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Consistent production of phenolic compounds by *Penicillium brevicompactum* for chemotaxonomic characterization

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Abstract

A consistently produced group of fungal secondary metabolites from *Penicillium brevicompactum* has been purified and identified as the Raistrick phenols. These compounds are shown to exist separately as an equilibrium mixture in aqueous solutions. The Raistrick phenols have all been included in the metabolite profile of *P. brevicompactum*. By means of thin layer chromatography-scanning and high performance liquid chromatography-UV diode array detection, the chromatographic and spectroscopic data can be used in the chemotaxonomic characterization of the fungus.

Introduction

Penicillium brevicompactum Dierckx produces a variety of known and unknown secondary metabolites, of which some only are produced under very specific conditions, whereas the production of others is less dependent on the substrate and other external factors (Campbell 1984; Frisvad & Filtenborg 1989; Frisvad & Filtenborg 1990). Campbell (1984) describes secondary metabolites as: 'products of normal cellular metabolism that are more restricted in their distribution, being found in less than every species in a single family'. Modern fungal taxonomy includes several aspects: a.o. classic morphological taxonomy, physiological taxonomy and chemotaxonomy. Chemotaxonomy may be based on the species specific production of both intra- and extracellular secondary metabolites, which gives the metabolite profile, that is unique for each fungal species. The metabolite profiles can be visualized by thin layer chromatography (TLC), TLC scanning (TLC/S) and high performance liquid chromatography-UV diode array detection (HPLC-DAD) (Frisvad & Filtenborg 1989; Frisvad & Filtenborg 1990; Filtenborg et al. 1980; Filtenborg et al. 1983; Frisvad & Thrane 1987). It is often seen that two different fungal species have one or more secondary metabolites in common, but other metabolites can discriminate between the two fungal species (Frisvad & Filtenborg 1989). Because of the consistent production of some secondary metabolites by *P. brevicompactum* (Frisvad & Filtenborg 1989; Frisvad & Filtenborg 1990), these metabolites become of significance to the characterization of this fungus.

Of the known secondary metabolites from *P. brevicompactum* mycophenolic acid is produced consistently (Frisvad & Filtenborg 1989; Frisvad & Thrane 1987). Mycophenolic acid was first isolated from *P. stoloniferum* Thom (= *P. brevicompactum*) by Gosio in 1896 and is one of the oldest known antibiotics (Danheiser et al. 1986). Both a

total synthesis (Danheiser et al. 1986) and a biosynthetic pathway (Colombo et al. 1982; Bowen et al. 1977) have been proposed. Mycophenolic acid has previously been included in the metabolite profile (Frisvad & Filtenborg 1989) and the chromatographic and spectroscopic data are therefore well known. Consistently produced are also some compounds which fluoresce blue in UV-light (254 nm). The working theory in the present study was that these compounds may be the Raistrick phenols, because they are reported to be produced by *P. brevicompactum* and are known for their strong fluorescence (Clutterbuck et al. 1932). The Raistrick phenols is a collective name for three closely related compounds:

- 2,4-dihydroxy-6-(2-oxopropyl) benzoic acid,
- 2,4-dihydroxy-6-(1-hydroxy-2-oxopropyl) benzoic acid, and
- 2,4-dihydroxy-6-(1,2-dioxopropyl) benzoic acid.

In this paper these three metabolites will be referred to as 'the ketone', 'the ketol' and 'the diketone', respectively: Fig. 1 shows formulae and conformations. These secondary metabolites and 3,5-dihydroxyphthalic acid were isolated from *P. brevicompactum* and identified in 1932–33 (Clutterbuck et al. 1932; Oxford & Raistrick 1933). Godin (1953 and 1955) reported the isolation of the Raistrick phenols, 3,5-dihydroxyphthalic acid, 2,4-dihydroxy-6-propyl benzoic acid and 5 unidentified phenolic compounds from *P. brevicompactum*. One or more of the Raistrick phenols have also been isolated from the fungal species *Ceratocystis ulmi* (Buisman) Moreau (Claydon et al. 1974), *C. ips* (Rumb) Moreau (Ayer et al. 1986) and *Alternaria kikuchiana* Tanaka (Kameda et al. 1973). Brevianamide A is also produced by *P. brevicompactum* (Birch 1971), but not as consistently as the former compounds. The production is dependent on both the substrate and on the isolate (Frisvad & Filtenborg 1990). Brevianamide A has a yellow fluorescence in UV-light (366 nm) and is, dispite its inconsistent production, included in the metabolite profile and used in the chemotaxonomic characterization of *P. brevicompactum*.

The aim of the present work was to identify and characterize the consistently produced compounds from *P. brevicompactum* which give rise to the blue fluorescent spots in UV-light (254 nm) and to include their chromatographic and spectroscopic data together with data for mycophenolic acid and brevianamide A in the metabolite profile of *P. brevicompactum*.

Materials and methods

Metabolite standards

Standards (brevianamide A, mycophenolic acid and grisefulvin) of fungal secondary metabolites were obtained from a collection of fungal secondary metabolites held at the Department of Biotechnology, The Technical University of Denmark.

Reagents

All solvents were of analytical reagent grade, except for the acetonitrile used for analytical HPLC, which was of HPLC grade (Merck no. 14291).

Chromatographic equipment

Analytical HPLC was performed on a Hewlett-Packard HP 1090M High Performance Liquid Chromatograph equipped with two pumps, a built-in Photodiode Array Detector (DAD) and an external computer control (HP 9000 Model 310). A 100 mm × 4.0 mm I.D. Nucleosil 5 μm C_{18} reversed phase column was used (Macherey-Nagel & Co., Duren, Germany). The flow rate was 2.0 ml/min and injections (10 μl) were made with an autosampler injector system (Frisvad & Thrane 1987).

Preparative HPLC was performed on a 440 mm × 37 mm I.D. LiChroprep 40–63 μm C_{18} reversed phase column (Merck) with a Shimadzu CECIL 2012 variable wavelength UV detector and an external pump. Fungal extracts (see later) (650 mg) dissolved in as little eluent as possible

were injected manually on the column. The flow-rate was 20 ml/min and the detector monitored at 220 nm.

Analytical TLC was performed on 0.25 mm silica gel 60 precoated plates with and without fluorescent indicator (Merck no. 5715 and Merck no. 5721, respectively) and the reflectance spectra were recorded on a CAMAG TLC Scanner II. Preparative TLC was performed on glass plates coated with 1.0 mm silica gel 60 PF_{254} (Merck no. 7747).

Other equipment

IR spectra were recorded on a Perkin Elmer 1720 FTIR, MS spectra were recorded on V.G. trio 2 equipment and NMR spectra were recorded on a Bruker AC-250 MHz instrument.

Fungi and growth conditions

For metabolite profile analysis by TLC and HPLC-DAD and for checking for consistent production, 10 isolates of *Penicillium brevicompactum* Dierckx (IBT 3482, 4342, 5951, 6483, 6497, 6500, 6504, 6603, 6609, 10040) and 1 isolate of *P. echinulatum* (Raper & Thom) Fassatiova (IBT 5110) were grown on Czapek yeast autolysate agar (CYA) (Pitt 1979) and yeast extract sucrose agar (YES) (Scott et al. 1970) for 7 days at 25° C in the dark. For isolation and purification of secondary metabolites *P. brevicompactum* (IBT 6500) was grown as a surface culture on liquid glucose-amino acid medium (GAM) (Salemink et al. 1965) at 25° C in the dark for 25 days.

Purification of metabolites

The liquid medium (GAM) was filtered and rotary evaporated to dryness under vacuum. To the residue was added 100 ml 5% Sodium bicarbonate and 100 ml ethyl acetate per 1000 ml evaporated medium. The 2 layers were separated and the ethyl acetate layer was washed twice with 25 ml 5% Sodium bicarbonate. The ethyl acetate layers were discarded. The pooled aqueous layers were adjusted to pH 3 with 4 N hydrochloric acid and extracted 3 times with 1/3 vol. ethyl acetate. The pooled ethyl acetate layers were dried with Magnesium sulphate, filtered and rotary evaporated to dryness under vacuum. The residue was dissolved in as little water as possible and the metabolites separated into three fractions on preparative, reversed-phase HPLC with water/methanol/acetic acid (100 : 10 : 1, 200 : 35 : 2 and 100 : 100 : 1, v/v/v). The fractions collected were each rotary evaporated to dryness under vacuum and dissolved in acetone. Each fraction was purified on preparative TLC silica gel plates using TEF (toluene/ethyl acetate/90% formic acid, 5:4:1, v/v/v) as eluent. The bands were removed from the TLC plate and extracted 3 times each with 3 vol. of 80° C hot water. The water was evaporated and each metabolite was recrystallised from ethyl acetate. The identity of the metabolites was confirmed by comparing IR, MS and NMR spectra of the purified metabolites with literature data (Claydon et al. 1974; Kameda et al. 1973; Grove & Pople 1979). The purified metabolites were subjected to the same analyses (see later) as the agar plugs and the extracts in order to compare and identify metabolites in the agar medium and the extract.

Metabolite profile analyses

Agar plugs (4 mm in diameter) were taken directly from 7 days old Petri dish cultures and placed on two TLC plates (application line: 25 mm from the bottom) together with an external standard (griseofulvin), for metabolite profile analysis by TLC. The TLC plates were eluted (developing time: 15 min) in two different systems: TEF (toluene/ethyl acetate/90% formic acid, 5:4:1, v/v/v) and CAP (chloroform/acetone/2-propanol, 85:15:20, v/v/v) (Frisvad & Thrane 1987). After drying the plates were scanned by a TLC Scanner for reflectance spectra.

Five Petri dishes of each substrate (CYA and

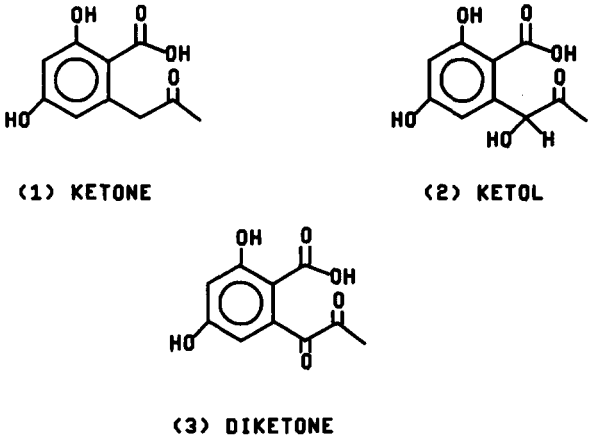


Fig. 1. The structures of the Raistrick phenols: 2,4-dihydroxy-6-(2-oxopropyl) benzoic acid (1), 2,4-dihydroxy-6-(1-hydroxy-2-oxopropyl) benzoic acid (2) and 2,4-dihydroxy-6-(1,2-dioxopropyl) benzoic acid (3).

YES) with 7 days old cultures were extracted for metabolite profile analysis by HPLC. The agar substrate was extracted once with 150 ml chloroform/methanol (2:1, v/v) and once with 150 ml ethyl acetate and 3 ml formic acid (98%) in a Colworth Stomacher 400. The extracts were filtered, pooled and rotary evaporated to dryness under vacuum. The residue was dissolved in 3 ml acetonitrile and defatted twice with 3 ml hexane. The hexane layers were discarded. The acetonitrile extract was analysed by HPLC-DAD using an acidic acetonitrile/water gradient (Frisvad & Thrane 1987).

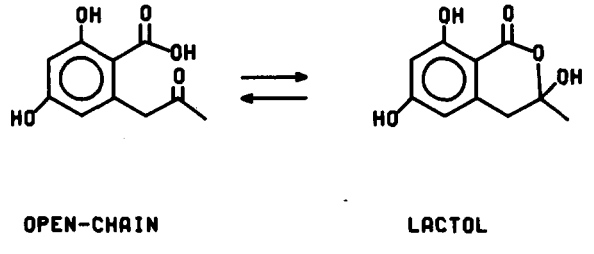


Fig. 2. The structures of the two tautomers of 2,4-dihydroxy-6-(2-oxopropyl) benzoic acid (the ketone).

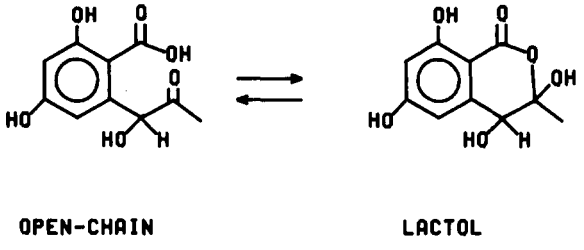


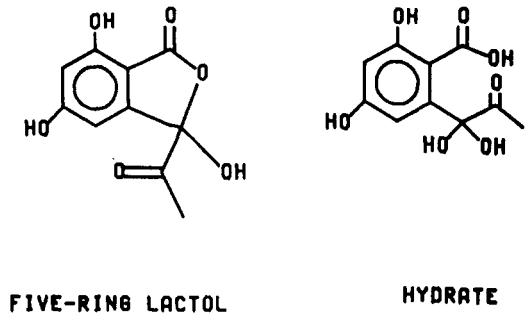
Fig. 3. The structures of the two tautomers of 2,4-dihydroxy-6-(1-hydroxy-2-oxopropyl) benzoic acid (the ketol).

Results and discussion

Purification and identification

The metabolites of *Penicillium brevicompactum*, responsible for the most conspicuous blue fluorescence spots in UV light (254 nm), were shown to be the three Raistrick phenols (the ketone, the ketol and the diketone). (Fig. 1). The IR- and MS-spectra of the purified, crystalline metabolites were identical with the literature data of the Raistrick phenols (Claydon et al. 1974; Kameda et al. 1973; Grove & Pople 1979). This suggests that both the ketone and the ketol exist in solid form as their lactor forms, and that the diketone exists as a five-ring lactol (Figs 2, 3, 4). Grove & Pople (1979) reported that the diketone in organic solvents is present as the open chain tautomer, and that the ketone in both the solid state and in solution exists in the lactol form. Also, the ketol exists in the lactol form in solution according to Grove & Pople (1979). The major difficulty was to determine which of the possible tautomeric forms (Figs 2, 3, 4) the three phenols actually adopted in aqueous solution (e.g. in growth medium, extracts and standards analysed in aqueous eluents) which are the conditions for metabolites in our analyses.

The results of the analyses of the Raistrick phenols in aqueous solution by NMR showed that the majority of the ketol, under all conditions, exists as the lactol in agreement with Grove & Pople (1979). Only about 2% (on peak height basis) of the ketol exists as the open chain form and could only be detected by HPLC-DAD. Regarding the ketone, the results indicate an equilibrium between the



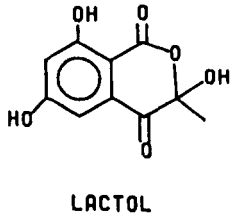


Fig. 4. The structures of the three tautomers of 2,4-dihydroxy-6-(1,2-dioxopropyl) benzoic acid (the diketone).

lactol and the open chain form, where the open chain form is dominant (Ratio 1:10). This finding, however, does not agree with Grove & Pople (1979), who detected exclusively the lactol form. Kameda et al. (1973) report, though, that the open chain form can be detected in small amounts (about 5%) by NMR in organic solvent. In the present study the diketone was never found as the open chain form with two ketone functions. This tautomer should give a UV spectrum quite different from that observed, because of its two 'extra' conjungations. Instead, the HPLC-DAD showed an equilibrium between the hydrate and the lactol form of the diketone. One of the UV spectra of the purified diketone mixture in the acetonitrile/water gradient resembled that of the ketone. This indicates that one of the diketone tautomers must have a similar chromophore. This, and a very short retention time, suggests that one of the diketone tautomers, at least under aqueous conditions, exists as the more polar hydrate, which was confirmed by NMR. Smaller amounts of the lactol of the diketone have been detected (Ratio 1:3).

It was shown by means of model compounds (orsellinic acid, 6-methoxy mellein, 5'-hydroxyasperentin and zearalenone) with similar chemical structures and thereby similar chromophores, that it was possible to differentiate between the open chain form and the lactol form by their UV spectra. The spectrum of a lactol form shows an additional shoulder at 227 nm, as compared to the spectrum of a open chain form (Fig. 5). The lactol form of the diketone shows a similar shoulder, but a 253 nm, because of the bathochromic shift the overall spectrum exhibits.

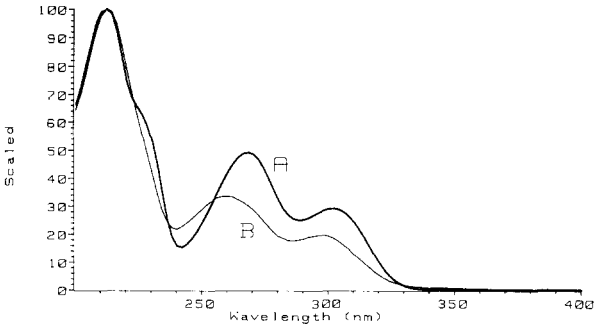


Fig. 5. The ultraviolet spectra of the two tautomers of the ketol. Spectrum A is the lactol tautomer and spectrum B is the open chain tautomer.

Metabolite profile

In the light of the abovementioned findings, concerning the chemical structures of the 6 tautomers of the Raistrick phenols, and using the described methods, the chromatographic and spectroscopic data of the metabolites in the metabolite profile of *P. brevicompactum,* could be listed in Table 1.

Table 1 contains the results of the metabolite profile analyses of agar plug, extract, standards and purified metabolites by TLC/S and HPLC/DAD. The first column gives the ratios between the two tautomers of each metabolite, except for mycophenolic acid and brevianamide A. In the second and third column are listed the R_f values relative to griseofulvin (R_{fg}) (x 100) in two TLC systems (TEF and CAP) for both tautomers of each metabolite as far as they could be detected. The lactol form of the ketone and the hydrate of the diketone have almost identical R_{fg} values in TEF and appear as one spot, when both are present in agar plugs and extracts.

Table 1. Chromatographic and spectroscopic characteristics of metabolites in the metabolite profile of *Penicillium brevicompactum*.

Metabolite	Ratio[a]	R_f value relative to griseofulvin (x 100) (R_{fg})		Absorption maximum (*), shoulder(sh) and minimum (nm) and relative absorbance (%)				RI[b]
		TEF	CAP	Reflectance spectra nm	%	Ultraviolet spectra nm	%	
Ketone open-chain	10	124	55	214*	55	214*	100	719
				237	30	239	18	
				266*	100	263*	37	
				287	72	287	18	
				299*	78	298*	21	
Ketone lactol	1	146	ND[c]	214*	30	214*	100	807
						228sh	63	
				238	4	240	13	
				269*	100	271*	63	
				289	60	292	26	
				300*	64	300*	28	
Ketol open-chain	1	ND	ND	ND		212*	100	717
						240	22	
						261*	33	
						287	18	
						298*	20	
Ketol lactol	50	93	38			212*	100	698
				226*	69	227sh	57	
				239	62	242	15	
				268*	100	269*	48	
				285	90	288	24	
				301*	95	302*	28	
				352sh	29			
Diketone hydrate	3	144	41	234*	89	214*	100	680
				245	88	239	18	
				257*	90	261*	46	
				275	77	279	15	
				296*	100	296*	23	
				345sh	59			
Diketone lactol	1	165	ND	208	13	221	50	855
				238*	100	239*	100	
				253sh	80	253sh	67	
				271	34	275	18	
				295*	54	298*	25	
				319	36	323	18	
				345*	45	347*	22	
Mycophenolic acid		146	77			216*	100	985
				227sh	89	237	17	
				255*	100	251*	25	
				282	80	273	3	
				314*	90	304*	12	
				340sh	51			
Brevianamide A		71	82	200	64	207	66	877
				235*	100	233*	100	
				251	86			
				262*	90	265sh	22	
				287	47	288	1	
				314*	53			
				331	51			
				399*	93	407*	13	

[a] The ratios between the two tautomers of each metabolite are calculated on basis of the areas from the HPLC-DAD.
[b] Retention indices are calculated according to Frisvad and Thrane (1987). [c] ND – not detected.

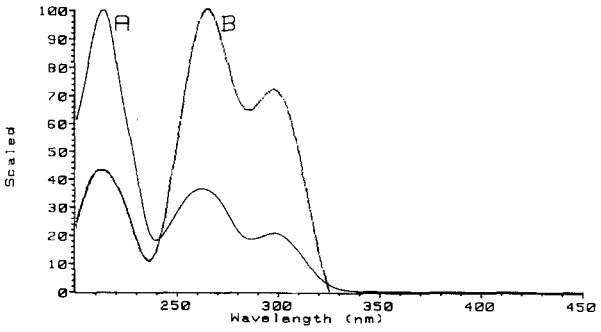


Fig. 6. The ultraviolet spectrum (A) and the reflectance spectrum (B) of the open chain tautomer of the ketone.

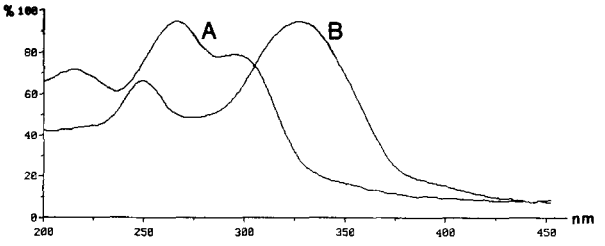


Fig. 7. The reflectance spectra of two spots with the same R_{fg} value. Spectrum A is the open chain tautomer of the ketone from *P. brevicompactum* and spectrum B is penechin from *P. echinulatum*.

They could only be recognized as two different compounds on HPLC-DAD, because of their different Retention Indices. The fourth and fifth column contain the maximum (marked with *) and minimum absorptions of the reflectance and ultraviolet spectra of all tautomers, except the open chain form of the ketol, which could not be detected on TLC. The Retention Indices calculated from the alkylphenones are given in the sixth column.

Recording of ultraviolet spectra is an established discipline, whereas reflectance spectra are not yet very widely used. The advantage of the TLC Scanner is that it is possible to determine reflectance spectra directly on the TLC plate by comparison with a substance free area on the plate (Jork et al. 1990). The wavelength maxima of the reflectance spectra often correspond to those in the ultraviolet spectra of the same substance in solution, as seen in Fig. 6 for the open chain form of the ketone, where the wavelength maxima are similar, in contrast to the relative absorbance. Traces of absorbent and solvent can cause either bathochromic shifts (for ketones and aldehydes) or hypsochromic shifts (for phenols) (Jork et al. 1990). The TLC Scanner can, however, be employed as a quick aid to characterization of e.g. fungal metabolites, particularly when standards are chromatographed on the same plate. The reflectance spectra can be used to determine whether two spots with the same R_f value are identical or not. In the present work the TLC Scanner made it possible to discriminate between two different fungal species by their reflectance spectra. Figure 7 shows the reflectance spectra of two spots (from *P. brevicompactum* and *P. echinulatum*) with same R_{fg} value, one is a penechin derivative from *P. echinulatum*, the other is the open chain form of the ketone from *P. brevicompactum*. Both spots show the same blue fluorescence in UV-light at 254 nm.

Conclusion

The fact that the Raistrick phenols can be extracted as their sodium salts with aqueous Sodium bicarbonate, should favour the open chain forms of the metabolites with their free carboxylic group but since the ketol in its lactol form is extracted too, this suggests that the Raistrick phenols, at least in their lactol forms, are more soluble in water than in ethyl acetate. Regarding the ultraviolet spectra of the Raistrick phenols, it is possible to distinguish between the lactol form and the open chain form of both the ketone and the ketol. The spectra of the hydrate and the lactol form of the diketone are quite easy to distinguish, because of the extra conjungation of the lactol form, which causes the bathochromic shift of the spectrum compared with the spectrum of the hydrate. Differentiation among the lactol forms or open chain forms is only possible because of their distinct Retention Indices, since their chromophores, and thereby their ultraviolet spectra, are identical, except for the lactol form of the diketone.

In the present work the maximum wavelengths of the reflectance spectra could only partly be compared to those of the ultraviolet spectra, if the

relative absorbances were neglected, because the spectra are recorded after two different principles. The correlation between the spectra from the TLC Scanner and from the HPLC-DAD is not reliable enough to be used a proof of identity between spot and peak. The TLC Scanner can, however, be used for proof of identity between different spots with the same R_{fg} values on TLC (e.g. between spots from extracts and standards and between different fungal isolates).

A chemotaxonomic analysis of fungal isolates, identified as *P. brevicompactum* by their morphological characteristics, always shows a conspicuous pattern of metabolites on TLC, fluorescing blue and violet in UV light (Filtenborg & Frisvad 1980; Frisvad & Filtenborg 1990). The chromatographic and spectroscopic data of these metabolites (R_{fg} values in several TLC-systems, Retention Indices, ultraviolet spectra and reflectance spectra) should all be used in combination and not used alone (e.g. only Retention Indices for identification of peaks in HPLC). Using them separately may lead to misidentification of metabolites. By using the entire data set of the metabolites in the profile, together with known standards of fungal metabolites, a greater certainty in recognition of metabolites, and thereby identification of fungal species, can be achieved.

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