



Development and validation of an HPLC-DAD method for guanosine determination in *Hericium erinaceus* water extracts for quality control applications

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Received: 12 February 2026 / Accepted: 12 March 2026 / Published Online: 23 March 2026

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Abstract A precise and reproducible high-performance liquid chromatography method with diode-array detection (HPLC-DAD) was developed for the quantification of guanosine in *Hericium erinaceus* water extract (HEW). Chromatographic separation was performed using an amide column under gradient elution, with a mobile phase comprising 0.1% trifluoroacetic acid in water and acetonitrile, and UV detection at 260 nm. The method was validated according to ICH guidelines and demonstrated excellent specificity, linearity ($r^2 = 0.9996$), accuracy (recovery 97.57%–103.91%), and precision (intra-day RSD 0.86%, inter-day RSD 2.57%). The limits of detection and quantification were 0.80 and 2.43 µg/mL, respectively. Application of the validated method revealed that HEW contained significantly higher guanosine levels than those reported in other mushroom species. This work establishes the first ICH-compliant HPLC-DAD method specifically tailored for *H. erinaceus*, providing a robust analytical tool for quality control and standardization of guanosine-rich extracts, and supporting future industrial and pharmacological applications.

Keywords Guanosine · *Hericium erinaceus* · ICH guidelines · Method validation · UV spectrum

Introduction

Mushrooms produce a wide range of bioactive compounds, including polysaccharides [1], terpenoids, triterpenoids, phenolic compounds, flavonoids, sterols, various vitamins such as B-group and D₂ [2], and nucleosides such as guanosine [3]. Oyster mushrooms (*Pleurotus* species) provide diverse health benefits, including antioxidant, anti-diabetic, and immunomodulatory properties [4]. Medicinal mushrooms such as *Polyporus umbellatus* and *P. alveolaris*, which contain various polypeptides and cytotoxic steroids, exhibit anti-cancer, anti-bacterial, anti-inflammatory, anti-fungal, immunostimulating, and hepatoprotective effects [5].

Hericium erinaceus, commonly known as lion's mane, is widely recognized for its nutritional, medicinal, and neuroprotective properties due to its array of bioactive compounds [6], including nucleosides such as guanosine [7]. To ensure the quality and efficacy of *H. erinaceus* materials, the development of validated analytical methods targeting these marker compounds is essential.

Despite growing evidence for the neuroprotective and metabolic roles of guanosine, its quantitative assessment in *H. erinaceus* has been largely overlooked. Most previous chromatographic studies have focused primarily on adenosine or cordycepin [8,9]. Analytical techniques such as high-performance liquid chromatography coupled with diode-array detection and mass spectrometry (HPLC-DAD-MS) have been developed for nucleosides in the New Zealand native mushroom *H. novae-zealandiae* [10]. However, a dedicated and validated procedure specifically for *H. erinaceus* remains unavailable. Establishing a validated method for guanosine quantification is therefore crucial for defining quality markers and supporting the functional standardization of mushroom-derived

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products. Recent metabolomics-based studies have emphasized the importance of chemical markers and regional variation for ensuring consistent quality of herbal materials, highlighting the need for reliable analytical tools in natural product standardization [11]. HPLC-DAD has proven to be a reliable and sensitive technique for the separation and quantification of nucleosides in complex matrices such as mushroom extracts, owing to its high selectivity and reproducibility [3,12]. Given that chemical compositions can vary even within the same genus, establishing an analytical procedure specifically tailored to *H. erinaceus* is crucial. This study addresses this need by developing and validating a precise, accurate, and reliable HPLC-DAD method for the quantification of nucleosides in *H. erinaceus*.

Among nucleoside compounds, guanosine is a biologically active purine nucleoside with diverse and significant neuroprotective properties [13]. It modulates glutamatergic transmission, reduces oxidative stress [9,14], and activates key neuronal survival pathways, making it a promising therapeutic candidate for neurological disorders [7-9,15]. Given its neuromodulatory and cytoprotective potential, the reliable quantification of guanosine in *H. erinaceus* is essential for establishing links between chemical composition and biological function [16-18].

Validating an HPLC-DAD method for guanosine quantification is crucial for ensuring analytical accuracy, precision, and sensitivity, particularly in light of the growing interest in functional foods and nutraceuticals derived from *H. erinaceus*. Regulatory authorities, including the Ministry of Food and Drug Safety, the Food and Drug Administration, and the European Medicines Agency, emphasize that robust method validation, strict quality control, and adherence to Good Manufacturing Practices are necessary to guarantee the safety, efficacy, and consistent production of such products before market approval [19].

This introduction lays the foundation for method development and validation by addressing both the challenges and potential of HPLC-DAD for nucleoside analysis in *H. erinaceus*. A validated quantification approach is essential for connecting the mushroom's biochemical profile to its biological activities, thereby providing a scientific foundation for future research and the standardization of high-quality functional products.

Materials and Methods

Plant materials

The dried fruiting bodies of *H. erinaceus* used in this study were cultivated and supplied by Gold Farm Agricultural Corporation, Gimcheon, Republic of Korea.

Instruments and reagents

An Agilent 1260 Infinity II Quaternary Pump (Santa Clara, CA,

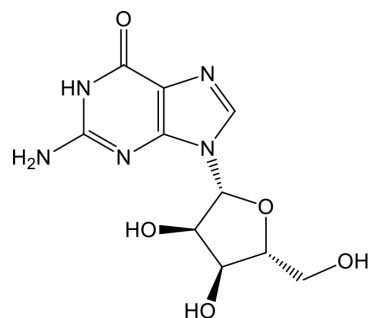


Fig. 1 Chemical structure of guanosine

USA) equipped with a pump, autosampler, TSKgel Amide-80 column (4.6×250 mm, 5 μ m; Tosoh Corp., Tokyo, Japan), and a DAD wide-range detector (Santa Clara, CA, USA) was used for the analysis. HPLC-grade acetonitrile (ACN) was purchased from Scharlau (Barcelona, Spain), and water and methanol (MeOH) were purchased from Honeywell (Burdick and Jackson, Muskegon, MI, USA). HPLC-grade trifluoroacetic acid (TFA) was provided by Thermo Scientific (Waltham, MA, USA). Guanosine was supplied by the Natural Product Institute of Science and Technology (www.nist.re.kr), Anseong, Republic of Korea (Fig. 1).

Preparation of extracts

Sequential hot-water extraction followed by spray drying was used to produce the *H. erinaceus* water extract (HEW) samples (CNS Pharm Korea Co., LTD, Seoul, Republic of Korea). Raw material was first extracted with 25 volumes of water at 97 °C for 4 h, filtered through a 25- μ m filter, and concentrated below 60 °C. A second extraction was performed using the same solvent ratio at 97 °C for 2 h, followed by identical filtration and concentration. The combined extracts were sterilized at 85 °C for 30 min and then spray-dried (inlet temperature 180±10 °C; outlet temperature 90±10 °C).

Preparation of samples and standard solutions for HPLC-DAD

A guanosine standard stock solution (1 mg/mL) was prepared by accurately weighing 10 mg of guanosine, transferring it to a 10 mL volumetric flask, and filling the flask to volume with 50% MeOH. The solution was sonicated until completely dissolved and filtered through a 0.2 μ m polyvinylidene fluoride (PVDF) syringe filter. For the test solution, 200 mg of HEW was placed in a 10 mL volumetric flask, and 8 mL of 50% MeOH and water (v/v) was added. The mixture was ultrasonically extracted and brought to volume with 50% MeOH to obtain a 20 mg/mL stock solution. This HEW solution was centrifuged (10,000 rpm, 10 min, 4 °C), and the resulting supernatant was filtered through a 0.2 μ m PVDF syringe filter. The filtered sample solution was then appropriately diluted with 50% MeOH prior to HPLC analysis.

HPLC-DAD conditions

An HPLC-DAD analysis was performed on a reversed-phase HPLC system equipped with a DAD detector using a TSKgel Amide-80 column (4.6×250 mm, 5 µm). The flow rate was maintained at 1.0 mL/min, and the column oven temperature was set to 30 °C. The sample was dissolved in 50% MeOH at a concentration of 20 mg/mL. Gradient elution was conducted using 0.1% TFA in water (solvent A) and ACN (solvent B) under the following conditions: 0-10 min, 5% A; 10-30 min, 5-45% A; 30-35 min, 45% A; 35-40 min, 45-5% A; 40-45 min, 5% A. The injection volume was 10 µL, and UV detection was performed at 260 nm. A column temperature of 30 °C was selected because preliminary evaluations indicated improved peak symmetry and retention stability compared with lower temperatures. Maintaining a constant temperature also minimized run-to-run variability, enhancing reproducibility during method validation.

Method validation

The newly proposed HPLC-DAD method was rigorously validated for specificity, linearity, limits of detection and quantification (LOD and LOQ), specificity, accuracy, and precision in accordance with the guidelines of the International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use (ICH) (Guideline IHT, 2015) [20].

The specificity of the HPLC-DAD method was evaluated to ensure that guanosine quantification was not influenced by the diluent or by any other components present in the HEW matrix. Chromatograms of three solutions were compared: the blank (50% MeOH), a guanosine standard solution (25 µg/mL), and the HEW test solution (20 mg/mL). The blank chromatogram showed no interfering peaks at the retention time of guanosine. The guanosine peak in the HEW was confirmed by matching its retention time with that of the standard. Additionally, peak purity was verified using the DAD, which demonstrated that the UV-Vis absorption spectrum of the analyte (200-400 nm) in the extract was identical to that of the guanosine standard at 260 nm.

Linearity was assessed using a five-point calibration curve. Standard solutions were prepared by serial dilution of the guanosine stock solution (1 mg/mL) with 50% MeOH to yield final concentrations of 12.5, 25, 50, 100, and 200 µg/mL. Each concentration was injected in triplicate under the optimized HPLC conditions. A calibration curve was then constructed by plotting the

mean peak area (Y) against guanosine concentration (X, µg/mL) using linear regression. The coefficient of determination (r^2) was calculated to evaluate linearity. LOD and LOQ were determined based on the standard deviation (SD) of the intercept (σ) and the slope (S) using the formulas: LOD = 3.3 (σ /S) and LOQ = 10 (σ /S).

Accuracy was evaluated using a spike-and-recovery test at three levels (low, medium, and high). Triplicate sets of unspiked *H. erinaceus* samples (used to determine endogenous guanosine concentration, B) and spiked samples, A, prepared by adding 25, 50, or 100 µg/mL of a guanosine standard (the theoretical added concentration, C), were analyzed by HPLC-DAD. Percent recovery was then calculated using the following formula:

$$\text{Recovery (\%)} = \frac{A - B}{C} \times 100$$

Precision was evaluated in terms of repeatability and intermediate precision. Prior to the assessment, a calibration curve was constructed by serially diluting the guanosine stock solution (1 mg/mL) with 50% MeOH to obtain concentrations ranging from 12.5 to 200 µg/mL. The HEW stock solution (20 mg/mL) was used as the test sample. Repeatability was determined by Analyst A, who performed five replicate injections ($n=5$) of the sample solution. Intermediate precision was assessed by Analyst B, who independently performed the same procedure using five additional independent replicates to evaluate inter-analyst variability. Precision was expressed as the relative standard deviation (RSD, %) of the quantitative results obtained from these measurements.

Calibration curve

A calibration curve was constructed by plotting the concentrations of the standard solutions against their corresponding peak areas. The correlation coefficient (r^2) was used to confirm the linearity of the curve, which was subsequently applied to determine guanosine concentrations in the samples. Calibration functions were established based on peak area (Y), concentration (X, µg/mL), and the mean $\pm$ SD of triplicate injections ($n=3$) (Table 1).

Results and Discussion

The development and validation of the proposed HPLC-DAD method for guanosine quantification in HEW yielded reliable and

Table 1 Calibration curve and validation parameters for guanosine

Compound	t_R	Range (µg/mL)	Calibration equation	r^2	LOD (µg/mL)	LOQ (µg/mL)
Guanosine	23.9	12.5-200	$Y = 23.644X - 23.166$	0.9996	0.80	2.43

t_R = retention time

Y = peak area, X = concentration of the standard (µg/mL)

r^2 = correlation coefficient for the five data points in the calibration curve ($n=3$)

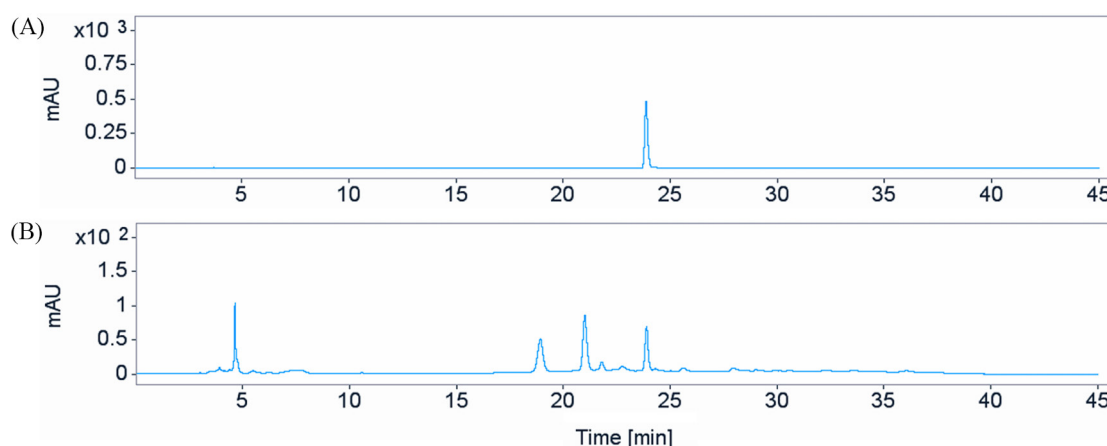


Fig. 2 HPLC-DAD chromatograms of (A) standard guanosine and (B) HEW

robust results.

Specificity was confirmed by comparing the UV spectra of the guanosine standard with those of the HEW sample, which showed identical absorption profiles. The chromatograms further demonstrated that the guanosine standard and the guanosine peak in the test solution shared the same retention time (Fig. 2). Importantly, no interfering peaks originating from the mushroom matrix were detected at or near the guanosine retention time, confirming high specificity and effective chromatographic separation (Fig. 3). The sharp and symmetrical peak shape also supports the robustness of the chromatographic conditions and demonstrates excellent selectivity for guanosine, enabling precise quantification even within the complex mushroom matrix. Additionally, peak purity was verified using the DAD, which demonstrated that the extract was identical to that of the guanosine standard at 260 nm (Fig. 3). Furthermore, the excellent spike-and-recovery results effectively excluded the possibility of significant co-elution (Table 2). While this approach robustly supports specificity according to ICH guidelines, future validation efforts incorporating LC-MS could provide orthogonal mass-based confirmation to completely rule out minor co-eluting impurities within such a complex natural product matrix.

The method exhibited excellent linearity across the tested concentration range, with coefficients of determination (r^2) between 0.9996 and 0.9997 ($n=3$), meeting the acceptance criteria for analytical method validation (Table 1). The LOD and LOQ were 0.80 and 2.43 $\mu\text{g/mL}$, respectively, indicating that the method is highly sensitive (Table 1).

Accuracy was evaluated through spiking experiments at different concentration levels. The recovery rates for guanosine in spiked HEW ranged from 97.57% to 103.91% ($n=3$) (Table 2). These results confirm that the method is highly accurate and reliable for quantifying guanosine in the complex *H. erinaceus* matrix. For intra-day precision ($n=5$), the mean guanosine content was 1.446

mg/g with an RSD of 0.86%. For inter-day precision ($n=5$), the mean content was 1.447 mg/g with an RSD of 2.57% (Table 3). Both RSD values fall well within acceptable limits, demonstrating that the method is precise and reproducible for routine analysis.

A previous study reported that guanosine concentrations in other mushrooms, including water extracts of *Lactarius salmonicolor* (0.368 mg/g), *Craterellus fallax* (0.191 mg/g), and *Tuber borchii* (0.204 mg/g), were considerably lower (Camcı & Buyuktuncel, 2025). The concentration of guanosine measured in HEW in this study was 1.45 mg/g. As reported in previous studies, guanosine content varies widely among edible fungi [21]. The reliable quantification of this neuroprotective nucleoside in food and supports the need for a precise analytical method for its quality control [22]. It should be noted that while guanosine is a primary metabolite and lacks the strict species-specificity typically required for taxonomic identification of botanical raw materials, its selection as a single quantitative marker in this study is robustly justified. Given its significant neuroprotective bioactivity, quantifying this purine nucleoside in *H. erinaceus* is essential for functional quality control. This approach ensures the batch-to-batch consistency and standardization of the extract's pharmacological efficacy. It is important to note that levels of bioactive compounds such as guanosine can vary depending on factors including mushroom strain, cultivation conditions, anatomical part used, and extraction parameters [23,24]. When compared with previously reported HPLC methods for nucleosides in other mushroom species, the validation parameters obtained in this study were comparable to or better than those in earlier reports, confirming the method's suitability for routine analytical applications.

A straightforward and reproducible HPLC-DAD method was developed for the quantification of guanosine in HEW. Validated according to ICH guidelines [25], this study fills an important methodological gap by establishing a robust and reliable procedure specifically tailored to *H. erinaceus*. Although analytical techniques

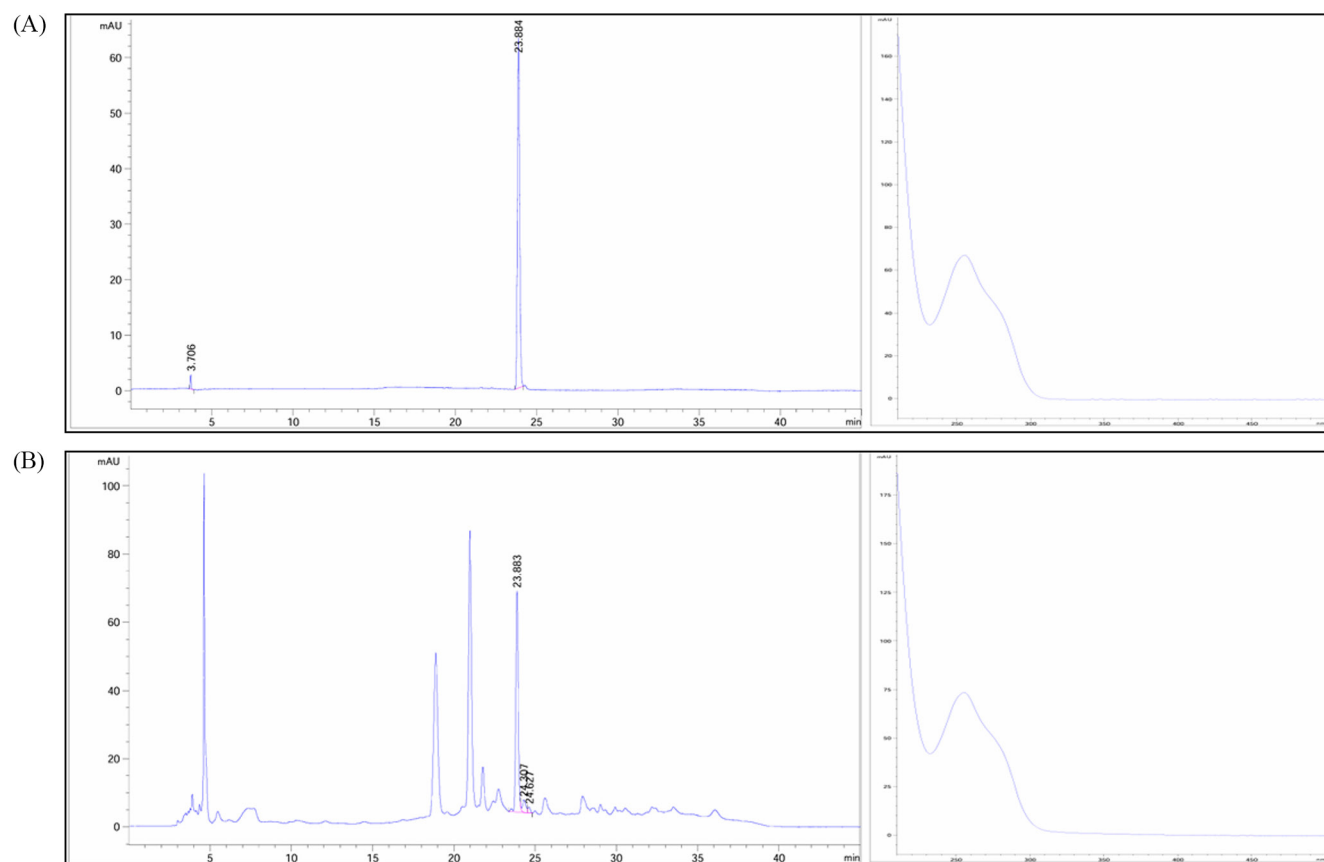


Fig. 3 HPLC-DAD chromatograms and UV spectra at 260 nm of (A) standard guanosine and (B) the guanosine peak in HEW

Table 2 Accuracy analysis statistics for the determination of guanosine

Compound	Spike amount (mg/mL)		Recovery (%)			Average (%)	RSD (%)
	Sample	Guanosine	1 st	2 nd	3 rd		
Guanosine	10.0	0.025	103.07	103.61	103.91	103.53	0.41
		0.05	103.69	103.33	103.02	103.35	0.33
		0.1	97.57	97.79	97.85	97.74	0.15

have been reported for related mushroom species, including an HPLC-DAD-MS method for nucleosides in *Hericium* sp. [10], no dedicated and fully validated method for *H. erinaceus* has previously been available. This study represents the first validated analytical method specifically for the quantification of guanosine in *H. erinaceus*. The present study appears to be the first report of an ICH-compliant HPLC-DAD procedure capable of accurately quantifying guanosine in HEW, achieving full analysis within an efficient 45-minute runtime. To demonstrate its practical applicability, the method was successfully applied to commercial HEW, where a substantial guanosine peak was detected and identified through retention time matching with an authentic standard at 260 nm [26].

This validated procedure provides the high level of reproducibility and consistency required for routine quality control, extending its

relevance beyond basic standardization. Its application is critical for optimizing industrial production processes and for the formulation of *H. erinaceus*-based functional foods. The method can be directly employed to guide the optimization of extraction parameters, which have been shown to significantly enhance the yield of bioactive compounds such as guanosine. Furthermore, given that guanosine biosynthesis is influenced by cultivation and stress conditions, this robust method may also facilitate the screening and selection of high-guanosine strains for industrial use [27]. Moreover, the analytical framework established herein serves as an important benchmark. A key advantage of this method is its use of an LC-MS compatible mobile phase, which enables seamless integration with more advanced analytical platforms. Future research should capitalize on this compatibility to move beyond basic quantification

Table 3 Intra- and inter-day precision for the determination of guanosine

Sample concentration (mg/mL)	Guanosine content (mg/g)	
	Intra-day (<i>n</i> =5)	Inter-day (<i>n</i> =5)
10.0	1.449	1.424
	1.435	1.417
	1.430	1.419
	1.458	1.484
	1.454	1.491
Average content (mg/g)	1.4455	1.4471
SD	0.0124	0.0372
RSD (%)	0.8551	2.5724
Average content (mg/g)	1.4463	
SD	0.0011	
RSD (%)	0.0779	

and pursue the structural elucidation of guanosine derivatives from *H. erinaceus*. Such work would connect analytical chemistry with functional mycology by linking specific structural variations to bioactivity. This approach is essential for deepening our understanding of pharmacological mechanisms and for advancing the development of *H. erinaceus* as a standardized functional food or nutraceutical.

The validated HPLC-DAD method established in this study offers a sensitive, accurate, and reproducible approach for guanosine quantification in *H. erinaceus*. In addition to ensuring analytical precision, the method supports industrial standardization and quality control of *H. erinaceus*-based nutraceuticals. Its LC-MS compatibility also lays the groundwork for future research on guanosine derivatives and their pharmacological significance. To the best of our knowledge, this is the first study to establish an ICH-validated HPLC-DAD method specifically optimized for quantifying guanosine in HEW.

A validated and optimized HPLC-DAD analytical method was established for the quantification of guanosine in HEW. Validation following ICH guidelines confirmed the method's specificity, linearity, accuracy, and precision, demonstrating its reliability for quantifying guanosine within a complex mushroom matrix without analytical interference. The analysis showed that HEW contains a substantially higher concentration of guanosine than other reported medicinal mushrooms, highlighting *H. erinaceus* as a significant source of this bioactive nucleoside. Accordingly, this method provides an essential tool for raw material standardization and the quality control of commercial nutraceutical products, supporting the quantitative optimization of extraction and production processes. Moreover, the use of an LC-MS-compatible mobile phase creates a foundation for future metabolomic investigations and structural elucidation studies. This compatibility will allow for the structural

characterization of guanosine derivatives and the exploration of their structure-activity relationships, thereby connecting analytical chemistry with the functional evaluation of *H. erinaceus*.

Acknowledgments This study was supported by technical assistance from Gyeonggido Business & Science Accelerator (GBSA), Suwon, Republic of Korea.

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