Milling affects volatile compound recovery in functional mushroom extracts

Dingding Xuan, Sajni Shah, Dr. Eric Janusson, Dr. Markus Roggen- Controlled Chemistry

Introduction

Fritsch milling equipment is an alternative to uncontrolled milling for sample preparation. Here we show a general method and assay for volatile constituents of functional mushroom extracts can be dramatically affected by milling conditions.

Hand grinders, food processors, coffee grinders, and other milling methods with limited control settings are standard in laboratories. However, many biomolecules of interest are labile and could be consumed during these processes. Therefore, instruments that provide little control of milling parameters may yield inaccurate results for compound discovery and quantification. We investigated whether particle size, mill time, and blade speed influence analyte recovery and subsequent measurement of compound concentration in functional mushrooms. We used Lion's Mane (*Hericium erinaceus*) as the test substrate and a Fritsch P11 bladed mill to precisely control the various milling factors. Using the most abundant components of these mushrooms (leucine, myrcene, alpha-hydroxyisobutyric acid, 2-acetylbutyrolactone, 2-diethylaminoethyl acetate, and mannitol), we monitored their concentration resulting from various milling procedures.

Experimental

Lion's Mane mushroom extracts were prepared through a simple sample preparation and extraction analytical workflow. Experimental variables were minimized to emphasize differences in milling conditions. The extracts were surveyed by GCMS for compound discovery and quantification.

Sample Preparation

Vacuum-dried whole Lion's Mane mushrooms were donated by Nammex (Gibsons, BC). Whole, dried, Lion's Mane mushrooms were weighed, sectioned using a razor, milled using a Fritsch P11 bladed mill, and then run through a 7mm particle filter. Batches of material were milled at various rates programmed into the P11 mill (see milling parameters in Table 1). An aliquot of each batch of milled material was taken for particle size analysis and a separate aliquot was taken for liquid extraction.

Extraction

Milled Lion's Mane (1.0g) was weighed into 20mL scintillation vials. The extraction was performed with 20.0 mL of a 1:1 mixture of HPLC grade methanol and deionized water containing 0.5% (v/v%) formic acid (Fisher Optima) at room temperature for 3 hours. The extracts were decanted, and an aliquot was filtered with 0.20 µm Nylon syringe filters into glass vials for analysis.

Table 1. Milling parameters for sample preparation experiments on Lion's Mane

Conditions	Mill Speed (RPM)	Mill Time (s)	Replicates
Slow-Short	2000	30	3
Slow-Mid	2000	60	3
Slow-Long	2000	90	3
Mid-Short	5000	30	3
Fast-Short	8000	30	3

GCMS Analysis

The samples were analyzed by GCMS (Agilent Intuvo 9000 GC, 5975 MSD) using an oven ramp program designed to survey a broad range of analytes (Table 2). The injection volume was 1.0 µL and the GC column used was an HP-5ms ultra inert 30 m fused silica column. Suitable compounds were extracted from the GCMS raw data and identified using the Agile 2 peak identification algorithm. Compound fragmentation patterns, extracted ion areas, and retention times were extracted using MS-DIAL and assigned through comparison to our proprietary spectral database.

Results

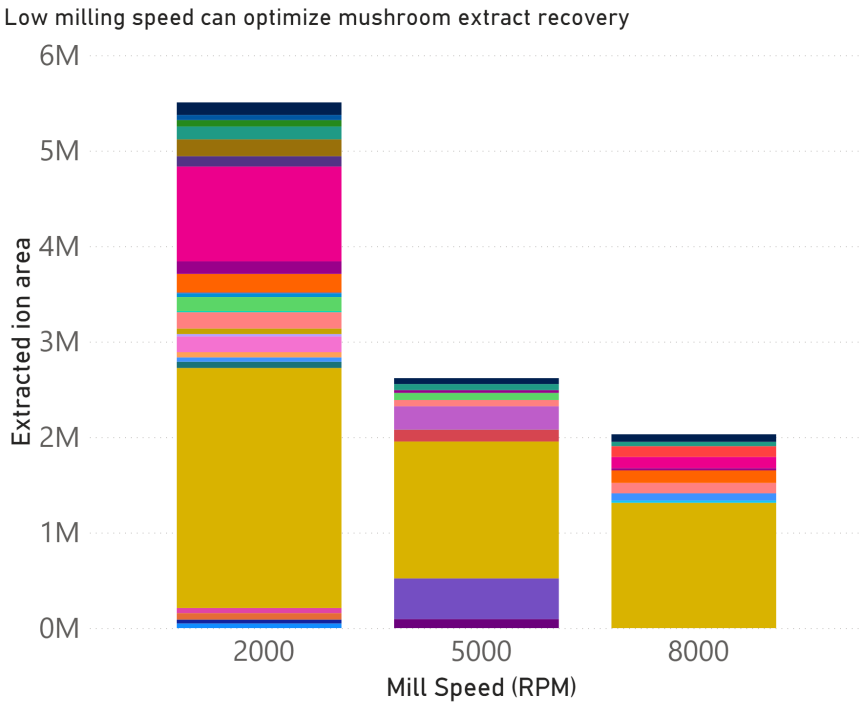

Figure 1. Molecules recovered from extraction after milling at three different speeds programed into the P11 mill. Mill time fixed at 30 sec.

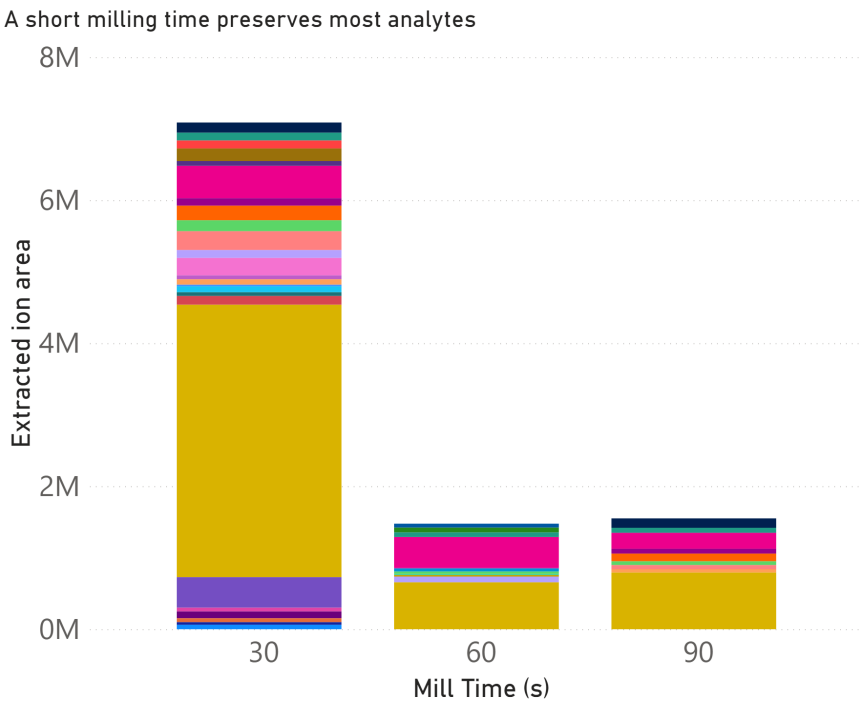

Figure 2. Molecules recovered from extraction after milling for different time periods. Mill speed fixed at 2000 RPM

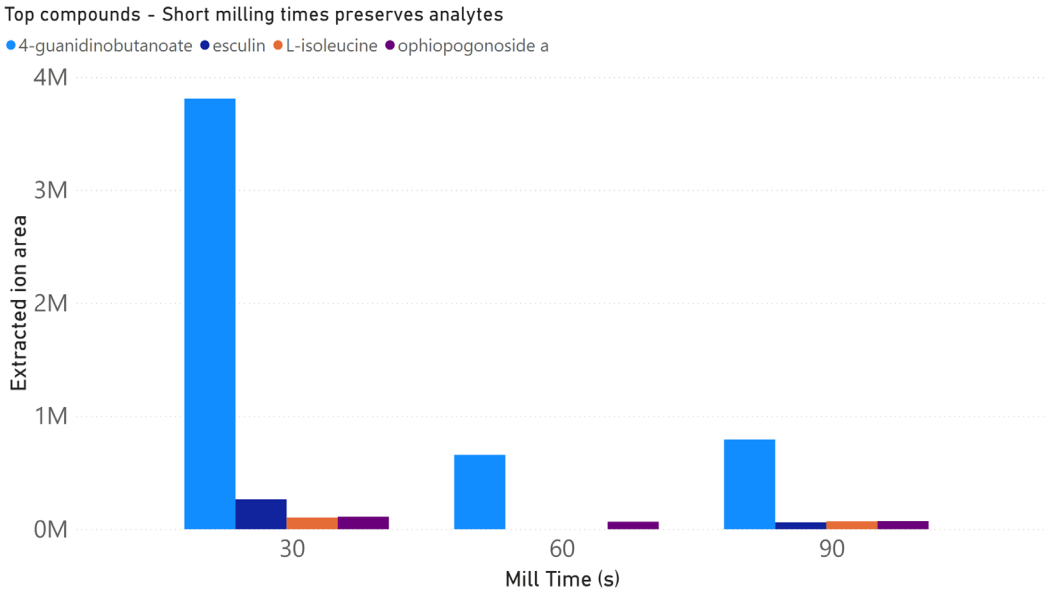

Figure 3. The top molecules identified at all mill times and speeds. Here we show their concentrations at three mill times at 2000 rpm.

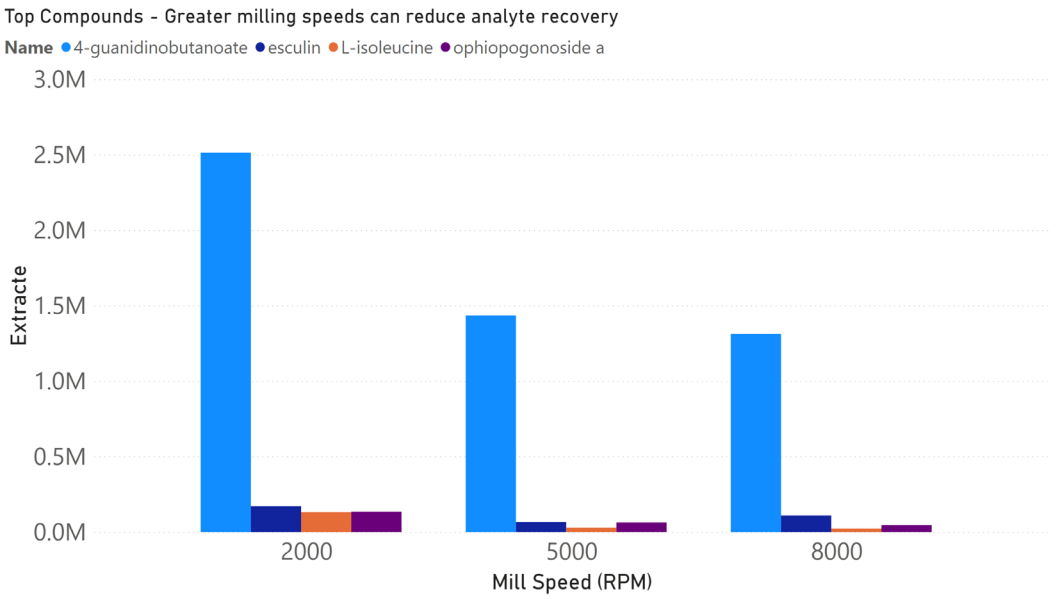

Figure 4.The top molecules identified at all mill times and speeds. Here we show their concentrations at three mill speeds for 30 seconds.

Conclusions

The experiments above demonstrate that precise control of mill conditions is important in mushroom sample preparation. Lion's mane mushroom samples were milled with a Fritsch P11 mill set to different parameters of mill speed and time, and then underwent liquid extraction. The analyte composition of the extracts was then identified as shown in figures 1 and 2. Our results show that mill speed and time correspond to changes in mushroom extract analyte composition. We could show that mill settings had a direct influence on analyte recovery from the mushroom (figures 3 and 4). 4-guanidinobutyrate is found in edible mushrooms and is a known fungal metabolite in many species.[1,2] *Hericium erinaceus* is known to have 3.3% of its protein content made up of L-isoleucine.[3] Meanwhile, to the best of our knowledge, esculin and Ophiopogonoside A have not yet been described in these mushrooms. Among these compounds, lower mill speeds and shorter mill times corresponded to increased recovery. This indicates a Fritsch P11 mill would allow researchers to optimize their sample prep to ensure the highest yields.

References

1. PubChem Compound Summary for CID 500, 4-Guanidinobutyric acid. *National Center for Biotechnology Information* https://pubchem.ncbi.nlm.nih.gov/compound/4-Guanidinobutyric-acid (2022).

2. Li, J., Wu, H., Wang, L., Huang, Y. & Wang, L. Key taste components in two wild edible Boletus mushrooms using widely targeted metabolomics. *Biochemical Systematics and Ecology* **96**, 104268 (2021).

3. Aparicio-Razo, M. & González-Pérez, M. ANALYSIS OF BIOMOLECULES OF THE FUNGUS HERICIUM ERINACEUS THROUGH THE THEORY OF ELECTRON TRANSFER OF QUANTUM CHEMISTRY AND ITS RELATIONSHIP WITH THE PRIMARY AMINO ACIDS. **9**, 139–147 (2020).

Supporting

Table 2

Name	Abundance in all experiments
4-guanidinobutanoate	5261052.235
L-leucine	1110933.091
3-amino-1,2-propanediol	425572.75
esculin	345937.8184
L-beta-homoleucine	326320.0547
tetrahydrofuran	272256.9375
dodecamethylcyclohexasiloxane	270464.666
cadaverine	244824.6094
ophiopogonoside A	243731.9395
hexazinone	240513.2041
citropen	223926.4727
4-oxo-3-phenyl-6-propyl-4h-chromen-7-yl acetate	195539.752
L-isoleucine	183783.2715
dehydroandrographolide	181819.543
N,N-dibutylnitrous amide	176344.2031
myrcene	134515.9443
4-hydroxybenzaldehyde	122221.9766
alpha-hydroxyisobutyric acid	120649.4336
N,N'-dicyclohexylthiourea	112791.6953
2-hydroxybutyric acid	94918.07813
2-((7-acetamido-1,2,3-trimethoxy-9-oxo-5,6,7,9-tetrahydrobenzo[a]heptalen-10-yl)amino)-n-(4-(5-(methoxymethyl)-1h-1,2,4-triazol-3-yl)phenyl)acetamide	90886.3457
cyclododecanol	86997.33765
6-methoxy-4-methyl-2h-chromen-2-one	82209.84376
phenaceturic acid	66982.5

1-myristoyl-2-hydroxy-sn-glycero-3-phosphate (sodium salt) sodium salt	66748.45361
1-[(2e,4e)-6,7-dihydroxy-2,4-octadienoyl]prolyl-n-methylvalyl-n2-methylalaninamide	62692.64649
butanal	54538.58203
pyruvaldehyde	53863.91406
2-methylbutyrylglycine	53269.54297
cinchonine	52899.89649
sinapic acid	48724.09424
diffractaic acid	44918.83301
l-2,3-diaminopropionic acid	38335.02344
[(2r,3r,4s,5r,6s)-3,4,5-tris(acetyloxy)-6-[(7-tert-butyl-5,8-dihydroxy-1,4-dioxo-3-{[(2s,3r,4s,5r,6r)-3,4,5-tris(acetyloxy)-6-[(acetyloxy)methyl]oxan-2-yl]sulfanyl}-1,4-dihydronaphthalen-2-yl)sulfanyl]oxan-2-yl]methyl acetate	36675.40137
pseudolaric acid a-o-beta-d-glucopyranoside	31419.36426
dl-coniine	28856.19677
ganoderol b	28060.43164
cyanate	25044.28906
allo-threonine	23335.97266
4-acetamidobutanoate	22061.03906
nerolidol	22051.06641
pseudopyronine b	21448.30469
dioctyl phthalate	21001.31372
daphnetin	20752.16382
mannitol	19570.46094
protocetraric acid	18046.0166
octylamine	16933.83765
n-acetyl-b-alanine	16850.11523
n-octyl-2-pyrrolidone	16671.71484
L-norleucine	14774.7041
flucarbazone	14044.42285
isoamylamine	12573.57324
bis(2-ethylhexyl) phthalate	12386.82568
2-amino-3-methylvaleric acid	11756.65332
formiminoaspartate	11709.78027
1-dodecyl-2-pyrrolidinone	11695.82129
sakuranetin	11362.86719
3-methyl-2-oxobutanoate	11235.17969
1-dodecanamine	11142.62036
isocucurbitacin b	10466.6709
guanidine	10229.51465
lutein	9612.479492
benzoic acid	9459.237305

lacosamide	8988.265625
dimethyl malate	8386.848145
1,2-dilauroyl-sn-glycero-3-phosphate monosodium salt	5801.692871
norfluoxetin	5688.64502
metformin	5674.167969
loureirin a	5657.355713
trimethylamine	5535.165039
mexiletine acetate	5474.612305
1-myristoyl-2-hydroxy-sn-glycero-3-phosphoethanolamine	5455.387695
norfluoxetine	4984.3125
dl-norgestrel	3903.630127
aminoadipic acid	3554.445068
ethanolamine	2852.979736
diisopropyl phthalate	2370.876465
4-oxobutanoate	1833.481812
1,5-diaminopentane	268.3061523