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Simultaneous quantification of free amino acids and 5′-nucleotides in shiitake mushrooms by stable isotope labeling-LC-MS/MS analysis

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ABSTRACT

Evaluation of free amino acids (FAAs) and nucleotides in various food matrices has been a widely studied topic in recent years. Here, a fast and efficient strategy for the simultaneous analysis of 20 FAAs and six 5′-nucleotides, using stable isotope labeling-liquid chromatography coupled with tandem mass spectrometry (SIL-LC-MS/MS) is proposed. The method was validated with respect to selectivity, linearity, limits of detection (LOD) and quantification (LOQ), recovery, precision, and stability. LOQs of most FFAs were lower than 1 ng/mL, and 5′-nucleotides were in the range of 5–20 ng/mL. FAAs and 5′-nucleotides in ten shiitake mushrooms from different cultivate areas were further analyzed. Results showed that the contents of cytidine 5′-monophosphate, adenosine 5′-monophosphate, lysine, threonine, arginine were significantly different. Principal component analysis showed clear discrimination of origins, seasons and species. Thus, the proposed method is suitable for the fast discrimination of species and geographical origins of shiitake mushrooms.

1. Introduction

Amino acids, the basic units of proteins and polypeptides, play an essential role in several metabolic processes of living organisms (Delgado-Povedano, Calderon-Santiago, Priego-Capote, & Luque de Castro, 2016). Besides proteins, plants and fruits possess amino acids in their free form, which mostly show a typical pattern (Silva et al., 2004). Free amino acids (FAAs) are particularly interesting because they are not only responsible for the savory taste of food, but have various physiological and pharmacological activities (Ming, Li, Huo, Wei, & Chen, 2014). Nucleotides are an essential element in many biochemical processes, which constitute the basic molecules of nucleic acids-DNA and RNA (Hess & Greenberg, 2012). Quantification of nucleotides is of fundamental interest in numerous applications such as energy metabolism, biochemical processes, or in understanding the mechanism of nucleoside analog compounds (Cohen, Jordheim, Megherbi, Dumontet, & Guitton, 2010). Analysis of FAAs and 5′-nucleotides is essential in metabolic research studies in a wide variety of medical and biopharmaceutical applications (Johnsen et al., 2011; Zhai, Chen, Zhu, & Lu, 2015). For closely related to the savory taste and beneficial effects of food, FAAs and 5′-nucleotides in Finnish forest mushrooms were detected by Manninen, Rotola-Pukkila, Aisala, Hopia, and Laaksonen (2018). Kong et al. (2017) investigated FAAs, organic acids, and nucleotides in commercial vinegar and classified different kinds of vinegar.

Numerous methods have been developed to quantify amino acids and nucleotides. The traditional approach for FAAs is derivatization with photometric or fluorimetric detection. Reversed-phase high-performance liquid chromatography (HPLC) for the separation of amino acids is the preferred routine in many studies (Akhlaghi, Ghaffari, Attar, & Hoor, 2015), which can guarantee adequate separation and high sensitivity. However, reagent interference, instability of derivative, long preparation time and difficulties in derivatization to specific amino acids are the disadvantages of ordinary HPLC method (Zhai et al., 2015). For these reasons, Takach, O'Shea, and Liu (2014) established a high-throughput UPLC-MS/MS method to quantify 44 amino acids within 5 min in rat and mouse biological matrices. Gao et al. (2016) developed a hydrophilic interaction liquid chromatography coupled with electrospray tandem mass spectrometry for direct analysis of FAAs in the soil. The use of LC-MS/MS eliminates the derivatization step and allows for overlapping amino acid retention times thereby shortening the analysis time.

Separations of nucleosides are usually accomplished by two general methods: chromatography and electrophoresis. UPLC-MS/MS was applied to profiling twenty endogenous nucleosides and nucleotides in the cancer cell (Zhu et al., 2018). Capillary electrophoresis coupled with ultraviolet (UV) and/or mass spectrometry (MS) detection has been widely used for the analysis of nucleosides and nucleotide in the food industry (Phan et al., 2017).

There is considerable interest in the quantification of FAAs and

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nucleotides in various fields, such as nutritional quality evaluation, clinical disease diagnostics, and primary metabolites analysis. However, due to the similar polarity of amino acids and nucleotides, it is difficult to separate them completely by a C18 column. Several recent publications still quantify FAAs and 5′-nucleotides separately (Li et al., 2018; Manninen et al., 2018), the two-injection method not only waste precious samples but also increase the workload and analysis time. Quantifying FAAs and nucleotides in a single run could simplify sample preparation, reduce the use of organic reagent and improve the efficiency of equipment.

LC–MS/MS is increasingly used for quantification of complex metabolites with high resolution and sensitivity. However, co-eluting matrix components have a detrimental effect on analysis, since they can cause ion suppression or enhancement of target analyte (Van Eeckhaut, Lanckmans, Sarre, Smolders, & Michotte, 2009). Possible solutions can be used to eliminate matrix effects, which include diluting the samples, reducing inject volumes and purifying the sample before injection (Arrivault et al., 2015; Van Eeckhaut et al., 2009). However, these options might lead to an inadequate sensitivity for low abundance metabolites. Recently, stable isotope labeling (SIL) strategy has been developed for quantification of metabolites and proteomics with the MS-based platform (Arrivault et al., 2015; Zheng et al., 2017). Because stable isotopes did not change the physicochemical properties of the molecule, the stable-isotope-labeled internal standards (SIL-ISs) behave in the same way as the analytes during extraction (Arrivault et al., 2015). Furthermore, the ionization efficiencies could be considered equally. Therefore, SIL-IS could eliminate the matrix effect and correct the analytes loss during the extraction procedure. This study aimed to develop a stable isotope labeling-liquid chromatography coupled with tandem mass spectrometry (SIL-LC-MS/MS) method to quantify 20 FAAs and six 5′-nucleotides simultaneously and validated it in shiitake mushrooms. Furthermore, ten shiitake mushrooms from different cultivate areas were analyzed to validate the practicability of the proposed method.

2. Materials and methods

2.1. Materials

Ten kinds of dried shiitake mushrooms were provided by Baoshan Fu Group of Agricultural Science and Technology Do. Ltd. (Yunnan, China), which were stored in a dry condition before further analysis. All shiitake mushrooms were cultivated in the same year. Detail information was listed in the Supplemental material Table S1. Only the cap of mushroom was used in the analysis.

Methanol and acetonitrile (HPLC gradient grade) were purchased from Spectrum Chemical Mfg. Corp (Gardena, CA, USA). Inosine 5′-monophosphate (IMP, 99%), cytidine 5′-monophosphate (CMP, 99%), guanosine 5′-monophosphate (GMP, 99%), uridine 5′-monophosphate (UMP, 99%) and adenosine 5′-monophosphate (AMP, 99%) were purchased from Sigma Aldrich (St. Louis, MO, USA.). L-tryptophan (98.5%), L-methionine (98.5%), L-tyrosine (98.5%), glycine (98.5%), L-serine (98.5%), L-arginine (98.5%), L-lysine (98.5%), L-isoleucine (98.5%), L-leucine (98.5%), L-proline (98.5%), L-cysteine (98.5%), L-valine (98.5%), L-Alanine (98.5%) were purchased from Sangon Biotech (Shanghai) Co., Ltd. (Shanghai, China). Xanthosine 5′-monophosphate (XMP, 99%), glycine-^{15}N (^{15}N-Gly, 98 atom%), L-aspartic acid-^{15}N (^{15}N-Asp, 99 atom%), L-glutamic acid (99.5%), formic acid (98%, HPLC gradient grade) were obtained from Aladdin Reagent Co., Ltd (Shanghai, China). L-phenylalanine (99%), L-threonine (99%), L-glutamine (99%), L-aspartic acid (99%), L-asparagine (98.5%), L-histidine (99%) were purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). Cytarabine-$^{13}C_3$-5′-monophosphate ($^{13}C_3$-CMP, 99 atom%) were obtained from TRC (Toronto, Canada).

Stock solutions were prepared in methanol–water (50:50, v/v) and stored at −20 °C. Working solutions were prepared by using serial dilution of the stock solution with 0.1% formic acid–water (v/v) on the day of analysis. The mixed standard solution at a concentration of 200.00 ng/mL for each compound was used as quality control (QC).

2.2. Sample preparation

Samples was prepared according to a previously reported procedure with some modifications (Chen et al., 2015). Several extraction procedures and parameters were tested to maximize the extraction efficiency. The optimized procedure was described. Dried mushrooms were ground into fine powder. Ten milligrams of sample powder was suspended in 1 mL deionized water and shook for 1 min at 80 °C. After centrifugation at 22,400*g* for 10 min, the supernatant was diluted to an appropriate concentration and an aliquot of 100 μL internal standard mixture was added. Deproteinization was performed by the addition of 1:8 (v/v) ice-cold methanol and incubating at 4 °C for 10 min. After centrifuged at 22,400*g* for 10 min, the supernatant was collected and evaporated by a high-speed vacuum concentrator (Labogene, Scan Speed 40, Denmark). The condensate was redissolved in 1 mL.0.1% formic acid–water (v/v) and centrifuged at 22,400*g* for 10 min before injection. All assays were performed in triplicate.

2.3. Equipment and chromatographic condition

Quantification of FAAs and 5′-nucleotides were performed by HPLC (Shimadzu LC-20AD, Japan) coupled with a triple quadrupole mass spectrometer (AB Sciex, 4000 Qtrap System, America). The separation of analytes was achieved on an AB Sciex AAA C18 column (4.6 mm × 150 mm, 5 μm). Mobile phase A was 0.1% (v/v) formic acid in water, mobile phase B was 0.1% (v/v) formic acid in acetonitrile. The following gradient elution was applied: 0–0.5 min, 2% B; 0.5–10.0 min, 2–25% B; 10.1–12.0 min, 90% B; 12.1–18 min, 2% B. The flow rate was 0.8 mL/min. The analytical column was thermostatted at 30 °C. The injected volume was 10 μL.

Tandem mass (MS/MS) detection was performed in a positive mode using multiple reaction monitoring (MRM). The source parameters were: entrance potential (EP): 10 V; spray voltage: 5500 V; curtain gas: 20 psi; desolvation temperature (TEM): 600 °C; ion source gas1 and gas2 were set at 60 psi. High purity nitrogen was used as the gas source and curtain gas. MRM parameters were optimized by injecting individual standard solutions into mass spectrometer and listed in Table 1 and Supplemental material Table S2.

2.4. Method validation

The assay was validated for selectivity, calibration curves, limits of detection (LOD) and quantification (LOQ), recovery, precision, and stability, respectively. Validation was carried out by sample 1. The instrument blank was conducted using water and analyzed at the beginning of the sequence. A QC sample was analyzed at every 12 injections to monitor the instrument stability.

2.4.1. Selectivity

The selectivity of the method was evaluated by analyzing QC sample in MRM mode and comparing retention time and two *m/z* transitions for each analyte.

2.4.2. Calibration curves, LOD and LOQ

Mixed working solutions with 0.10, 1.00, 10.00, 20.00, 50.00, 100.00, 200.00, 500.00, 700.00, 1000.00 ng/mL were prepared to construct the calibration curves (three replicates per concentration). The calibration curves were plotted by the peak area ratio of quantified ion to specific SIL-IS versus concentration. The linearity was tested by verifying the coefficient of determination (R^2) of analytes. LOD and LOQ were evaluated as three and ten times of the signal-to-noise ratio (S/N), respectively.