Chapter 15

Metabolomics Protocols for Filamentous Fungi

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Abstract

Proteomics and transcriptomics are established functional genomics tools commonly used to study filamentous fungi. Metabolomics has recently emerged as another option to complement existing techniques and provide detailed information on metabolic regulation and secondary metabolism. Here, we describe broad generic protocols that can be used to undertake metabolomics studies in filamentous fungi.

Key words: Systems biology, Metabolomics, Metabolome, Metabolite, Fungi, Derivatisation, GC-MS, LC-MS, NMR, SPE

1. Introduction

Metabolomics is the most recent of the “omics techniques to be embraced by functional genomics” (1–4). The metabolome is defined as the quantitative complement of low-molecular-weight metabolites present in a cell under a given set of physiological conditions (5, 6). Whilst often viewed as a complementary technique to transcriptomics and proteomics, metabolomics does offer distinct advantages (5–7). First, the metabolome is directly related to the phenotype; metabolic fluxes are regulated not only by gene expression, but also by post-transcriptional and post-translational events (Fig. 1). Second, changes in the metabolome are amplified relative to changes in the transcriptome or proteome. It is also true that the technology involved in metabolomics is generic, as a given metabolite, unlike a transcript or protein, is the same in every organism that contains it. Consequently, the metabolomics data will directly highlight metabolic pathways and processes differentially regulated,

Fig. 1. General schematic of the omics pathway.

and by consequence, the genes and proteins responsible for their synthesis. In broad terms, the field can be broken into two distinct forms, targeted and non-targeted. In targeted metabolomics, we know the metabolite we are looking for and thus can optimise the extraction methods and instrumentation accordingly. Good examples of targeted metabolomics include the analysis of mycotoxins in food samples. The primary focus of non-targeted metabolomics is to determine the relative concentration of as many metabolites as possible in the sample at the time of sampling. Unlike DNA or proteins that are comprised of a defined set of building blocks, the variation of metabolites in a living cell in terms of chemical properties is enormous ranging from small and polar (e.g. hexoses) and to large and non-polar (e.g. lipids). To maximise the coverage of metabolites, different technologies are often employed and these are described briefly below.

Gas chromatography-mass spectrometry (GC-MS) is a versatile technique that is well suited to non-targeted metabolite profiling of volatile and thermally stable polar and non-polar metabolites (8). Classes of metabolites routinely measured by GC-MS include sugars, amino acids, phosphorylated metabolites, organic acids, lipids and amines. Typically, most of these compounds are non-volatile and thus require comparatively complex derivatisation stages to be included during sample preparation to confer volatility and thermal stability. There are several configurations of mass spectrometers that are typically used in combination with GC, including linear quadrupole, ion trap and time-of-flight. Throck Watson and David Sparkman (2007) provide comprehensive details of each of these platforms, and Dunn (9) provides an elegant overview of each with a focus on metabolomics. The protocol listed below describes the use of a single linear quadrupole mass spectrometer (10–13).

Liquid Chromatography-Mass Spectrometry (LC-MS) is another technique commonly used in metabolomics. Sample preparation for LC-MS is simple and it caters well for compounds that are not well suited for GC-MS (large, thermally labile, non-volatile etc.). However, LC is technically demanding, requiring the use of large amounts of MS-grade solvents. These are not only expensive to purchase, but also to dispose of. Technical reproducibility is not trivial, particularly compared with GC, and retention time drift is also problematic. There is also the issue that no single column is suitable for a wide range of analyte classes. Although this is true for GC, it is significantly more so for LC. To date in fungi, LC-MS has been typically used to examine and screen for secondary metabolites (14) rather than non-targeted scanning of primary metabolites. Secondary metabolites are typically unsuited to GC-MS as they are often too large, particularly after derivatisation. The method below outlines a robust technique for screening secondary metabolites using liquid chromatography with an ion trap mass spectrometer.

The third metabolomics technique to be considered in this chapter is Nuclear Magnetic Resonance (NMR). NMR is a spectroscopic technique that identifies, particularly, atom–atom bonds, and so differs fundamentally from the methods reviewed above which are MS-based. Like all techniques, there are strengths and weaknesses to consider when using NMR for metabolomics (15, 16). NMR is a non-biased and non-destructive technique that requires very little sample preparation. This lessens the likelihood of sample loss or the introduction of artefacts into the preparation. NMR also has the potential for high-throughput fingerprinting, allowing for the analysis of hundreds of samples per day. It is only paramagnetic nuclei that can be detected, but as these include ^1H , ^{13}C , ^2H , ^{14}N , ^{15}N , ^{17}O , ^{19}F , ^{23}Na , ^{29}Si , ^{31}P and ^{35}Cl , all biomolecules are well covered. The significant drawback with NMR compared with MS-based methods, however, is sensitivity. It has been reported that NMR is only half as sensitive as MS techniques, thus precluding the detection of low-abundance metabolites (17). These strengths and weaknesses aside, NMR has been extensively used for metabolic fingerprinting, particularly in the medical fields. Studies are now starting to emerge exploiting its strengths in the fungal field.

2. Materials

Use only analytical grade solvents or higher grade if available. Always use high purity milli-Q filtered water. The volume of solutions and number of each consumable required will depend on the number of replicates/extracts used. All of the required safety protocols and MSDSs must be followed

2.1. Materials for GC Analysis

2.1.1. Harvesting of Fungal Tissue and Quenching of Metabolism

Flask Culture

1. 5- or 10-mL Pipette with tip, one per replicate.
2. 50-mL Falcon tubes, one per replicate.
3. Forceps.
4. Quenching solution; -40°C 75% methanol in water.
5. Rinsing solution; -40°C 50% methanol in water.
6. Dry ice, enough to submerge tubes of quenching & rinsing solutions.
7. 50-mL Syringes, one per replicate.
8. Cotton gauze 100 cm^2 , one per replicate.
9. Spatula.
10. Liquid nitrogen, enough to submerge falcon tubes.
11. Freeze dryer.

Plate Culture

1. Scalpel, handle size 3.
2. Round edge scalpel blade, size 10 (to fit handle).
3. 2-mL Snap-lock microcentrifuge tubes (see Note 1).
4. Liquid nitrogen, enough to submerge 2-mL microcentrifuge tubes.
5. Freeze dryer.

2.1.2. Extraction of Metabolites from Fungal Tissue

Cell Disruption and Metabolite Isolation

1. Spatula.
2. 2-mL Safe-lock microcentrifuge tubes (see Note 1).
3. Weight scale—capable of measuring mg quantities.
4. Methanol.
5. Milli-Q water.
6. Ribitol (>99% purity).
7. Tissue lyser—equipped with capsule to hold 2-mL tubes (QIAGEN; see Note 2).
8. Liquid nitrogen.
9. Ice.
10. Vortex.
11. Microcentrifuge tubes (1.5–2 mL).
12. Bench-top microcentrifuge.
13. Pipettes.

Drying Metabolites

1. Speedivac vacuum concentrator.
2. Freeze dryer (see Note 3).

2.1.3. Derivatisation and GC-MS Analysis of Fungal Metabolites

1. Methoxyamine HCl, 20 mg per sample.
2. Pyridine, 10 mL would be sufficient.
3. Thermomixer.

4. *N*-Trimethylsilyl-*N*-methyl trifluoroacetamide (MSTFA), 30 μ L per sample.
5. Hexane.
6. Alkanes (see Note 4).
7. Glass vials; 1 mL.
8. Glass insert; 100 μ L.
9. Crimp caps.
10. Crimper.
11. GC-MS equipped with an autosampler and syringe capable of 1 μ L injections.

2.1.4. Deconvolution and Identification of Metabolites from GC/LC-MS Data

1. Deconvolution and analysis software.
2. Multivariate analysis software, capable of PCA.
3. Statistical analysis software.

2.2. Materials for LC Analysis

2.2.1. Extraction of Fungal Secondary Metabolites

1. 100% Methanol, AR grade (or MS grade).
2. Milli-Q water.
3. 5% Methanol in Milli-Q water.
4. Hexane, LC-MS grade or better.
5. Sodium chloride, AR grade.
6. Griseofulvin (internal standard).
7. 50-mL Falcon tubes.
8. Bench-top centrifuge and 50-mL tube adaptors.
9. Vortex.
10. Sonic waterbath (optional).
11. 40-mL amber glass vials.
12. Bulk C18 Solid Phase Extraction (SPE) material.
13. 8-mL empty SPE reservoirs with frits.
14. 500 mg C18 SPE columns or cartridges.
15. 50-mL syringe.
16. Glass wool.
17. Nitrogen drying manifold.
18. Pipettes and filter tips.

2.2.2. LC-MS Analysis of Fungal Metabolites

1. Microcentrifuge tubes (1.5 mL).
2. Bench-top microcentrifuge.
3. Amber glass autosampler vials with caps.
4. 100- μ L limited volume glass inserts.
5. Water, LC-MS grade.

6. Acetonitrile, LC-MS grade.
7. Formic acid, (>99% pure).
8. HPLC-MS system with autosampler.
9. RP18 column with guard column.

2.3. Materials for NMR Analysis

2.3.1. Culturing and Culture Harvesting

1. Carbon substrate (^{13}C -Labelled optional (see Note 5)) to add to fungal growth medium. For natural abundance spectra add e.g. 40 mM glucose.
2. Make a 250-mM solution of the required standard in 1 mL deuterium oxide. Lyophilise to dryness. Prior to NMR spectrum acquisition, resuspend thoroughly in 1 mL deuterium oxide, centrifuge at maximum speed in a bench-top microcentrifuge to pellet any insoluble matter, and add 700 μL to a NMR tube.
3. Sterile mortar and pestle (1 per sample).
4. Liquid nitrogen.
5. Methanol:water Solution: make a 70:30 methanol:water solution and store at -20°C . Each sample requires 30 mL.
6. Sterile glass funnel lined with Whatman #1 paper filter, placed in a 50-mL Falcon tube standing in ice (1 per sample).
7. Rotary evaporator.
8. One 10 mL tube per sample.
9. Freeze dryer.
10. Deuterium oxide.
11. Internal reference compound (optional but recommended) (see Note 6).
12. Two 1.5 mL eppendorf tubes per sample.
13. Bench-top microcentrifuge.
14. Clean NMR tubes (1 per sample).
15. There are a number of NMR analysis software platforms for viewing and annotating spectra.

3. Methods

The following procedures were performed on *in vitro* cultures of the filamentous ascomycete *Stagonospora nodorum*. Briefly, the fungus was grown by inoculation of both liquid minimal medium and solid agar plate medium with 25 mM glucose as the carbon source. Flask cultures were grown in the dark for 1 week at 22°C with shaking at 150 rpm. Plate cultures were grown for 1–2 weeks with a 12-h white-light regimen at 22°C (18). For statistical significance of the metabolomic data, the preparation of eight biological

replicates is recommended. The appropriate controls should be included (see Note 7). It is not the purpose of this protocol to include operation of LC/GC-MS or NMR equipment. Platform-specific training will be required for any of those instruments.

3.1. GC Methods

3.1.1. Harvesting Fungal Tissue and Quenching of Metabolism (see Note 8)

Flask Culture

1. Pipette 10 mL of culture (cut tapered tip off pipette tip if required) into a 50-mL falcon tube containing 20 mL Quenching Solution.
2. (In advance) Remove the plunger from a 50-mL syringe, fold a 100-cm² square of gauze 3 times and line the eluting end of the syringe with the folded gauze. Pour the entire contents of the falcon tube into the syringe, place the plunger back in the end and elute the solution into a fresh falcon tube (if the analysis of extracellular metabolites/media components is required).
3. Carefully remove the plunger, pour 10 mL rinsing solution over the mycelia resting on the gauze, return the plunger and elute the washing solution for discarding.
4. Using forceps carefully remove the gauze from the syringe and scrape the mycelia into a fresh falcon tube with a spatula.
5. Freeze the mycelia in liquid nitrogen.
6. Lyophilise the fungal tissue in a freeze dryer.

Plate Culture

1. Using a scalpel equipped with a round-edged blade, scrape the mycelia from the plate whilst avoiding disruption of the growth medium.
2. Scrape the mycelia from the scalpel blade into a microcentrifuge tube.
3. Freeze the mycelia in liquid nitrogen.
4. Lyophilise the fungal tissue in a freeze dryer.

3.1.2. Extraction of Metabolites from Fungal Tissue

The following protocol describes the extraction of metabolites from lyophilised fungal tissue regardless of the culture conditions or harvest method. The data analysed in this protocol uses metabolites extracted from 2 mg of lyophilised fungal tissue (see Note 8). The extraction steps should be performed at low temperatures wherever possible.

1. Transfer at least 2 mg of dried fungal tissue to a 2-mL safe-lock microcentrifuge tube and record the weight of the transferred tissue.
2. To the tube, add 685 μ L of methanol and shake vigorously for 2 min in a tissue lyser at 30 Hz (see Note 2).
3. Remove from the lyser and to the tube add 75 μ L of water containing 1.25 μ g ribitol (internal standard) per 1 mg of fungal tissue and return the tube to the tissue lyser for a further 2 min.

4. Freeze the tube in liquid nitrogen by gripping the tube with forceps in an upright position until the suspension is frozen.
5. Remove from the liquid nitrogen and gently open the lid to the tube as soon as possible, to avoid the lid bursting off as the tube warms. Allow the suspension to thaw on ice.
6. Vortex the tube briefly and centrifuge at $20,000 \times g$ for 2 min to collect the cell debris.
7. Transfer the entire supernatant to a fresh microcentrifuge tube being careful not to disturb the pellet.
8. To the remaining pellet, add 250 μL 90% methanol. Place the tube back in the shaker for a further 2 min.
9. Remove the tube from the tissue lyser and again centrifuge at $20,000 \times g$ for 2 min.
10. Add the supernatant to that previously collected and discard the pellet.
11. Mix the combined supernatants by vortex and transfer a 2 mg equivalent (see Note 9) of supernatant to a fresh tube.
12. Dry the extracted samples in 200–300- μL glass inserts placed inside 1.5-mL plastic tubes using a centrifugal evaporator (see Note 3).
13. If the analysis of extracellular components is required, the eluate collected during the harvest should be vortexed and 100 μL (the amount used may need to be adjusted depending on the concentration of media components remaining in the extracellular eluate) transferred to a fresh microcentrifuge tube containing 2.5 μg ribitol and drying the contents in a centrifugal evaporator (see Note 3). Follow the steps as per the metabolite extracts from this point.

3.1.3. Derivatisation and GC-MS Analysis of Fungal Metabolites

Various options are available for derivatisation. The method below uses MSTFA which is suitable for most classes of compounds. Other derivatisation techniques are described in detail in Halket and Zaiken (19).

1. Dry the extracted samples (approximately 2 mg equivalent of tissue) in 200–300- μL glass inserts placed inside 1.5-mL plastic tubes using a centrifugal evaporator.
2. Add 20 μL of methoxyamine hydrochloride and shake for 2 h (1,250 rpm, 37°C) (see Note 10 for online derivatisation procedure provided a Gerstel or CTC autosampler is available).
3. Add 30 μL of MSTFA and continue shaking (1,250 rpm, 30°C) for 30 min.
4. Finally add 5 μL of alkanes (6.2 $\mu\text{g}/\text{mL}$) to enable retention indices to be calculated in the data analysis stage.
5. Transfer glass inserts into autosampler vials and seal with crimp caps. Derivatised samples should be analysed within 2 days.

6. Place derivatised samples into the autosampler tray on the GC-MS and set up a sequence table specifying vial positions and sample names.
7. 1 μL injections are most commonly carried out in splitless and split injection modes depending upon sample concentration (see Note 11).
8. It is advised that methanol blanks be run every three samples in splitless injection mode and every six in split mode to minimise possible carryover of compounds remaining on the column to the subsequent analyses.
9. For comprehensive metabolomic profiling we suggest using the $5.6^\circ\text{C}/\text{min}$ oven temperature gradient (see Table 1 for parameters and mass spectrometer settings).
10. Tuning of the instrument and air/water checks should be undertaken before each experiment.

*3.1.4. Deconvolution
and Identification
of Metabolites from
the GC-MS Data*

The acquired GC-MS data can be analysed using the freely available AMDIS package (<http://chemdata.nist.gov/mass-spc/amdis/>) or a commercial software package such as AnalyzerPro™ (SpectralWorks Ltd., Runcorn, United Kingdom). AnalyzerPro identifies individual analytes from the GC-MS data which includes deconvoluting co-eluting and partially co-eluting peaks using the mass spectral

Table 1
GC-MS parameters for non-targeted metabolomics using a single-quad mass spectrometer

GC column	Varian factor 4 column (VF-5ms; 30 m $\times$ 0.25 mm $\times$ 0.25 μm + 10 m Ezi-guard) (see Note 12)
Autosampler	1 pre-wash with hexane 5 sample pumps prior to injection 5 post washes with methanol, 5 with hexane
GC oven gradient ($5.6^\circ\text{C}/\text{min}$)	70°C (hold 1 min), ramp to 76°C at $1^\circ\text{C}/\text{min}$, ramp to 325°C at $5.63^\circ\text{C}/\text{min}$ (hold for 3 min)
GC oven gradient ($15^\circ\text{C}/\text{min}$)	70°C (hold 1 min), ramp to 325°C at $15^\circ\text{C}/\text{min}$ (hold 3 min) (see Note 13)
Other GC parameters	Carrier gas flow (Helium) 1 mL/min at constant flow (see Note 14) Inlet temperature 250°C
Mass spectrometer parameters	Transfer line (auxiliary) 280°C Quadrupole mass analyser 150°C Source 250°C Solvent delay before MS turned on approximately 7.5 min (will require some adjustment with different instruments/columns) Scan m/z range 50–600, sample rate of 2^2 for the $5.6^\circ\text{C}/\text{min}$ method (2^1 for the $15^\circ\text{C}/\text{min}$ method)

information collected. With the construction of a “metabolite library” (see Note 15), analytes can be cross-referenced against it and identified as metabolites. An output of metabolite peak areas representing the abundance of each metabolite can be used for the subsequent analysis. The following describes this process of analysis.

1. Check the chromatography for overloading peaks. If required, individual analytes may need to be quantified on a less abundant ion.
2. Analytes that are not matched against a reference from the library should be identified as “unknowns” and labelled accordingly, incorporating identifiers such as the retention time, retention index and base peak in the label.
3. Identify analytes common to the test samples and the controls. Some of these are likely to have been introduced during preparation and should be eliminated from the data set.
4. Normalise the metabolite abundances to the ribitol (internal standard) by dividing the peak area of each metabolite by the peak area of the ribitol.
5. Scaling the data by $x = \log(x + 1)$ transformation of the ribitol-normalised data to reduce the impact of a large dynamic range of metabolite abundances within the data set.
6. Perform a principal component analysis (PCA) of the scaled data to identify major sources of variance in the data set, and thus the most influential metabolites.
7. Influential metabolites should be validated for statistical significance using the ribitol-normalised data.

3.2. LC Methods

3.2.1. Extraction Protocol for Secondary Metabolites

Culture Filtrate

1. For each sample, weigh 0.5 g of bulk SPE material (preferably water wettable, see Note 16) to a 50-mL tube. Activate with 3 mL methanol, remove excess, then wash and equilibrate twice with 5 mL milli-Q water (can be prepared beforehand, see Note 17). Carefully remove excess water and introduce the culture filtrate in ~40 mL batches. Shake horizontally for 20–30 min each.
2. Allow to stand for 1 min for SPE material to settle between renewing culture filtrate. Repeat until the desired volume of culture filtrate is processed.
3. While the culture filtrate is extracting, prepare one empty SPE reservoir with frit per sample.
4. Remove all culture filtrate from tubes. Add 5 mL milli-Q water, resuspend the SPE material and transfer to an empty SPE reservoir with frit.
5. Wash the SPE material with 5 mL 5% methanol. Elute with 3 mL 100% methanol (see Notes 16 and 17).

3.2.2. Mycelium

1. Weigh freeze-dried mycelium in a 50-mL tube, add 100 μ g Griseofulvin, 30 mL 100% methanol and shake for 2 h.
2. Filter the extract into a weighed 40-mL amber glass vial using a 50-mL syringe with ca 1 cm high glass wool stuffed into the eluting end (can be prepared beforehand). Squeeze the mycelium thoroughly. Dry the extracts under a stream of nitrogen and gentle heating (see Note 18).
3. Reconstitute dried extracts in 10 mL milli-Q water. Vortex thoroughly (or use a sonic water bath) to completely dissolve extracts.
4. Add sodium chloride to the extracts to facilitate phase separation (optional, see Note 19). Overlay with 10 mL hexane and vortex or shake vigorously. Centrifuge at maximum speed for 5–15 min.
5. Remove the hexane completely (see Note 20).
6. Load the cleared aqueous extracts onto 500 mg C18 SPE columns at a flow rate of 1–5 mL/min. Wash with 5 mL 5% methanol in milli-Q water. Elute with 3 mL 100% methanol into weighed 4-mL amber glass vials (see Note 21).

3.2.3. LC-MS Analysis of Metabolites

1. Dry the methanol extracts obtained from the SPE on the nitrogen drying manifold (see Note 18).
2. Resuspend in 300 μ L 5% acetonitrile, transfer to a reaction tube and centrifuge at maximum speed for 5–10 min (see Notes 16 and 21).
3. Transfer a 50- μ L aliquot into a glass vial containing a 100- μ L limited volume glass insert.
4. The parameters for an optimal HPLC separation and MS detection are highly platform dependent and need to be optimised for every standard, analyte or combination of those. We recommend using standard method development settings as recommended by the manufacturer of your instrument(s) and refer to further publications ([14](#), [20–25](#)).
5. Our laboratory typically uses an Agilent 1100 LC-MSD IonTrap. The HPLC mobile phase is water:acetonitrile 95:5 (both with 0.1% formic acid), flow rate 0.2 mL/min. We commonly use a 150 $\times$ 2.1-mm, 3- μ m GraceSmart C18 column with guard column and inject 1–5 μ L of sample, running non-injection blanks at the beginning of each sequence and every 3–4 samples to check for column contamination (Table 2).

3.3. NMR Methods

3.3.1. Methanol: Water Extraction of Polar Metabolites

1. Grow fungal culture in flask in preferred media and growth conditions.
2. Pre-cool a sterile mortar and pestle with liquid nitrogen.
3. Add the lyophilised fungal culture and grind under liquid nitrogen until it is reduced to a fine powder (see Note 22).

Table 2
Typical chromatography parameters for LC separation
of fungal secondary metabolites

Step	Time (min)	% A (water)	%B (acetonitrile)
Start	0.0	95	5
Initial hold	10.0	95	5
Gradient	60	0	100
Top hold	75	0	100

Post time: 15 min

Electrospray ionisation conditions “smart conditions” were activated with needle voltage 3,500 kV, drying gas temperature 325°C, drying gas flow 12 L/min

Trap parameters typically used: trap drive 100%, Ion count 30,000, maximum accumulation time 50 ms

4. Add 20 mL -20°C 70:30 methanol:water solution and continue grinding until the sample is resuspended.
5. Transfer the resuspended sample to a #1 Whatman paper-lined glass funnel and collect filtrate in a 50-mL Falcon tube standing in ice.
6. Wash the mortar and pestle twice with 5 mL methanol:water solution, and transfer to the funnel as in the previous step.
7. Set the water bath on the rotary evaporator to 45°C . Apply the sample and reduce the sample volume to 2–3 mL.
8. Transfer the sample to a pre-weighed 10 mL tube and snap freeze in liquid nitrogen.
9. With the lid loosened, removed or punctured, lyophilise the sample overnight in a freeze dryer.
10. Reweigh the 10 mL tube to determine the dry weight of the lyophilised sample and store at -80°C until ready for NMR spectroscopy.

3.3.2. Preparation for NMR Spectroscopy

1. Resuspend the sample thoroughly in 1 mL deuterium oxide by pipetting and vortexing as necessary and transfer to a pre-weighed 1.5-mL Eppendorf tube.
2. Centrifuge the sample at maximum speed in a bench-top microcentrifuge to pellet any particulate debris. Remove the supernatant to a second 1.5-mL tube.
3. Dry the first Eppendorf in a heating block and re-weigh to determine the weight of the pellet. The pellet weight is deducted from the dry weight of the sample determined from the 10-mL tube in step 9 above to give the true dry weight of the polar metabolites.

4. Centrifuge the sample in the second Eppendorf at maximum speed as before (see Note 23).
5. Transfer 700 μ L supernatant to a clean NMR tube (see Note 24).
6. Load the NMR tube into a NMR spectrophotometer and acquire a ^{13}C spectrum (see Note 25).
7. Once a spectrum has been acquired, the sample may be retrieved and stored for further analysis (see Notes 26 and 27).

4. Notes

1. Other microcentrifuge tubes can be used; however, some brands are prone to leaking during the metabolite extraction that follows.
2. If a tissue lyser is unavailable, steps should be taken to ensure thorough homogenisation of the fungal mycelia. Effectively lyophilised flask-cultured fungus may be more suitable than plate cultures, as the mycelia forms a finer suspension with the methanol more readily during metabolite extraction.
3. Highly concentrated metabolite extracts may require the combined use of a vacuum concentrator and a lyophiliser to dry. The lyophiliser is recommended as the extract will be more readily dissolved during later derivatisation steps. The extract however will not remain frozen in the lyophiliser if the methanol content is too high. Vacuum concentrating to remove the methanol is therefore required. The fungal extracts are likely to contain a high proportion of sugars; this method of drying the extract can also be used to prevent “caramelising” the metabolite extract.
4. The alkanes comprise a mixture of n-Decane, n-Dodecane, n-Pentadecane, n-Nonadecane, n-Docosane, n-Octacosane, n-Dotriacontane and n-Hexatriacontane in hexane, as internal retention index controls.
5. There is a wide range of ^{13}C labelled compounds available from e.g. Omicron Biochemicals, Sigma-Aldrich, Cambridge Isotope Laboratories etc. For labelled glucose we added 40 mM D-[1- ^{13}C]-glucose to the MM-C medium. In the case of labelled mannitol, which was a poor growth substrate for some mutant strains which we investigated, cultures were first grown for 2 days on MM-C with 40 mM glucose. The samples were harvested and washed to remove all medium and then resuspended in 50 mL MM-C with 20 mM D-[1- ^{13}C]-mannitol and cultured for a further 24 h
6. There have been a number of internal reference compounds used in fungal ^{13}C -NMR studies in order to act as a point of

reference for the chemical shifts of resonance peaks. These include acetone (26), acetate (27), EDTA (28–30), dioxane (31, 32), hexamethyldisiloxane (33), TMS (29, 34–38) and trimethylsilyl propionate (TMSP) (39). Deficiencies have been noted with each of these and the recommendation has been made that 2,2-dimethyl-2-silapentane-5-sulfonic acid (DSS) be used as the universal standard, due to its being insensitive to variations in temperature and pH, and the fact that it is chemically inert and has a single, sharp, unambiguous highfield resonance peak (40).

7. At various steps during the harvesting and extraction of metabolites, some thought must be given to the preparation of controls, which can be analysed by GC-MS along with the experimental samples. The subsequent data analysis will be made easier if analytes introduced during the preparation of samples for analysis can be quickly identified and excluded from the data set. Such controls include quenching solution, rinsing solution, media, and extraction solvents. These can be dried down and derivatised alongside the metabolite extracts for GC-MS analysis.
8. Harvesting and quenching by these methods allow the separation of mycelia from the growth media and snap freezing in less than 10 s.
9. The 2 mg equivalent of supernatant is calculated from the recorded weight of the fungal mycelia and a total extraction volume of 1,000 μL . For example, if the initial amount of fungal tissue was 2.5 mg, 800 μL of the extract would contain an equivalent of a 2 mg metabolite extract and 2.5 μg of ribitol. The amount of tissue used in the metabolite extraction will depend on the concentration of metabolites required for analysis, which will depend on the limits of detection of the individual analytical instrument used in the study.
10. Provided that a capable autosampler is available, online derivatisation can be used. This has the advantage of consistent derivatisation times before injection for all samples and is particularly important for large experiments. It should be noted however that it is important to keep reagents fresh (i.e. no older than 3 days).
11. The optimal split ratio has been described as the smallest ratio at which the compound present at highest concentration does not saturate the mass spectrometer detector (41). Overloading is evident when the bell shape curve of a peak becomes asymmetrical. In many biological samples, sugars are often present at concentrations many fold higher than the majority of other compounds. In such cases, overloading of peaks sometimes cannot be avoided if quantification of the less abundant metabolites is to be achieved; samples should therefore be run using a higher split ratio to accurately quantify the more abundant compounds.

12. A range of columns are available with various bonded phases designed for specific purposes. The Varian VF-5ms column is suitable for a wide range of applications however there are a number of columns with similar properties available from alternative suppliers.
13. The 15°C/min oven gradient can be used for higher-throughput analyses however this will be to the detriment of compound separation.
14. In order to obtain reproducible retention times for each compound across experiments on a single column, a standard (i.e. mannitol) can be used to lock the instrument method so that column pressure is adjusted to ensure elution of the standard at a fixed time. This enables the method to be re-locked following column maintenance. A lock time should be chosen to maintain the column flow close to 1 mL/min for optimal chromatographic resolution.
15. An efficient method for rapidly identifying observed analytes is using a metabolite library. The subsequent analysis of the generated metabolite profile will be aided by the use of a metabolite library generated by analysing purchased “standards” on the analytical instrument and under the same conditions as is being used for the metabolomic study. Some software such as AnalyzerPro will allow the creation of an “in-house” library which can be used to easily identify metabolites in the data.
16. The volume of solvent depends on the amount as well as the solubility of compounds and therefore needs to be determined empirically. Make sure SPE columns and bulk material never run dry as this will negatively affect recovery. Add water to SPE material slowly and carefully to excessive frothing.
17. Using water wettable C18 material (e.g. Alltech Prevail®) will reduce frothing and allows for omitting the preconditioning step with methanol. SPE can either be performed per individual sample using cartridges or columns with disposable syringes or in parallel on a vacuum manifold. Most manufacturers recommend an optimal flow rate of 1–5 mL/min for sample binding, but since binding of analytes occurred during shaking, the flow rate here can be faster.
18. This may take several hours, but allows for a great number of samples to be processed in parallel. Always adjust the Nitrogen nozzles to approximately 1–2 cm above extract surface and flow rate low enough that the extracts do not splash. Gentle heat (~40°C) may be applied to speed up evaporation. Alternatively, the methanol extracts can be filtered into 100-mL glass bulbs and dried on a rotary evaporator.
19. We found that methanol extracts of *S. nodorum* contain a large amount of lipids and fatty acids. These need to be removed

or they will interfere with the SPE and/or LC. If needed, they can be recovered from the hexane easily and analysed subsequently, e.g. by GC-MS (42). Adding sodium chloride to the extract facilitates phase separation in case foam comprised of extract/hexane/air or any other sort of interphase forms between hexane and the extract. The whole step may be omitted if the reconstituted extract is clear (i.e. coloured, yet translucent).

20. Make sure not to carry over any hexane to the SPE, as this will cause the analytes to elute prematurely from the SPE material.
21. Alternatively to removing particulates by centrifuging, Whatman Mini-UniPrep™ Syringeless Filters, which also act as autosampler vials, therefore speeding up the processing and reducing sample loss, can be used.
22. Fungal mycelium is amenable to grinding and should not require any grinding accessory. If the sample comprises a large proportion of spores or is otherwise difficult to reduce to a fine powder, then glass beads or acid washed sand may be added to assist in grinding.
23. Samples should always be centrifuged prior to loading into NMR tubes since any particulate matter will interfere with the spectrum acquisition. Samples may be coloured, but they should be translucent.
24. To clean NMR tubes, rinse several times with de-ionised water, followed by several rinses with 100% acetone. Dry the tubes in an oven at 70°C for 30 min. Do not leave the plastic lids on as these may melt.
25. It is not the purpose of this protocol to include operation of an NMR spectrophotometer. Platform-specific training will be required for the spectrophotometer to be used (as well, of course for any LC/GC-MS equipment).
26. To remove and retain a sample from an NMR tube, place a 1.5-mL eppendorf tube over the open end of the tube. Hold the eppendorf in your hand with the NMR tube oriented upside down along your wrist and forearm. Swing your arm once over your shoulder and the centrifugal force should be sufficient to transfer the sample into the eppendorf. It is advisable to make sure you have sufficient space to perform this manoeuvre, ensure other personnel are at a safe distance, and practice with water first to avoid losing a precious sample.
27. NMR is a non-destructive method and samples may be stored indefinitely for further analysis by NMR or other techniques. Samples may be stored at -80°C or may be lyophilised and stored at room temperature. If it is likely that the sample may be contaminated, 0.01% sodium azide may be added to prevent growth of contaminants as it is NMR invisible.

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