Safety Assessment of Mushrooms in Dietary Supplements by Combining Analytical Data with in Silico Toxicology Evaluation

By: Karen M. VanderMolen, Jason G. Little, Vincent P. Sica, Tamam El-Elimat, Huzefa A. Raja, Nicholas H. Oberlies, Timothy R. Baker, and Catherine Mahony

"Safety Assessment of Mushrooms in Dietary Supplements by Combining Analytical Data with in Silico Toxicology Evaluation." Karen M. VanderMolen, Jason G. Little, Vincent P. Sica, Tamam El-Elimat, Huzefa A. Raja, Nicholas H. Oberlies, Timothy R. Baker, and Catherine Mahony. *Food and Chemical Toxicology*, 2017, 103, 133-147. PMID: 28267567; doi: 10.1016/j.fct.2017.03.005

Made available courtesy of Elsevier: https://doi.org/10.1016/j.fct.2017.03.005

Abstract:

Despite growing popularity in dietary supplements, many medicinal mushrooms have not been evaluated for their safe human consumption using modern techniques. The multifaceted approach described here relies on five key principles to evaluate the safety of non-culinary fungi for human use: (1) identification by sequencing the nuclear ribosomal internal transcribed spacer (ITS) region (commonly referred to as ITS barcoding), (2) screening an extract of each fungal raw material against a database of known fungal metabolites, (3) comparison of these extracts to those prepared from grocery store-bought culinary mushrooms using UHPLCPDA-ELS-HRMS, (4) review of the toxicological and chemical literature for each fungus, and (5) evaluation of data establishing presence in-market. This weight-of-evidence approach was used to evaluate seven fungal raw materials and determine safe human use for each. Such an approach may provide an effective alternative to conventional toxicological animal studies (or more efficiently identifies when studies are necessary) for the safety assessment of fungal dietary ingredients.

Keywords: Human safety | Dietary ingredient | Supplements | Barcoding | Fungi | UHPLC-HRMS

Article:

***Note: Full text of article below**

Food and Chemical Toxicology 103 (2017) 133–147

Contents lists available at ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

Safety assessment of mushrooms in dietary supplements by combining analytical data with *in silico* toxicology evaluation

Karen M. VanderMolen [a, b, *], Jason G. Little [a, c], Vincent P. Sica [d, e], Tamam El-Elimat [d, f], Huzefa A. Raja [d], Nicholas H. Oberlies [d], Timothy R. Baker [e], Catherine Mahony [g]

[a] Product Safety and Regulatory Affairs, New Chapter, Inc., 90 Technology Dr, Brattleboro, VT 05301, United States
[b] Central Product Safety, The Procter and Gamble Company, Winton Hill Business Center, 6100 Center Hill Ave, Cincinnati, OH 45232, United States
[c] Personal Health Care, The Procter and Gamble Company, Mason Business Center, 8700 Mason-Montogomery Rd, Mason, OH 45040, United States
[d] Department of Chemistry and Biochemistry, University of North Carolina at Greensboro, Greensboro NC 27402, United States
[e] Research and Development, Corporate Functions Analytical, The Procter & Gamble Company, Mason Business Center, 8700 Mason-Montogomery Rd, Mason, OH 45040, United States
[f] Department of Medicinal Chemistry and Pharmacognosy, Faculty of Pharmacy, Jordan University of Science and Technology, Irbid 22110, Jordan
[g] Central Product Safety, Procter and Gamble Company Technical Centres Ltd, Egham, Surrey TW20 9NW, United Kingdom

ARTICLE INFO

Article history:
Received 16 November 2016
Received in revised form
30 January 2017
Accepted 2 March 2017
Available online 4 March 2017

Keywords:
Human safety
Dietary ingredient
Supplements
Barcoding
Fungi
UHPLC-HRMS

ABSTRACT

Despite growing popularity in dietary supplements, many medicinal mushrooms have not been evaluated for their safe human consumption using modern techniques. The multifaceted approach described here relies on five key principles to evaluate the safety of non-culinary fungi for human use: (1) identification by sequencing the nuclear ribosomal internal transcribed spacer (ITS) region (commonly referred to as ITS barcoding), (2) screening an extract of each fungal raw material against a database of known fungal metabolites, (3) comparison of these extracts to those prepared from grocery store-bought culinary mushrooms using UHPLCPDA-ELS-HRMS, (4) review of the toxicological and chemical literature for each fungus, and (5) evaluation of data establishing presence in-market. This weight-of-evidence approach was used to evaluate seven fungal raw materials and determine safe human use for each. Such an approach may provide an effective alternative to conventional toxicological animal studies (or more efficiently identifies when studies are necessary) for the safety assessment of fungal dietary ingredients.

1. Introduction

As a source of diverse secondary metabolites, fungi have enjoyed a long history of use in both culinary and medicinal applications (Das et al., 2010; Kalaivani et al., 2010; Paterson, 2006; Rios, 2011; Xu, 2012). While some of the chemically interesting and/or biologically active constituents are shared across all fungi (e.g, β-glucans (Dalonso et al., 2015)), others are exclusive to specific species or genera (Patakova, 2013; Xu, 2012), and some constituents may only be produced under distinct growing conditions (Wang et al., 2014). In general, many medicinal mushrooms have not been evaluated for their safe human use using modern analytical approaches. While history of use data should be carefully considered, and can provide a foundation for establishing safe use, some toxicological endpoints may be more opaque. Obtaining safety data for developmental and reproductive toxicity (DART), genotoxicity, and chronic endpoints can prove particularly difficult. Complicating the evaluation of such fungi, modern cultivation practices and preparations are rarely consistent with traditional medicinal uses. While fruiting bodies are most often the portion of the organism used in traditional Chinese medicine (TCM) (Bensky et al., 2004), commercial raw materials typically consist of the fungi's mycelium, which grows more quickly and is therefore less expensive to produce. In addition, the growing conditions can impact the secondary metabolite profile of fungi (Bills et al., 2008; Bode et al., 2002; Fiedurek et al., 1996; Miao et al., 2006; Mohanty

* Corresponding author. Central Product Safety, The Procter and Gamble Company, Winton Hill Business Center, 6100 Center Hill Ave, Cincinnati, OH 45232, United States.
E-mail addresses: vandermolen.k@pg.com (K.M. VanderMolen), little.j.6@pg.com (J.G. Little), sica.vp@pg.com (V.P. Sica), telimat@just.edu.jo (T. El-Elimat), haraja@uncg.edu (H.A. Raja), nicholas_oberlies@uncg.edu (N.H. Oberlies), baker.tr@pg.com (T.R. Baker), mahony.c@pg.com (C. Mahony).

http://dx.doi.org/10.1016/j.fct.2017.03.005

and Prakash, 2009; VanderMolen et al., 2013), which impacts profoundly the ability of investigators to bridge data generated from different raw materials or different suppliers, even of the same species.

Given these challenges, a multifaceted approach was developed to establish safe human use of fungi as dietary ingredients. The approach described here relies on five key principles to evaluate the safety of non-culinary fungi for human consumption. (1) A critical starting point in the safety review of fungal raw material is confirmation of identity. There exist numerous challenges associated with macro- and microscopic identification of fungi, not least of which is the subjectivity of the analysis. Morphological differences due to sexual and asexual states of the same species confuses the conventional taxonomical nomenclature, causing multiple names to be assigned to genetically identical species of fungi (termed 'pleomorphy') (Sugiyama, 1987). With advances in the speed and cost of genetic analysis, the issue of fungal identity can now be more adequately addressed by DNA identification (Raja et al., 2017). All fungal raw materials evaluated in this study were, as a first step, identified by sequencing the nuclear ribosomal internal transcribed spacer (ITS) region (commonly referred to as ITS barcoding) (Raja et al., 2017; Schoch et al., 2012). (2) The next step was to screen an extract of each fungal raw material against a database of known fungal metabolites, as a way to quickly identify known toxins. (3) This was followed by a comparison of these extracts to those prepared from store-bought culinary mushrooms. Mushrooms that are widely consumed and well established as food in broad and diverse populations can provide a relatively innocuous baseline with which to compare common constituents, and exclude shared components that should not raise a safety concern. (4) Available toxicological and chemical literature for each fungus was reviewed for available safety data. (5) Marketplace data were used to establish presence in the market.

This weight-of-evidence approach was used to evaluate seven fungal raw materials and determine safe human use for each; two of the raw materials are used as in-detail examples, while the results of the remaining raw materials are summarized. We believe that this comprehensive approach will provide an effective alternative to conventional toxicological animal studies (or identify when studies are necessary) for the safety assessment of fungal dietary ingredients.

2. Methods

2.1. Approach to safety evaluation

The assessment of each fungal raw material's safe use as a dietary ingredient was performed using a weight-of-evidence approach, with the following criteria: (1) DNA identification, (2) screening fungal raw material extracts against a database of known fungal metabolites (dereplication), (3) similarity to grocery store (culinary) mushrooms as determined by UHPLC-PDA-ELS-HRMS (ultrahigh–performance liquid chromatography–photodiode array–evaporative light scattering–high resolution mass spectrometry), (4) expert opinion and literature review, and (5) marketplace data. Detailed descriptions of each of these criteria are outlined below. Based on the strength and totality of evidence, conclusions were drawn as to the supportable use of the raw material as a dietary ingredient. A decision tree outlines the general approach used to evaluate the available evidence (Fig. 1).

2.2. Materials

Seven fungal raw materials were purchased from a commercial supplier, and were identified by the supplier as Chaga (*Inonotus obliquus*), Wild-Crafted Chaga (*Inonotus obliquus*), Turkey Tail (*Trametes versicolor*), Shiitake (*Lentinula edodes*), Maitake (*Grifola frondosa*), Reishi (*Ganoderma lucidum*) fruiting bodies and mycelium, and Cordyceps (*Cordyceps sinensis*). The Chaga, Turkey Tail, Shiitake, Maitake, and Cordyceps were grown on brown rice and consisted primarily of mycelium. The Wild-Crafted Chaga was harvested from its natural habitat by the supplier. The Reishi was composed 80:20 of two subcomponents: 80% was grown on brown rice and consisted primarily of mycelium, and 20% was fruiting bodies grown on sawdust (no sawdust is incorporated into the raw material). These subcomponents were obtained as separate materials, allowing us to analyze them individually. The supplier also provided a sample of the brown rice that was used to grow the fungi. A selection of dried culinary mushrooms (including Shiitake, Black Trumpet, Chanterelle, Crimini, and Porcini) were purchased from a local grocery store to use during the comparison of the fungal raw materials to store-bought mushrooms.

2.3. DNA identification of fungi samples

Methods for the identification of these fungal samples (including DNA extraction, PCR amplification, and Sanger sequencing of dry powdered mycelium and/or fruiting body) have been described in detail previously (Raja et al., 2017).

2.4. Extractions

All samples were extracted by adding 60 mL of 1:1 methanol-chloroform (MeOH–$CHCl_3$) to a 15 g aliquot of each powdered material and shaking overnight (~16 h) at 100 rpm at RT. The sample was filtered using vacuum, and the remaining residues were washed with 10 mL of 1:1 MeOH–$CHCl_3$. To the filtrate, 90 mL of $CHCl_3$ and 150 mL of H_2O were added; the mixture was stirred for 1 h and then transferred into a separatory funnel. The bottom (organic) and upper (aqueous) layers were drawn off and evaporated to dryness. The dried organic extract was re-constituted in 50 mL of 1:1 MeOH–CH_3CN and 50 mL of hexanes. The biphasic solution was stirred for 0.5 h and then transferred to a separatory funnel. The MeOH–CH_3CN layer was drawn off and evaporated to dryness under vacuum (see Fig. 2).

The extraction scheme was developed based on previous experience of the authors and consideration of the relevant literature, especially an exhaustive study by researchers with the National Cancer Institute (McCloud, 2010). Essentially, the polarity of 1:1 MeOH–$CHCl_3$ is such that most 'organic' soluble molecules are extracted efficiently.

2.5. UHPLC–PDA–ELS–HRMS analysis

Analysis was performed using an ultrahigh–performance liquid chromatography–photodiode array–evaporative light scattering–high resolution mass spectrometry (UHPLC–PDA–ELS–HRMS) method. A splitter was incorporated post–PDA to allow for simultaneous analysis by both the ELS detector (ELSD) and mass spectrometer. HRMS was performed on a Thermo LTQ Orbitrap XL mass spectrometer (ThermoFisher, San Jose, CA) equipped with an electrospray ionization (ESI) source. The source conditions were set to aquire (m/z 100–2000) positive-ionization mode with a capillary temperature of 300 °C, a source voltage of 4.0 kV, a capillary voltage of 20 V, and the tube lens set to 100 V. Dereplicated compounds were confirmed by performing tandem mass spectrometry (MS/MS) with a collision induced dissociation set to 30%. UHPLC was carried out on a Waters Acquity system [using a BEH C18 (2.1 × 50 mm, 1.7 μm) column (Waters Corp., Massachusetts, USA) equilibrated at 40 °C]. Negative-ionization mode was

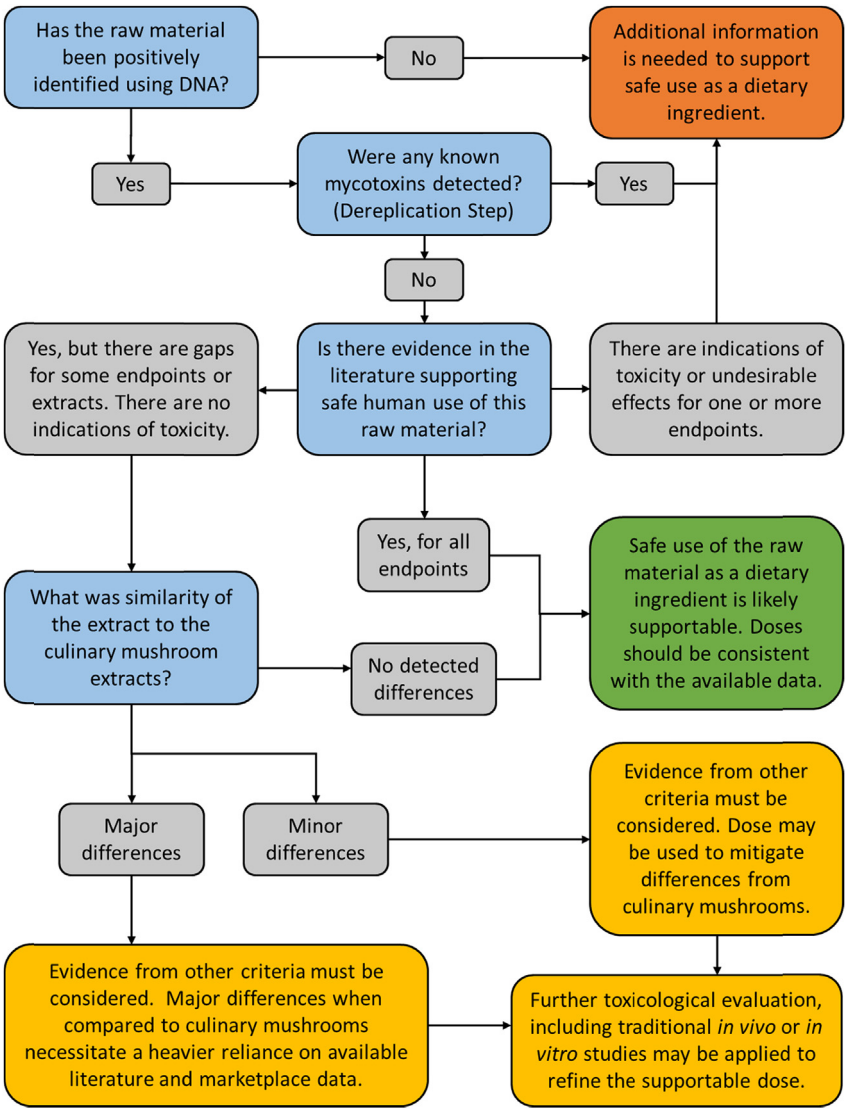


Fig. 1. Decision tree for evaluation of fungal raw materials.

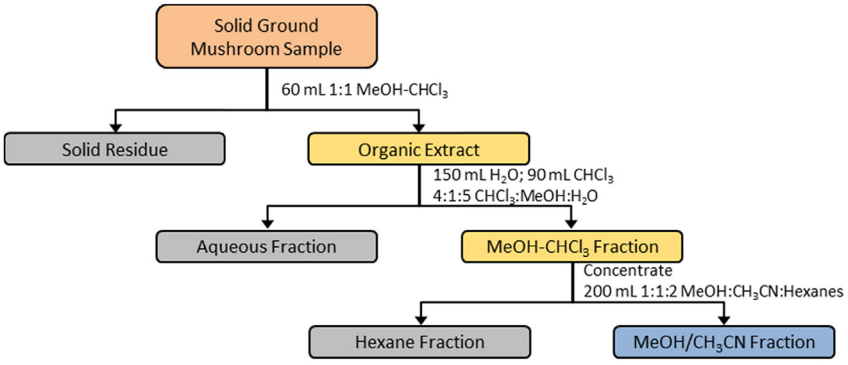


Fig. 2. Extraction scheme for fungal samples.

previously shown to not provide additional information (El-Elimat et al., 2013), and thus was not used. The mobile phase consisted of CH_3CN–H_2O (both acidified with 0.1% formic acid), which started at 15% CH_3CN for 1 min, increased linearly to 100% CH_3CN over 7 min, held for 1.5 min, and then returned to the starting conditions within 0.5 min. Analyses were performed using Fisher optima LC/MS grade CH_3CN and H_2O. An Acquity UPLC PDA detector was used to acquire UV/VIS spectra, which were collected from 200 to 500 nm with 4 nm resolution. An Acquity UPLC ELSD was used with a gas pressure of 40 psi, a nebulizer temperature of 40 °C, and a drift

tube temperature of 60 °C. The MeOH–CH_3CN extracts and mycotoxin standards were prepared in 1:1 MeOH–dioxane to a concentration of 2 mg/mL and 1 mg/mL, respectively.

While developing the method, it became clear that it would be necessary to ensure that both the ELSD and the mass spectrometer were reporting the information simultaneously in order to correlate the accurate masses (from the MS) with their corresponding contribution to the composition of the extract (from the ESLD). Since the flow from the UHPLC was split post-PDA to the two detectors, bufotenine was used as a standard to determine the lag (if any) between the signal from the ELSD to the signal of the mass spectrometer. The time difference between the two detectors was determined to be 0.11 min, allowing for accurate alignment of the data during analyses.

2.6. Dereplication

A database consisting of over 300 fungal secondary metabolites was previously assembled containing the retention times, UV data, HRMS data and MS/MS fragmentation patterns of each compound (El-Elimat et al., 2013). This database was comprised of compounds that were isolated from fungi largely due to their cytotoxic properties. In addition to those compounds, six known mycotoxins, specifically from mushrooms, were added to the database, including: ibotenic acid (**1**), muscimol (**2**), 2-amino-hexynoic acid (**3**), muscarine chloride (**4**), bufotenine (**5**), and phalloidin (**6**) (Fig. 3). Each fungal sample was prepared at 2 mg/mL and analyzed via the UHPLC-PDA-ELS-HRMS method described above. The raw data files were uploaded into ACD MS Manager (v 2015.2.5) with the add-in software Intellixtract (Advanced Chemistry Development, Inc., Toronto, Canada). Intellixtract rapidly deconvoluted the complex UHPLC-HRMS chromatograms and cross-referenced the pseudomolecular ion peaks with the database. Any matches in retention time and HRMS were then manually analyzed confirming that the retention time, UV data, HRMS and MS/MS matched the standard.

2.7. Comparison to culinary mushrooms

Using the UHPLC–PDA–ELS–HRMS methodology described in section 2.5, for each sample, all the peaks that contributed >0.33% ELSD of the total signal were examined. Each constituent *m/z* was checked against corresponding peaks in the store-bought culinary mushroom (Porcini, Crimini, Black Trumpet, Chanterelle, and Shiitake) and brown rice extracts. A "match" was assigned if the accurate mass (±5 ppm), retention time, and contribution to the ELSD signal were consistent with at least one of the culinary extracts.

After matches for summed mass spectral signal corresponding to each ELSD peak were determined, the peak was assigned a percent similarity based on the quantity of matched and unmatched significant ions in each spectrum. Because of the compound-to-compound variability in ionization/volatilization efficiency inherent to electrospray mass spectrometry, these percent similarities were assigned conservatively, with the estimates leaning towards "different" than culinary mushrooms. The percent similarity of each peak was then multiplied by the area of its corresponding ELSD signal. Thus, a peak considered to be 10% different (*i.e.*10% of the significant peaks could not be matched to either the culinary mushrooms or organic brown rice) with an ELSD Area of 33.0% would result in a 3.3% estimation of the mass of the mushroom extract being designated as different from the store-bought mushrooms. This process was then repeated for each ELSD peak, and the results were summed to provide a total %-difference for each mushroom. This %-difference was then used to estimate the mass of "different" mushroom metabolites expected in the proposed doses. While all compounds do not ionize to the same extent, enough ions were analyzed that the net differences should, in total, balance to statistically represent a reasonable estimation. Given this, we believe the combination of MS and ELSD data provided a pragmatic approach to examining the samples in a reproducible and thorough manner.

2.8. Literature review

A thorough review of the currently available toxicological and chemical literature for each fungus was performed. Traditional preparations and historical use, such as culinary or medicinal, were considered. As this paper is not intended to be a systematic review of the available literature, structured search methodology and inclusion and exclusion criteria were not used. The quality of studies obtained was evaluated on a case-by-case basis.

2.9. In-market data

The Dietary Supplements Labels Database (DSLD), maintained by the National Institutes of Health (NIH), was used to evaluate

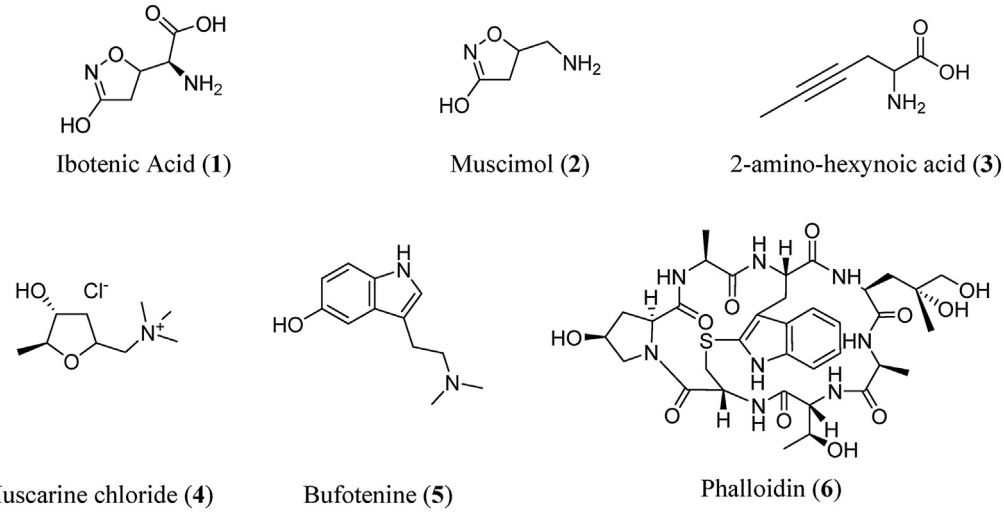


Fig. 3. The six known mycotoxins from mushrooms that were added to the fungal secondary metabolite database: ibotenic acid (1), muscimol (2), 2-amino-hexynoic acid (3), muscarine chloride (4), bufotenine (5), and phalloidin (6).

market presence in the United States. The common names and Latin binomials of each raw material were used as search terms. The number of brands, number of products, median, and maximum doses of each raw material were recorded.

3. Results

Each fungal raw material went through the hazard assessment process outlined above, using (1) DNA identification, (2) dereplication against known fungal metabolites, (3) comparison to store-bought culinary mushrooms, (4) review of the available literature, and (5) consideration of marketplace data. Due to space considerations, the Chaga and Reishi samples are used as examples for in-depth explanation of the assessment process. Results are summarized for the remaining fungal raw materials.

3.1. DNA identification of fungi samples

The store-bought Shiitake and Crimini mushrooms were confirmed by analysis of the ITS region to be *Lentinula edodes* and *Agaricus bisporus*, respectively. The store-bought Porcini was confirmed to be a *Boletus* spp, although the ITS region did not have >80% similarity with the *Boletus edulis* species. Likewise, the Black Trumpet was confirmed to be a *Craterellus* spp, although the ITS region did not have >80% similarity with the *Craterellus comucopioides* species. The analysis of the store-bought Chantarelle was unsuccessful. While a PCR product was obtained, it was not possible to acquire sufficiently high quality ITS data from the sequencing. A second sample from the same batch was processed in an identical fashion with the same results. These results are likely due to the age and/or processing of the material, resulting in degradation of the DNA.

The fungal raw materials (Chaga, Wild-Crafted Chaga, Turkey Tail, Shiitake, Maitake, Reishi, and Cordyceps) were also analyzed to confirm identity. The Chaga, Turkey Tail, Shiitake, and Maitake materials were confirmed to be *Inonotus obliquus*, *Coriolus versicolor*, *Lentinula edodes*, and *Grifola frondosa*, respectively. The Reishi mycelium and fruiting body raw materials shared identical ITS sequences; the results from the BLAST search shared >99% sequence similarity with *Ganoderma lucidum*. However, the ITS results suggested that the sample may be more closely related to the Chinese variety of *G. lucidum*, which has been recently given a new name: *G. sichuanense.* ITS sequence comparison of the *Cordyceps* raw material indicated that the fungus was not a *Cordyceps* species, but was instead *Tolypocladium inflatum.* The ITS sequences of *Cordyceps sinensis* (now *Ophiocordyceps sinensis*) were downloaded from GenBank and compared with those of *Tolypocladium inflatum*. Based on these results it was evident that the ITS sequences obtained from the "*Cordyceps*" raw material were more similar to those of *Tolypocladium inflatum* compared to *Ophiocordyceps sinensis*. A Randomized Axelerated Maximum Likelihood (RAxML) analysis also showed that ITS sequences from the "Cordyceps" raw material grouped with *Tolypocladium inflatum*, but not with sequences from *Ophiocordyceps sinensis*. The Wild-Crafted Chaga sample ITS comparison shared 99% similarity with *Cladosporium* spp., not *Inonotus obliquus*. Because the "*Cordyceps*" and "Wild-Crafted Chaga" raw materials were not the correct species, further assessment of these fungi was not pursued; however, these results emphasize the necessity of positive DNA identification when assessing a fungal raw material for use in a dietary supplement. Safety assessment continued for the identity-confirmed Chaga, Turkey Tail, Shiitake, Maitake, and Reishi raw materials.

Results of the ITS barcoding are summarized in Table 1, which has been adapted from previously published results (Raja et al., 2017).

3.2. Dereplication

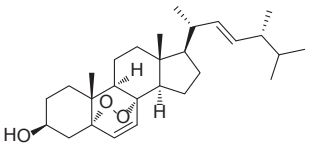
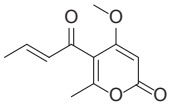
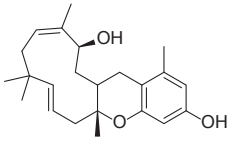
The six remaining fungal raw material extracts (Reishi mycelium, Reishi fruiting body, Maitake, Shiitake, Turkey Tail, and Chaga) were then subjected to a targeted UHPLC-PDA-HRMS/MS protocol that screened for the presence of cytotoxins and mycotoxins from a database of over 300 fungal secondary metabolites (El-Elimat et al., 2013). All of the fungal raw material extracts yielded matches for fungal metabolites from this database based on retention time, UV data, HRMS data, and MS/MS data (Table 2). The metabolite ergosterol peroxide was detected in each extract, but was also detected in the five store-bought culinary mushroom extracts (Porcini, Crimini, Blank Trumpet, Chanterelle, and Shiitake). Pyrenocine A (Sato et al., 1979, 1981) and pughiinin A

Table 1
List of fungi samples profiled in this study.

Binomial name	Common name	ITS barcoding ID	GenBank accessions[a]	GenBank query coverage[b]	GenBank percent similarity	Variable bases (%)
Trametes versicolor (*Coriolus versicolor*)	Turkey Tail	*Trametes versicolor* (*Coriolus versicolor*)	KT693226, KT693227	100%	100%	1.7%
Inonotus obliquus	Chaga	*Inonotus obliquus*	KT693232, KT693233	100%	99%	1.4%
Inonotus obliquus	Chaga (Sclerotia)	*Cladosporium* sp.	KT693272, KT693273	≥98%	99%	0.2%
Grifola frondosa	Maitake	*Grifola frondosa*	KT693234, KT693235	98%	99%	0%
Lentinula edodes	Shiitake	*Lentinula edodes*	KT693236, KT693237	100%	99%	2.9%
Wolfiporia cocos (*Poria cocos*)	Poria	*Wolfiporia cocos* (*Poria cocos*)	KT693238, KT693239	≥92%	93-95%	4.6%
Agaricus bisporus	Crimini	*Agaricus bisporus*	KT693240, KT693241	98%	99%	0.7%
Lentinula edodes	Shiitake	*Lentinula edodes*	KT693242, KT693243	98%	99-100%	2.9%
Ganoderma ludicum	Reishi	*Ganoderma sichuanense*	KT693248, KT693249	89%	99%	1.2%
Ganoderma ludicum	Reishi	*Ganoderma sichuanense*	KT693250, KT693251	89%	99%	1.2%
Ganoderma ludicum	Reishi	*Ganoderma sichuanense*	KT693252, KT693253	89%	99%	1.2%
Boletus edulis	Porcini	*Boletus shiyong*	KT693260, KT693261	≥80%	97-100%	0.8%
Craterellus comucopioides	Black Trumpet	*Craterellus comucopioides*	KT693262, KT693263	≥80%	97-100%	0%
Cantharellus cibarius	Chanterelle	No sequence obtained	N/A	N/A	–	–
Ophiocordyceps sinensis (*Cordyceps sinensis*)	Cordyceps	*Tolypocladium inflatum*	KT693266, KT693267	98%	99%	0.2%

[a] ITS sequences of these fungi were deposited in GenBank in a previous study (Raja et al., 2017).
[b] To be considered the same species, the ITS should have a ≥80% coverage and ≥97% similarity. For *Wolfiporia cocos*, we have assigned an accurate name on the product label based on 4.6% variable bases. Although this value is >3% we believe that this may be due to mushroom forming species having a higher weighted intraspecific ITS variability of 3.33% with a standard deviation of 5.62 (Nilsson et al., 2008). In addition, the extreme length of *W. cocos* ITS is due to insertions in the ITS1 and ITS2 regions (Lindner and Banik, 2008).

Table 2
Metabolites dereplicated in fungal raw materials.

Fungal sample	Dereplicated metabolites
All	 Ergosterol peroxide
Turkey Tail	*None*
Chaga	*None*
Reishi	*None*
Reishi Fruiting Body	*None*
Maitake	*None*
Shiitake	 Pyrenocine A (<0.10% ELSD signal)
	 Pughiinin A (<0.10% ELSD signal)

(Pittayakhajonwut et al., 2002) were detected in the Shiitake extract with the signal from the ELSD constituting <0.10% of the total ELSD signal.

3.3. Comparison to culinary mushrooms

An untargeted analysis of the six fungal raw material extracts (Reishi mycelium, Reishi fruiting body, Maitake, Shiitake, Turkey Tail, and Chaga) was performed by comparing the UHPLC-PDA-ELS-HRMS data for the eight extracts to the five store-bought mushrooms extracts (Porcini, Crimini, Black Trumpet, Chanterelle, and Shiitake) and the organic brown rice extract. For each of the eight fungal raw material extracts, the areas for each peak of the ELSD chromatograms were calculated. Any peak area totaling over 0.33% of the overall signal was considered for the analysis. The HRMS data was examined for each considered peak, and compared to the five store-bought mushrooms and organic brown rice. Unlike UV and MS, an ELSD system is a mass-dependent detector, thus it does not depend on spectral or physicochemical properties of the analytes (Eom et al., 2010; Vehovec and Obreza, 2010). Because the ELSD is mass dependent, the size of peaks is directly correlated to the mass of constituents within the peak.

The majority of the summed MS signals (abundant *m/z* peaks in the mass spectra) corresponding to those ELSD signals contained matching *m/z* values (within ±5 ppm) with at least one of the culinary extracts, according to the HRMS data. In other words, the majority of constituent masses found in each extract were found in one or more culinary mushroom extracts and/or the brown rice substrate extract.

3.3.1. Chaga

Over 97% of the total ELSD signal for the Chaga extract, which accounted for all chromatographic peaks with an area greater than 0.33%, was examined in detail using HRMS (Table 3). A comparison of base peak mass spectrometry chromatograms and ELSD chromatograms was made between the Chaga extract and the extracts of store-bought Porcini, Crimini, Blank Trumpet, Chanterelle, and Shiitake mushrooms, as well as an organic brown rice extract (Fig. 4).

An example of the analysis undertaken can be seen by comparing the peak at 6.06 min on the ELSD (0.40% of total signal; Table 3), which correlates to the peak at 5.96 min on the mass spectrometer (lag related to post-column tubing volumes after split). The summed mass spectrum at 5.96 min displays several prominent *m/z* signals (Fig. 5A). A partial mass spectrum encompassing the most intense masses of the organic Chaga extract was compared to the MS spectra of the extracts for the culinary mushroom samples (Fig. 5B). Exact mass chromatograms for each of these accurate masses (±5 ppm) were compared to the store-bought mushroom samples (Fig. 6). As can be seen in this figure,

Table 3
Chaga - Contribution of mass spectral features that differ from culinary mushrooms to the estimated difference of the raw material from food.

ELSD retention time (min)[a]	HRMS retention time (min)[b]	Area (ELSD)[c]	Area %[d]	Peak % difference[e]	Total % difference (peak diff. × area)[f]
6.06	5.96	2.16×10^7	0.4	0	0.0
7.94	7.84	3.50×10^9	64.3	0	0.0
8.00	7.90	5.36×10^8	9.8	0	0.0
8.07	7.97	3.02×10^8	5.6	0	0.0
8.37	8.27	8.75×10^7	1.6	0	0.0
8.62	8.52	1.77×10^7	0.3	0	0.0
8.41	8.31	6.95×10^8	12.8	0	0.0
8.73	8.63	7.20×10^7	1.3	0	0.0
8.81	8.71	4.74×10^7	0.9	0	0.0
8.89	8.79	3.76×10^7	0.7	0	0.0
Total Evaluated:			98.	Total Different:	0.0
Total Unevaluated:			2.0	Total Similar:	98.

(a, b): Retention times [ELSD(a) and HRMS(b)] peaks analyzed. (c, d): Peak area and corresponding area % of peaks analyzed. (e): Estimated percent difference of that peak as determined by comparing mass spectral features to culinary mushroom extracts. (f): The percent contribution of these differences to the raw material as a whole.

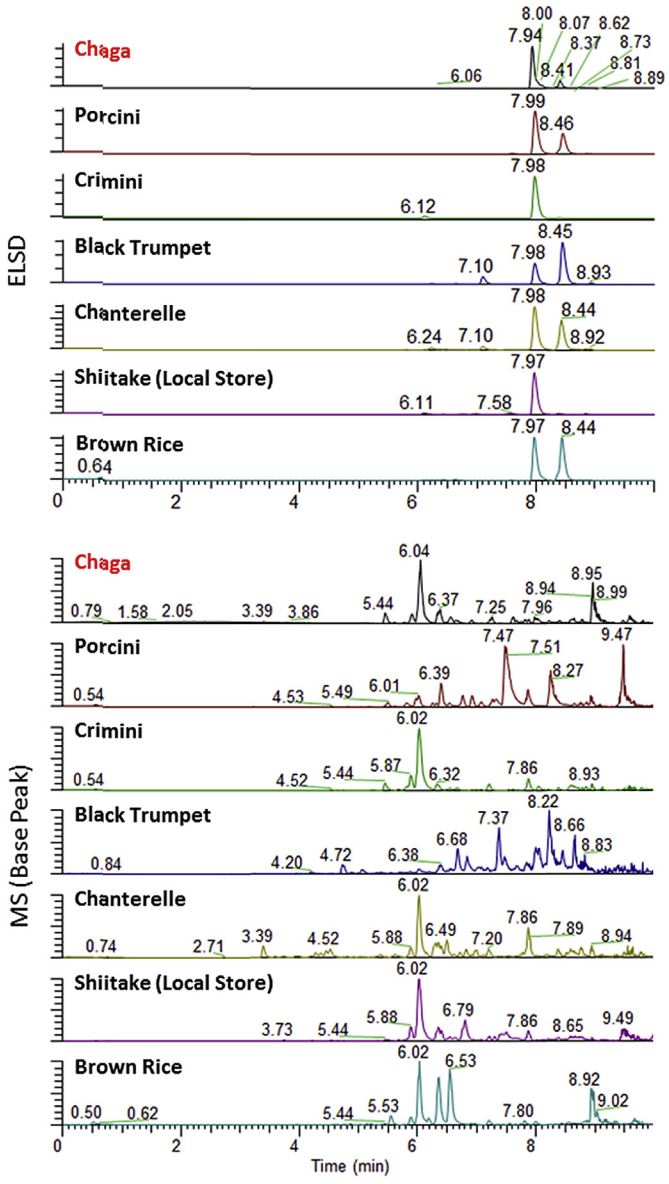


Fig. 4. Comparison of Chaga ELSD and base peak MS chromatograms to corresponding culinary mushroom and brown rice chromatograms. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

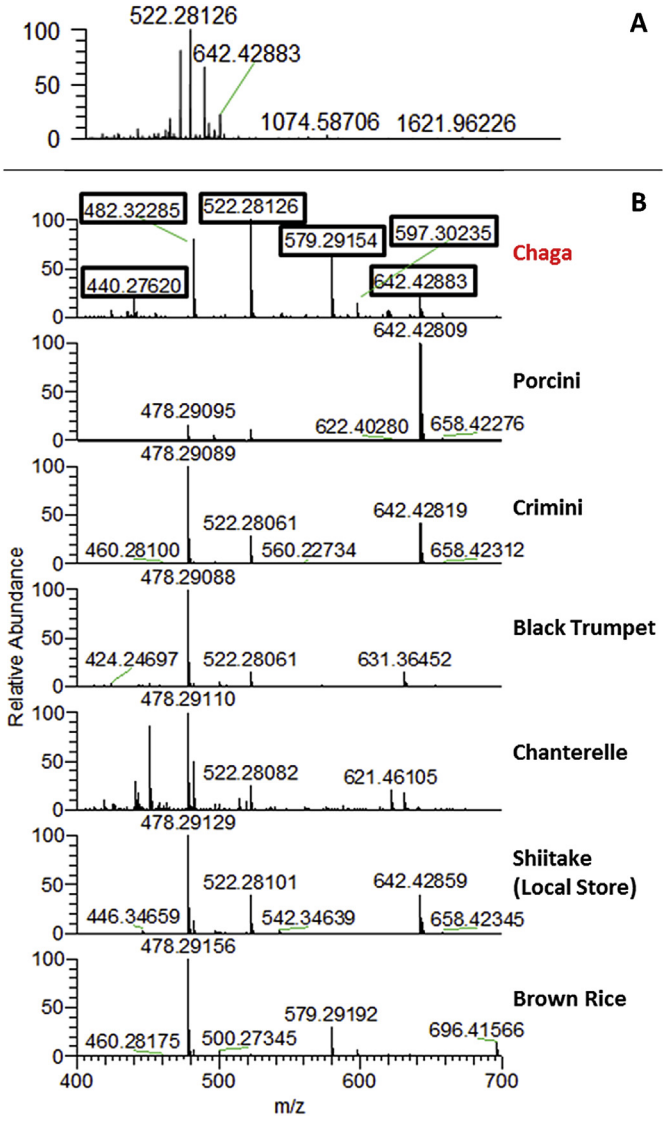


Fig. 5. Mass spectra at 5.96 min, unzoomed (A) and zoomed to examine prominent *m/z* (B).

for each of the examined prominent *m/z* signals in the peak at 5.96 min, matching accurate masses (±5 ppm) were identified in at least one of the culinary samples. This analysis of accurate mass chromatograms was performed in an identical manner for each of the ten ELSD signals that make up 98% of the Chaga extract's mass. The top HRMS signals correlating to each ELSD signal for the Chaga powder were all matched to signals found in a store-bought mushroom or brown rice, and the extract was determined to be approximately 98% similar to culinary mushrooms, with roughly 2.0% unevaluated due to low signal (Table 3). It is important to note that there was no attempt to identify the chemical constituents observed by this process. Rather, the assumption was made that if, following identical extraction from two fungi, analytes that had the same observed chromatographic retention time and accurate mass spectrometry signal, then they were identical for the purposes of hazard assessment.

3.3.2. Reishi

A similar analysis was performed on the Reishi mycelium extract and Reishi fruiting body extract. The mycelium powder and fruiting body powder are combined (80% mycelium and 20% fruiting body by mass) in the final commercial raw material, but the individual subcomponents were obtained, and chemically analyzed separately.

Over 95.0% of the ELSD signals were examined by HRMS for both the mycelium and fruiting body powders (Tables 3 and 4). While most HRMS signals were matched to signals in a culinary material, there were several instances that required deeper analysis. For example, when examining the peak at 8.27 min (Fig. 7B) in the Reishi mycelium extract, the majority of the signals were matched in relation to the extracts of the store-bought samples (Fig. 8). However, there was a signal at *m/z* 575.3332 (±5 ppm) which could not be matched to a mass spectral feature (*m/z* and RT combination) in any of the culinary extracts (Fig. 9).

This comparison was repeated for each mass spectral peak in the Reishi mycelium (Fig. 7), and each peak was assigned a percent-difference. In the example given, the ELSD peak at 8.27 min

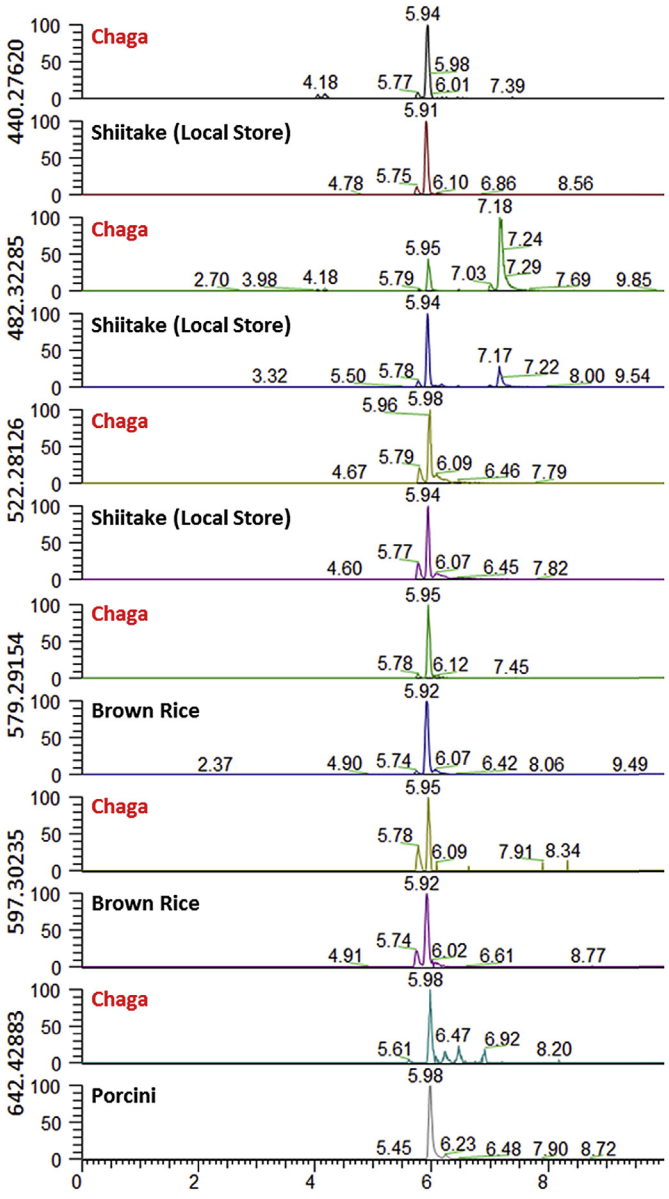


Fig. 6. Intensity-normalized exact mass chromatograms of Chaga, store-bought culinary mushrooms, and brown rice extracts at 5.96 min. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(Fig. 7B) was assigned a 20% difference, corresponding to approximately 1/6 of the peaks analyzed, rounded up (Table 4). The peak at 7.84 min (Fig. 7A) also had one non-matching HRMS signal, and was assigned a 20% difference; the peak at 8.32 min (Fig. 7C) had one non-matching HRMS signal, which represented a smaller percentage of the peaks evaluated, and was assigned a 15% difference. In all cases, the estimated "difference" was rounded up, in an effort to more conservatively estimate the similarity to the culinary samples. After these percent-differences were assigned, they were then multiplied by the area of each peak. Thus, the 20% difference of the peak at 7.94 min (Fig. 7A) was multiplied by the area of the peak, 37.51% (Table 4), to give a contribution to the total difference of 7.5%. Repeating this process for the other two peaks resulted in a contributed difference of 0.3% for the peak at 8.37 min, and 7.0% for the peak at 8.32 min. Adding these values together provided a total estimated difference of 16%. This calculation is made possible because of the mass dependence of the ELSD. In total, the Reishi mycelium extract was estimated to contain 80% similar material (compared to the culinary extracts), 16% different material, and 4.0% unevaluated (Table 4).

In the Reishi fruiting body, several analytes were detected that were unique to the sample and not found in the store-bought samples (Fig. 10). As with the Reishi mycelium, the exact mass chromatograms were compared to the store-bought samples, but there were many unmatched mass spectral features. An example is shown in Fig. 11. As a result of the numerous unmatched *m/z* in the extracted ion chromatograms (XICs), many of the ELSD peaks were assigned high degrees of percent-difference (Table 5). In total, the Reishi fruiting body was estimated to contain 58% similar material (compared to the culinary extracts), 37% different material, and 5.0% unevaluated (Table 5). These evaluations were then combined to adjust for the 80:20 ratio at which the mycelium and fruiting body powders are combined in the finished commercial raw material. In total, the Reishi raw material extract was estimated to contain 75% similar constituents, 21% different material, and 4.0% unevaluated material.

3.3.3. Shiitake, Maitake, Turkey tail

The comparisons to store-bought culinary mushrooms described above were performed in an identical manner for the Shiitake, Maitake, and Turkey Tail raw materials. Results are summarized in Tables 6, 7, and 8. All three showed very few differences from the culinary mushrooms; however, these data served to verify the general approach. The similarity of the Shiitake and Maitake raw materials to the culinary mushrooms was not unexpected, given their use as foods, but the analyses ensured that the mycelium were not substantially different from the fruiting bodies used as food.

3.3.4. Wild-Crafted Chaga and Cordyceps

Because the identity of these fungal raw materials were incorrect, as determined by the DNA analysis, neither further characterization work nor hazard assessment were pursued.

3.4. Literature review

As this paper is not meant to be a systematic review of the literature, results of our literature search are summarized here, with selected papers referenced. A complete list of references retrieved may be found in the supplemental information.

3.4.1. Chaga

Inonotus obliquus is found primarily on Birch trees in colder climates. While it is used in TCM (Zhong et al., 2009), the available toxicological data are limited. The polysaccharide fraction has been tested in an acute mouse assay (no mortality at a maximum dose of 5 g/kg body weight) (Haixia et al., 2009). An aqueous extract of the mycelium has been evaluated in rats for reproductive and developmental toxicity (neither fetal nor maternal toxicity at 2 mL/kg *i.p.*) (Anasiewicz et al., 1998). Ethyl acetate and methanolic extracts were not mutagenic in an Ames test (TA98 and TA100, up to 50 μg/plate) (Ham et al., 2009). An undefined extract was reported to not cause any toxic effects over five to six months in rats or rabbits at oral doses up to 0.3 g/kg body weight (Shikov et al., 2014) although the original reference could not be retrieved.

3.4.2. Reishi

This fungus has been used as a medicinal food in China, Japan, Taiwan, and other Asian regions for centuries (Paterson, 2006). Known in TCM as *ling zhi*, the fruiting bodies are used for a number of ailments, in doses of up to 15 g in decoctions, and up to 3 g in

Table 4
Reishi Mycelium - Contribution of mass spectral features that differ from culinary mushrooms to the estimated difference of the raw material from food.

ELSD retention time (min)[a]	HRMS retention time (min)[b]	Area (ELSD)[c]	Area %[d]	Peak % difference[e]	Total % difference (peak diff. × area)[f]
7.94	7.84	3.39×10^9	37.5	20	7.5
8.37	8.27	1.74×10^8	1.9	20	0.3
8.42	8.32	5.12×10^9	56.7	15	7.0
Total Evaluated:			96.	Total Different:	16.
Total Unevaluated:			4.0	Total Similar:	80.

(a, b): Retention times [ELSD(a) and HRMS(b)] peaks analyzed. (c, d): Peak area and corresponding area % of peaks analyzed. (e): Estimated percent difference of that peak as determined by comparing mass spectral features to culinary mushroom extracts. (f): The percent contribution of these differences to the raw material as a whole.

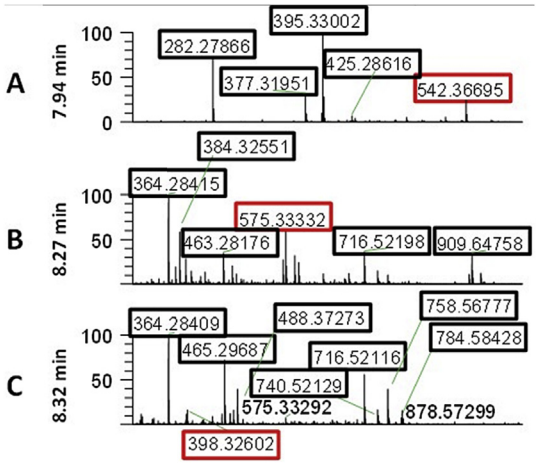


Fig. 7. The summed mass spectra at each retention time correlating to a prominent ELSD signal (>0.33%) with the mis-matching m/z values highlighted in red. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

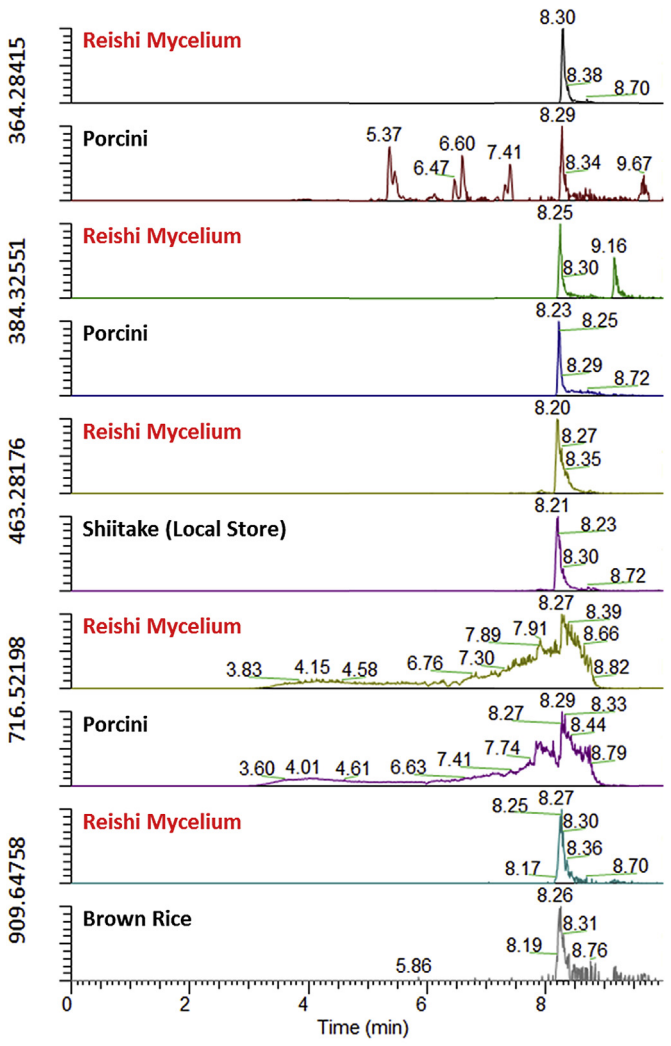


Fig. 8. Representative data of the matching intensity-normalized exact mass chromatograms for the Reishi mycelium extract with store-bought culinary mushrooms or brown rice extracts at 8.27 min from Fig. 7B. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

powdered form (Bensky et al., 2004). Numerous toxicological studies were available for *Ganoderma lucidum* (Reishi) preparations, including primarily acute and subacute studies. Two 90-day studies on the polysaccharide fraction (oral route) provided NOAELs (No Observed Adverse Effect Levels) of 1200 mg/kg/day (Wihastuti et al., 2014, 2015) and 2000 mg/kg/day (Chen et al., 2011); these NOAELs were the highest doses used in the studies. A number of mutagenicity (Ames) tests were available for a variety of preparations, including aqueous and methanolic extracts of mycelium, fruiting bodies, and spore powder. Genotoxicity studies included an *in vitro* chromosomal aberration assay and *in vivo* peripheral blood micronucleus tests of the polysaccharide fraction (Chen et al., 2011). Nine clinical trials were found in the literature, all reporting that *G. lucidum* preparations were well tolerated. One of these concluded that there were no negative liver, kidney, or DNA effects over the 4-week study, as assessed by monitoring the participants' aspartate aminotransferase, alanine transaminase, creatinine, and performing a comet assay (Wachtel-Galor et al., 2004). One of the 90-day studies included gross necropsy and histopathology of reproductive organs (Chen et al., 2011), though otherwise, traditional developmental and reproductive studies were lacking. In general, there was no evidence in the literature of any toxic effects.

3.4.3. Shiitake

The fruiting bodies of *Lentinula edodes* are a common food, particularly in Asia. Numerous toxicological studies covering the common endpoints are available (Supplemental Information). Perhaps unsurprisingly, minimal toxicity has been seen in systemic (Yoshioka et al., 2010) and DART (Frizo et al., 2014) studies via the oral route. Mutagenicity (von Wright et al., 1982) and genotoxicity (de Lima et al., 2001; Miyaji et al., 2006) assays have all reported negative results. Several clinical trials were found in the literature, reporting only mild, transient gastrointestinal symptoms (Levy et al.; Spierings et al., 2007; Yoshioka et al., 2009).

3.4.4. Maitake

Like Shiitake, Maitake is a common food. Conventional toxicology studies are more limited than that found for Shiitake, but include several subchronic (up to 120 days) studies in mice (oral) (Kodama et al., 2002; Nanba, 1997) and a chronic (13 months) study

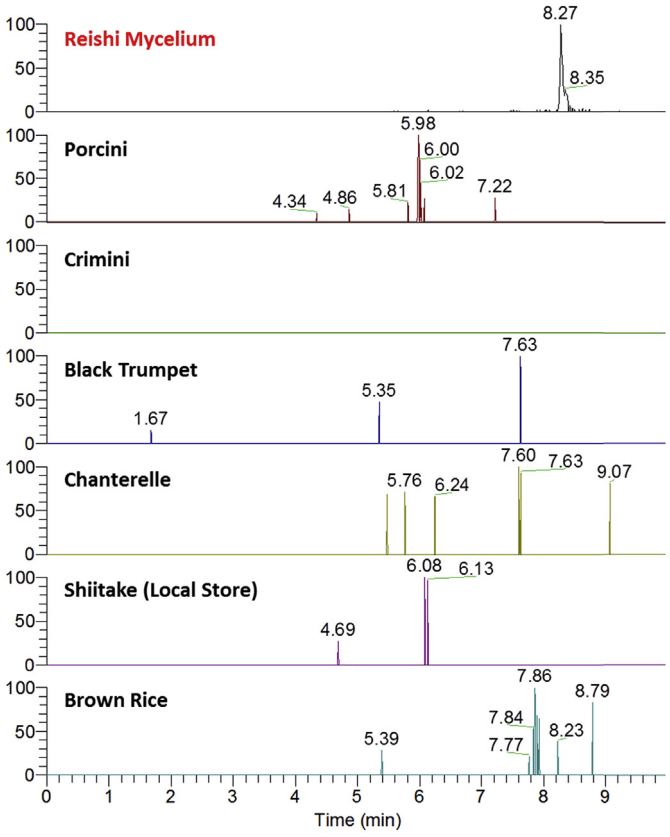


Fig. 9. Representative data of a mis-match for the intensity-normalized exact mass chromatogram of 575.3332 (±5 ppm) in Reishi mycelium against the store-bought culinary mushrooms and brown rice extracts at 8.27 min from Fig. 7B. This suggests the presence of an analyte unique to Reishi Mycelium, relative to the store bought samples. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

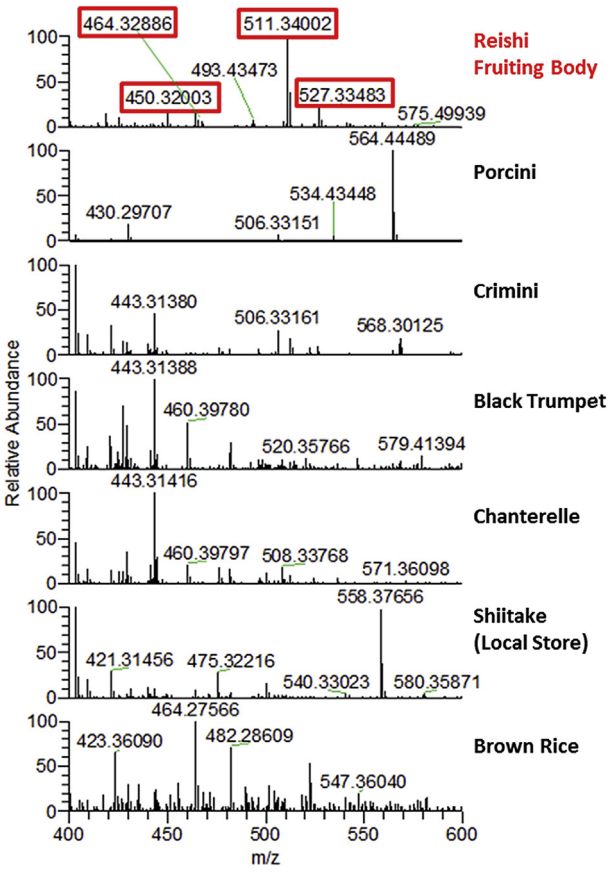


Fig. 10. Mass spectra showing high levels of mis-matched m/z comparing the Reishi fruiting body and culinary extracts at 7.05 min (MS).

in mice (oral) (Nanba, 1997). None of these reported toxicity at any dose. Two *in vitro* cytotoxicity assays probing embryotoxicity (mouse embryonic fibroblast (BALB/3T3) cells) and neurotoxicity (mouse neuroblastoma (N2a) cells) reported no toxicity at the highest dose) (Phan et al., 2013). Three clinical trials were found (Deng et al., 2009; Glauco et al., 2004; Wesa et al., 2015), including two on a hot water-ethanol extract of the fruiting bodies; adverse events included mild nausea, and a grade I allergic reaction (rash and pruritus).

3.4.5. Turkey tail

There is some discrepancy in mushroom reference books concerning the edibility of Turkey Tail, though it appears that those that list it as inedible do so because of its fibrous texture and unpalatability, rather than any known toxicity (Cotter, 2014; Lamaison and Polese, 2008; Meuninck, 2015; Phillips et al., 2010; Russell, 2006; Spahr, 2009). One of its constituents, Polysaccharide-K, displays anticancer effects *in vitro* (Fisher and Yang, 2002), and is being studied as an adjuvant in the treatment of some cancers. As a result, some systemic (Hor et al., 2011; Lai et al., 2011), DART (Ng and Chan, 1997), and even clinical studies (Chu et al., 2002; Ito et al., 2004; Ohwada et al., 2004; Tsang et al., 2003), on the safety of aqueous extracts and concentrated polysaccharide fractions are available. Results reported in these studies indicate that the extract is not embryotoxic, abortifacient, genotoxic, or clastogenic. NOAELs reported for the polysaccharide extract include 1000 mg/kg/day (mice, oral, 28-day) (Lai et al., 2011) and 5000 mg/kg/day (rats, oral, 28-day) (Hor et al., 2011); both of these represent the highest dose tested. Studies on other preparations and extracts are limited (Knezevic et al., 2015; Torkelson et al., 2012; Zhang et al., 2015).

3.5. In-market data

To gauge in-market presence of each fungal raw material, the DSLD (Knezevic et al., 2015) was searched for products containing similar ingredients. The DSLD is maintained by the Office of Dietary Supplements and the U.S. National Library of Medicine, and contains a comprehensive record of the label information for dietary supplements in the United States market.

3.5.1. Chaga

The most common Chaga ingredient on labels in the DSLD was reported as “Chaga” (41 products). Other ingredients, 9 in total, included variations of “Chaga Extract,” “Chaga Mushroom,” and “Organic Chaga.” There were, in total, 70 products that reported some version of “Chaga” on the label; this number is likely inflated, as some products may include more than one version of the ingredient. A total of eight distinct brand names were reported. Some products did not report a dose, instead listing only the dose of a proprietary blend in which Chaga was included. Others listed the dose of an extract, but not the equivalence. Of the doses available in the DSLD, the maximum was 1000 mg, while the median was 120 mg.

3.5.2. Reishi

The most common Reishi ingredients were “Reishi” (109

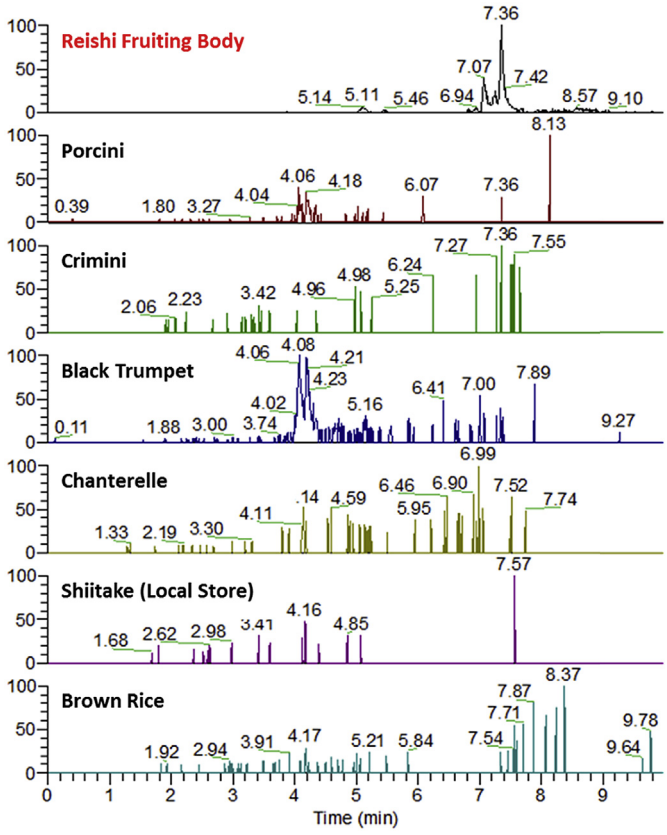


Fig. 11. Intensity-normalized exact mass chromatograms for 450.32003 (±5 ppm), showing no match between the Reishi fruiting body peak at RT 7.05 (MS) to any of the culinary extracts.

products), "Reishi Mushroom" (82 products), and "Organic Reishi" (24 products). Other ingredients listed included variations of these ingredient names, as well as variations of "Reishi Extract." The total number of products listed was 315; however there a high degree of overlap, as some products contain more than one "Reishi" ingredient. Within these products, 28 unique brand names were found. Most products did not report the dose of the ingredient, instead listing only the dose of a proprietary blend in which Reishi was included. Of the doses reported, the maximum dose was 4500 mg, and the median dose was 100 mg.

3.5.3. Shiitake

The most common Shiitake ingredients were "Shiitake" (98 products), and "Shiitake Mushroom" (43 products). A total of 223 products were listed, although some products likely include multiple versions of the "Shiitake" ingredients. Within the "Shiitake" and "Shiitake Mushroom" ingredients, 45 distinct brand names were found. Most products did not report the dose of the ingredient, instead listing only the dose of a proprietary blend in which Shiitake was included. Of the doses reported, the maximum dose was 1500 mg, and the median dose was 50 mg.

3.5.4. Maitake

The most common Maitake ingredients were "Maitake" (99 products), and "Maitake Mushroom" (48 products). A total of 267 products containing a Maitake ingredient were listed, although some products may include multiple versions, thus slightly inflating this number. Within the "Maitake" and "Maitake Mushroom" ingredients, 48 distinct brand names were identified. Most products did not report the dose of the ingredient, instead listing only the dose of a proprietary blend in which Maitake was included. Of the doses reported, the maximum dose was 900 mg, and the median dose was 60 mg.

Table 5
Reishi Fruiting Body - Contribution of mass spectral features that differ from culinary mushrooms to the estimated difference of the raw material from food.

ELSD retention time (min)[a]	HRMS retention time (min)[b]	Area (ELSD)[c]	Area %[d]	Peak % difference[e]	Total % difference (peak diff. × area)[f]
6.20	6.10	4.80×10^7	1.3	50	0.6
6.66	6.56	1.02×10^8	2.7	100	2.7
6.71	6.61	1.42×10^8	3.7	95	3.6
6.87	6.77	2.95×10^8	7.8	95	7.4
7.15	7.05	4.97×10^7	1.3	100	1.3
7.35	7.25	9.91×10^8	26.0	50	13.
7.57	7.47	7.71×10^7	2.0	90	1.8
7.85	7.75	2.93×10^8	7.7	0	0.0
7.96	7.86	1.07×10^9	28.0	0	0.0
8.37	8.27	4.87×10^7	1.3	10	0.1
8.43	8.33	4.28×10^8	11.2	50	5.6
8.65	8.55	1.34×10^7	0.4	25	0.1
8.83	8.73	7.42×10^7	2.0	50	1.0
Total Evaluated:			95	Total Different:	37
Total Unevaluated:			5.0	Total Similar:	58

(a, b): Retention times [ELSD(a) and HRMS(b)] peaks analyzed. (c, d): Peak area and corresponding area % of peaks analyzed. (e): Estimated percent difference of that peak as determined by comparing mass spectral features to culinary mushroom extracts. (f): The percent contribution of these differences to the raw material as a whole.

Table 6
Shiitake - Contribution of mass spectral features that differ from culinary mushrooms to the estimated difference of the raw material from food.

ELSD retention time (min)[a]	HRMS retention time (min)[b]	Area (ELSD)[c]	Area %[d]	Peak % difference[e]	Total % difference (peak diff. × area)[f]
6.87	6.77	4.91×10^7	0.4	0	0.0
7.57	7.47	5.49×10^7	0.4	0	0.0
7.96	7.86	5.52×10^9	41.2	0	0.0
8.38	8.28	3.80×10^8	2.8	0	0.0
8.43	8.33	7.08×10^9	52.9	0	0.0
8.91	8.81	4.19×10^7	0.3	0	0.0
Total Evaluated:			98	Total Different:	0.0
Total Unevaluated:			2.0	Total Similar:	98

(a, b): Retention times [ELSD(a) and HRMS(b)] peaks analyzed. (c, d): Peak area and corresponding area % of peaks analyzed. (e): Estimated percent difference of that peak as determined by comparing mass spectral features to culinary mushroom extracts. (f): The percent contribution of these differences to the raw material as a whole.

Table 7
Maitake - Contribution of mass spectral features that differ from culinary mushrooms to the estimated difference of the raw material from food.

ELSD retention time (min)[a]	HRMS retention time (min)[b]	Area (ELSD)[c]	Area %[d]	Peak % difference[e]	Total % difference (peak diff. × area)[f]
7.96	7.86	1.02×10^{10}	38.7	0	0.0
8.36	8.26	9.10×10^{8}	3.5	25	0.9
8.46	8.36	1.46×10^{10}	55.6	0	0.00
8.84	8.74	1.30×10^{8}	0.5	50	0.3
8.9	8.8	1.36×10^{8}	0.5	0	0.00
Total Evaluated:			99	Total Different:	1.0
Total Unevaluated:			1.0	Total Similar:	98

(a, b): Retention times [ELSD(a) and HRMS(b)] peaks analyzed. (c, d): Peak area and corresponding area % of peaks analyzed. (e): Estimated percent difference of that peak as determined by comparing mass spectral features to culinary mushroom extracts. (f): The percent contribution of these differences to the raw material as a whole.

Table 8
Turkey Tail - Contribution of mass spectral features that differ from culinary mushrooms to the estimated difference of the raw material from food.

ELSD retention time (min)[a]	HRMS retention time (min)[b]	Area (ELSD)[c]	Area %[d]	Peak % difference[e]	Total % difference (peak diff. × area)[f]
7.83	7.76	453×10^{6}	28.2	0	0.00
8.25	8.18	200×10^{6}	12.5	0	0.00
8.32	8.25	716×10^{6}	44.6	0	0.00
8.76	8.69	532×10^{4}	0.3	0	0.00
8.86	8.79	346×10^{5}	2.2	25	0.5
8.99	8.92	213×10^{5}	1.3	0	0.00
9.19	9.12	120×10^{6}	7.5	0	0.00
9.36	9.29	106×10^{4}	0.7	50	0.3
9.68	9.61	551×10^{4}	0.3	0	0.00
9.96	9.89	178×10^{5}	1.1	0	0.00
Total Evaluated:			99	Total Different:	1.0
Total Unevaluated:			1.0	Total Similar:	98

(a, b): Retention times [ELSD(a) and HRMS(b)] peaks analyzed. (c, d): Peak area and corresponding area % of peaks analyzed. (e): Estimated percent difference of that peak as determined by comparing mass spectral features to culinary mushroom extracts. (f): The percent contribution of these differences to the raw material as a whole.

3.5.5. *Turkey tail*

The most common Turkey Tail ingredient was listed as "Turkey Tails," and included only 17 products; the total number of products containing some type of Turkey Tail ingredient was 39. Including all reported Turkey Tail ingredients, 12 distinct brands were identified. The highest reported dose of Turkey tail was 1 g, and the median (of 11 reported doses) was 120 mg.

4. Discussion

In the absence of comprehensive toxicological studies, a weight of evidence approach must be taken when evaluating raw materials for safe inclusion in dietary supplements. Of primary importance is a positive identification of the fungal raw material. The difficulty of relying solely on macro- or microscopic techniques was demonstrated when two of the eight fungal raw materials under investigation proved to be misidentified (Raja et al., 2017).

The dereplication results provided a targeted approach for the safety assessment of the fungal raw materials, in that known fungal metabolites and mycotoxins were screened for directly. Ergosterol peroxide was detected in all of the fungi, including the store-bought culinary mushrooms. Ergosterol peroxide had been previously reported to be found in edible mushrooms (it is a sterol found in the cell membranes of all fungi), and therefore was not considered a harmful component from any of the mushrooms at dietary intake levels (Kobori et al., 2007; Krzyczkowski et al., 2009). While the Shiitake mycelium raw material (but not the store-bought fruiting body) also contained pyrenocine A and pughiinin A, neither of these compounds have reported toxic effects except to plants. Furthermore, neither were found in considerable quantities based on the ELSD signals (<0.10% of the total ELSD signal), indicating that their levels could be controlled by adjusting the dose of the raw materials. No known mycotoxins were detected in any of the raw materials.

The confirmation of identity and dereplication steps represent a screening stage in the overall process of assessing the safety of fungal raw materials. They are necessary, but very binary, steps that must be taken to ensure that the material purchased is the same being evaluated. A misidentified raw material cannot be safely used in a product; likewise, detection of any known mycotoxins immediately requires further risk assessment. Chemically comparing the fungal raw materials to culinary mushrooms represents a much more nuanced perspective that depends on an accepted safe exposure to dietary ingredients in a genetically diverse and significantly broad population. "Different" metabolites do not necessarily represent harm, but a lack of toxicological literature on that material could elevate the risk of using a material with a metabolomic profile different from food mushrooms. The purpose of comparing the fungal raw materials to foods on a constituent level was to more completely characterize and quantify this risk. Where insufficient literature is available, these now-quantified differences could be controlled and minimized by adjusting dose in the final product.

The chemical comparison of the MS peaks was both very defined and somewhat subjective. Matching retention time, accurate mass, and contribution to peak area (*i.e.*, by matching the size and shape of chromatographic peaks) is an accepted and reliable way confirm that two unknown constituents are the same (El-Elimat et al., 2013). In other words, it was reasonable to assume identical molecular species were present in two samples when the retention and accurate mass signals were identical. The identity of the analyte was not considered to be crucial, only its presence in the commonly consumed culinary mushrooms. While unique components could be analyzed further, to inform decision-making or refine dose, such analyses were not within the scope of this study.

In contrast, choosing a cutoff threshold for which MS peaks to compare proved challenging. For some samples, it was clearly evident which *m/z* signals contributed to the ELSD signal based on

the intensity abundances and comparison of narrow mass chromatogram peaks to ELSD peaks, thus only those *m/z* were analyzed (Fig. 7A). For spectra that were less clear, the top six most intense peaks were selected (Fig. 7B). However, in rare instances, the lower abundant signals of the top six peaks were too similar in intensity to determine which were significant, thus up to eight peaks were selected and analyzed further (Fig. 7C). While this selection process was flexible in terms of numbers of *m/z* peaks considered, it provided a more thorough analysis then selecting a set number of peaks and ignoring signals similar in intensity.

Assessing the safety of the fungal raw materials requires expert knowledge and judgement. Strong evidence of safe human use would theoretically consist of positive DNA identification, no detected mycotoxins, a very high degree of similarity to culinary mushrooms, thorough information in the literature on the chemistry and safety of the material, and/or broad market prevalence. Weak evidence of safety could result from data gaps in the literature, significant degrees of difference from culinary mushrooms, and/or limited market presence. Major data gaps in the literature, a high degree of difference from the analyzed culinary mushrooms on a constituent-level basis, and/or little or no documented market presence would suggest inadequate evidence of safety and more traditional toxicological studies would be required. Further analytical investigations focused on differences would in such cases yield a better understanding of the constituent profile and help inform design of further studies. An incorrectly identified fungal raw material, detection of a mycotoxin, known serious adverse events associated with the fungus in the marketplace, and/or indications of toxicity in the safety literature would indicate that the safe use of that fungal raw material could not be supported without out further investigation.

A summary of the weight-of-evidence approach used for the raw materials in this study can be found in Table 9.

While evaluating the fungal raw materials, the most straightforward were those that are often considered food, and/or had a high degree of similarity to the culinary materials. For example, both Shiitake and Maitake are commonly eaten as foods, and Shiitake, at least, has a wealth of available toxicological data supporting its safe use. The apparent prevalence in the marketplace, the lack of reported adverse events, as determined by the literature review and the very high degree of similarity between their mycelium growths (the raw materials investigated) and the culinary fruiting bodies to which they were compared give confidence that these materials are safe for consumption at doses consistent with dietary intakes of culinary mushrooms.

The literature search results were much more limited for Turkey Tail and Chaga, necessitating a heavier reliance on other data. While there were no indications of toxicity, particularly for aqueous extracts of the two mushrooms, conventional toxicological data were not available for most endpoints and non-aqueous preparations.

Table 9
Weight-of-evidence summary for evaluated raw materials.

Raw material	Identity verified by DNA	Dereplication	Similarity to grocery store mushrooms	Prevalence in the market	Published literature	Weight of evidence evaluation
Chaga	Yes	No toxins detected	98% similar 2% unevaluated	70 products, median dose 120 mg	Limited toxicological data. No reported toxic effects.	Chemically very similar to food. Likely poses no greater risk than culinary mushrooms, particularly if unevaluated portion is minimized through dose.
Reishi (Mycelium + Fruiting Bodies)	Yes	No toxins detected	75% similar 21% “different” 4% unevaluated	315 products, median dose 100 mg	Long history of use as medicinal food in Asian countries. Subchronic and acute studies available. Nine clinical trials, one of which monitored safety endpoints. Traditional developmental and reproductive studies lacking. Limited genotoxicity data available. No evidence of toxic effects.	Not a high similarity to culinary mushrooms; necessitates greater reliance on substantial history of use and available toxicological data.
Shiitake	Yes	No toxins detected	98% similar 2% unevaluated	223 products, median dose 50 mg	Long history of use as food (fruiting body). Numerous toxicological studies available, showing minimal toxicity.	Chemically very similar to food. Existing history of use and available toxicological data indicate no greater risk than culinary mushrooms.
Maitake	Yes	No toxins detected	98% similar 1% “different” 1% unevaluated	267 products, median dose 60 mg	Long history of use as food (fruiting body). Subchronic oral studies available. Limited data on other endpoints in literature.	Chemically very similar to food. Likely poses no greater risk than culinary mushrooms, particularly if “different”/unevaluated portion is minimized through dose.
Turkey Tail	Yes	No toxins detected	98% similar 1% “different” 1% unevaluated	39 products, median dose 120 mg	Aqueous extracts heavily studied, results indicating no apparent toxicity. Studies on other extracts are limited.	Chemically very similar to food. Some toxicological data available. Likely poses no greater risk than culinary mushrooms, particularly if “different”/unevaluated portion is minimized through dose.
Wild Chaga	No	———— assessment not continued ————				Weight of evidence evaluation not possible without positive identification.
Cordyceps	No	———— assessment not continued ————				Weight of evidence evaluation not possible without positive identification.

Chaga ingredients were more prevalent in the marketplace than some of the other materials assessed (up to 70 products, as opposed to 39 listed for Turkey Tail), but not as prevalent as Reishi (up to 315 products). In general, fungal ingredients appear not to be particularly popular in the market; searches of the DSLD for "Ginger" and "Turmeric" for instance provided hits for up to 1660 products and 1082 products, respectively. However, Chaga does appear in the marketplace at relatively high doses (up to 1000 mg), and there were no data found in the literature that indicated associated adverse events. Combined with the fact that there were no detectable differences from the culinary ingredients, it was determined that further work was not necessary to support its safe use at doses consistent with those currently in the marketplace. Likewise, the Turkey Tail had a very high degree of similarity to the culinary ingredients, and certain preparations are being studied in humans, with no apparent toxic effects (Chu et al., 2002; Ito et al., 2004; Ohwada et al., 2004; Tsang et al., 2003). While it does not appear to have as large a market presence as the other fungal ingredients studied, it is, in general, considered edible (Cotter, 2014; Russell, 2006; Spahr, 2009) though unpalatable. Based on the available weight of evidence, its safe use can likely be supported without further studies, especially if the dissimilarity from culinary ingredients is minimized by using a smaller dose.

The Reishi raw material was the most different, when compared to the culinary ingredients, but there was substantially more literature (Bensky et al., 2004; Chen et al., 2011; Wachtel-Galor et al., 2004; Wihastuti et al., 2014, 2015) to support its safe use in supplements (see Supplemental Information for the complete list of references evaluated). It appeared to have the broadest market presence of any of the fungal materials studied (up to 315 products), with a number of different preparations available to consumers. It is reasonable that the long history of use, presence in the marketplace, and abundant toxicological literature outweighs the dissimilarity from common food mushrooms.

Evaluating complex mixtures for human safety, particularly one that could vary from lot-to-lot, presents a number of challenges. Maintaining consistent methods of production and preparation becomes critical to yielding a similar product. While known toxins can theoretically be screened for through dereplication (El-Elimat et al., 2013), this requires a thorough and maintained database of constituents to compare against. Importantly, it does not include unknown toxins, necessitating further steps to ensure safe consumption. Likewise, chemical comparison to food can provide evidence of safety if there is a very high degree of similarity, but dissimilarity does not necessarily indicate toxicity. Toxicological literature for botanicals and traditional medicines can be sparse, and often not directly address the preparation of interest; this challenge is probably even greater for those based on fungal preparations, due to the differences in growing conditions between traditionally used materials and commercially available ingredients. Using a weight of evidence approach, including existing literature, analytical, and *in silico* tools, provides an alternative approach to safety assessment, minimizing or even eliminating the need for animal testing in making decisions for safe human use.

5. Conclusions

The use of the integrated approach and particularly chemical constituent comparison to common food mushrooms provides a novel approach to addressing the safety of fungal raw materials. In the absence of the usual suite of toxicological studies, a comparison to common food can be made, with the ultimate conclusion being that the materials with high degrees of similarity are no more a risk to the consumer than that of food. Components may be identified either in the dereplication step or the chemical comparison step that require further evaluation, but the systematic approach of comparing the chemical profiles to that of something inherently low-risk (food) allows these constituents to be prioritized. In many cases, as with ergosterol peroxide, these components can be excluded using solely literature resources.

It is important to note that this method of evaluation is specific to the particular cultivation of the fungi and the dose evaluated. Constituents in the fungal raw material that differ from available culinary mushrooms may be restricted to safe levels by adjusting dose. Changing the cultivation process of the fungus can change the secondary metabolome profile, thus changing the levels of any constituents of concern. This would necessitate a reevaluation of raw material to ensure that any new metabolites are considered and restricted to safe levels if appropriate. However, these same limitations apply to a more traditional suite of toxicological tests; a change in the raw material raises questions as to whether the studies sufficiently address the safety of the new material.

Incorporating analytical and *in silico* tools to the safety assessment of natural materials offers a number of tangible benefits. In addition to cost and time savings, there is a reduced demand on the number of animals required to ensure the safe human use of the material. Both initial evaluations and comparisons to new or similar materials can be performed in an expedited and less resource intensive fashion.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.fct.2017.03.005.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.fct.2017.03.005.

References

Anasiewicz, A., Gogacz, M., Wäojcicki, T., Bentkowski, M., 1998. Influence of the extract from mycelium of Inonotus obliquus on development of foetuses of rats. Teratol 58, 21A.
Bensky, D., Clavey, S., Stöger, E., 2004. Chinese Herbal Medicine: Materia Medica, third ed. Eastland Press, Seattle, WA.
Bills, G.F., Platas, G., Fillola, A., Jimenez, M.R., Collado, J., Vicente, F., Martin, J., Gonzalez, A., Bur-Zimmermann, J., Tormo, J.R., Pelaez, F., 2008. Enhancement of antibiotic and secondary metabolite detection from filamentous fungi by growth on nutritional arrays. J. Appl. Microbiol. 104, 1644–1658.
Bode, H.B., Bethe, B., Hofs, R., Zeeck, A., 2002. Big effects from small changes: possible ways to explore nature's chemical diversity. ChemBioChem 3, 619–627.
Chen, S.N., Nan, F.H., Chen, S., Wu, J.F., Lu, C.L., Soni, M.G., 2011. Safety assessment of mushroom beta-glucan: subchronic toxicity in rodents and mutagenicity studies. Food Chem. Toxicol. 49, 2890–2898.
Chu, K.K., Ho, S.S., Chow, A.H., 2002. Coriolus versicolor: a medicinal mushroom with promising immunotherapeutic values. J. Clin. Pharmacol. 42, 976–984.
Cotter, T., 2014. Organic Mushroom Farming and Mycoremediation: Simple to Advanced and Experimental Techniques for Indoor and Outdoor Cultivation. Chelsea green Publishing, White River Junction, Vermont.
Dalonso, N., Goldman, G.H., Gern, R.M., 2015. Beta-(1–>3), (1–>6)-Glucans: medicinal activities, characterization, biosynthesis and new horizons. Appl. Microbio. Biot. 99, 7893–7906.
Das, S.K., Masuda, M., Sakurai, A., Sakakibara, M., 2010. Medicinal uses of the mushroom Cordyceps militaris: current state and prospects. Fitoterapia 81, 961–968.
de Lima, P.c.L.A., Delmanto, R.D., Sugui, M.M., da Eira, A.F., Salvadori, D.M.F., Speit, G., Ribeiro, L.R., 2001. Letinula edodes (Berk.) Pegler (Shiitake) modulates genotoxic and mutagenic effects induced by alkylating agents in vivo. Mutat. Res. Genet. Toxicol. Environ. Mutagen 496, 23–32.
Deng, G., Lin, H., Seidman, A., Fornier, M., D'Andrea, G., Wesa, K., Yeung, S., Cunningham-Rundles, S., Vickers, A.J., Cassileth, B., 2009. A phase I/II trial of a polysaccharide extract from Grifola frondosa (Maitake mushroom) in breast cancer patients: immunological effects. J. Cancer Res. Clin. Oncol. 135, 1215–1221.
El-Elimat, T., Figueroa, M., Ehrmann, B.M., Cech, N.B., Pearce, C.J., Oberlies, N.H., 2013. High-resolution MS, MS/MS, and UV database of fungal secondary

metabolites as a dereplication protocol for bioactive natural products. J. Nat. Prod. 76, 1709–1716.
Eom, H.Y., Park, S.Y., Kim, M.K., Suh, J.H., Yeom, H., Min, J.W., Kim, U., Lee, J., Youm, J.R., Han, S.B., 2010. Comparison between evaporative light scattering detection and charged aerosol detection for the analysis of saikosaponins. J. Chromatogr. A 1217, 4347–4354.
Fiedurek, J., Gromada, A., Jamroz, J., 1996. Effect of medium components and metabolic inhibitors on beta-galactosidase production and secretion by Penicillium notatum 1. J. Basic Microbiol. 36, 27–32.
Fisher, M., Yang, L.X., 2002. Anticancer effects and mechanisms of polysaccharide-K (PSK): implications of cancer immunotherapy. Anticancer Res. 22, 1737–1754.
Frizo, Í., Pickler, T.B., Grotto, D., Gerenutti, M., 2014. Alterations in the reproductive performance of the female rats and fetotoxicity of Lentinula edodes (Shiitake). Reprod. Toxicol. 48, 25.
Glauco, S., Jano, F., Paolo, G., Konno, S., 2004. Safety of Maitake D-fraction in healthy patients: assessment of common hematologic parameters. Altern. Complement. Ther. 10, 228–230.
Haixia, C., Lingling, F., Peng, D., Xin, Z., Xueming, L., 2009. Acute toxicity evaluation and compositional analysis of a polysaccharide from the medicinal mushroom Inonotus obliquus, Bioinformatics and Biomedical Engineering, 2009. ICBBE 2009. 3rd International Conference on, pp. 1–4.
Ham, S.S., Kim, S.H., Moon, S.Y., Chung, M.J., Cui, C.B., Han, E.K., Chung, C.K., Choe, M., 2009. Antimutagenic effects of subfractions of Chaga mushroom (Inonotus obliquus) extract. Mutat. Res. 672, 55–59.
Hor, S.Y., Ahmad, M., Farsi, E., Lim, C.P., Asmawi, M.Z., Yam, M.F., 2011. Acute and subchronic oral toxicity of Coriolus versicolor standardized water extract in Sprague-Dawley rats. J. Ethnopharmacol. 137, 1067–1076.
Ito, K., Nakazato, H., Koike, A., Takagi, H., Saji, S., Baba, S., Mai, M., Sakamoto, J., Ohashi, Y., Study Group of Immunochemotherapy with, P.S.K.f.C.C, 2004. Long-term effect of 5-fluorouracil enhanced by intermittent administration of polysaccharide K after curative resection of colon cancer. A randomized controlled trial for 7-year follow-up. Int. J. Colorectal Dis. 19, 157–164.
Kalaivani, M., Sabitha, R., Kalaiselvan, V., Rajasekaran, A., 2010. Health benefits and clinical impact of major nutrient, red yeast rice: a review. Food Bioproc. Tech. 3, 333–339.
Knezevic, A., Zivkovic, L., Stajic, M., Vukojevic, J., Milovanovic, I., Spremo-Potparevic, B., 2015. Antigenotoxic effect of Trametes spp. extracts against DNA damage on human peripheral white blood cells. Sci. World J. 2015, 146378.
Kobori, M., Yoshida, M., Ohnishi-Kameyama, M., Shinmoto, H., 2007. Ergosterol peroxide from an edible mushroom suppresses inflammatory responses in RAW264.7 macrophages and growth of HT29 colon adenocarcinoma cells. Br. J. Pharmacol. 150, 209–219.
Kodama, N., Komuta, K., Nanba, H., 2002. Can maitake MD-fraction aid cancer patients? Altern. Med. Rev. 7, 236–239.
Krzyczkowski, W., Malinowska, E., Suchocki, P., Kleps, J., Olejnik, M., Herold, F., 2009. Isolation and quantitative determination of ergosterol peroxide in various edible mushroom species. Food Chem. 113, 351–355.
Lai, C.H., Teng, J.F., Hsu, T.H., Lin, F.Y., Yang, P.W., Lo, H.C., 2011. 28-day oral safety evaluation of extracellular polysaccharopeptides produced in submerged culture from the Turkey tail medicinal mushroom Trametes versicolor (L.:Fr.) Pilat LH-1 in mice. Int. J. Med. Mushrooms. 13, 227–236.
Lamaison, J.-L., Polese, J.-M., 2008. The Great Encyclopedia of Mushrooms. H. F. Ullmann, United States.
Levy, A.M., Kita, H., Phillips, S.F., Schkade, P.A., Dyer, P.D., Gleich, G.J., Dubravec, V.A., Eosinophilia and gastrointestinal symptoms after ingestion of shiitake mushrooms. J. Allergy Clin. Immun. 101:613–620.
Lindner, D.L., Banik, M.T., 2008. Molecular phylogeny of Laetiporus and other brown rot polypore genera in North America. Mycologia 100, 417–430.
McCloud, T.G., 2010. High throughput extraction of plant, marine and fungal specimens for preservation of biologically active molecules. Molecules 15, 4526.
Meuninck, J., 2015. Basic Illustrated Edible and Medicinal Mushrooms. Falcon-Guides, Guilford, Connecticut.
Miao, L., Kwong, T.F., Qian, P.Y., 2006. Effect of culture conditions on mycelial growth, antibacterial activity, and metabolite profiles of the marine-derived fungus Arthrinium c.f. saccharicola. Appl. Microbio. Biot. 72, 1063–1073.
Miyaji, C.K., Poersch, A., Ribeiro, L.R., Eira, A.F., Colus, I.M., 2006. Shiitake (Lentinula edodes (Berkeley) Pegler) extracts as a modulator of micronuclei induced in HEp-2 cells. Toxicol. In Vitro 20, 1555–1559.
Mohanty, S.S., Prakash, S., 2009. Effects of culture media on larvicidal property of secondary metabolites of mosquito pathogenic fungus Chrysosporium lobatum (Moniliales: moniliaceae). Acta Trop. 109, 50–54.
Nanba, H., 1997. Maitake D-fraction: healing and preventive potential for cancer. J. Orthomol. Med. 12, 43–49.
Ng, T.B., Chan, W.Y., 1997. Polysaccharopeptide from the mushroom Coriolus versicolor possesses analgesic activity but does not produce adverse effects on female reproductive or embryonic development in mice. Gen. Pharmacol.-Vasc. S. 29, 269–273.
Nilsson, R.H., Kristiansson, E., Ryberg, M., Hallenberg, N., Larsson, K.-H., 2008. Intraspecific ITS variability in the kingdom Fungi as expressed in the international sequence databases and its implications for molecular species identification. Evol. Bioinform. Online 4, 193–201.
Ohwada, S., Ikeya, T., Yokomori, T., Kusaba, T., Roppongi, T., Takahashi, T., Nakamura, S., Kakinuma, S., Iwazaki, S., Ishikawa, H., Kawate, S., Nakajima, T., Morishita, Y., 2004. Adjuvant immunochemotherapy with oral Tegafur/Uracil plus PSK in patients with stage II or III colorectal cancer: a randomised controlled study. Br. J. Cancer 90, 1003–1010.
Patakova, P., 2013. Monascus secondary metabolites: production and biological activity. J. Ind. Microbiol. Biot. 40, 169–181.
Paterson, R.R.M., 2006. Ganoderma - a therapeutic fungal biofactory. Phytochem 67, 1985–2001.
Phan, C.W., David, P., Naidu, M., Wong, K.H., Sabaratnam, V., 2013. Neurite outgrowth stimulatory effects of culinary-medicinal mushrooms and their toxicity assessment using differentiating Neuro-2a and embryonic fibroblast BALB/3T3. BMC Complement. Altern. Med. 13, 261.
Phillips, R., Kibby, G., Foy, N., Phillips, R., 2010. Mushrooms and Other Fungi of North America, second ed. Firefly Books, Buffalo, NY.
Pittayakhajonwut, P., Theerasilp, M., Kongsaeree, P., Rungrod, A., Tanticharoen, M., Thebtaranonth, Y., 2002. Pughiinin A, a sesquiterpene from the fungus Kionochaeta pughii BCC 3878. Planta Med. 68, 1017–1019.
Raja, H.A., Baker, T.R., Little, J.G., Oberlies, N.H., 2017. DNA barcoding for Identification of Consumer-Relevant Mushrooms: a partial solution for product certification? Food Chem. 214, 383–392.
Rios, J.L., 2011. Chemical constituents and pharmacological properties of Poria cocos. Planta Med. 77, 681–691.
Russell, B., 2006. Field Guide to Wild Mushrooms of Pennsylvania and the Mid-Atlantic. Pennsylvania State University Press, University Park, Pa.
Sato, H., Konoma, K., Sakamura, S., 1979. Phytotoxins produced by onion pink root fungus, Pyrenochaeta terrestris. Agric. Biol. Chem. 43, 2409–2411.
Sato, H., Konoma, K., Sakamura, S., Furusaki, A., Matsumoto, T., Matsuzaki, T., 1981. X-ray crystal structure of pyrenocine A, a phytotoxin from Pyrenochaeta terrestris. Agric. Biol. Chem. 45, 795–797.
Schoch, C.L., Seifert, K.A., Huhndorf, S., Robert, V., Spouge, J.L., Levesque, C.A., Chen, W., 2012. Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi. Proc. Natl. Acad. Sci. U. S. A. 109, 6241–6246.
Shikov, A.N., Pozharitskaya, O.N., Makarova, M.N., Makarov, V.G., Wagner, H., 2014. Bergenia crassifolia (L.) Fritsch–pharmacology and phytochemistry. Phytomedicine 21, 1534–1542.
Spahr, D.L., 2009. Edible and Medicinal Mushrooms of New England and Eastern Canada. North Atlantic Books, Berkeley, Calif.
Spierings, E.L.H., Fujii, H., Sun, B., Walshe, T., 2007. A phase I study of the safety of the nutritional supplement, Active Hexose Correlated Compound, AHCC, in healthy volunteers. J. Nutr. Sci. Vitaminol. 53, 536–539.
Sugiyama, J., 1987. Pleomorphic Fungi: the Diversity and its Taxonomic Implications. Kodansha. Elsevier, Tokyo, Amsterdam, New York.
Torkelson, C.J., Sweet, E., Martzen, M.R., Sasagawa, M., Wenner, C.A., Gay, J., Putiri, A., Standish, L.J., 2012. Phase 1 clinical trial of Trametes versicolor in women with breast cancer. ISRN Oncol. 2012, 251632.
Tsang, K.W., Lam, C.L., Yan, C., Mak, J.C., Ooi, G.C., Ho, J.C., Lam, B., Man, R., Sham, J.S., Lam, W.K., 2003. Coriolus versicolor polysaccharide peptide slows progression of advanced non-small cell lung cancer. Respir. Med. 97, 618–624.
VanderMolen, K.M., Raja, H.A., El-Elimat, T., Oberlies, N.H., 2013. Evaluation of culture media for the production of secondary metabolites in a natural products screening program. AMB Express 3, 71.
Vehovec, T., Obreza, A., 2010. Review of operating principle and applications of the charged aerosol detector. J. Chromatogr. A 1217, 1549–1556.
von Wright, A., Knuutinen, J., Lindroth, S., Pellinen, M., Widén, K.G., Seppä, E.L., 1982. The mutagenicity of some edible mushrooms in the ames test. Food Chem. Toxicol. 20, 265–267.
Wachtel-Galor, S., Tomlinson, B., Benzie, I.F.F., 2004. Ganoderma lucidum ('Lingzhi'), a Chinese medicinal mushroom: biomarker responses in a controlled human supplementation study. Br. J. Nutr. 91, 263–269.
Wang, L.X., Lu, Z.M., Geng, Y., Zhang, X.M., Xu, G.H., Shi, J.S., Xu, Z.H., 2014. Stimulated production of steroids in Inonotus obliquus by host factors from birch. J. Biosci. Bioeng. 118, 728–731.
Wesa, K.M., Cunningham-Rundles, S., Klimek, V.M., Vertosick, E., Coleton, M.I., Yeung, K.S., Lin, H., Nimer, S., Cassileth, B.R., 2015. Maitake mushroom extract in myelodysplastic syndromes (MDS): a phase II study. Cancer Immunol. Immunother. 64, 237–247.
Wihastuti, T.A., Sargowo, D., Widodo, M.A., Heriansyah, T., Soeharto, S., Anita, K.W., A'ini, N.Q., 2015. Effects of subchronic exposure of PSP *Ganoderma lucidum* on renal function and histopathology feature in *Rattus novergicus* Wistar strain. Natl. J. Physiol. Pharm. Pharmacol 5.
Wihastuti, T.A., Sargowo, D., Widodo, M.A., Heriansyah, T., Soeharto, S., Ardi, A.I., 2014. Subchronic toxicity of Ganoderma lucidum polysaccharide peptide (PsP) to liver physiology and histopathology imaging of liver on Rattus norvegicus strain Wistar rats. Biomed. Pharmacol. J 7.
Xu, Z.-H., 2012. Chemistry of fungal products. In: Xu, R., Ye, Y., Zhao, W. (Eds.), Introduction to Natural Products Chemistry. CRC Press, pp. 27–53.
Yoshioka, Y., Tamesada, M., Nagayama, A., 2009. The safety of excessive intake of the food containing extract of cultured *Lentinula edodes* mycelia (L.E.M.) in healthy adult volunteers. Jpn. J. Complement. Altern. Med. 6, 9–15.
Yoshioka, Y., Tamesada, M., Tomi, H., 2010. A repeated dose 28-day oral toxicity study of extract from cultured Lentinula edodes mycelia in Wistar rats. J. Toxicol. Sci. 35, 785–791.
Zhang, Y., Fraatz, M.A., Muller, J., Schmitz, H.J., Birk, F., Schrenk, D., Zorn, H., 2015. Aroma characterization and safety assessment of a beverage fermented by Trametes versicolor. J. Agric. Food Chem. 63, 6915–6921.
Zhong, X.-h., Ren, K., Lu, S.-j., Yang, S.-y., Sun, D.-z., 2009. Progress of research on Inonotus obliquus. Chin. J. Integr. Med. 15, 156–160.