Rapid Analysis of Crude Fungal Extracts for Secondary Metabolites by LC/TOF-MS – A New Approach to Fungal Characterization

Application

Food

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Abstract

A novel approach to studying the production of secondary metabolites by fungi using LC/TOF-MS has been developed. Fungi grown on culture media are solvent-extracted and directly analyzed by LC/TOF-MS. Searching against a database of 465 secondary metabolites, mycotoxins and other compounds of interest can be readily identified. The methodology was validated by spiking culture media with 20 mycotoxin standards and identifying these toxins in the crude solvent extracts. Subsequently, using seven different fungi from culture collections, after culturing for 7 to 14 days in three different media, anticipated metabolites were readily identified.

Introduction

From a food safety perspective there is a need to characterize molds (fungi) isolated from agricultural products, as these may represent a potential source of mycotoxin contamination in food.

Traditionally, this fungal characterization has been based on classical mycology, involving culturing the fungi on different media and then classifying depending on morphological and growth behavior characteristics. However, such classification can be time-consuming and is somewhat subjective, being dependant on the skill and experience of the mycologist. Additionally, such typing of fungi only provides anecdotal evidence about actual profiles of secondary metabolites, as it is based on previously observed secondary metabolism of particular fungal species. This empirical approach is further confounded by the fact that fungi of the same species can be both toxigenic and nontoxigenic; that is, some readily produce mycotoxins, but some otherwise indistinguishable fungi of the same species are genetically incapable of toxin production. Classification of fungal species alone therefore provides no real insight into mycotoxin production.

In the past, direct analysis of fungal culture media for the presence of mycotoxins has of necessity involved “target” analysis with the inevitable assumption as to which toxins should be sought. However, LC/TOF-MS offers new possibilities for studying the behavior of fungi with regard to toxin production. Providing that efficient extraction from the medium of toxins with widely differing polarity can be demonstrated, the specificity of TOF-MS means that any further sample clean-up is not necessary. Furthermore, targeted analysis is also unnecessary as the instrument can provide accurate mass measurement of molecular ions of any components detected in an LC run, and these can be identified by searching a database of exact masses of relevant secondary metabolites.

In this note we describe suitable conditions for extraction of secondary metabolites from cultured fungi and LC/TOF-MS conditions for subsequent analysis. The methodology has been validated by spiking aflatoxins, ochratoxin A, trichothecenes, zearalenone, and fumonisins into various growth media, and demonstrating good recovery from the media at low levels and subsequent identification by searching against a database of 465 secondary metabolites. The methodology has been applied to one *Penicillium* species and six *Aspergillus* species, which were obtained from a culture collection, and their secondary metabolites have been compared with the anticipated toxin profiles.

Experimental

All analytical work was performed using an Agilent 6210 TOF-MS coupled to an Agilent 1200 Series HPLC. The separation of mycotoxins and other fungal metabolites was also carried out using an HPLC system (consisting of vacuum degasser, autosampler with thermostat, binary pump, and DAD system) equipped with a reversed-phase C18 column (ZORBAX Eclipse XDB 100 × 2.1 mm, 1.8 µm). The TOF-MS was equipped with a dual-nebulizer electrospray source, allowing continuous introduction of reference mass compounds. The instrument was scanned from *m/z* 100 to 1,000 for all samples at a scan rate of 1 cycle/sec in 9,429 transient/scan. This mass range enabled the inclusion of two reference mass compounds, which produced ions at *m/z* 121.0508 and 922.0097. The injected sample volume was 5 µL.

The HPLC analysis used a mobile phase of acetonitrile and 2 mM ammonium acetate in an aqueous solution of 1% formic acid at a flow rate of 0.3 mL/min. The gradient elution started with 15% acetonitrile and reached 100% acetonitrile in 20 min. The column was washed with 100% acetonitrile for 5 min. and equilibrated for 5 min between chromatographic runs. UV spectra were obtained using diode array detection scanning every 0.4 sec from 200 to 700 nm with a resolution of 4 nm. The optimum TOF-MS conditions are given in Table 1. The data recorded were processed with Analyst-QS software with accurate mass application. The database of 465 mycotoxins and other fungal metabolites was created in Excel from reference sources [1,2], which were easily adapted to use in a search capacity using Agilent software.

Table 1. LC/MS-TOF Operational Conditions in ESI+ Ion Mode

Parameter	
Capillary voltage	3000 V
Nebulizer pressure	40 psig
Drying gas	10 L/min
Gas temperature	300 °C
Fragmentor voltage	150 V
Skimmer voltage	60 V
OCT* RF	250 V
OCT* DC	37.5 V
Mass range (*m/z*)	100–1000
Reference masses	121.050873; 922.009798

*Octapole

Fungal Extraction

Well-characterized isolates of *A. paraciticus (NRRL 2999)*, were obtained from the USDA culture collection and isolates of *A. flavus, (200198), A. ochraceus (200700), A. oryzae (200828), A. niger (200807), A. fumigatus (200418),* and *P. citrinum (501862)* were obtained from the TÜBITAK Mamara Research Center culture collection. Fungi were inoculated onto malt extract agar (MEA), potato dextrose agar (PDA), and yeast extract sucrose agar (YES) in petri dishes. After allowing the fungi to grow for 7 to 14 days at 25 °C, typical prolific growth of fungal colonies was observed on the surface of the media. Samples of fungal hyphae, together with underlying culture media, were taken by vertically cutting two 6-mm diameter plugs using a cork borer. The plugs were transferred to 5-mL disposable screw-cap bottles. Extraction conditions were modified from previous published methods [3,4]. One of the plugs was extracted twice with 2 mL ethyl acetate with 1% formic acid and then 2 mL isopropanol. The second plug was extracted twice with 2 mL ethyl acetate with 1% formic acid and then 2 mL acetonitrile, followed by 1 min vortexing and 30 min total ultrasonication. The extracts were filtered and evaporated gently under a nitrogen stream. The residues in both cases were dissolved in 1 mL methanol, ultrasonicated for 10 min and passed through a 0.2-µL disposable filter prior to HPLC analysis.

Results and Discussion

Optimization of LC/TOF-MS Conditions

The most important instrumental parameters, which were capillary voltage, nebulizer pressure, drying gas, gas temperature, and skimmer voltage, were initially optimized by autotune to achieve

maximum sensitivity. However, the fragmentor voltage also needed to be optimized to provide maximum structural information, which sometimes required a compromise. Optimization was carried out by varying the fragmentor voltage in the range of 55 to 250 V without changing any other conditions. The fragmentor voltage that provided minimum fragmentation was found at 150 V.

To validate the whole procedure, 20 commercially available standards (aflatoxins B_1, B_2, G_1, and G_2; aflatoxin M_1; ochratoxin A; zearalenone; 4-deoxynivalenol; 3-acetyldeoxynivalenol; 15-acetyldeoxynivalenol; diacetoxyscirpenol; fusarenone X; neosolaniol; fumonisins B_1, B_2, and B_3; nivalenol; HT-2 toxin; T2 toxin; and kojic acid) and internal standard (benzophenone) were mixed together. Using positive electrospray, the accurate masses of protonated molecule ions, retention times, and UV spectra were obtained in each case.

Construction of Database of Accurate Masses of Fungal Metabolites

An Excel spreadsheet was constructed containing the exact mass data for each of the 465 mycotoxins and fungal metabolites, together with their empirical formulas [1,2]. Theoretical monoisotopic exact masses of the compounds were calculated based on their molecular formula using an Excel spreadsheet (called "Formula DB Generator" and provided with the Agilent TOF) and put into csv (comma-separated values) format for use by the Agilent TOF automated data analysis software. The csv file is searched automatically by the LC/TOF-MS instrument at the completion of the sample run and a report is generated on compounds that were found in the database. The creation of the data analysis method is done using a data analysis editor. The editor allows selection of adducts (for example, in positive ion H^+, NH_4^+, Na^+, etc.) and neutral losses to be searched automatically, as well as mass accuracy and retention time tolerances, report options, and other search and detection criteria. Retention times are not required but if they are known add a degree of confidence to the identification.

We use samples of various growth media that had been spiked with the standard mixture of 20 mycotoxin standards to determine retention times. The standards were injected 10 times to establish the repeatability of those retention times. The criteria used for identification were a fit for the accurate mass of the M+1 ion to a mass tolerance of ± 5 ppm, a retention time match to ± 0.2 min (if standards available), a minimum peak height count, which is called the compound threshold of 1,000 counts (or a signal-to-noise ratio of ~10:1 or 0.06% relative volume), and, if present, good correspondence (to ± 5 ppm) with predicted adducts and neutral fragment losses.

Method Validation by Spiking and Analysis

Based on the above detection criteria, all 20 standards were correctly identified when spiked at 25 to 100 µg/kg into growth media, and analyzed as described above.

Utilization of the Method to Determine Metabolite Production from Well-Characterized Fungi

Rather than simply looking at theoretical situations with spiked growth media, the above technique was applied to the real situation of well-characterized fungi being cultured on various media. One *Penicillim* species and six *Aspergillus* species were grown on three different media. Using the simple solvent extraction described above, the crude extracts were directly analyzed by LC/TOF-MS. By way of illustration, Figure 1 shows the total ion chromatogram for an *A. flavus* extract indicating about 20 components detected. The peak eluting at 8.9 min on database searching was found to have an accurate mass of *m/z* 313.0712. Based on the $M+H^+$ ion this corresponded to aflatoxin B_1 with a 0.2 ppm mass match as compared to the database exact mass for aflatoxin B_1.

The software uses a molecular feature (MFE) algorithm that removes all ions that are not real peaks and groups the real ions into "molecular features." Those molecular features can be characterized by their relationship with each other and adducts, dimers, trimers, etc., and their isotopes (depicted as +1, +2, etc.) are deduced. The molecular features and accurate mass measurement of these species for the peak at a retention time of 8.9 min identified as aflatoxin B_1 are summarized in Table 2. Selecting a molecular feature, the software will calculate possible empirical formulas and score the isotopes for the "fit" to the proposed formula; this is also shown in Table 2. The formula with the score of 100 is that of aflatoxin B_1. This formula then can be automatically translated to a Web connection search with NIST, ChemIndex, and Medline. The search results in NIST indicated the formula and structure of aflatoxin B_1 as illustrated in Figure 2.

In addition to the identification of aflatoxin B_1 as a secondary metabolite from *A. flavus*, this fungi was also found to produce aflatoxin B_2, aflatoxin B_3, and aflatoxin G_1, which are consistent with

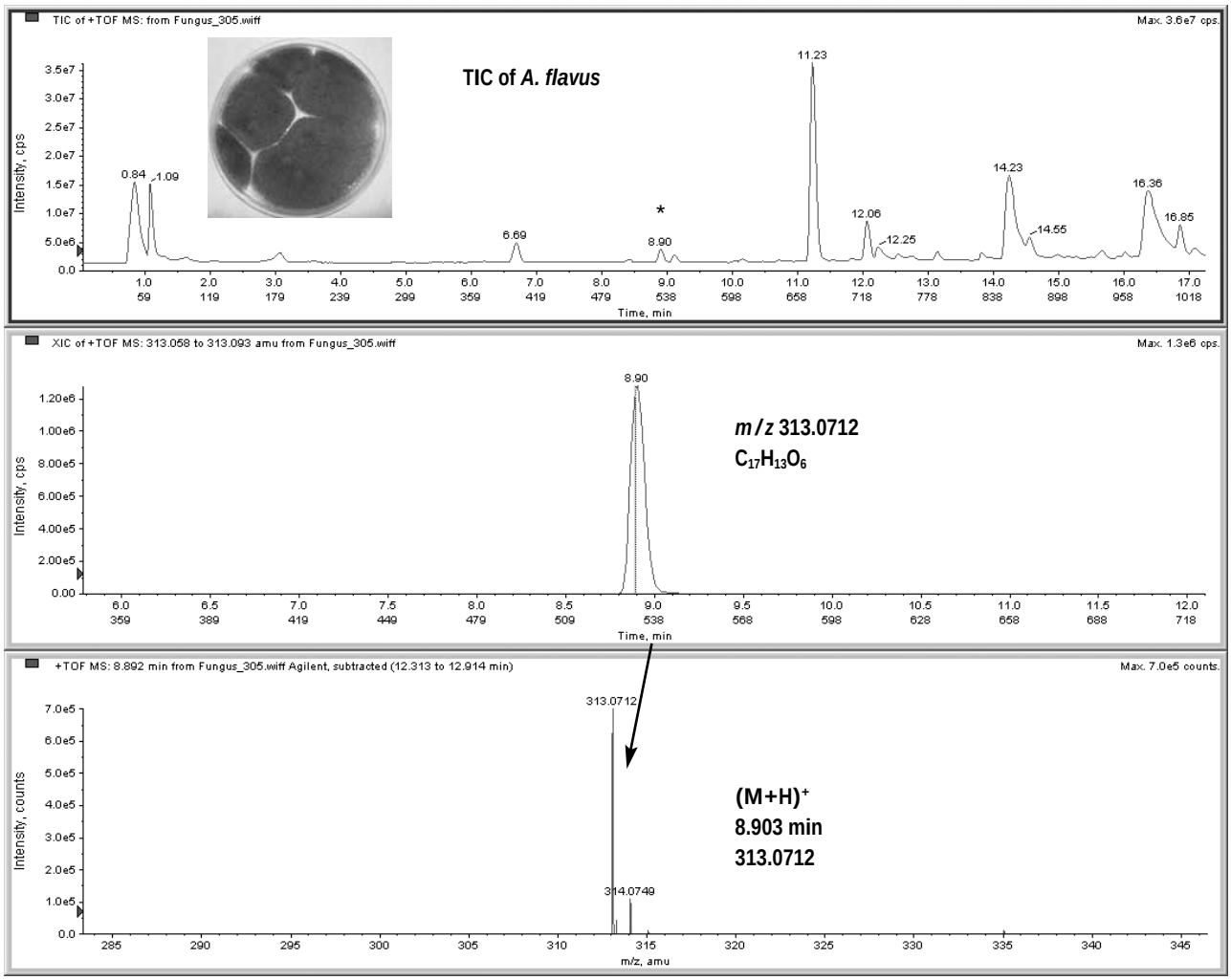

Figure 1. **Analysis of an extract from *A. flavus* by LC/TOF-MS illustrating:**
(a) Total ion chromatogram (TIC) with * peak corresponding to aflatoxin B_1,
(b) Extracted ion chromatogram from *m/z* 313.058 to 313.093 for aflatoxin B_1,
(c) Full-scan spectrum showing accurate mass with 0.2 ppm error for M+1 ion for aflatoxin B_1.

Table 2. Typical Clusters Seen in ESI+ LC/MS-TOF on the Peak Retention Time of 8.90 min, *m/z* 313.0706

MFE
Feature #27 (RT = 8.903)

Species	RT	*m/z*	Mass	Abundance	Width
M	8.903		312.0633	5541933	0.088
M+H	8.903	313.0706	312.0633	4035186	0.09
M+H+1	8.903	314.0744		622147	0.088
M+H+2	8.902	315.0766		74349	0.09
M+H+3	8.906	316.0795		7943	0.085
M+Na	8.904	335.0529	312.0637	86580	0.094
M+Na+1	8.904	336.0563		15898	0.097
M+Na+2	8.916	337.0593		1848	0.091
2M+H	8.906	625.1357	312.0642	741	0.049
2M+Na	8.902	647.1164	312.0636	226965	0.061
2M+Na+1	8.902	648.1202		65639	0.063
2M+Na+2	8.900	649.1228		14941	0.065
2M+Na+3	8.899	650.1256		2677	0.058
2M+Na+4	8.897	651.1290		257	0.048

MFE

Composition's chemical formula	dm(Da)	dm(ppm)	dm(ppm)	DBE	Score
$C_{17}H_{12}O_6$	0.0001	0.2	0.2	12	100
$C_{18}H_8N_4O_2$	0.0014	4.5	4.5	17	77
$C_{14}H_4N_{10}$	–0.0013	–4.2	4.2	18	68
$C_9H_{16}N_2O_8S$	–0.0006	–1.9	1.9	3	58
$C_{13}H_8N_6O_4$	–0.0026	–8.4	8.4	13	55

NIST
National Institute of Standards and Technology

Standard Reference Data Program | Data Gateway | Online Databasis | Chemistry WebBook

Aflatoxin B_1

- **Formula:** $C_{17}H_{12}O_6$
- **Molecular weight:** 312.27
- **IUPAC International Chemical Identifer:**
  - InChI=1/C17H12O6/c1-20-10-6-11-14(8-4-5-21-17(8)22-11)15-13(10)7-2-3-9(18)12(7)16(19)23-15/h4-6,8,17H,2-3H2,1H3
- **CAS Registry Number:** 1162-65-8
- **Chemical structure:**

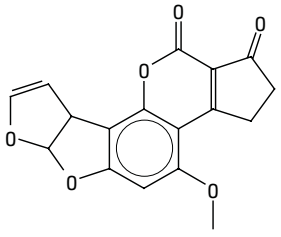

Figure 2. Database search result for emprical formula using NIST (Medline and ChemIndex results were the same but are not given here). Note molecular weights should not be searched in these databases as they are often the "average" molecular weight and not the monoisotopic weight.

known metabolic behavior. In Table 3 the screening results from the database search with a 5 ppm tolerance are shown with the accurate masses of some other peaks, which corresponded to known compounds. Kojic acid and methoxysterigmatocystin, which are a good match, are both well-known fungal metabolites that might be expected to be found from *A. flavus*. A good match was also found for cinnamic acid, which is not known as a metabolite.

When this new approach was applied in a preliminary study of a total of seven different fungi obtained from culture collections and grown on three different media, the results shown in Table 4 were obtained. In most cases the predicted metabolites were found, which gives good confidence in the methodology. Some of these initial results showed that predicted mycotoxins were not detected and unexpected metabolites were found. The possibility of a misidentified culture exists or that metabolites not previously reported were detected. While this demonstrates the power of the approach, these results do need to be followed up with more in-depth study.

Future Prospects

The use of accurate mass LC/TOF-MS combined

Table 3. Results of Automated Mycotoxin Database Search for *A. flavus* Extract (Extraction compound list is sorted in ascending order of retention time within 5 ppm error. Benzophenone was used as an internal standard.)

Mass Value = 142.03					
Formula	**Compound**	**Mass**	**Error (mDa)**	***Error (ppm)**	**Ret. Time Error**
$C_6H_6O_4$	Kojic acid	142.03	–0.10	–0.7	–
Mass Value = 148.05					
Formula	**Compound**	**Mass**	**Error (mDa)**	**Error (ppm)**	**Ret. Time Error**
$C_9H_8O_2$	Cinnamic acid	148.05	–0.08	–0.5	–
Mass Value = 328.06					
Formula	**Compound**	**Mass**	**Error (mDa)**	**Error (ppm)**	**Ret. Time Error**
$C_{17}H_{12}O_7$	Aflatoxin G_1	328.06	1.01	1.4	–0.05
Mass Value = 354.07					
Formula	**Compound**	**Mass**	**Error (mDa)**	***Error (ppm)**	**Ret. Time Error**
$C_{19}H_{14}O_7$	5-Methoxysterigmatocystin	354.07	0.99	2.8	–
Mass Value =312.06					
Formula	**Compound**	**Mass**	**Error (mDa)**	***Error (ppm)**	**Ret. Time Error**
$C_{17}H_{12}O_6$	Aflatoxin B_1	312.06	–0.05	–0.2	–0.11
Mass Value =312.06					
Formula	**Compound**	**Mass**	**Error (mDa)**	***Error (ppm)**	**Ret. Time Error**
$C_{17}H_{14}O_6$	Aflatoxin B_2	314.08	0.06	0.2	0.06
Mass Value = 338.08					
Formula	**Compound**	**Mass**	**Error (mDa)**	***Error (ppm)**	**Ret. Time Error**
$C_{19}H_{14}O_6$	Methylsterigmatocystin	338.08	–0.16	–0.5	–
Mass Value = 182.07					
Formula	**Compound**	**Mass**	**Error (mDa)**	***Error (ppm)**	**Ret. Time Error**
$C_{13}H_{10}O$	Benzophenone	182.07	0.73	4.0	–0.15

Table 4. A Comparison of Detected and Predicted Metabolites from Culture Collection Fungi Grown in MEA, YES, and PDA Medium

	Fungi						
Metabolites	*P. citrinum*	*A. flavus*	*A. paraciticus*	*A. niger*	*A. fumigatus*	*A. oryzae*	*A. ochraseus*
AFB1		✱ √	✱ √				
AFB2		✱ √	✱ √				
AFB3		✱ √	✱				
AFG1		✱ √	✱ √				
AFG2		✱	✱ √				
KA	✱ √	✱ √	✱ √		✱	✱	
MST		✱ √	√				
5-MST		√					√
OTA				✱ √			✱
RO-A					√		
FU-B					✱ √		
MA				✱ √			
AA		✱					√
Nig				✱ √			
Ter							√
Cit	✱						

√ - metabolites detected by LC/TOF-MS; ✱ - metabolites predicted to be present

Key:

AFB1	Aflatoxin B_1 etc.	FU-B	Fumigaclavine B
KA	Kojic acid	MA	Malformin (peptides)
MST	Methylsterigmatocystin	AA	Aspergillic acid
5-MST	5-methoxysterigmatocystin	Nig	Nigragillin
OTA	Ochratoxin A	Ter	Terrein
RO-A	Roquefortine A (isofumigaclavine A)	Cit	Citrinin

with database searching is a powerful example of a new, versatile identification technique that can be used in targeted analysis. In the area of fungal metabolites, the potential to screen fungi for a range of metabolites for which dedicated methods are not available has been demonstrated. This approach offers new possibilities for fungal typing based on metabolite production and rapid screening of agricultural products for mycotoxins of food safety interest. Where previously unknown metabolites are detected, although LC/TOF-MS can provide some insight, further work with a hybrid quadrupole time-of-flight LC/MS system (LC/QTOF-MS) will be required for structural elucidation.

Conclusions

A simple and rapid method has been developed using LC/TOF-MS to determine the profile of secondary metabolites produced by fungi under various culture conditions. The approach has been validated by spiking representative metabolites into solid cultures and demonstrating good recovery and identification by searching accurate masses against a metabolite database. Results for a range of well-characterized fungi from a culture collection showed that the anticipated toxins could be readily detected.

References

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