LC-MS/MS Determination of Gyromitrin in Mushrooms as a Method to Identify False Morel Mushrooms

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Abstract

False morel mushrooms have been responsible for severe intoxication, vomiting, diarrhea, jaundice, convulsions, coma and even death. Gyromitrin is the major toxin contained in these fresh mushrooms. The current method to determine gyromitrin in false morels is by physical examination. This method needs extensive technical training and FDA needs a more modern chemical testing method to accurately determine gyromitrin in the false morels to support the FDA regulatory program. Acetonitrile extraction and salting-out sample cleanup method was used to extract gyromitrin spiked into three different blank mushrooms. The sample extract was directly injected on an LC-MS/MS instrument to determine gyromitrin in the sample. A calibration curve with matrix-matched standard, composed of baby bella mushroom extract, was used for quantification of gyromitrin in this study. Retention time against the authentic standard and at least two MRM transitions were monitored to achieve true positive identification of gyromitrin in the sample. The average recovery for gyromitrin at 0.4, 4, and 40 µg/g (n = 18) ranged from 81-106%, with a relative standard deviation of ≤ 8%. This method may be used to replace the physical examination technique to identify false morel mushrooms.

Introduction

Gyromitra esculenta is one of the mushrooms known as false morel mushrooms. It is harvested and eaten by many people in northern Europe and also in the USA. It is poisonous, even fatal, unless properly dried and boiled. The principal poisonous ingredient is gyromitrin (acetaldehyde-N-methyl-N-formylhydrazone, AMFH). Gyromitrin hydrolysis in the stomach yields *N*-methyl-formylhydrazine (MFH), which is further metabolized to monomethylhydrazine (MMH)[1] (Figure 1). The LD50 value of gyromitrin has been estimated to be 340 mg/kg for mice, whereas the LD50 value of N-methylhydrazine for mice is much lower at 33 mg/kg [2]. It has been suggested that MMH is the active substance responsible for intoxication following the consumption of false morels[2].

The typical gyromitrin content in fresh false morel mushrooms is between 40 and 732 mg/kg (wet weight) [3] and in dried mushroom between 0.05 and 0.5% [4]. These mushrooms have caused severe poisoning and even deaths in humans[5]. Clinical data are characterized primarily by vomiting and diarrhea, followed by jaundice, convulsion and coma (Hendricks, 1940)[6].

Intact molecules of gyromitrin in false morel mushrooms was extracted and analyzed by GC-FID on a glass capillary gas chromatograph along with eight other N-methyl-N-formylhydrazone analogs[7]. Larrson and Eriksson explained an analytical method to identify false morel mushrooms by extracting gyromitrin from the sample and measuring MMH after derivatization with thin-layer chromatography[1]. The method is time-consuming and only semi-quantitative. MMH is too polar, and poorly retained in a conventional reversed-phase HPLC. The lack of UV chromophore made detection challenging with a UV detector. Mass spectrometry detection cannot be used to identify MMH due to the interference encountered in the very low molecular weight range. Derivatization followed by GC[3] and HPLC[8, 9] separation has been reported. These procedures are complicated and time-consuming. Hydrophilic interaction liquid chromatography (HILIC) was used to analyze hydrazine and methyl hydrazine without derivatization steps [10, 11] . Mass spectrometer was used to identify gyromitrin in false morels [12, 13]. The current method to identify the false morel mushrooms is by physical examination (Figure 2). This method needs extensive technical training and FDA needs a more modern chemical testing method to quickly and accurately identify the false morel mushrooms.

The purpose of this study is to develop a quick and accurate method to determine gyromitrin in mushrooms by LC-MS/MS and to use gyromitrin as a biomarker for the identification of false morel mushrooms.

Experimental

Chemicals and Reagents

1) Chemical standard of gyromitrin (97% purity) was obtained from Toronto Research Chemicals (Toronto, Ontario Canada).
2) Acetonitrile, methanol and water of HPLC grade were purchased from Fluka (Buchs, Switzerland.).
3) QuEChERS salt kits containing 6 g of $MgSO_4$ and 1.5 g of NaCl were purchased from UCT, Inc, (Bristol, PA).
4) EDP 3 electronic pipettes at different capacities (0-10 µL, 10-100 µL, and 100-1000 µL) were purchased from Rainin Instrument LLC (Oakland, CA) and were used for standard preparation and fortification.
5) Mobile phase A was HPLC grade water and mobile phase B was HPLC grade methanol. The extraction solvent was HPLC grade acetonitrile.

Standard Preparation

1) A stock solution of gyromitrin standard (10 mg/mL) was prepared in acetonitrile (corrected for % purity). A set of working standard solutions at 1000, 100, and 10 µg/mL in acetonitrile was prepared from the stock solution. These solutions were used to fortify a set of three 2 g blank samples by pipetting 80 µL to achieve the fortification levels of 40, 4, and 0.4 µg/g, respectively.
2) A set of calibration standard solutions at 10, 25, 50, 100, 250, 500, and 1,000 ng/mL in acetonitrile and blank sample matrices were prepared from the working standard (100 µg/mL).

Sample Preparation and Extraction Procedure

Three different types of mushroom (baby bella, whole white, and portabella) were obtained from a local market. The samples were cut into small pieces with a knife and blended with dry ice in a blender/homgenizer Blixer 3 (Robot Coup, Inc, Jackson, MS) with pulsed action until contents were uniform and had the consistency of a fine powder. Homogenate was placed in a 1-quart plastic cup, loosely sealed and stored in a freezer (-20 °C) overnight to allow the carbon dioxide to dissipate, then sealed until the time of analysis.

1) On the extraction day, thaw the samples to room temperature and weigh in triplicate at 2 ± 0.1 g each into 50-mL polypropylene centrifuge tubes (Fisher Scientific, Pittsburgh, PA).
2) Fortify the blank samples with 80 µL of 10, 100, 1000 µg/mL of the gyromitrin solutions at the levels of 0.4, 4, and 40 µg/g, mix briefly on a vortex mixer, and let to stand at room temperature for one hour.
3) Add purified water (5 mL) to each tube using an automatic pipette and vortex briefly to disperse the samples into the liquid phase.
4) Add acetonitrile (10 mL) to each tube using an automatic pipette.
5) Cap the tube tightly and shake for 10 min on a SPEX 2000 Geno grinder (SPEX Sample Prep LLC, Metuchen, NJ) at 2,000 stroke/min.
6) Add the QuEChERS salts into the tubes and shake for another 10 min on the SPEX 2000 Geno grinder. The tubes were then centrifuged at 4,130 rpm (3,000 x g) for 10 min using a Q-Sep 3000 centrifuge (Restek, Bellefonte, PA).
7) Transfer the sample extract (top layer) into an autosampler vial and capped. 1µL of the sample extract was injected into the LC-MS/MS system.

Instrumentation

1 Liquid Chromatograph/Mass spectrometer- The instrument was equipped with two LC-20AD pumps, a Sil-20AC autosampler, and a CTO-20AC column oven (Shimadzu, Kyoto, Japan), coupled with a 6500 Q-TRAP mass spectrometer from AB SCIEX (Foster City, CA). The Analyst software (version 1.6) was used for instrument control and data acquisition. Nitrogen and air from TriGas Generator (Parker Hannifin Co., Haverhill, MA) were used for nebulizer and collision gas in LC-MS/MS. The MS determination was performed in positive mode with the

following MS source conditions: curtain gas (CUR) of 30 psi, ion spray voltage (ISV) of 5500 volts, collisionally activated dissociation gas (CAD)- high, nebulizer gas (GS1) of 60 psi, heater gas (GS2) of 60 psi, and source temperature (TEM) of 500 °C. Analyte-specific MS-MS conditions and LC retention times for the analytes are shown in Table 1.

2 HPLC column: A Kinetex XB-C18 100 A (2.6 µm, 100 x 2.1 mm) analytical column and a C18 SecurityGuard guard column (4 x 3 mm) both from Phenomenex (Torrance, CA) were used for HPLC separation at 40 °C with a sample injection volume of 1 µL. The mobile phase was 5% B from 0 - 0.5 min at a flow rate of 0.4 mL/min then ramped up to 100% B from 0.5 – 4 min to elute the analytes. Mobile phase was left at 100 % B for another 3 min to flush the column. The column was then equilibrated with 5% B at the same flow rate for a total run time of approximately 12 min. A diverter valve connected between the HPLC column and the MS interface was used to direct the LC eluent to waste from 0 – 2 min and 3.5 – 12 min.

Results and Discussion

Chromatography Optimization

A standard solution of gyromitrin at 1 µg/mL in acetonitrile was infused into the mass spectrometer to determine the product ions (Figure 3) of precursor ion (m/z = 101) and optimum values for the MS parameters (Table 1). Three reversed-phase columns were evaluated including a) Kinetex XB-C18 100 A (2.6 µm, 100 x 2.1 mm) from Phenomenex (Torrance, CA) , b) Xterra MS C-18 (2.5 µm, 50 mm x 2.1 mm) from Waters (Milford, MA), and c) ACE 3 C18 (3 2.5 µm 50x3 mm) from Mac-Mod (Chadds Ford, PA), Gradient elution of water/methanol containing 10 mM ammonium formate and 0.1% formic acid was used during the evaluation period. This mobile phase combination has been used successfully for pesticide residue analysis in food. A standard solution of gyromitrin in acetonitrile at 1 µg/mL was injected on the columns with a gradient elution from 5% methanol to 90 % methanol in five minutes at a flow rate of 0.4 mL/min. The resulting peak shapes were evaluated. All three columns gave decent peak shapes which eluted within 5 min. The Kinetex XB-C18 gave the lowest column backpressure; therefore, it was chosen for later work. However, there were two issues with this column. Firstly, the peak shape was not symmetrical and not consistent. Upon closer examination, the gyromitrin standard exhibited a coelution of two peaks when it was monitored with different MRM transitions (Figure 4). Secondly, gyromitrin is not stable under acidic conditions and may degrade to monomethyl hydrazine (Figure 1). The gyromitrin standard in acetonitrile with 0.1% formic acid exhibited a smaller peak response than the gyromitrin standard in acetonitrile at the same concentration. To eliminate these issues, the mobile phase was modified by eliminating the ammonium formate and formic acid all together. This modification significantly improved the peak shape and stability of gyromitrin in the sample during the analysis.

Sample Extraction

The QuEChERS extraction and cleanup procedure has been used widely for pesticide screening in food. The method is quick and effective and should be suitable for a moderately polar molecule like gyromitrin[14]. The original QuEChERS method[14] (no acid added) was chosen to minimize the

degradation of gyromitrin in the extraction step. During the salting-out step, most of the water-soluble pigments and polar interferences in the mushrooms were extracted into the bottom layer of the aqueous portion (Figure 5). Gyromitrin was extracted into the top layer of acetonitrile which was almost colorless and easily transferred into an autosampler vial.

Evaluation of Matrix Effects

Matrix suppression was evaluated to determine the effectiveness of the sample extraction and cleanup utilized in this method. Matrix effect (%ME) in the sample extract was calculated as the slope of calibration curve of analyte in sample matrix divided by the slope of calibration curve of analyte in solvent multiplied by 100 (Figure 6). A value of 100% means that no matrix effect is present. If the value is less than 100%, it means that there is matrix suppression. If the value is more than 100%, it means that there is matrix enhancement. The matrix effects of three blank mushroom samples were found to be 77, 80, and 83%, respectively. Figure 5 is an example of calibration curves of gyromitrin in acetonitrile and in mushroom blank extract no 1 (baby bella). Because there was significant matrix effect, a calibration curve with matrix-matched standard (baby bella) was used for quantification of gyromitrin in this study.

Method Performance

Gyromitrin was extracted from the samples with 5 mL of water and 10 mL of acetonitrile using a SPEX 2000 Geno grinder at 2,000 stroke/min for 10 min. This solvent was used in pesticide analysis in food with satisfactory results [14]. The Geno grinder provides vigorous shaking action for efficient extraction, and it can accommodate up to 15 50-mL tubes at a time. After the salting out step, most of the water-soluble pigments were extracted into the bottom aqueous layer and gyromitrin was partitioned into the top clear acetonitrile layer. The mobile phase of water and methanol without acid gave reproducible peak response and peak shape of gyromitrin throughout the run. The Kinetex column is a fused-core stationary phase that produced sharp symmetrical peaks at a lower back pressure than the fully porous stationary phase options (column 2 and 3 in this study). The diverter valve was used to bypass the column effluent before and after the gyromitrin retention window to keep the Ion-spray interface clean.

Method performance was evaluated by spiking three blank mushroom samples (in duplicate) with gyromitrin at 0.4, 4, and 40 μg/g and analyzing on three different days. The results were used to determine accuracy (recovery %) and precision (RSD %) of the method using the matrix-matched standard curve of the baby bella mushroom from 10 to 1000 ng/mL. Table 2 shows the recovery data of gyromitrin in three different blank mushrooms performed on three different days. Method performance was summarized in Table 3. The calibration curve was a linear fit with 1/x weighing, and they all showed satisfactory linearity with a coefficient of determination (R^2) of more than 0.995. The chromatograms of gyromitrin standard in acetonitrile at 100 ng/mL and in blank mushroom no 1 spiked at 4 μg/g are shown in Figure 7. The LC-MS/MS chromatograms are free from background interference. The m/z ratio of confirmation ion (101/73) over the quantification ion (101/60) of the gyromitrin found in the sample matched with the m/z ratio found in the standard (within ± 20%)[15]. The third m/z at 101/58 was also used as an additional confirmation MRM

transition. The method detection limit (MDL) was calculated according to the FDA guidelines with 7 replicates of the low spike at 30 ng/g. The MDL was calculated by multiplying the standard deviation of 7 replicates with a student t value at a confidence level of 99% with a degree of freedom of 6 (3.14). The MDL was calculated as 13 ng/g. The method quantification limit (MQL) was three times the MDL at 39 ng/g.

Application of the Method

The method was tested to identify the presence of gyromitrin in a dried false morel sample kindly provided by the Swedish Food Agency (Figure 2). This was a non-official sample fro research purposes. It is very difficult to identify if this sample is a false morel mushroom by just physical examination as the samples were significantly transformed from their original fresh appearance during the drying process. The basic criteria used to distinguish false mushroom is not applicable at this stage. Only one gram of ground mushroom was used due to the low moisture content. Figure 7 shows that this sample contained 130 ng/mL of gyromitrin in the sample extract. Unlike the baby bella mushroom, the false morel mushroom has an interference peak which eluted near the gyromitrin peak when it was monitored at 101/58 m/z transition. However, the first two MRM transition for the false morel mushrooms, *m/z* 101/60 and *m/z* 101/73, showed no interfering peaks at 130 ng/mL gyromitrin, which is adequate for confirmation of identity. The method found 1.3 µg/g of gyromitrin in this dried false morel sample. This value is much less than the value reported in the literature and is because only the free from of gyromitrin can be extracted from the mushroom. In order to extract the bound form of gyromitrin, an acid hydrolysis procedure must be used to transform gyromitrin to monomethylhydrazine and then converted back to gyromitrin[12]. This method has, however, proven that it can identify the presence of gyromitrin in the false morel mushrooms which meets objective of this study.

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Figure 1 Gyromitrin is converted to in vivo to N-Methyl-N-formylhydrazine, and then N-Methylhydrazine

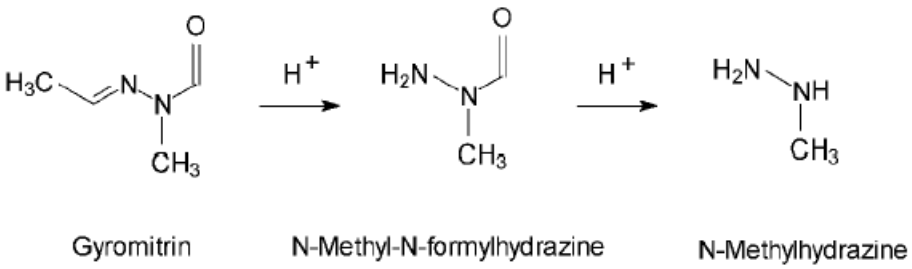
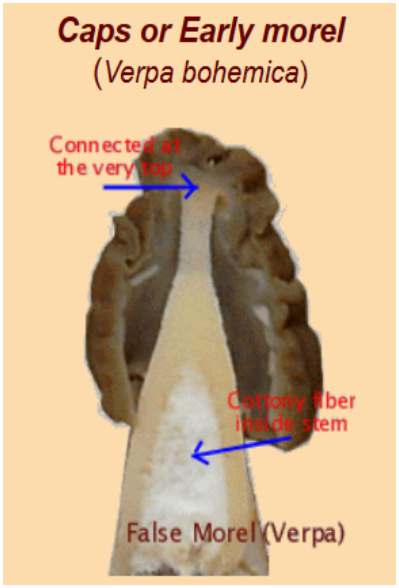


Figure 2 Physical Characteristics of True Morel and False Morel

A) True Morel *
- hollow inside
- honey comb structure
- cap connected to stem

B) False Morel *
- cotton fiber in stalk
- cap only connect at the top

C) Dried False Morel



* Picture credit from https://www.michiganmorels.com/morels2.shtml

Figure 3 Product ion scan of gyromitrin (precursor = 101 m/z) in positive mode with the collision energy of 16.

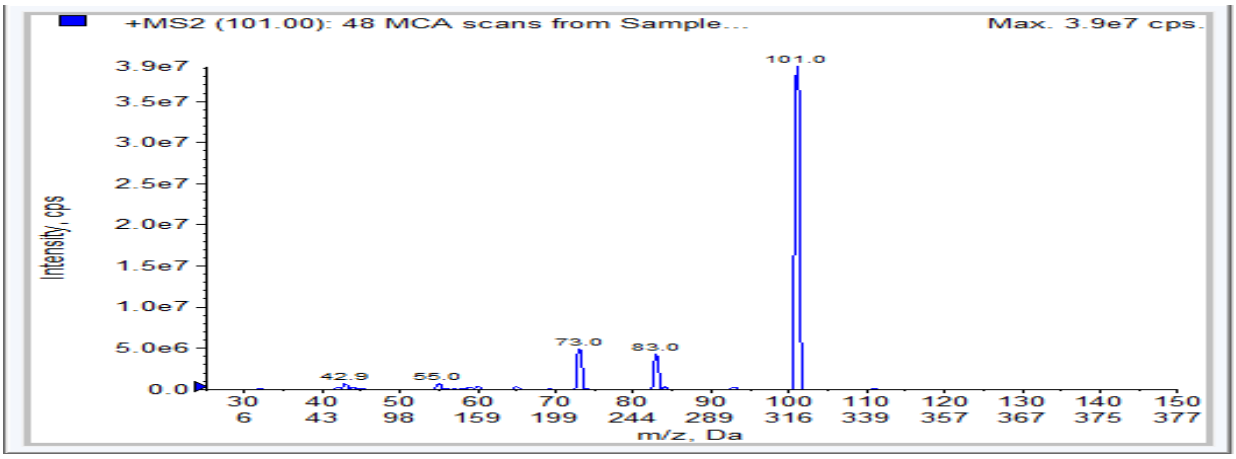


Figure 4 Chromatogram of standard gyromitrin under acidic mobile phase monitored at different precursor/product ions

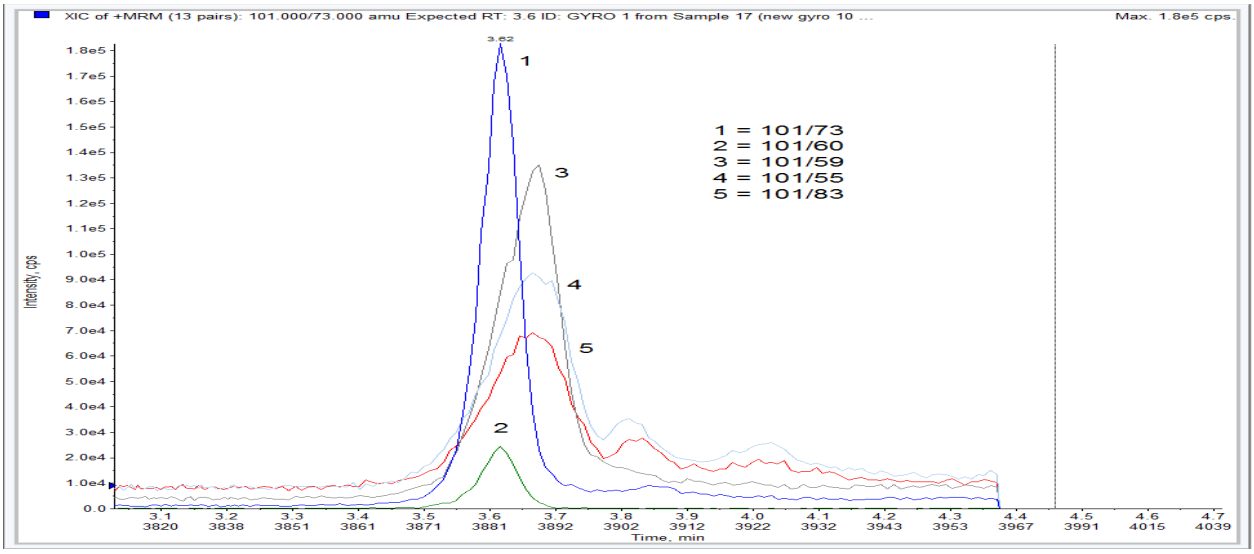


Figure 5 Picture of the sample tube after being shaken with MgSO4/NaCl salt mixture and centrifugation. Gyromitrin was extracted and partitioned into the top clear layer of the acetonitrile

Figure 6 Calibration curves of gyromitrin in acetonitrile and in the blank mushroom extract

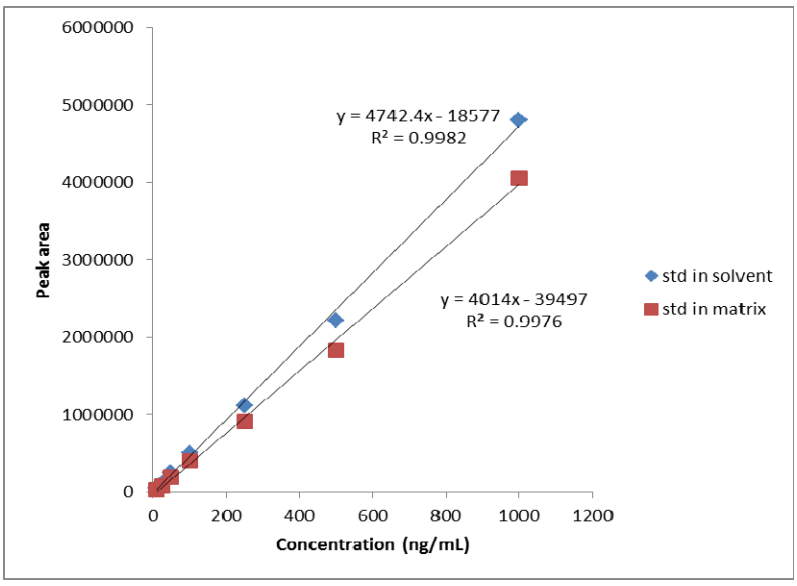


Figure 7 Chromatogram of gyromitrin in 1) acetonitrile (100 ng/mL), 2) blank mushroom 1 extract (80 ng/mL), and 3) false morel extract. (130 ng/mL found) [A = MRM transition 101/60, B = MRM transition 101/73, C = MRM transition 101/58]

1 2 3

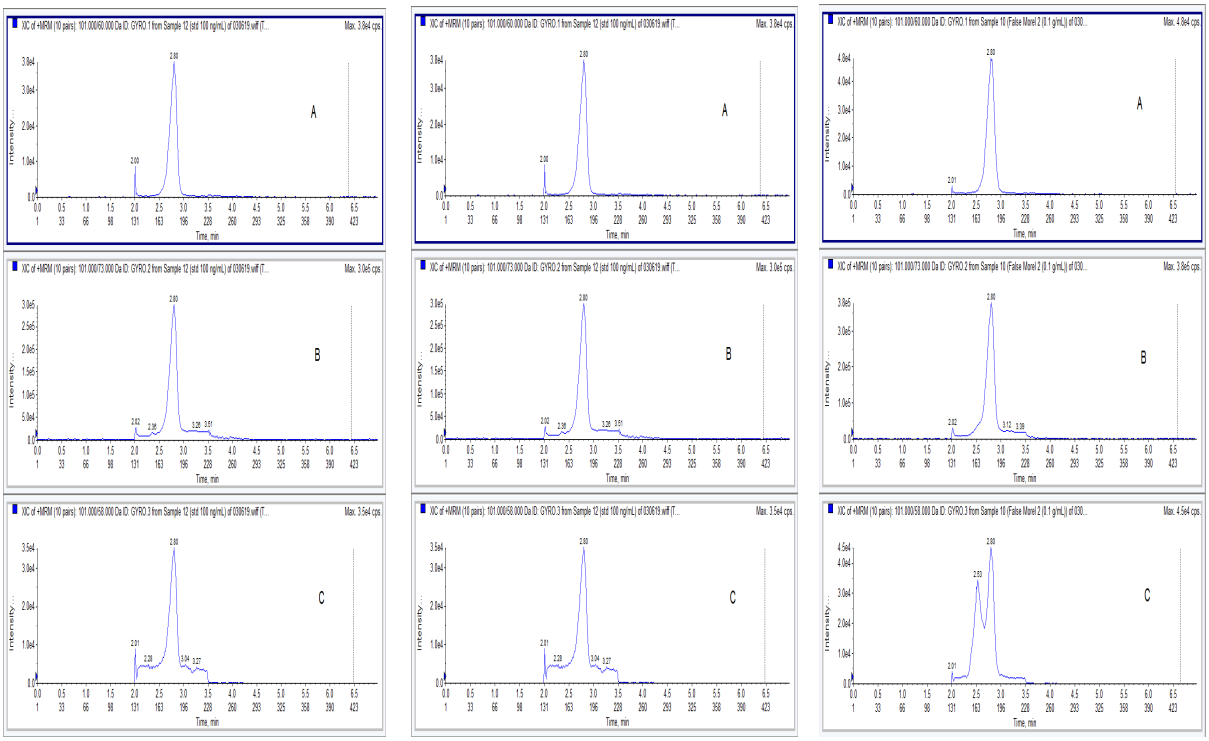


Table 1. Retention time and MS conditions for LC-MS/MS analysis.

Analyte	Precursor Ion (m/z)	Product Ion (m/z)	DP	CE	EP	CXP	Retention Time (min)
Gyromitrin.1 *	101	60	150	16	10	9	2.8
Gyromitrin.2	101	73	150	14	10	9	2.8
Gyromitrin.3	101	58	150	29	10	9	2.8
Gyromitrin.4	101	43	150	16	10	9	2.8
Gyromitrin.5	101	59	150	29	10	9	2.8

Compound dependent parameters: DP = declustering potential, CE = collision energy, EP = entrance potential, CXP = collision cell exit potential * this transition was used for method quantification.

Table 2 Recovery data of three mushroom blank samples spiked with gyromitrin on three different days

Spike level µg/g	Matrix	Recovery (%) Day 1		Recovery (%) Day 2		Recovery (%) Day 3	
0.4	1	83	93	93	83	92	81
	2	97	92	93	97	93	90
	3	95	96	91	94	93	90
4	1	82	95	95	81	96	83
	2	99	97	96	99	98	83
	3	94	95	88	93	81	81
40	1	95	109	87	97	100	86
	2	105	92	99	86	88	97
	3	104	106	96	91	99	105

Table 3 Method performance summary for all three different matrices at three levels

Parameter	Fortification level (µg/g)		
	0.4	4	40
Intraday (day 1)			
average recovery (n=6)	93	94	102
std dev	5.1	6.0	6.8
RSD (%)	5.5	6.4	6.7
Interday			
average recovery (n=18)	91	91	97
std dev	4.6	7.0	7.4
RSD (%)	5.0	7.8	7.6

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