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Exploring the biotechnological potential of endophytic fungi using online automated sample preparation GC-MS

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Introduction

Metabolomics investigates the metabolite composition of a cell type, tissue, or biological fluid via analysis of the complete set of metabolites also known as the metabolome. In fact, metabolites are the intermediates or end products of multiple enzymatic reactions and therefore are the most informative representations of the biochemical activity of an organism.

Metabolic profiling can be carried out by two main approaches: targeted and untargeted. Targeted metabolomics performs hypothesis-driven experiments to focus exclusively on the quantification of predefined sets of metabolites with a high level of precision and accuracy. On the other hand, untargeted metabolomics carries out simultaneous measurement of a large number of metabolites from each sample, providing the global metabolic profile. Untargeted studies are characterized by the generation of large amounts of complex data which require high performance bioinformatics and statistical tools to interpret results for the identification of compounds and for the semi-quantitative evaluation of those compounds in the investigated samples.

Since preparation of extensive sample sets is required to allow successful differentiation between sample types, analytical data quality is essential to highlight true biological variability. This is the ideal scenario where automation can provide the needed robustness and reproducibility to achieve good quality data.

Furthermore, automated sample preparation on-line solutions (i.e. MPS autosampler mounted or linked with a dedicated instrumental technique such as GC-MS or LC-MS) allow the use of the PrepAhead function existing within the GERSTEL Maestro software. The PrepAhead function allows to perform the sample preparation for each sample immediately preceding the GC-MS injection whilst the previous sample is running. This not only offers a very time-effective option eliminating operator downtime but also ensures that freshly derivatised samples are analysed promptly reducing the risk of degradation due to lifetime.

In this study, an untargeted approach was applied to investigate the metabolic diversity of fungal endophytes to provide valuable data in support of a grant application for the University of Newcastle. Fungal endophytes can produce a broad array of metabolites. They have reported metabolic capabilities that include degradation of cellulose and plastics, as well as the biosynthesis of potential biofuel molecules, anti-bacterial and anti-fungal chemicals. This application note describes the whole untargeted metabolomics workflow applied for this study, including the development of the automated sample preparation, data processing and results interpretation.

Methoximation followed by silylation (MOX-TMS), the most commonly adopted derivatisation method for GC-MS metabolomics, was fully automated for the preparation and analysis of the samples. Data were processed using Agilent Mass Hunter softwares (Qualitative Analysis, Unknown Analysis and Quantitative analysis) in combination with the statistical tools offered by Agilent, Mass Profiler Professional (MPP) software for chemometrics.

Instrumentation

Autosampler: GERSTEL MPS xt Dual Rail, Left MPS 10 µL syringe /Right MPS 250 µL syringe

Modules: Agitator, GERSTEL MultiPosition Vortexer (mVorx), GERSTEL MultiPosition Evaporation Station (mVAP), Anatune CF-200 Robotic Centrifuge

GC-MS: Agilent GC 7890- MSD 5975C, Inert Ion Source EI, Agilent GC 7890-Q-TOF 7200, Removable Ion Source EI (RIS)

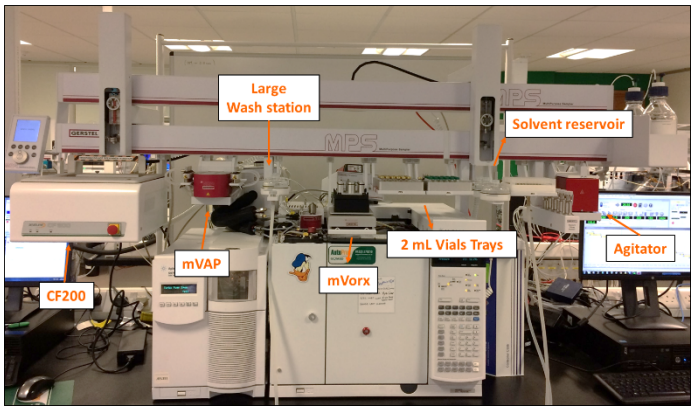


Figure 1: Online fully automated solution for the direct MOX-TMS derivatisation and GC-MS MSD analysis of freeze dried fungi

Methods

Samples

Six replicates of three species of endophytic fungi (3, 11 and 21) were grown in potato dextrose media for 4 days for a total of 18 samples. Cells were washed in deionised water to remove media and quenched in 50% cold methanol. Samples were spun to collect pellets which were then homogenised and freeze dried for analysis.

Automated sample preparation

Approximately 2 mg of freeze dried fungus was weighed into a 2mL high recovery vials. 100µL of MOX solution (16 mg/mL methoxyamine hydrochloride in pyridine) was firstly added to the sample and the mixture was incubated at 30 °C for 90 min. Once methoximation was completed, 140µL of silylating reagent (MSTFA + 1% TMCS) was added to the sample and reacted for 30 minutes at 37°C. After centrifugation for 1 minutes at 4500 rpm, 2 µL of the top organic layer was directly injected on the GC-MSD. Samples were randomised to reduce bias contributions and three procedural blank were also prepared to take into account the background.

Once injected on the GC-MSD system the extracts were transferred to the GC/Q-TOF system to benefit of the sensitivity and accurate mass information provided by the Q-TOF analyser.

Figure 2 shows the PrepAhead function in the timeline for the preparation of 12 samples. The multi-coloured bands represent the sample preparation and the orange bands the GC run-time. Duration of the whole process was 9 hours and 18 min.

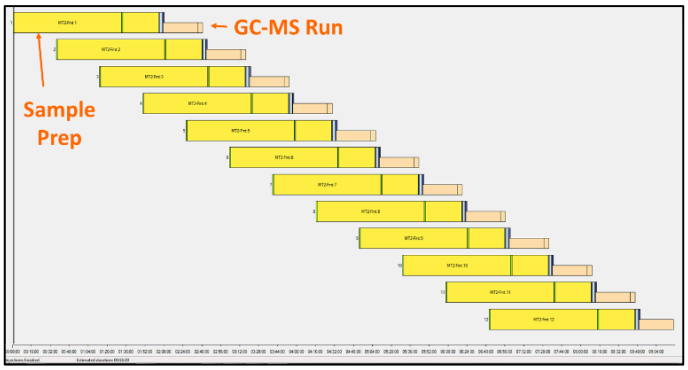


Figure 2: Timeline preview for the online automated sample preparation and GC-MS analysis of 12 fungi samples by MOX-TMS.

GC-MS analysis

GC-MS (MSD):

Column: HP-5MS Ultra inert 30 m x 0.25 mm x 0.25 µm
Injection mode: Pulsed Splitless at 250 °C
Flow: 1 mL/min
GC ramp: 50 °C held for 2 min, 8 °C/min to 320 °C, held for 9.25 min
Runtime: 45min
Auxiliary temperature: 300 °C
Inert Source, EI mode at 300°C, Mass range 50-650 *m/z*

GC-MS (Q-TOF):

Column: HP-5MS Ultra inert 60 m x 0.25 mm x 0.25 µm
Injection mode: Split 1:10
Flow: 1 mL/min
GC ramp: 40 °C held for 2 min, 7°C/min to 300 °C, held for 6 min
Auxiliary temperature: 300 °C
Removable Ion Source (RIS) in Electron impact (EI) mode at 250 ºC
Collision cell: Nitrogen as collision gas 1.5 mL/min
QTOF in 2GHz mode, scan range 50-650 m/z

Results and Discussion

Metabolomics workflow step 1: Data analysis

Data were firstly inspected using Agilent Mass Hunter Qualitative Analysis software for data quality control. Figure 3 shows the comparison of the total ion chromatograms (TICs) obtained for the same sample run on the GC-MS single quadrupole and on the GC-MS Q-TOF. Chromatograms are shown on the same Y axis scale to put emphasis on the increased sensitivity achieved using the time of flight analyser.

Data were then processed using Agilent Mass Hunter Unknown Analysis software to perform deconvolution and library search of the detected components.

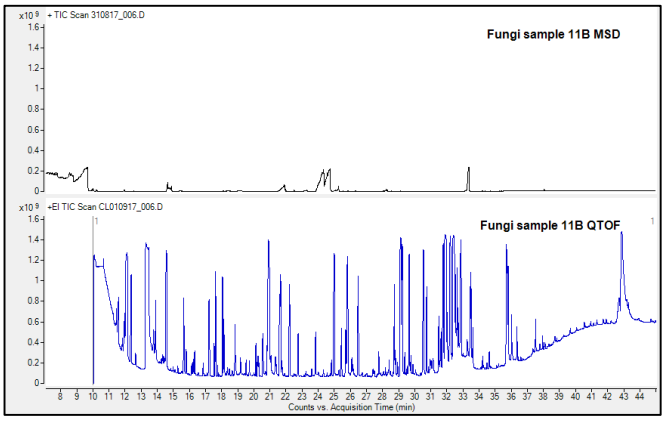


Figure 3: TICs for the fungi sample 11B by GC-MS (MSD) analysis (top) and GC-MS (Q-TOF) analysis (bottom)

Table 1 summarises the total components, the hits and the blank subtracted components for all the samples for both the MSD data and the QTOF data.

	MSD			QTOF		
Sample	Total components	Hits	Blank subtracted	Total components	Hits	Blank subtracted
11A	267	92	24	342	140	62
11B	326	130	19	375	146	74
11C	294	117	22	381	144	65
11D	278	107	22	365	133	68
11E	279	124	29	373	148	54
11F	287	122	20	328	130	64
21A	283	88	27	359	143	75
21B	250	63	38	352	115	81
21C	288	89	29	327	121	71
21D	258	90	26	345	136	72
21E	231	95	29	302	135	64
21F	234	93	31	286	121	63
3A	295	119	29	371	174	65
3B	232	75	34	285	119	77
3C	321	119	23	371	148	73
3D	336	128	30	417	167	73
3E	285	90	32	390	151	66
3F	278	116	26	387	167	65

Table 1: Total components, hits and blank subtracted components found in both MSD and Q-TOF data using Agilent Mass Hunter Unknown Analysis

Deconvoluted data were converted to Compound Exchange Format files (CEF) to be exported to Agilent Mass Profiler Professional (MPP) software for statistical interpretation of the data.

Metabolomics workflow step 2: Statistical interpretation

Once CEF were imported, data were processed in MPP to perform the following: experiment grouping, filtering according to abundance, retention time and mass, retention time alignment, baselining, significance testing and fold change. This process allows to recognise what in MPP software are called entities. An entity (aka compound or component) is a molecular entity for which retention time, mass and abundance have been detected. Entities can be identified or unidentified.

Once data have been processed, several statistical analysis tools are available for the evaluation and investigation of the data.

Principal components analysis (PCA) and clustering are two very powerful tools to investigate the data. PCA is a visual way to explore the variance in the data set and it helps in the identification of patterns. Clustering allows to organise entities and experimental conditions based on the similarity of their abundance profiles. Figure 4 shows the PCA obtained for both the MSD and QTOF data. The three fungi species and the procedural blanks separate nicely in different clusters.

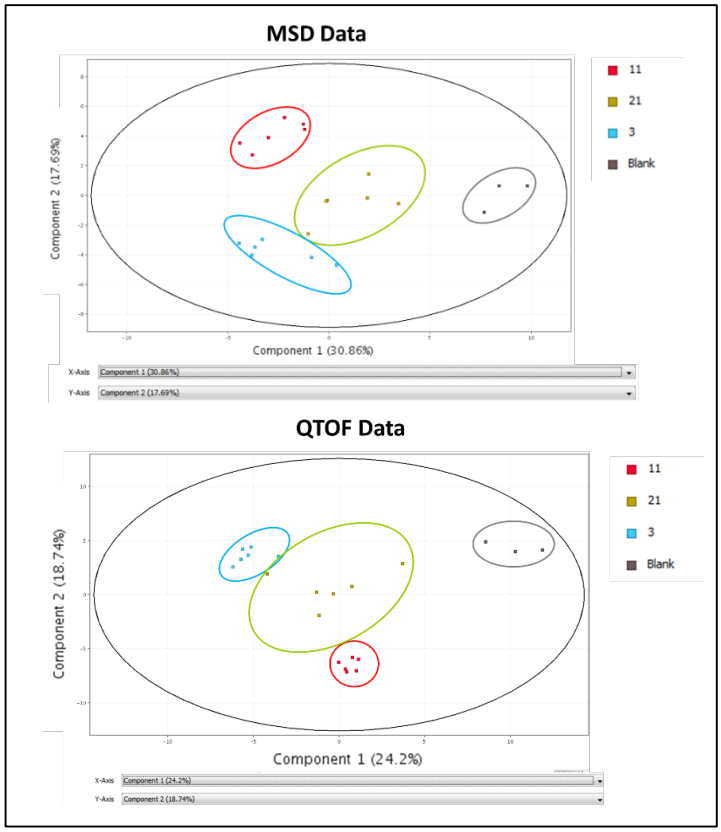


Figure 4: PCA obtained for the analysis of fungi samples by GC-MS MSD (top) and GC-MS Q-TOF (bottom)

Figure 5 shows the results obtained for one of the clustering algorithm, hierarchical. The output is a dendrogram or tree diagram. Similar entities are grouped on branches from the same nodes and entities are displayed in a heat map. Hierarchical is an unsupervised analysis meaning that grouping is solely done on abundance profiles disregarding any grouping information provided during the data processing. As shown in Figure 5, the three fungi species and blank separate in different group in the dendogram and clusters of entities unique to a particular species can be spotted in the heat map.

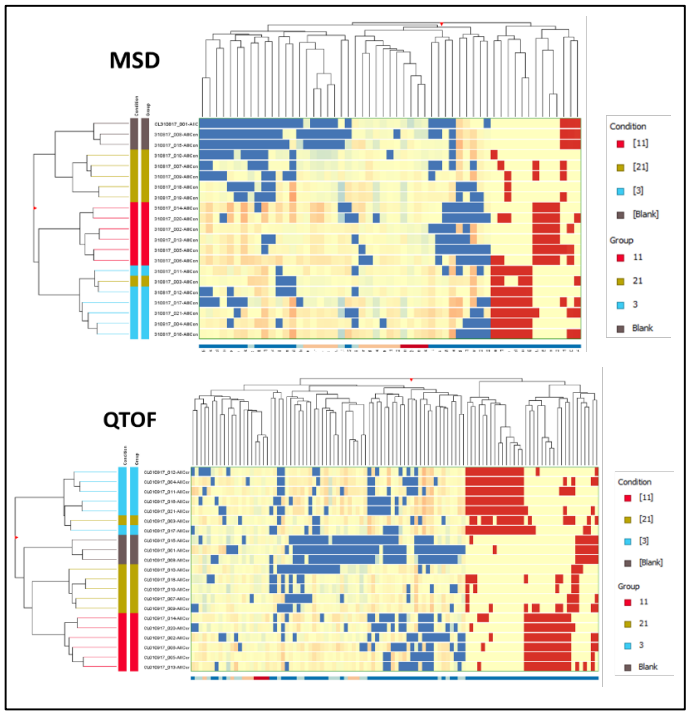


Figure 5: Hierarchical dendrogram obtained for the analysis of fungi samples by GC-MS MSD (top) and GC-MS Q-TOF (bottom)

Another useful algorithm within MPP is the" Find Unique Entities" option which will produce a Venn diagram representing entities shared by the investigated groups and entities unique to the groups. A list of the entities related to each group can be generated and further investigated. As shown in the Venn diagram for the fungi dataset in Figure 6, each fungi species revealed unique entities: 21 entities for group 11, 15 entities for group 21 and 24 entities for group 3.

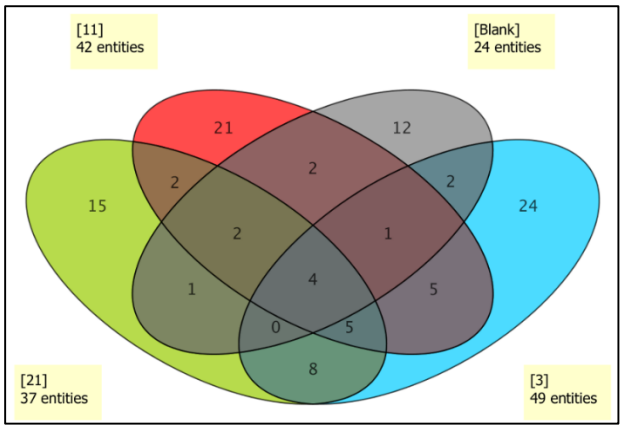


Figure 6: Venn diagram for the investigated fungi species obtained using the "Find Unique entities" tool in MPP

The obtained list of unique entities for each fungi species was further examined going back to Agilent Mass Hunter Qualitative Analysis Software to confirm legitimacy.

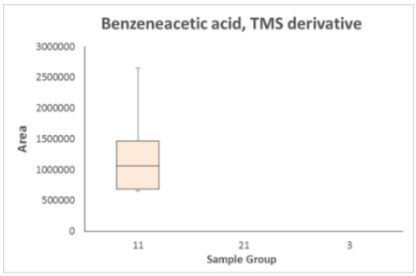
Metabolomics workflow step 3: Semi-quantitative data evaluation

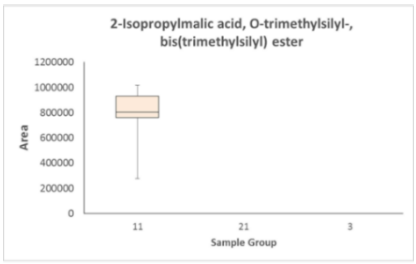
Suggested unique entities were confirmed using Mass Hunter Qualitative Analysis by checking retention time, EICs profiles for the most abundant ion and match factor for library search. These entities were further examined for semi quantitative evaluation. A quantitative method was generated for each of the target entity to allow integration of the peaks and confirmation of identity based on quantifier/qualifier ratios. Two qualifiers were selected per entity. Table 2 summarises the number of entities which were confirmed present in the samples, the entities which were found unique of the group and the entities which were upregulated in the group.

Group	MPP Unique Components	Confirmed present	Confirmed unique	Confirmed upregulated
11	21	11	3	3
21	15	9	0	0
3	24	10	4	2

Table 2: Unique components confirmed present, unique or upregulated in the three fungi species groups

Figure 7 and 8 show the box plot graphs for the peak areas of the entities found unique or upregulated in the 11 and 3 fungi groups, respectively.





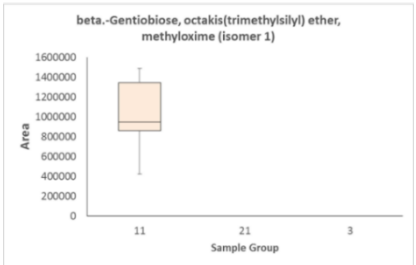
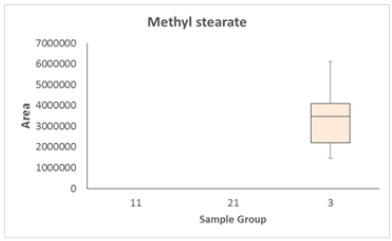
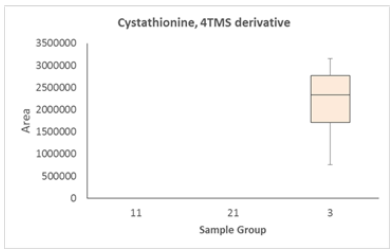
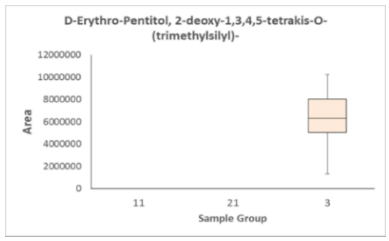


Figure 7: Boxplot graphs for the unique entities in fungi species group 11







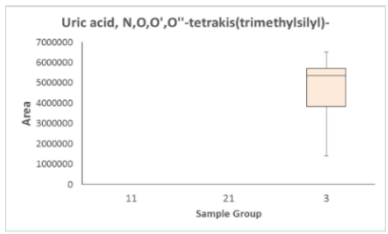


Figure 8: Boxplot graphs for the unique entities in fungi species group 3

Conclusions

A robust and time effective solution for the automated direct MOX-TMS derivatisation and GC-MS analysis of freeze dried fungi samples was developed in our labs and applied to 18 real samples. Control of the analytical variability using automated sample preparation allowed to generate a high quality dataset which could effectively highlight the biological variability of the three fungi species by means of powerful chemometrics tools. The three investigated fungi species were separated in different clusters in both PCA and hierarchical dendogram. Unique entities characteristic of the single species were detected, identified and evaluated semi-quantitatively.

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