4-diene-2,10-dione, (5,6,6-trimethyl-)	C14H22O2	29.508	29.458	222	2.22
160	2,6-diisopropylnaphthalene	C16H20	29.577	29.550	212	1.26

Continued

Table I Continued

Peak	Chemical name	Chemical structure	RT (min)	RI (min)	MW	Abundance (%)
161	2,6,10,15-tetramethyl-heptadecane	C21H44	29.709	29.642	296	3.25
162	2,6,10-trimethyl-dodecane	C15H32	29.767	29.742	212	0.89
163	1-[2,3-O-Isopropylidene-5-deoxy-5-azido-β-d-ribofuranosyl]	C11H15N7O4	29.847	29.808	309	0.55
164	6-methyl-2-undecene	C12H24	29.892	29.875	168	0.14
165	Pentacosane (C25)	C25H52	29.950	29.925	282	0.11
166	Unknown		30.017	29.975		0.27
167	1,2,3-trimethyl-4-propenyl-naphthalene	C16H18	30.090	30.050	210	0.68
168	Octadecane(C18)	C18H38	30.142	30.125	254	0.15
169	2-oxo-tetradecanoic acid (methyl ester)	C16H30O3	30.208	30.175	270	1.07
170	2(3H)-phenanthrenone, 4,4a,9,10-tetrahydro-4a-methyl	C15H16O	30.274	30.233	212	1.97
171	6-methoxy-2-(1-buten-3-yl)naphthalene	C15H16O	30.326	30.300	212	1.30
172	4-methyl-heptadecane	C18H38	30.375	30.358	254	0.32
173	Heptadecane (C17)	C17H36	30.458	30.425	240	0.25
174	3-methyl-heptadecane	C18H38	30.483	30.467	254	0.60
175	2-methyl-1-octadecene	C19H38	30.552	30.525	266	0.69
176	3,5,3,5-tetramethylbiphenyl	C16H18	30.650	30.608	210	0.20
177	3,5-di-tert-butyl-4-hydroxybenzaldehyde	C15H22O2	30.675	30.667	234	0.14
178	unknown		30.750	30.717		0.24
179	5-octadecene	C18H36	30.758	30.750	252	0.25
180	3-octadecene	C18H36	30.865	30.792	252	6.59
181	Unknown		30.908	30.892		0.27
182	2,6,10,14-tetramethyl-hexadecane	C20H42	30.942	30.917	282	0.88
183	2-methyl-eicosane	C21H44	30.992	30.975	296	0.11
184	unknown		31.108	31.050		0.40
185	Isopropyl ester, 1-methylethyl ester, isopropyl Myristate	C17H34O2	31.185	31.142	270	0.55
186	unknown		31.342	31.308		0.20
187	unknown		31.350	31.350		0.10
188	Octacosane	C28H58	31.433	31.408	394	0.14
189	2,3-dimethyl-heptadecane	C19H40	31.517	31.492	268	0.09
190	2,6-dimethyl-heptadecane	C19H40	31.592	31.567	268	0.09
191	unknown		31.680	31.642		0.30
192	Buty octyl ester	C20H30O4	31.765	31.708	334	0.49
193	unknown		31.825	31.808		0.02
194	1-docosene	C22H44	31.892	31.867	308	0.13
195	unknown		32.000	31.958		0.34
196	7,9-di-tert-butyl-1-oxaspiro (4,5) deca 6,9-diene-2,8-dione	C17H24O3	32.325	32.275	276	1.03
197	14-methyl-pentadecanoic acid, methyl ester	C17H34O2	32.342	32.342	270	0.45
198	3,5-di-tert-butyl-4-trimethylsiloxytuluene	C18H32OSi	32.517	32.392	292	3.64
199	2-methyl-1-tetradecene	C15H30	32.704	32.558	210	0.31
200	*n*-hexadecanoic acid	C16H32O2	32.775	32.725	256	0.23
201	Dibutylphthale	C16H22O4	32.833	32.792	278	0.99
202	3-methylbutyl ester, pentadecanoic acid	C15H30O2	32.942	32.917	242	0.13
203	5-eicosene	C20H40	32.950	32.942	280	0.01
204	2,4-diacetoxy-3-methyl-1-(trityloxymethyl)pentyl ester	C32H36O7	32.958	32.950	532	0.10
205	9-Eicosane	C20H40	33.033	32.983	280	3.44
206	Unknown		33.050	33.050		0.15
207	Octadecyl ester	C20H40O2	33.183	33.167	312	0.09
208	3,4-dihydro-4,4,7,8-tetramethyl-cumarin-6-ol	C13H16O3	33.492	33.475	220	0.10
209	1-hexadecanol	C16H34O	34.009	33.975	242	0.32
210	1-decanethiol	C10H22S	34.142	34.108	174	0.13
211	Octadecanoic acid, methyl ester	C19H38O2	34.375	34.350	298	0.07
212	N,N-*bis*[2-trimethylsiloxyethyl] ethanamine	C12H31NO2Si2	34.525	34.500	277	0.03
213	Unknown		34.708	34.683		0.17
214	Arachidic acid	C20H40O2	34.775	34.750	312	0.05
215	Octyl-cyclodecane	C18H36	34.942	34.900	252	0.19
216	3-eicosane	C20H40	34.997	34.958	280	1.36
217	Acetic acid, octadecyl ester	C20H40O2	35.150	35.125	312	0.18
218	6-cyclohexyl-tridecane	C19H38	35.550	35.517	266	0.07
219	2-methylhexadec-1-ene	C17H34	36.550	35.525	238	0.10
220	Benzyl butylphthalate	C19H20O4	36.708	35.683	312	0.11
221	Dioctyl ester, hexanedioic acid	C22H42O4	36.816	35.758	370	1.87
222	5-cyclohexyl-tridecane	C19H38	37.267	37.242	266	0.08
223	Pentatriacontane	C35H72	37.692	37.667	492	0.05
224	1,2-benzenedicarboxylic acid, diisooctyl ester	C24H38O4	38.130	38.058	390	3.92
225	Unknown		38.250	38.225		0.09
226	1-tricosene	C23H46	38.485	38.450	308	0.27
227	Unknown		38.883	38.842		0.10
228	1-chloro-heptacosane	C27H55Cl	39.300	39.267	414	0.07
229	Trifluoroacetic acid (*n*-octadecyl ester)	C20H37F3O2	40.158	40.125	366	0.12
230	13-docosenamide, Erucylamide	C22H43NO	40.267	40.233	337	0.12
231	2,6,10,14,18-pentamethyl-2,6,10,14,18-eicosapentaene	C25H42	40.358	40.333	342	0.18
232	1,3,5-*tris*(2,2-dimethylpropyl)-2-iodo-4-methyl-benzene	C22H37I	41.125	41.042	428	0.37

0.25 mm × 0.25 μm, the oven program had an initial temperature of 50°C for 0.5 min, then a 10°C/min run to 245°C, with a final hold at 245°C (5 min); the injector temperature was kept at 240°C (splitless) and the detector temperature was 260°C. Three replications were done with each column in these experiments.

The carrier gas was nitrogen (N2) at a linear flow-rate of 25 cm/s. The MS detector was operated at 194°C. The scan

Table II
Volatile Compounds Identified from *T. harzianum* using *n*-Butanol Solvent

Peak	Chemical name	Chemical structure	RT (min)	RI (min)	MW	Abundance (%)
1	2-methyl-1-butanol	C5H12O	5.274	5.258	88	1.13
2	Butanoic acid, Butyl ester	C8H16O2	5.325	5.308	144	0.63
3	1,1-dibutoxyethane, 1,1-[ethylidenebis(oxy)]bis-butane	C10H22O2	5.374	5.350	174	1.95
4	1-dodecene	C12H24	5.475	5.450	168	0.25
5	3-hydroxy-2-butanone	C4H8O2	5.942	5.883	88	0.67
6	1,1-dibutoxy-butane	C12H26O2	6.092	6.050	202	0.14
7	N,N-dimethyl-formamide	C3H7NO	6.500	6.458	73	0.51
8	1,1-dibutoxy-2-propanone	C11H22O3	6.950	6.892	202	11.23
9	2-butoxy-ethanol	C6H14O2	7.383	7.342	118	0.58
10	1-tetradecene	C14H28	7.925	7.900	196	0.41
11	Ethanoic acid	C2H4O2	8.171	8.150	60	56.88
12	unknown		9.369	9.325		1.42
13	unknown		9.895	9.817		18.19
14	1-chloro-hexadecane	C16H33Cl	10.575	10.542	260	0.44
15	2,5-furandione, dihydro-3-methylene	C5H4O3	11.450	11.400	112	1.05
16	2,2,2-trifluoro-acetamide	C2H2F3NO	12.033	11.992	113	0.21
17	unknown		12.425	12.408		0.04
18	2,4-dimethylbenzaldehyde	C9H10O	12.575	12.525	134	0.12
19	unknown		13.017	12.983		0.15
20	2-butoxyethyl acetate	C8H16O3	13.083	13.058	160	0.09
21	β phenylethyl alcohol	C8H10O	13.842	13.800	122	0.38
22	1-hydroxy-2- propanone	C3H6O2	14.175	14.133	74	0.22
23	Carbolic acid, phenol	C6H6O	15.008	14.983	94	0.05
24	unknown		15.267	15.233		0.10
25	unknown		16.667	16.617		0.73
26	4H-pyran-4-one	C6H8O4	17.733	17.692	144	0.09
27	3,5-*bis*(1,1-dimethylethyl)phenol	C14H22O	18.083	18.033	206	0.67
28	1-pyrrolidinamine	C4H10N2	18.317	18.267	86	0.31
29	isosorbide	C6H10O4	18.833	18.783	146	0.57
30	Hexadecanoic acid	C20H40O2	19.058	19.025	312	0.11
31	3H-pyrazol-3-one	C6H10N2O	19.992	19.950	126	0.08
32	2H-pyran-2-one	C6H10O3	20.442	20.392	130	0.44
33	2-propenyl ester, Pentanoic acid	C8H14O2	20.600	20.567	142	0.05
34	6 β hydroxyl-α-pentyl-3-oxa-a-homo-5α-androstane-4,17-dione	C24H38O4	22.108	22.075	390	0.11

Table III
Volatile Compounds Identified from *T. harzianum* using Methanol Solvent

Peak	Chemical name	Chemical structure	RT (min)	RI (min)	MW	Abundance (%)
1	1-methoy-2-propanone	C4H8O2	5.367	5.292	88	2.23
2	Glacial acetic acid	C2H4O2	8.117	8.083	60	50.26
3	1,3-butanediol	C4H10O2	9.313	9.275	90	2.50
4	2,3-butanediol	C4H10O2	9.870	9.800	90	33.77
5	3-methyl-2,5-furandione	C5H4O3	11.442	11.383	112	1.18
6	Phenylethyl alcohol	C8H10O	13.849	13.808	122	2.20
7	Carbamic acid, phenyl ester	C7H7NO2	15.033	14.992	137	0.53
8	Decahydro-1,6-dimethyl-naphthalene	C12H22	16.672	16.625	166	2.67
9	2,3-dimethyl-oxirane, *cis*-	C4H8O	17.758	17.725	72	0.26
10	1-aminoacetyl- piperazine	C6H13N3O	18.343	18.300	143	1.03
11	N-aminopyrrolidine	C4H10N2	18.859	18.817	86	1.74
12	unknown		20.475	20.425		1.62

range was from 35 to 450 m/z at a scan rate of 0.50 scan/s. Solvent delay was 15 min. To check the purity of each GC peak, MS was taken at various parts of each peak. Normal saturated hydrocarbons (C7–C30), cyclohexane, cyclopentane, fatty acids, alcohol, benzene derivatives and other compounds were putatively identified by mass spectral database search (NIST/EPA/NIH) followed by matching of MS data. All volatiles showing mass spectra with match factors $\geq$ 90% were put on a "positive list" of tentatively identified metabolites.

Reagents

Ethyl acetate, *n*-butanol, *n*-hexane and methanol (high purity 99.99%) were obtained from Baker (USA) and checked by GC–MS before the analysis for volatile metabolites.

Results and Discussion

The objective of this study was to develop a simple and reliable method for the investigation of volatile metabolites excreted by the cultured *T. harzianum* (under typical solvents); some parameters have been chosen during method development. Cultivation of the fungus was carried out under incubation to avoid oxygen limitation during fungus growth and to allow the sample extraction, chromatographic separation and MS detection. To reduce the risk of variations in the mixtures by manual handling, extraction procedures were performed before chemical mixture and GC–MS analysis. The GC–MS data was deconvoluted using the NIST software and the measured mass spectra was matched to entries in the compound library.

According to these criteria, over 278 fungal metabolites were identified from the *T. harzianum* sample. The volatile

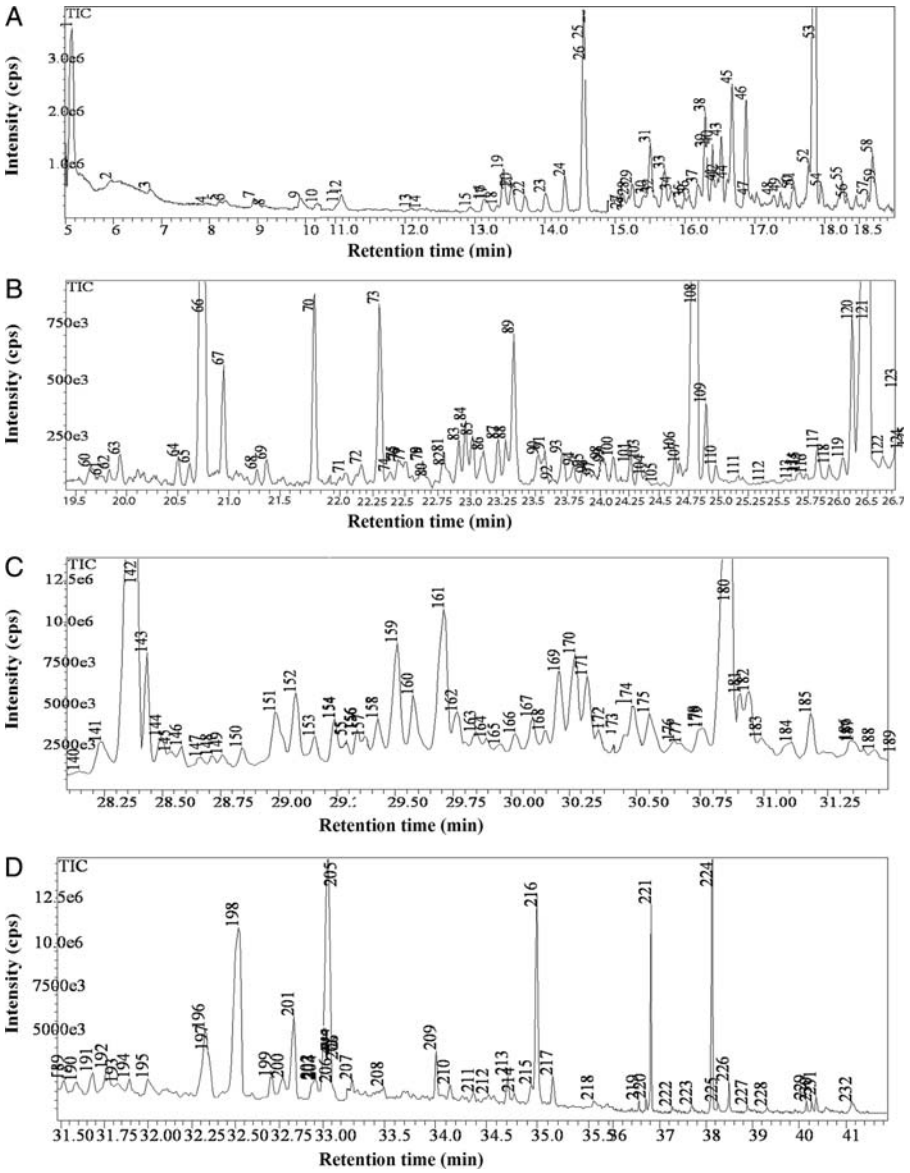

Figure 2. GC–MS of volatile compounds identified from the cultured *T. harzianum* (FA1132) isolated using a capillary column with nonpolar stationary phases; the identified peaks are shown in Table I.

compounds detected in the culture samples constitute members of the compound classes of alkanes, alcohols, ketones, pyrones (lactones), furanes, monoterpenes, and sesquiterpenes for which the fungal origin has been previously reviewed (3, 31). The compositions of all compounds are presented in Tables I, II and III; numerous minor peaks were found (< 0.1%) and could not be identified. Primarily, hydrocarbons, fatty acids, alcohol and benzene derivatives were identified from *T. harzianum* (FA1132) including cyclohexane, cyclopentane and other compounds that were found among the volatile metabolites.

Separation of compounds isolated from *T. harzianum* was conducted with three different capillary columns. The first column (BP1) allowed reasonable retention of the low volatile metabolites, but other components were not separated and passed through into the second column (BP10) and third column (BPX70) while the volatile metabolites were detected. Figures 2, 3 and 4 illustrate the chromatograms of the volatile metabolites obtained from nonpolar, medium polar and high polar columns. The column separation of many compounds, including some *cis* and *trans*-isomers of 1-ethyl-4-methyl-cyclopentane and 1-methyl- 2-(4-methylpentyl) cyclopentane and other volatile compounds were putatively identified in different groups by using three different capillary column of GC–MS based on their chemical structure, such as simple aromatic metabolites, terpenes, isocyano metabolites, some polyketides, butenolides and pyrones. Over 278 volatile compounds were identified during the experiment. None of these compounds has been reported earlier by any researchers. Sivasithamparam and Ghisalberti (4) reported that *Trichoderma* species were known to produce over 40 different metabolites that contributed to their mycoparasitic and antibiotic actions.

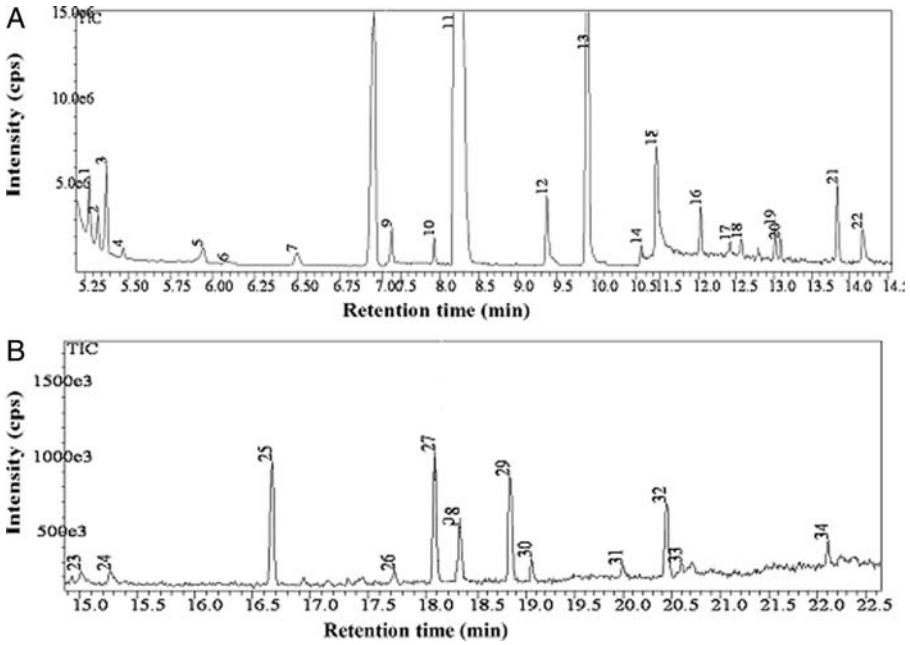

Figure 3. GC–MS of volatile compounds identified from the cultured *T. harzianum* (FA1132) isolated using a capillary column with medium polar stationary phases; the identified peaks are shown in Table II.

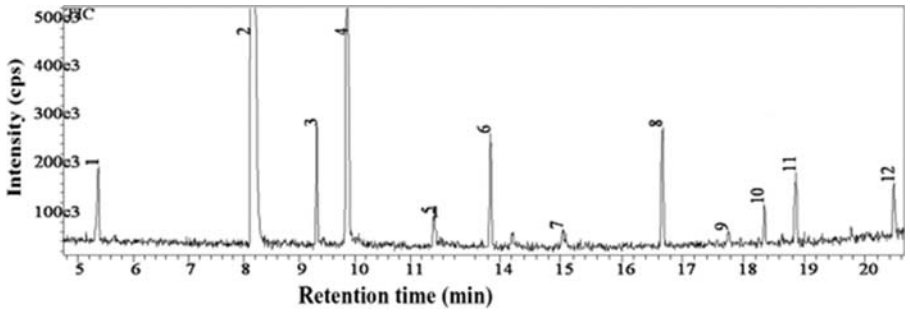

Figure 4. GC–MS of volatile compounds identified from the cultured *T. harzianum* (FA1132) isolated using a capillary column with high polar stationary phases; the identified peaks are shown in Table III.

Identification of volatile metabolites of *T. harzianum* include commonly obtained *n*-hydrocarbons in the C–C range with a predominance of C (Table I). According to these results, alkane hydrocarbons from C7 to C25 were thus identified, with a predominance of C12–C16 (Figure 2). These results essentially differ from published data concerning of all the alkanes. The contents of C and 7- and 8-methyl-C dominate and vary from 60 to 97% of the total hydrocarbons. Their hydrocarbon content is rather low in comparison with photobiont hydrocarbons.

The secondary metabolites with antibiotic activities that are produced by *T. harzianum* are classified in different groups based on their chemical structure and include non-volatile (e.g., peptaibols) and volatile (e.g., simple aromatic metabolites, terpenes, the isocyano metabolites, some polyketides, butenolides and pyrones) compounds (5). Some volatile compounds are associated with the antagonistic ability of *T. harzianum*, but none has been identified as a sole responsible agent of such biocontrol activity. One of the most abundant metabolites that was identified in this study was 6-pentyl-alpha-pyrone (6-PP), which was originally characterized by Collins and Halim (32), and identified as one of the key bioactive compounds of several species, e.g., *T. harzianum* and *T. koningii* as reviewed by Hanson (33). This is the most important volatile compound that was obtained from pyrone (peak 32 in Figure 3). In Figure 3, peak 32 is identified as 6-*n*-pentyl-6H-pyran-2-one (6PP). This compound is a nontoxic flavoring agent that was chemically synthesized for industrial purposes before its discovery as a natural product. 2-Nonanone and 2-undecanone have been described to be produced by *T. aureoviride* IMI 91968 (34); 3-octanone, 3-octanol, beta-sesquiphellandrene, 2-pentylfuran and 1-octen-3-ol have been detected in cultures of *T. atroviride* CCM F-536 (formerly classified as *T. viride*) and *T. atroviride* I2 (8). 2-Heptanone has previously been reported for *T. viride* (isolate T60) (12); phenylethyl alcohol is reported to be produced by *T. harzianum* 201 (35).

6-PP has been detected before in cultures of *T. atroviride* ATCC 74058 (9, 36) and other *Trichoderma* species (37). The

biological effects of 6-PP are numerous: it has been shown to reduce the production of the mycotoxin deoxynivalenol by *Fusarium graminearum* (38), and to exert antifungal properties by reducing the mycelial growth rate of *Rhizoctonia solani* and *F. oxysporum f. sp. lycopersici* (39). Since Vinale *et al.* (5) reported that 6-PP has a regulatory effect on wheat seedlings (growth promotion at low concentrations and growth inhibition at high concentrations of 6-PP). In comparison to control plants, tomato plants developed more vigorously and had a more extensive root system after their leaves had been sprayed with 6-PP (5).

To the best of our knowledge, this is the first application using three different capillary columns via three kinds of solvents (nonpolar, medium polar and high polar) to putatively identify a massive number of volatile compounds from filamentous fungus *T. harzianum* strain FA1132, obtaining a few molecular molecules and volatile compounds such as simple aromatic compounds, some polyketides such as pyrones and butenolides, volatile terpenes and isocyane metabolites. These are all relatively nonpolar and medium polar substances with a significant vapor pressure. Polar metabolites have high molecular weight, which, like peptaibols, allows for successful separation by using high polar column BPX70 stationary phases.

Conclusions

Volatile metabolites are highly involved in the multifaceted interactions between filamentous fungi and their living environment. Thus, analytical methods for the identification of volatiles are required to study their formation and function in biological interactions. The present study successfully separated hydrocarbons and other volatile compounds by using three different capillary columns with different nonpolar, medium polar and high polar stationary phases. Because the cultivation of fungi can be carried out directly on a GC–MS, measurement is recognized in a fully programmed way, and the presented approach offers an interesting and powerful tool for the study of the dynamic range of volatile metabolites. Based on specific criteria such as mass spectral match factors and retention indices, more than 278 different volatile compounds are identified that had not yet been ascribed to *T. harzianum*.

Acknowledgements

The authors acknowledge the valuable contributions of the late Prof. Dr. Faridah Abdullah throughout this study. This paper is therefore dedicated to her memory.

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