Characterization of Metabolites in *Stachybotrys chartarum* by LC/TOF-MS

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Introduction

There is an increasing concern about the adverse health effects associated with fungal growth in indoor environments and *Stachybotrys* is a fungus that has become notorious as a mycotoxin producer that can cause animal and human mycotoxicosis. Indeed, over the past 15 years in North America, evidence has accumulated implicating this fungus as a serious problem in homes and buildings and one of the causes of the "sick building" syndrome. Macrocyclic trichothecenes (satratoxins, roridins and verrucarins) seem to be the causative components of toxicoses for farm animals eating contaminated hay.

In addition to the macrocyclic trichothecenes, species of *Stachybotrys* can produce a number of secondary metabolites such as trichodermol, trichodermin; atranones including their dolabellane precursors; spirocyclic drimanes. Some of these metabolites can cause skin irritation or have immunosuppressant effects.

This work describes a novel multi-residue method using LC/TOF-MS for the analysis of several tricothenes and a number of secondary metabolites also known to be harmful. This study characterized *S. chartarum* by combining pathogenic characteristics such as haemolysis of red blood cell with metabolite production.

Experimental

Table 1. Analytical condition of LC/TOF-MS

HPLC	: Agilent 1200
Column	: ZORBAX Extend C18(100mm,2.1mm,1.8 μm)
Oven temp	: 40 °C
Mobile phase	: A=MeOH , B=10 mM CH_3COONH_4 30%A-----(30min)----70%A
Flow rate	: 0.2 mL/min
Injection	: 5 μL
MS	: Agilent 6220 Accurate-Mass TOF LC-MS
Ionization	: ESI (positive ion mode)
Nebulizer gas	: 345 kPa
Dry gas	: 10 L/min at 350 °C
Fragmentor	: 120V
Mass range	: 100-1000 Da
Reference	: *m/z*=112.050873, *m/z*=922.009798
Resolution	: >10000 at *m/z*=121

Experimental

Sample preparation

Thirty-seven *S. chartarum* isolates were inoculated on potato dextrose agar and inoculated plates were incubated at 25 °C for 3 weeks. The spore and hypha were separated from cultures on the agar and extracted with 80% methanol for 24 hours. For the analysis of target compounds, crude extracts were purified using MuitiSep #226.

Screening target compounds by exact mass database

Extracts were analyzed by LC/TOF-MS and screening of target secondary metabolites was performed using exact mass database with the parameters shown in Tables 2 & 3.

Table 2. Target screening parameter

Target mass	: $(M+H)^+$, $(M+MH^4)^+$
Charge	: Single charge ion
Mass window	: 0.01 Da
Relative mass error	: 5 ppm

Table 3. Exact mass database of fungus metabolites

Compounds	Formula	MW	Compounds	Formula	MW
3,4 Epoxy-6-hyroxy dolabella-7,12 diene-one	C20H30O3	318.21949	Spirodihydrobenzofuranlactam 1	C23H31NO4	385.23439
6-Hydroxydolabella-3,8,12 trien-14-one	C20H30O2	302.22458	Spirodihydrobenzofuranlactam 2	C25H35NO5	429.25150
Atranone A	C23H32O6	418.21889	Spirodihydrobenzofuranlactam 2	C25H35NO5	429.25150
Atranone B	C23H34O7	446.23045	Spirodihydrobenzofuranlactam 3	C28H39NO6	485.27770
Atranone F	C24H32O7	432.21488	Spirodihydrobenzofuranlactam 3	C28H39NO6	485.27770
Atranone C	C24H32O6	416.21990	Spirodihydrobenzofuranlactam 4	C28H37NO6	515.25190
Atranone G	C26H34O8	482.22540	Spirodihydrobenzofuranlactam 4	C28H37NO6	515.25190
Dolabellane diterpene	C20H30O2	302.22458	Stachybocin A	C52H70N2O18	882.50300
Epi-Roridine E	C29H38O8	514.25667	Stachybocin A	C52H70N2O18	882.50300
Glotoxin	C13H14N2O4S2	326.03950	Stachybocin D	C52H70N2O12	914.49290
Hydroxy-Roridin E	C29H38O9	530.25158	Stachybocin D	C52H70N2O12	914.49290
Hydroxy-Roridin E	C29H38O9	530.25158	Stachybotrylactone	C23H30O5	386.20930
Iso-Roridine E	C29H38O8	514.25667	Stachybotrylactone	C23H30O5	386.20930
Iso-Roridine H	C29H38O8	512.24102	Trichodermin	C19H26O4	292.18249
Iso-satratoxin F	C29H34O10	542.21528	Trichodermin	C17H24O4	292.16746
iso-Verrucarin J	C27H32O8	484.20978	Trichodermol	C15H24O3	250.16689
L671 776	C23H32O5	388.22580	Trichodermol	C15H22O3	250.15589
Roridin L2	C29H38O9	530.25158	Trichoverrin A	C29H40O9	532.26723
Roridine A	C29H40O9	532.26723	Trichoverrin A	C23H32O7	420.21480
Roridine E	C29H38O8	514.25667	Verrucarin A	C27H34O9	502.22028
Roridine H	C29H38O8	512.24102	Verrucarin B	C27H32O9	500.20463
Satratoxin G	C29H36O10	544.23985	Verrucarin B	C27H32O9	500.20463
Satratoxin H	C29H36O9	528.23593	Verrucarin J	C27H32O8	484.20972
Spirodihydrobenzofuranlactam 1	C23H31NO4	385.22438			

Characterization of *S. chartarum* by multivariate statistics

Mass features (metabolites) were extracted from all observed ions in 37 extracts by using Molecular Feature Extractor (MFE) by the parameters shown in Table 4. Extracted metabolites were filtered by T-test (P value<0.05) and fold analysis (Average>2 times). For multivariate statics, a data matrix consisting of 37 objects (S. chartarum isolates) and variables (metabolites) was constructed and analyzed.

Table 4. Molecular Feature Extraction parameters

Peak filter	: >100
Compound filter	: >5000
Extraction range Da	: Retention time: 3-45min mass :130-900
Isotope spacing	: 0.0025Da+5ppm
Charge state	: Single charge ion
Ion species	: $(M+H)^+$, $(M+NH4)^+$, $(M+Na)^+$, $(M+K)^+$

Target Analysis of Secondary Metabolites

Clean up by MultiSep#226

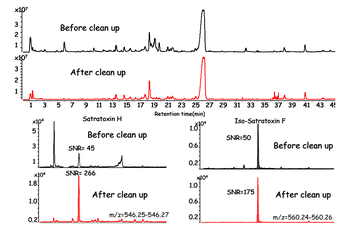


Figure 1. TIC and MC of crude and final fungi extract

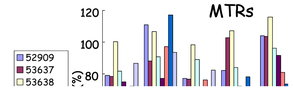
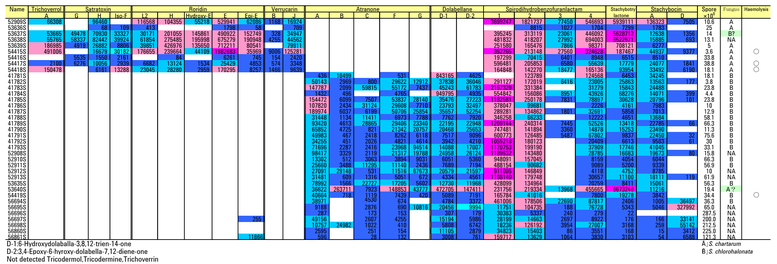


Figure 2. Recoveries of macrocyclic trichothecenes in *Stachybotrys chartarum* in clean up step

Table 6. Abundance of target secondary metabolites in *Stachybotrys* spore



Characterization of Secondary Metabolites by Multivariate Statistics

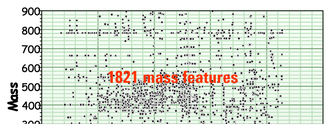


Figure 3. Mass plot by MFE

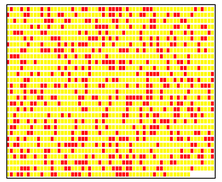


Figure 4. Abundance plot of 1821 mass features

Results and Discussion

Filtering by volcano plot

1821 metabolites were characterized to 32 and 72 metabolites by combining pathogenic characteristics such as haemolysis of red blood cell and MTRs production. These results were shown in Fig. 5 and 6.

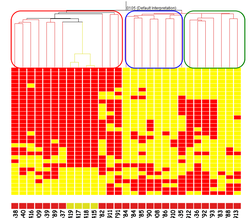


Figure 6. Dendrogram of 32 metabolites and 37 extracts produced by cluster analysis

Figure 5. Abundance plot of unique metabolites by haemolysis (left) and MTRs production (right).

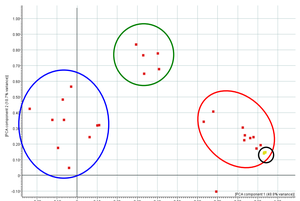


Figure 7. PCA plot of 37 extracts (Haemolysis)

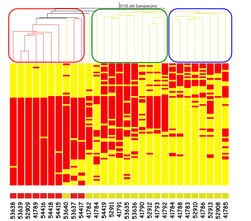


Figure 8. Dendrogram of 75 metabolites and 37 extracts produced by cluster analysis

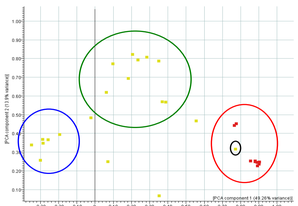


Figure 9. PCA plot of 37 extracts (MTRs production)

Conclusions

Target analysis of secondary metabolites

- Clean-up step by MultiSep#226 was useful to reduce chemical noise in *Stachybotrys* extract.
- 25 metabolites were identified by exact mass database.
- 28 *S. chlorohalonate* produced five atranones and their precursors, but did not produce macrocyclic trichothecenes.
- 9 *S. chartarum* produced MTRs, always including SH, iso-SF, RL2,RE,RH,epi-R and VJ, whereas SG, hydroxy-RE, VB and TA were occasionally detected.

Characterization of secondary metabolites

- 1821 mass features (metabolites) were extracted by MHE.
- 32 and 75 metabolites were characterized by volcano plot.
- Results of the cluster analysis and PCA based on the abundance of 32 metabolites showed three clusters
- One cluster with 11 extracts contained four extracts shown to induce haemolysis.