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Screening of polypeptide toxins as adulteration markers in the food containing wild edible mushroom by liquid chromatography-triple quadrupole mass spectrometry

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ABSTRACT

An accurate and sensitive method was established for the determination of polypeptide toxins amanitins and phallotoxins in the food containing wild edible mushrooms by liquid chromatography-triple quadrupole mass spectrometry. The toxins in sample matrix were extracted with hydrochloric acid solution (0.5% in water, *v/v*), cleaned by HLB cartridge and separated by C18 chromatographic column under basic condition (pH = 10.5). Amanitins and phallotoxins were monitored by an electrospray ionization source (ESI) under negative and positive modes, respectively, in one injection cycle. Basic mobile phase condition was applied to suppress the ionization efficiency of sodium ion adducts and improve that of the hydrogen ion adducts under ESI mode. The calculated limits of detection in sample matrix were 0.002–0.005 mg kg^{-1} and the limits of quantification were 0.005–0.01 mg kg^{-1}. The linear range was 0.01–2 mg kg^{-1} with a correlation coefficient >0.996. The average recoveries at three spiking levels were 72.6%–91.8% with relative standard deviations of 5.2%–10.1% (n = 6). The developed method was applicable for the adulteration screening of inedible *Amanitas* in the food containing wild edible mushroom and the applicable adulteration markers were α-amanitin, β-amanitin and phallacidin.

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1. Introduction

Economically motivated adulteration (EMA) with inedible compounds or undeclared substitutions is a hot topic in the field of food safety (Everstine, Spink, & Kennedy, 2013; MacMahon, Begley, Diachenko, & Stromgren, 2012; Moore, Spink, & Lipp, 2012). This kind of adulterations included 1) the illegal addition of inedible or cheap material to decrease the cost, such as the melamine events in dairy products (Abernethy & Higgs, 2013; Draher, Pound, & Reddy, 2014; Finete, Gouvea, Marques, & Netto, 2015; Xu et al., 2009) and the horse meat events in beef (Burns et al., 2016; Giaretta, Di Giuseppe, Lippert, Parente, et al., 2013; Kreuz et al., 2012; Premanandh, 2013); 2) the illegal addition of industry dye in food to improve the sensory quality for making higher profits, such as the Sudan red events (Haughey, Galvin-King, Ho, Bell, & Elliott, 2015; Rajabi, Sabzalian, Barfi, Arghavani-Beydokhti, & Asghari, 2015); 3) the illegal addition of addiction plant material to improve the tasting quality for attracting consumers, such as the pericarpium papaveris events (Yan, Zhao, & Li, 2005). Besides the negative impact to the food production and consumption, even direct damage to human health was reported such as the illegal addition of melamine to dairy products as protein adulterant (Everstine et al., 2013; Ke et al., 2010).

China has more than half of all fungi species in the world. Wild edible mushrooms are popular as they are delicious in taste, rich in nutrition or even beneficial to health and beauty (Badalyan et al., 1996; Badalyan et al., 1996). The normal wild edible mushrooms in China include *Boletus edulis, Boletus luteus, Boletus queletiischulz, Termitornyces albuminosus, Morchella esculenta, Cantharellus cibarius, Dictyophora, Russula alutacea, Lactarius volemus, Hericium erinaceus, Tricholoma matsutake, Cordyceps sinensis* et al. Fresh wild mushrooms are easy to go bad and difficult to save and transport. They are normally dried or prepared to dried powder and can be sold to farther areas beyond the place of origin. Besides mixed with the other materials, the powdered mushrooms can be directly used as food materials, especially as flavorings. Adulteration of some inedible wild mushrooms is possible for it's hard to distinguish the difference in powdered formation.

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Amanitas are one of the most possible adulterants for the high output and special delicacy. Although some of the *Amanitas* are nontoxic and can be considered for consumption in folk, some of them are extremely lethal toxicity (Gonmori, Fujita, Yokoyama, Watanabe, & Suzuki, 2011; Vetter & Vetter, 2014). It's hard to distinguish the toxic and nontoxic *Amanitas* from the shape. Some of the poisonous cases are the result of ingestion in mistake (Chen, Zhang, & Zhang, 2014; Gonmori et al., 2011). Moreover, the concentration and the species of the toxins would change under different weather conditions and production environments as reported by (Garcia et al., 2015).

The toxins from *Amanitas* include polypeptide compounds such as amanitins and phallotoxins besides muscarine or the amino acid original toxins such as ibotenic acid, muscimol (Gonmori et al., 2011; Vetter & Vetter, 2014). High levels of polypeptide toxins were reported in species of *Amanita* genus such as *Amanita phalloides*, *Amanita virosa*, *Amanita verna Amanita ocreata, Amanita bisporigera, Amanita suballiacea, Amanita tenuifolia and Amanita hygroscopica* (Gonmori et al., 2011; Kaya et al., 2013). α-Amanitin, β-amanitin, γ-amanitin, phallacidin and phalloidin were the major polypeptide toxins found in *Amanitas* (Kaya et al., 2013; Vetter & Vetter, 2014) and were used as the markers for adulteration screening of inedible *Amanitas* in the food containing wild mushroom in this study. The other analogues such as amaninamide, amanin, phallisin, and viroidin et al. (Clarke, Lloyd, & Robb, 2012; Jansson, Fredriksson, Herrmann, & Nilsson, 2012; Sgambelluri et al., 2014) were found as well in some *Amanita* species. These analogues were not applicable as the adulteration markers for the standards were not commercially available in the market. *Amanitas* is not allowed to be a commercial product, or in the ingredient of commercial product, because it's difficult to identify the toxic and non-toxic *Amanitas*. Therefore, once the toxic peptide was detected, the adulteration should be considered if the contamination by mistake can be excluded.

Amanitins and phallotoxins are lethal mushroom toxins containing polypeptide groups of bicyclic octapeptides and bicyclic heptapeptides, respectively. The median lethal dose (LD_{50}) was reported to 0.3–0.7 mg kg^{-1} for amanitins and 1.5–3 mg kg^{-1} for phallotoxins (Wieland, 1983). These toxins can induce the inhibition of RNA polymerase II (Nguyen et al., 1996). The major toxicity symptoms include fulminant hepatic insufficiency, kidney failure or even death (Chen et al., 2014; Gonmori et al., 2011). Poisonous cases were reported in recent years because of ingestion of wild mushroom in mistake (Chen et al., 2014; Gonmori et al., 2011). The screening study of illegal adulterant in the food containing wild mushroom, not only can strike such food fraud, can also effectively prevent the potential poisoning.

The structures of α-amanitin, β-amanitin, γ-amanitin, phallacidin and phalloidin are shown in Fig. 1. The powerful techniques for analysis of mushroom toxins were liquid chromatography with ultraviolet or diode array detector (LC-UV or LC-DAD) (Hu, Zhang, Zeng, & Chen, 2012; Kaya et al., 2013; Sgambelluri et al., 2014; Vargas, Bernal, Sarria, Franco-Molano, & Restrepo, 2011), liquid chromatography-triple quadrupole mass spectrometry (LC-MS/MS) (Leite et al., 2013; Nomura et al., 2012; Tanahashi et al., 2010; Yee, Woods, Poppenga, & Puschner, 2012) and liquid chromatography-time of flight mass spectrometry (LC-TOF-MS) (Clarke et al., 2012; Ishii et al., 2014; Yoshioka et al., 2014). LC-UV or DAD is a good tool for quantitative determination of toxins in higher concentrations in pure *Amanitas*. LC-TOF-MS was used for the screening of target and non-target mushroom toxins (Clarke et al., 2012; Jansson et al., 2012; Sgambelluri et al., 2014). LC-MS/MS was well applied to quantitatively analyze polypeptide mushroom toxins at trace levels in bio-materials (Helfer, Meyer, Michely, & Maurer, 2014; Leite et al., 2013; Tomkova, Ondra, & Valka, 2015) and was used to screen polypeptide toxins as adulteration markers in the food containing wild edible mushrooms in this study.

The purpose in this study was to 1) develop a convenient and effective sample preparation method by improving the procedures of extraction and clean-up of polypeptide toxins in food; 2) screen some applicable markers from the polypeptide toxins to distinguish the adulteration of inedible *Amanitas* in the food containing wild edible mushrooms; 3) obtain an accurate and sensitive instrument measurement method by optimizing the LC elution conditions to suppress the ionization efficiency of sodium ion adducts $[M+Na]^+$ and improve that of the hydrogen ion adducts ($[M+H]^+$); 4) verify the adulteration markers in real sample analysis.

2. Materials and methods

2.1. Chemicals and materials

All reagents and solvents were of analytical grade unless specified.

Polypeptide toxins α-amanitin, β-amanitin, γ-amanitin, phallacidin and phalloidin were supported by Alexis Corporation (San Diego, CA, USA). HPLC-grade methanol and dichloromethane were provided by Merck (Darmstadt, Germany). Ammonia solution (25%) and hydrochloric acid (HCl, 36–38%) were purchased from Hangzhou Liren Medicine (Hangzhou, China) and Zhejiang Zhongxing Chemicals (Jinhua, China), respectively. Formic acid was purchased from ROE Scientific Inc. (New Castle, DE, USA). HLB solid phase extraction (SPE) cartridge was supported by Waters (60 mg, 3 mL, USA). Ultrapure water was prepared using a Millipore system (Bedford, MA, USA).

Samples containing wild edible mushroom were obtained by online shopping originated from different provinces in China.

2.2. Preparation of the standards solution

Pure α-amanitin, β-amanitin, γ-amanitin, phallacidin or phalloidin was dissolved in methanol to obtain the single standard stock solution with a concentration of 100 μg mL^{-1}. A 10 μg mL^{-1} of mixture standards working solution was prepared by mixing 1 mL each standard stock solution and diluting to 10 mL with methanol. The calibration curve of the standard mixture was prepared at the concentrations of 1, 2, 5, 10, 20, 50, 100, and 200 ng mL^{-1} in polypeptide toxins free sample matrix. The solvent used for preparing calibration solution was 10% (*v*/*v*) of methanol/water mixture.

2.3. Sample preparation

About 1 g of a homogenized sample was vortexed for 0.1 min with 15 mL of HCl solution (0.5% in water, *v*/*v*) and sonicated for 15 min at room temperature. The total volume was then made up to 20 mL mark with the same HCl solution and the mixture was vortexed for 0.1 min. The sample mixture was then centrifuged at 8000 rpm for 2 min. About 8 mL of the supernatant was transferred to another test tube, vortexed with 2 mL of dichloromethane for 0.1 min and centrifuged at 8000 rpm for 2 min. An aliquot of 2 mL of the water phase (up layer) was loaded onto a HLB cartridge, which was aforehand conditioned with 2 mL of methanol and followed by 2 mL of water. Then 2 mL of water was used to wash the cartridge and the eluent was discarded. The cartridge was then vacuumed for 2 min. After that, the analytes were eluted with 3 mL of methanol. The methanol eluent was evaporated to dryness under gentle stream of nitrogen at 40 °C and the residue was redissolved with 1 mL of 10% (*v*/*v*) of methanol/water mixture by vortexing for 0.1 min. The final mixture was centrifuged at 14,000 rpm for 5 min and the supernatant was measured by LC-MS/MS.

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