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HPLC-MS and ^{1}H NMR-based metabolomic profiling of *Amanita muscaria extract* reveals several unique components

Backgrounds

Nuclear magnetic resonance (NMR)-based approaches have been used to identify metabolites in complex mixtures but this approach is hindered by the complexity of the mixtures, spectral overlapping and incomplete comparative databases. Similarly, high-performance liquid chromatography approaches require the use of specific and high-quality standards for comparison. In this study, we compared NMR and HPLC approaches in the analysis of a complex commercially-available extract (AME-1) from *Amanita muscaria*, an edible mushroom used for medicinal purposes. Specifically, it has been postulated that this mixture contains muscimol and complex sugars, but these have not yet been identified and accurately quantified.

Methods

NMR Spectroscopy: AME-1 was diluted 100 times with D_2O containing TMSP (3-(trimethylsilyl) propionate-2,2,3,3-d4) for NMR measurement. NMR experiments were performed on a Varian Direct Drive VNMRS 600 spectrometer, operating at a magnetic field strength of 14.1 T (599.49 MHz proton frequency) and equipped with an autoX dual broadband probe. One-dimensional (1D) 1H NMR spectra were measured for all samples using 1D 1H with water suppression sequence (NOESY 1D) at 298 K [1]. Metabolites were identified and quantified using Chenomx NMR Suite 8.6 Professional (ChenomX Inc., Edmonton, AB, Canada). For some metabolites, such as trehalose, GABA, and muscimol, the identification was further confirmed by spiking NMR analysis.

HPLC-MS Conditions: HPLC-MS with electrospray ionization (ESI) was conducted on an Agilent 1260 HPLC system. Analyte detection was achieved using an Agilent Single-Quad MSD (G6135B) with an electrospray ionization (ESI) source. HPLC separation was achieved using different columns depending on the target analyte to be analyzed. Identification and quantification were achieved by comparison of peak retention time, spiking LC, and area of reference standards.

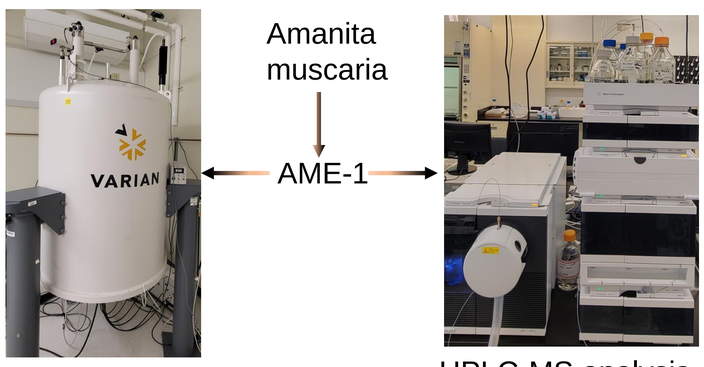

Figure 1. Overview of the workflow of NMR and HPLC-MS experiments. VNMRS 600 spectrometer (left) and Agilent 1260 LCMS system (right) were used for the analysis of AME-1.

Results

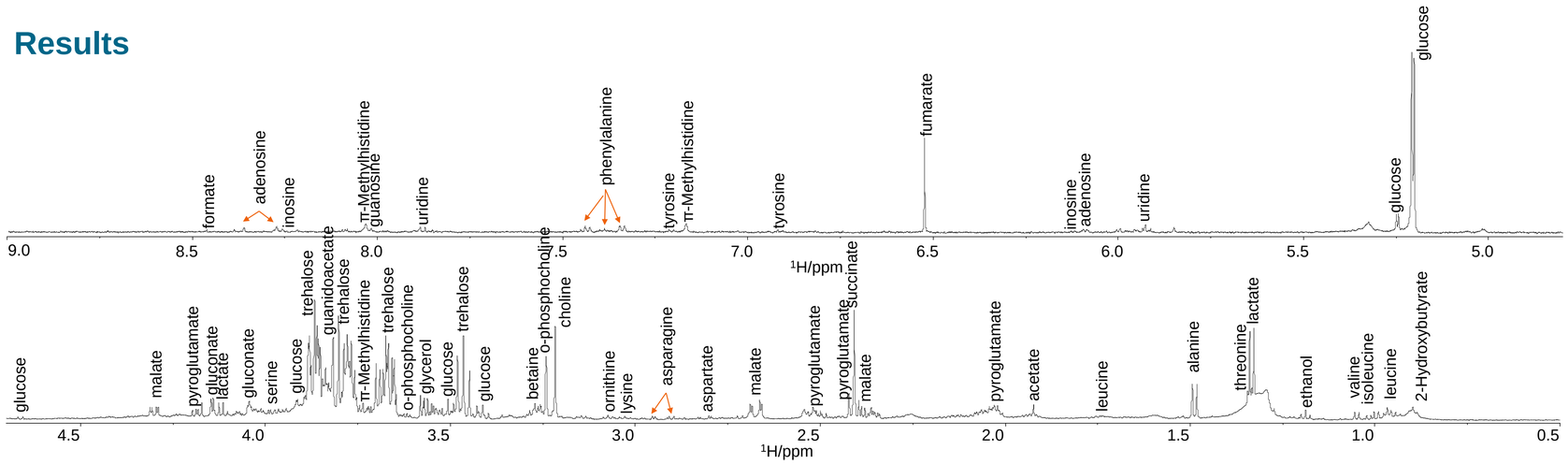

Figure 2. Annotated 1D ^{1}H NMR of AME-1 (100X diluted) at 600 MHz. The aliphatic region (bottom) and the aromatic region (top) of the NMR spectrum are shown. The identified compounds are labeled above each of the corresponding peaks.

	Compound Name	Concentration (mg/ml)	Compound group
1	Trehalose	47.34351	Carbohydrate
2	Gluconate	11.46889	Carboxylic acid
3	Malate	11.00074	Carboxylic acid
4	Glucose	10.38385	Carbohydrate
5	Pyroglutamate	8.34954	Amino acid and derivative
6	Lactate	5.62099	Carboxylic acid
7	Guanidoacetate	5.47489	Amino acid and derivative
8	2-Phosphoglycerate	4.00401	Carboxylic acid
9	Fumarate	3.71308	Carboxylic acid
10	Glycerol	3.49205	Carbohydrate
11	Serine	3.11171	Amino acid and derivative
12	Homoserine	3.07687	Amino acid and derivative
13	Succinate	2.70662	Carboxylic acid
14	Alanine	2.70032	Amino acid and derivative
15	Threonine	2.54679	Amino acid and derivative
16	Arginine	2.40396	Amino acid and derivative
17	Isocitrate	2.29391	Carboxylic acid
18	Mannitol	2.22976	Carbohydrate
19	2-Hydroxyglutarate	2.19795	Carboxylic acid
20	Proline	2.17711	Amino acid and derivative
21	Aspartate	2.00582	Amino acid and derivative
22	Glucarate	1.99423	Carboxylic acid
23	Asparagine	1.90767	Amino acid and derivative
24	π-Methylhistidine	1.8559	Amino acid and derivative
25	O-Phosphocholine	1.77889	Amino acid and derivative
26	myo-Inositol	1.76007	Amino acid and derivative
27	Galactonate	1.42209	Carboxylic acid
28	Citrate	1.32179	Carboxylic acid
29	Glutamine	1.27142	Amino acid and derivative
30	Choline	1.18858	Amino acid and derivative
31	Leucine	1.16217	Amino acid and derivative
32	Taurine	1.14763	Amino acid and derivative
33	Uridine	1.12088	Nucleoside
34	Phenylalanine	1.07539	Amino acid and derivative
35	Ornithine	0.9542	Amino acid and derivative
36	2-Aminoadipate	0.92345	Amino acid and derivative
37	Carnitine	0.85597	Amino acid and derivative
38	Valine	0.8552	Amino acid and derivative
39	2-Hydroxybutyrate	0.83176	Carboxylic acid
40	3-Methylglutarate	0.82423	Carboxylic acid
41	Fucose	0.80762	Carbohydrate
42	Lysine	0.78212	Amino acid and derivative
43	Adenosine	0.7456	Nucleoside
44	Ethylene glycol	0.71753	Carbohydrate
45	Guanosine	0.68544	Nucleoside
46	N-Acetylcysteine	0.59891	Amino acid and derivative
47	Isoleucine	0.57977	Amino acid and derivative
48	3-Hydroxy-3-methylglutarate	0.55776	Carboxylic acid
49	Azelate	0.54396	Carboxylic acid
50	Ethanol	0.50815	Carbohydrate
51	2-Oxocaproate	0.50364	Carboxylic acid
52	Tyrosine	0.49465	Amino acid and derivative
53	Muscimol	0.47922	Amino acid and derivative
54	Glycine	0.43991	Amino acid and derivative
55	Inosine	0.42915	Nucleoside
56	2-Hydroxyvalerate	0.40873	Carboxylic acid
57	2-Hydroxy-3-methylvalerate	0.39912	Carboxylic acid
58	Malonate	0.35693	Carboxylic acid
59	Acetate	0.32247	Carboxylic acid
60	Nicotinate	0.31516	Carboxylic acid
61	cis,cis-Muconate	0.27143	Carboxylic acid
62	Histidine	0.26686	Amino acid and derivative
63	4-Aminobutyrate	0.26296	Amino acid and derivative
64	Betaine	0.25302	Amino acid and derivative
65	Methionine	0.14772	Amino acid and derivative
66	2-Methylglutarate	0.14176	Carboxylic acid
67	Formate	0.09526	Carboxylic acid
68	Propylene glycol	0.08066	Carbohydrate

Table 1. List of metabolites identified in AME-1 by ^{1}H NMR-based profiling. In total, 68 metabolites were measured, quantified, and categorized into 4 groups: free amino acids and derivatives (32), carbohydrates (8), carboxylic acids (24), and nucleosides (4).

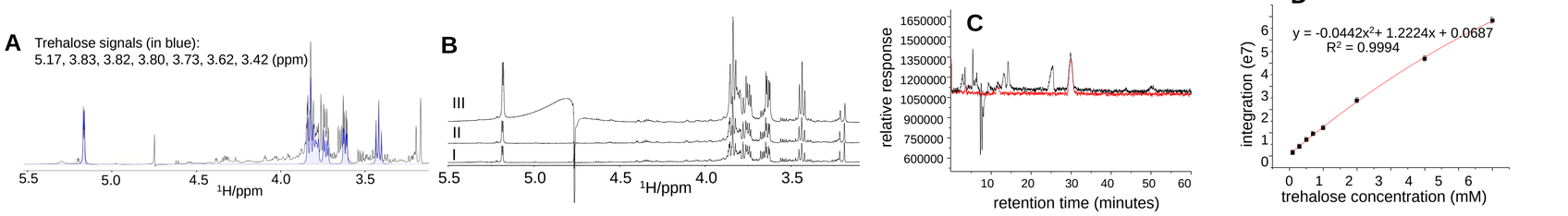

Figure 3. Analysis of trehalose in AME-1 by NMR and HPLC. (A) Expanded region of ^{1}H NMR of AME-1 highlighting the signals of trehalose (in blue color). **(B)** ^{1}H NMR of AME-1 before (I) and after trehalose spiking (II: with 0.7 mM trehalose; III: with 4mM trehalose). The intensity of signals centered at 5.17, 3.83, 3.80, 3.73, 3.62, and 3.42 ppm was increased when trehalose was added. **(C and D)** HPLC-MS chromatograms of trehalose standard **(C, red)** and 100X dilute AME-1 **(C, black)** showing the separation of trehalose from other metabolites. The mass spectrometer was operated in scan mode. **(D)** Trehalose calibration curve. Standards containing varying concentrations of trehalose were injected onto the LC-MS with SIM mode. Data presented as mean ± SD for n=4.

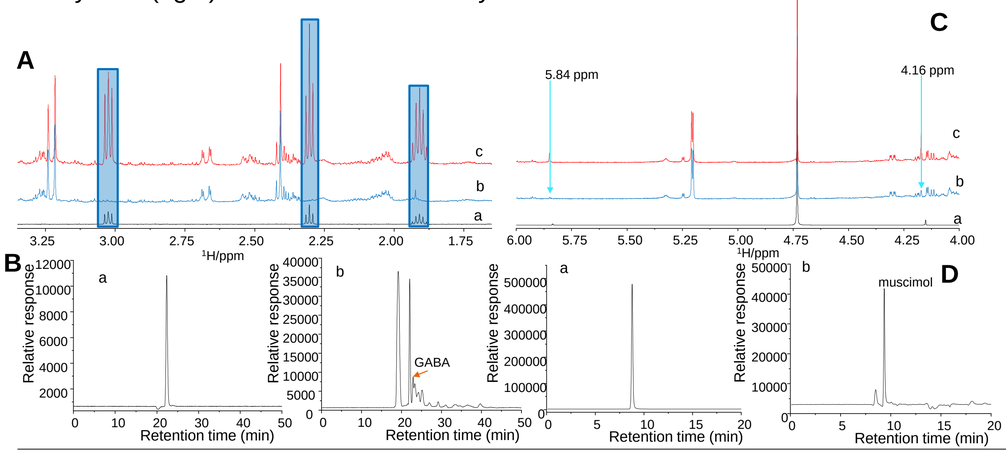

Figure 4. Analysis of GABA and muscimol in AME-1 by NMR and HPLC. (**A**) ^{1}H NMR of GABA (a), AME-1 before (b) and after (c) spiking with GABA. The blue area lighting the signals of GABA. (**B**) HPLC-MS chromatogram of GABA: single ion monitoring mode chromatogram of GABA standard (a) and AME-1 50X dilute (b) with m/z 87.1. (**C**) ^{1}H NMR of muscimol (a), AME-1 before (b) and after (c) spiking with muscimol. The signals at 5.84 and 4.16 ppm are due to muscimol. (**D**) HPLC-MS chromatogram of muscimol: single ion monitoring mode chromatogram of muscimol standard (a) and AME-1 50X dilute (b) with m/z 98.1. The signals of GABA and muscimol in HPLC-MS were further confirmed by the spiked HPLC experiments.

Conclusions

In this study, HPLC-MS and ^{1}H NMR-based metabolomics were used to detect and quantify water-soluble metabolites in AME-1 [2,3]. The NMR data shows that amanita muscaria has very high content of carbohydrates, such as trehalose, glucose, glycerol, and mannitol. Trehalose is the most abundant metabolite in the extract. Of the identified amino acids and derivatives, eight of 9 essential amino acids were found in AME-1 (except tryptophan).Owing to the high sensitivity of HPLC-MS, we were able to detect the GABA and muscimol signals in the extract sample.

Acknowledgements

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References

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2. Wagner, A., Pehar, M., Yan, Z., and Kulka M.: **Front. Pharmacol.** 2023, **14:**1102465. doi:10.3389/fphar.2023.1102465
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