Food Chemistry 278 (2019) 729–737

Contents lists available at ScienceDirect

Food Chemistry

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

Development and validation of an HPLC-DAD-MS method for determination of four nucleoside compounds in the New Zealand native mushroom *Hericium* sp.

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ARTICLE INFO

Chemical compounds:
Cytidine (PubChem CID: 6175)
Uridine (PubChem CID: 6029)
Adenosine (PubChem CID: 60961)
Guanosine (PubChem CID: 6802)

Keywords:
Nucleoside
Cytidine
Uridine
Adenosine
Guanosine
HPLC
Hericium sp.

ABSTRACT

To identify and quantify the content of nucleoside compounds in the New Zealand native edible mushroom *Hericium* sp., a high-performance liquid chromatography coupled with a triple quadrupole detector mass method was developed and validated. Four nucleoside substitutes, namely cytidine, uridine, adenosine, and guanosine, were identified. Optimization was conducted to study the effect of extraction method type, solvent pH, and extraction time. The optimal conditions were obtained using ultrasonic treatment in water at pH 3.8 for 30 min. For chromatographic separation, a C_{18} column was applied using 0.1% formic acid (pH 3.4) as the mobile phase with detection at 260 nm. The total concentration of the four nucleoside compounds was high, at 10.7 mg/g dry weight, indicating a potential benefit for human health. The excellent validation results based on selectivity, linearity, precision, accuracy and robustness revealed the reliability of the newly developed analytical method, which could be applied routinely in research laboratories.

1. Introduction

Edible mushrooms are good sources of nutraceutical compounds, including polysaccharides (β-glucans, dietary fibers), terpenes, peptides, glycoproteins, minerals, unsaturated fatty acids, and antioxidants (e.g., phenolic compounds, tocopherols and ascorbic acid) (Rathore, Prasad, & Sharma, 2017). In addition, mushrooms are also known to exhibit health effects based on their antimicrobial activities to viruses, bacteria and fungi, as well as possessing anti-inflammatory, antitumor, antidiabetic, antithrombotic, hepatoprotective and hypolipidemic properties (Rathore et al., 2017).

Hericium sp. (pekepekekiore) is a New Zealand native edible mushroom used in Māori culture (Fuller, Buchanan, & Roberts, 2004). Although yet to be named, it is a member of *Hericium* genus which has been a research focus among medicinal mushrooms for decades. From this genus, *H. erinaceus,* an indigenous mushroom in Eastern Asia, is widely known for its nutritional and culinary value, as well as its medical uses for centuries, e.g., for treating neurasthenia and general debility (Büssing & Hübner, 2009). Scientific studies have validated the presence of several bioactivities from *H. erinaceus*, including neuroprotection (Zhang et al., 2016), liver protection (Cui et al., 2016), anti-oxidant (Jiang, Wang, & Zhang, 2016), antimicrobial (Liu, Li, Shang, Zhang, & Tan, 2016), anticancer, immunomodulating (Li et al., 2014), as well as anti-inflammation activity (Yao et al., 2015). In addition to most of the above, several studies have focused on the polysaccharide components of this mushroom (Chen et al., 2016; Cheng, Tsai, Lien, Lee, & Sheu, 2016) that it shares with many other culinary-medicinal mushrooms. Furthermore, literature has shown that some bioactive compounds have been found exclusively present in the *Hericium* genus and this has attracted the attention of the pharmaceutical industry. These compounds include erinacine (Kawagishi et al., 1996; Kenmoku, Shimai, Toyomasu, Kato, & Sassa, 2002; Saito et al., 1998; Tsai-Teng et al., 2016; Wolters, Schembecker, & Merz, 2015), hericenone (Ma, Ma, & Ruan, 2012), and erinacol (Kenmoku, Tanaka, Okada, Kato, & Sassa, 2004).

Although many studies have been performed on the *Hericium* genus, especially on *H. erinaceus*, much less is known of the New Zealand *Hericium* sp. While the health benefits and the underlying mechanisms of polysaccharides from the *Hericium* genus have been extensively

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https://doi.org/10.1016/j.foodchem.2018.11.115
Received 14 September 2018; Received in revised form 9 November 2018; Accepted 22 November 2018
Available online 30 November 2018

investigated, little attention has been paid to the nucleosides that may be present in these mushrooms. Nucleosides are known to participate in a large number of biological processes. They thus play a vital role in the storage, transfer, and expression of genetic information. Moreover, nucleosides, such as cytidine, uridine, adenosine, guanosine, thymidine and inosine, are involved in other metabolic functions, such as forming part of biosynthetic routes, operating in the transfer of chemical energy, as components of some co-enzymes, and playing an important role as biological regulators (Domínguez-Álvarez et al., 2017). Nucleoside compounds were reported to be one of the most important active ingredients in mushrooms with medicinal effects (Büssing & Hübner, 2009; Gao et al., 2007; Ranogajec, Beluhan, & Šmit, 2010; Rathore et al., 2017; Yang, Lv, Zhang, & Xia, 2012). As an example, nucleosides are regarded as the main bioactive ingredients in *Cordyceps sinensis*, a very popular traditional Chinese medicine with highly medicinal and commercial value (Shashidhar, Giridhar, Udaya Sankar, & Manohar, 2013). They were reportedly found in the regulation and modulation of various physiological processes in the human body *via* the purinergic and/or pyrimidine receptors (Jacobson et al., 2004). Many potential bioactivities such as relieving physical fatigue, induction of nerve growth factor synthesis, anti-tumor growth and immunomodulation, have been attributed to these active components (Domínguez-Álvarez et al., 2017). They have also been reported to contribute to the improvement of memory and learning abilities (Kuś, Włodarczyk, & Tuberoso, 2018). Nucleosides were considered to assist brain function, affect immunomodulation, influence fatty acid metabolism contributing to iron absorption in the gut, and improve gastrointestinal tract repair after damage (Schlimme, Martin, & Meisel, 2007; Yamamoto, Wang, Adjei, & Ameho, 1997). In clinical applications, single use or combinations of nucleoside analogs have been applied to the treatment of more than 20% patients undergoing anticancer chemotherapy (Shelton et al., 2016). Based on literature about the *Hericium* genus, only one nucleoside compound, namely adenosine, has been reported from submerged cultures of *H. erinaceus* (Ofosu, Yu, Wang, & Li, 2016). None was reported for other species in this genus. The study of nucleosides in this native New Zealand *Hericium* sp. provides scientific data for this chemical constituent and concerning bioactivity of this species, which currently has limited information and application.

The aim of the present study was to identify, develop and validate a reliable analytical method that will provide accurate qualification by HPLC–electrospray ionization–triple quadrupole mass spectrometry [(HPLC–ESI–MS (TQ)] and quantification by HPLC–DAD of nucleoside compounds in *Hericium* sp. The effect of different sample preparation parameters, i.e. types of extraction, solvent pH, and extraction time, on the yield of nucleoside compounds was investigated using a multilevel orthogonal array design (OAD) strategy.

2. Materials and methods

2.1. Materials

Nucleoside reference standards: cytidine, uridine, adenosine, guanosine, inosine, adenine, beta-thymidine, guanine, and xanthosine, each with purity > 98% were purchased from Sigma-Aldrich, Christchurch, New Zealand. Acetonitrile (ACN, HPLC grade), methanol (MeOH, HPLC grade) was obtained from Macron Chemicals, Radnor, PA; formic acid (chromatography grade) and potassium phosphate monobasic (KH_2PO_4) were from Sigma-Aldrich, Auckland, New Zealand; and highly purified water (H_2O) was prepared by a Milli-Q purification system (Thermo Fisher, Waltham, MA). Freshly cultivated specimens of *Hericium* sp. Auckland, May 2016 (ICMP 21483) provided by a mushroom grower in Napier, New Zealand, were freeze dried and powdered prior to analysis.

2.2. Preparation of standard stock and working solutions

Standard stock solution was prepared by dissolving cytidine (20 mg), uridine (20 mg), adenosine (30 mg) and guanosine (20 mg) into 25 mL water (thereby containing 800 μg of cytidine, 800 μg of uridine, 1200 μg of adenosine and 800 μg of guanosine per mL). Calibration standards were prepared by measuring appropriate volumes of the resulting solution and diluting to the required concentration with highly purified water. The calibration working solutions were prepared daily and the stock solutions were stored at 4 °C prior to experiments. The rest of the five reference standards, inosine, adenine, beta-thymidine, guanine, and xanthosine were dissolved in water and prepared the same way as the above four standards to make concentrations of 30 μg/mL each.

2.3. Instrumentation and conditions

The chromatographic analyses were carried out using an Agilent 1100 HPLC system consisting of an in-line vacuum degasser G1322A, quaternary pump G1311A, autosampler G1313A, column oven G1316A, and a diode array detector G1315A. The ChemStation for LC & LC/MS System was used for data acquisition and analysis. A reversed-phase (RP) Waters SymmetryShield C_{18} column (250 × 4.6 mm, 5 μm) was used for separation at 40 °C. Formic acid at 0.1% was employed as mobile phase at a flow rate of 1.0 mL/min. The detection wavelength was 260 nm and the injection volume was 6 μL.

Qualitative analysis was performed on an Agilent 1290 HPLC system consisting of a quaternary pump G4204A, multi-sampler G7167B, column oven G1316C, diode array detector G4212A, and a triple quadrupole LC/MS 6460. The ChemStation for LC & LC/MS System was used for data acquisition and analysis. The mass spectrometer conditions were: electrospray ionization source; positive polarity; nitrogen drying gas; drying gas temperature – 250 °C; drying gas flow – 11 L/min; capillary voltage – 3500 V; collision energy – 35 eV. The scanning was established in production mode. All the other conditions were the same as for HPLC as above.

2.4. Optimization of sample extraction

The orthogonal array design (OAD) design was applied in this study focusing on the effects of the three most important variables: A: solvent pH (2.7, 3.8, 4.8, and 5.8); B: types of extraction (ultrasonic at room temperature, 35 kHz and water bath 100 °C); C: extraction time (20 min, 30 min, and 45 min). The experiment plan based on OAD and the level settings of individual factors are shown in Table 1. The total four nucleoside compounds concentration (TNC) of each trial was calculated and was used to evaluate the efficiency and to optimize the experimental conditions. All samples were tested in three repeats.

Samples (200 mg) were dissolved in 25 mL of deionized water at different pH values (using formic acid to adjust the pH) and treated in an ultrasonic bath (Type DT106; Bandelin, Berlin, Germany) or water bath (100 °C) for different extraction times (Table 1). The extraction solutions were filtered and passed through a 0.22-μm membrane (MicroAnalytix, Auckland, New Zealand). Samples were kept in the dark and at 4–5 °C until HPLC analysis.

2.5. Analytical method validation

Analytical method validation was conducted as described in International Conference on Harmonization (ICH, 2005); Association of Official Analytical Chemists (AOAC, 2002); and US Food and Drug Administration (FDA, 2000).

2.5.1. Selectivity and system suitability

The purity of the peaks from the *Hericium* sp. extract and the four reference standards were evaluated by checking the DAD spectra at three different points of the peaks. System suitability was assessed by examining the outcomes from resolution; selectivity; USP tailing; number of theoretical plates; and the relative standard deviation, RSD, of the retention times of the peaks obtained.

Table 1
Optimization of nucleoside extraction.

A) Orthogonal array design (OAD) and results for the three variables studied

Exp No.	A (pH)	B (method)	C (min)	TNC (mg/g DW)
1	5.8	Ultrasonic	45	9.54 ± 0.10
2	2.7	Bath	20	2.76 ± 0.13
3	3.8	Bath	30	6.00 ± 0.08
4	3.8	Ultrasonic	45	10.08 ± 0.09
5	5.8	Bath	20	7.57 ± 0.08
6	2.7	Ultrasonic	20	8.12 ± 0.04
7	4.8	Ultrasonic	30	9.21 ± 0.10
8	4.8	Ultrasonic	20	9.21 ± 0.05
9	4.8	Bath	20	4.69 ± 0.06
10	2.7	Bath	45	2.82 ± 0.05
11	2.7	Ultrasonic	30	8.10 ± 0.08
12	5.8	Ultrasonic	20	9.55 ± 0.11
13	4.8	Bath	45	4.72 ± 0.06
14	3.8	Bath	20	5.92 ± 0.06
15	3.8	Ultrasonic	20	9.94 ± 0.05
16	5.8	Bath	30	7.50 ± 0.09

A, pH value of solvent; B, type of extraction method, ultrasonic treatment or bath treatment; C, extraction time. TNC indicates total nucleosides content reported as average total amount in dry weight (mg/g DW) (n = 3).

B) F values from the OAD optimization

	Sum of squares	*F*-value
A	15.405	7.372*
B	131.832	63.083*
C	1.743	0.834

A, pH value; B, type of extraction; C, extraction time.
* $p < 0.05$.

2.5.2. Linearity

The mixed standard solutions comprising the four reference standards were prepared at five concentrations: from 6.504 to 48.780 μg/mL for cytidine; from 6.44 to 48.30 μg/mL for uridine; from 9.60 to 72.00 μg/mL for adenosine; and from 6.488 to 48.660 μg/mL for guanosine. The five standard solutions were analyzed one after another in the HPLC–DAD according to their concentrations from the lowest to the highest for calibration purposes. The corresponding peak areas of cytidine, uridine, adenosine and guanosine were obtained from the built-in ChemStation software. These standards were injected and analyzed in duplicate. Data were submitted to regression analysis using the least squares method.

2.5.3. Precision

The inter-day precision (intermediate precision) was studied by comparing the analysis performed on two different days, whereas assay for the intra-day variation was performed on the same day. The precision of the method was expressed in terms of % RSD (relative standard deviation) of peak area. Six replicates of a 200 mg sample were analyzed, to evaluate the precision of the developed method.

2.5.4. Accuracy

Recovery was determined by spiking known quantities of the standards into the sample before extraction, at three different concentration levels within the analytical concentration range of the curve. Three independently prepared samples at each concentration were analyzed using the method developed. The accuracy of the method was expressed as percent recovery (100% × calculated concentration/theoretical concentration).

2.5.5. Limit of quantitation (LOQ) and limit of detection (LOD)

The limit of detection (LOD) and limit of quantitation (LOQ) were calculated from the equations: LOD = 3.3σ/S and LOQ = 10σ/S, respectively, where σ is the standard deviation of the y-intercept of the regression lines and S is the slope of the calibration curve.

2.5.6. Method robustness

The samples (n = 6) were prepared based on the optimized conditions after the trial as stated in Section 2.4 and conditions with small, deliberate variations in chromatography parameters as follows: using an alternative column (Waters Symmetry RP-C18 (250 mm × 4 mm i.d., 5 mm)); an altered column temperature (30 °C); various mobile phase pH values (condition 1: formic acid solution pH 2.6; condition 2: formic acid solution pH 4.2); a different mobile phase composition (methanol: 0.1% formic acid, 2:98) and a diverse flow rate (condition 1: 0.85 mL/min, condition 2: 1.20 mL/min). The total concentrations of four nucleoside compounds were analyzed by ANOVA and Tukey test, to provide an indication of method robustness during normal usage.

2.6. Statistical analysis

All results were expressed as the mean of at least three replicates ± SD. The resulting data were subjected to a one-way ANOVA performed with IBM SPSS Statistics 23. Differences between means at $p < 0.05$ level were considered significant. Excel 2013 (Microsoft Office) was also used for statistics and a 5% level of significance was selected.

3. Results and discussion

3.1. Optimization of sample extraction

As nucleosides are highly polar compounds, liquid–liquid extraction applying water as the extraction solvent is considered to be the appropriate way to extract the analytes from the mushroom sample. In addition, as nucleosides are usually alkaline in nature, the yields of nucleoside compounds at different pH values of solvent should be compared, to study the extraction efficiency. Furthermore, to increase extraction efficiency, it is also common to conduct extraction assisted by temperature (Phat, Moon, & Lee, 2016; Ranogajec et al., 2010) and other parameters such as ultrasonic treatment (Gao et al., 2007; Yang et al., 2012). Therefore, extraction type, pH of solvent and extraction time, were investigated to optimize the extraction efficiency. Orthogonal array design (OAD), a type of factorial design, has been used to optimize experimental conditions with fewer numbers of experiments (Guo et al., 2007; Wang, Liao, Lee, Huang, & Tsai, 2008). OAD is particularly advantageous in rapid characterization of complicated processes using fewer experiments. The OAD optimization of the extraction conditions was based on the maximum total nucleoside compounds yield of the sample. Three parameters — A (solvent pH value), B (type of extraction) and C (extraction time) — were chosen from other measurements (for example, ratio of water/ethanol, ratio of sample/solvent, data not shown) prior to OAD optimization. This helped narrowing down the ranges of parameters tested. After implementing the 16 experimental trials based on the OAD 16 matrix, the average amount of total nucleosides at all three individual factors was obtained at the set levels (Table 1A).

To verify whether the effect of individual factors on extraction efficiency is statistically significant, an ANOVA test was applied to analyze and interpret the experimental data obtained from the OAD optimization. The statistical significance of each extraction parameter was evaluated by the *F*-value and the results were summarized (Table 1B). Based on the mean extraction yields of total nucleosides, the impact of the three parameters decreased in the order of: B (type of extraction) > A (solvent pH) > C (extraction time), according to the *F*-values. The type of extraction (B) was found to be the most significant

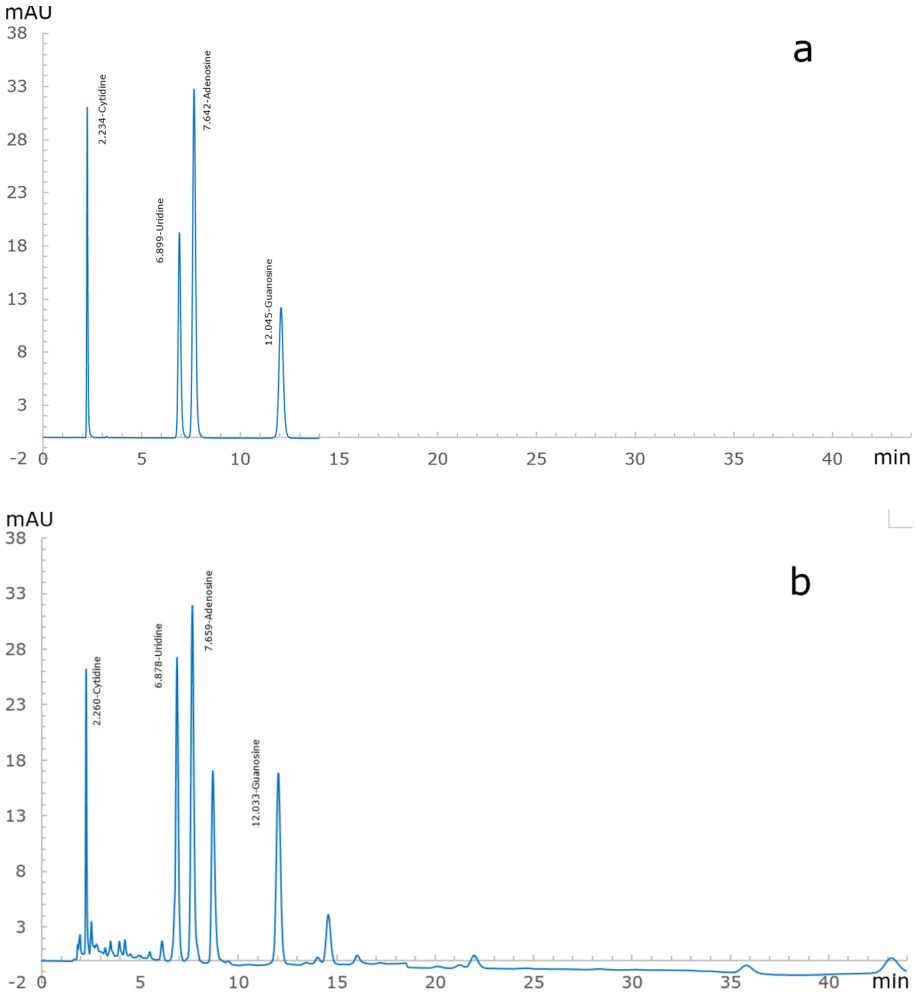


Fig. 1. Chromatogram of: a) standards solution of cytidine, uridine, adenosine and guanosine, and b) the identified nucleoside compounds in the *Hericium* sp. extract. (Chromatographic conditions: Waters SymmetryShield RP-18 column (250 mm × 4 mm i.d., 5 mm), detection at 280 nm, injection volume 6 µL, flow rate 1.0 mL/min, 40 °C, 0.1% formic acid as mobile phase).

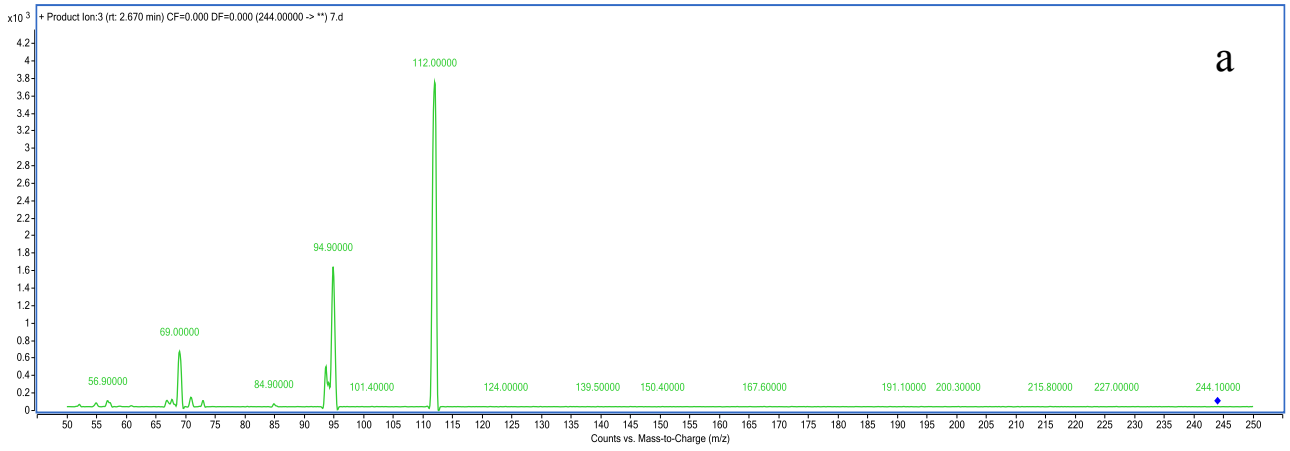
parameter (at the 95% confidence level), followed by pH value (A). No significant difference was observed in the extraction time (at the 95% confidence level) compared to the other two experimental variables ($p > 0.05$). The total nucleoside concentrations (TNC) are as shown in Table 1A. Considering the extraction efficiency, the cost of energy and the feasibility of the experiment, the optimal conditions for extraction were therefore obtained by using 25 mL of water at pH 3.8 (adjusted by formic acid) and applying ultrasonic treatment for 30 min.

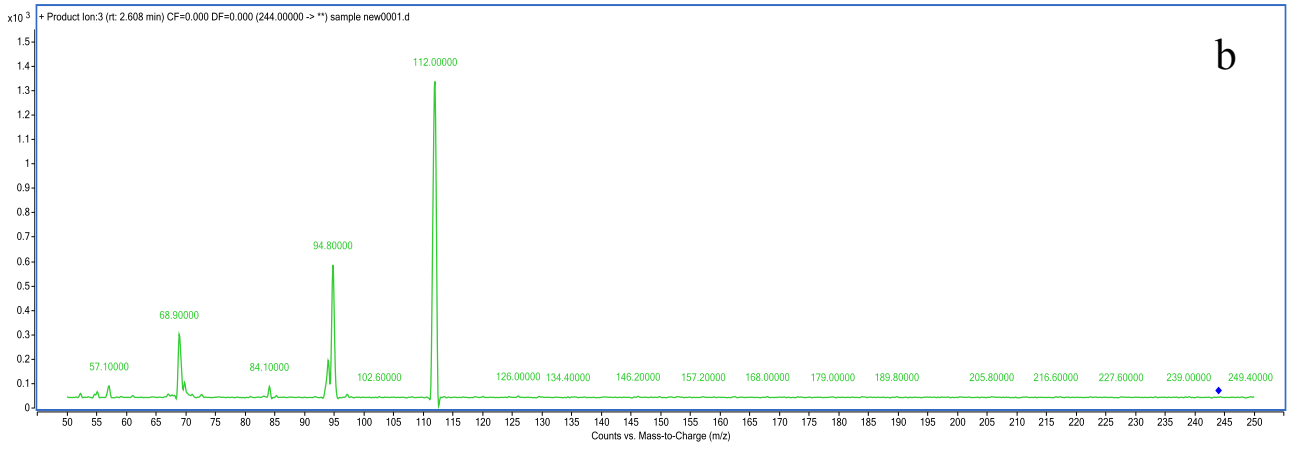
3.2. HPLC method development

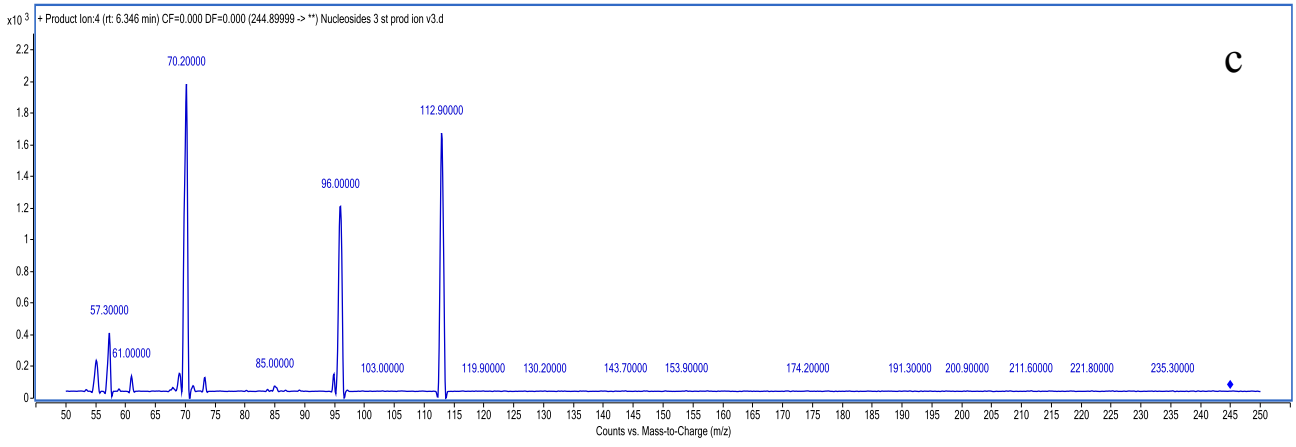
For development of the analytical method, previous research concerning the determination of nucleoside compounds from other mushrooms (Ranogajec et al., 2010) was consulted. This included the study of Ofosu et al. (2016) where one of the nucleoside compounds, adenosine, was reported. The HPLC mobile phase gradient was optimized on a selected Waters column. To achieve the best separation and shortest analysis time, several mobile phase systems were tried and the MeOH: 0.05% formic acid system with an optimal gradient of 100% water phase was found to achieve better performance than other mobile phase systems, including the MeOH: water system and the MeOH: KH_2PO_4 solution system. Two Waters C18 columns, Symmetry and SymmetryShield, were put into trials and the latter was chosen because of its high endurance in the 100% water phase. A total of nine nucleoside compound reference standards were prepared to aid the identification of nucleoside compounds in our sample. Out of those, four nucleoside compounds were identified in the sample, namely cytidine, uridine, adenosine and guanosine. The other five nucleosides (inosine, adenine, beta-thymidine, guanine, and xanthosine) were not detected in the *Hericium* sp. extract. The representative chromatogram of the standards and the *Hericium* sp. extract are as shown in Fig. 1a and b.

3.3. Nucleoside compounds structure confirmation

From Fig. 1b, four compounds were eluted from the *Hericium* sp. extract, holding the same retention times as those of the four reference standards, namely cytidine, uridine, adenosine and guanosine. Since only adenosine had been reported to be present in the culture medium of *H. erinaceus* (Ofosu et al., 2016), an HPLC–TQ method was applied to confirm the structure of the four compounds identified from the HPLC, by comparing the fractions from the targeted peaks in the sample with those of the related standards (Fig. 2a–h). It can be seen that there were three fragments (112.0, 94.8 and 68.9) produced at 2.6 min by the eluted compounds from the *Hericium* sp. extract (Fig. 2a), similar to those from the cytidine standard (112.0, 94.9 and 69.0, Fig. 2b). In addition, the intensity ratios of these fragments from the *Hericium* sp. extract were also similar to those from the cytidine. For example, the intensity ratio between the 112.0 and 94.8 fragments from the *Hericium*







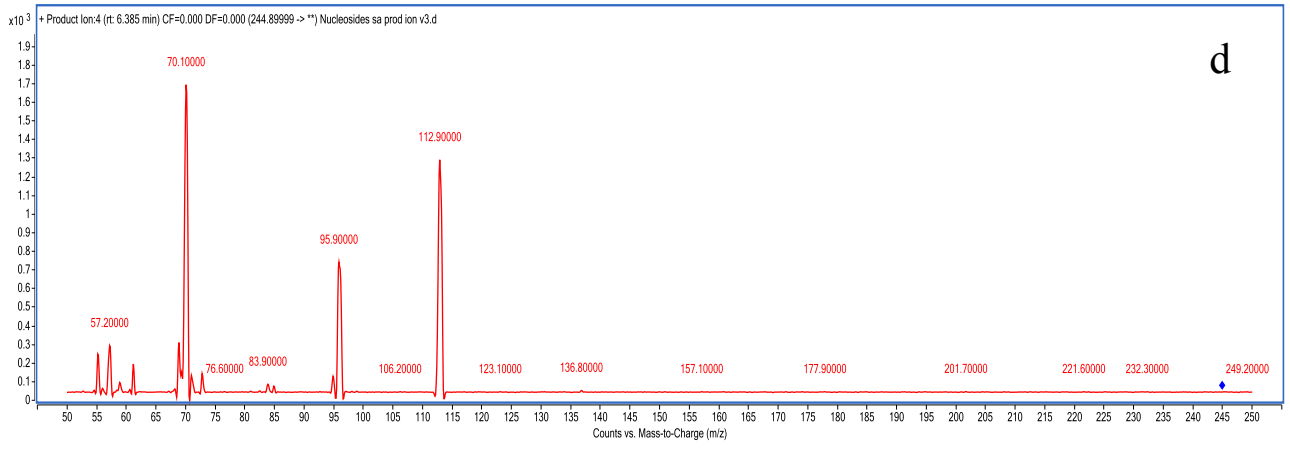


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Fig. 2. Ion spectra of four standards and the related targeted compounds in the *Hericium* sp. extract. From top to bottom, a. cytidine standard (M 243.2, RT 2.67 min); b. *Hericium* sp. extract (M 243.2, RT 2.61 min); c. uridine standard (M 244.9, RT 7.00 min); d. *Hericium* sp. extract (M 244.9, RT 6.98 min); e. adenosine standard (M 268, RT 7.74 min); f. *Hericium* sp. extract (M 268, RT 7.76 min); g. guanosine standard (M 284, RT 12.13 min); and h. *Hericium* sp. extract (M 284, RT 12.16 min).

sp. extract was about 2, which was very close to that obtained from the reference cytidine standard between 112.0 and 94.8. The same results were observed for the other three nucleoside compounds (uridine, adenosine and guanosine). This provides strong evidence that the four eluted compounds that were sharing the same retention time as the standards also had the same chemical structures as the standards. In this way, cytidine, uridine, adenosine and guanosine were identified from the *Hericium* sp. extract using the HPLC–TQ method.

3.4. Analytical method validation

3.4.1. Selectivity and system suitability

The purity of the four peaks from the *Hericium* sp. extract and the related reference standards was reviewed by comparing the DAD spectra obtained from three points (rising, apex and declining) of the peak. The results showed that the spectra of the three points were identical, revealing that no other compounds co-eluted. The system suitability indicated reliable chromatographic conditions (Supplementary Material 1) with the outcomes of resolution > 2; selectivity > 1; USP tailing < 1.5; number of theoretical plates > 7000, according to the US FDA (FDA, 1994).

3.4.2. Linearity, LOQ and LOD, precision and accuracy

Five concentrations of mixed reference solutions were prepared and analyzed for cytidine (6.504–48.780 μg/mL), uridine (6.44–48.30 μg/mL), adenosine (9.6–72.0 μg/mL), and guanosine (6.488–48.660 μg/mL) in duplicate, to examine linearity. The linear regression formula and the correlation coefficient were acquired (Table 2). A linear model was achieved according to the figure of the calibration curve (area *versus* concentration; Supplementary Material 2) and residuals, which satisfied the requirement of homoscedasticity and were randomly scattered (Supplementary Material 3). The statistical data for regression are shown in Table 2.

3.4.3. Precision and accuracy

The repeatability (i.e. the precision under the same conditions over a short interval of time, namely one day) and the intermediate precision (i.e. the variation on two different days) were investigated and calculated. The low values of % RSD (< 2.70%) revealed good precision of the developed HPLC method in terms of inter- and intra-day variation (Table 3).

The results in Table 3 also show that the method is accurate within the recovery range of 97%–103% (with RSDs less than 3.00%), which falls within the generally accepted range of 95–105% and could be considered as high accuracy. There is no official criterion set for recovery rate in a quantitative analysis. The AOAC guideline (AOAC, 2002) directs that the recovery rates rely on the analyte concentration in the sample, and the lower the concentration, the bigger the tolerance. The reasonable recovery rate ranges from 95 to 102%, 92 to 105%, 90 to 108% and 85 to 110% for analyte at concentrations of 10%, 1%, 0.1% and 0.01%, respectively. The recovery results (97%–103%) gained from this study therefore meet the guideline.

3.4.4. Method robustness

The robustness of this newly developed analytical procedure is measured by its capacity to remain unaffected by small and deliberate variations in method parameters as stated in Section 2.5.6. Impacts on the chromatograph performance and nucleoside concentrations were evaluated by slightly changing the column type, column temperature, mobile phase composition, pH of the mobile phase, and flow rate. Small changes in column temperature, pH of mobile phase, and flow rate did not result in statistically significant differences in the total nucleoside concentration determined ($p > 0.05$). Thus, it can be concluded that the method is robust enough for these parameters. However, varying the mobile phase composition by introducing as little as 2% methanol could cause the elution of cytidine at very early stages (less than 1 min) and this was too fast to be detected. This observation indicates that the mobile phase composition must be strictly controlled at 100% water to ensure reliability of the method.

3.5. Concentrations of four nucleoside compounds

Table 4 summarizes the contents of nucleoside compounds as determined in the New Zealand *Hericium* sp. using the validated method with the working range of each nucleoside substitute. The total amount of the four detected nucleosides was 10.7 mg/g, more than 1% of the dry weight.

It was reported that mushrooms that showed antitumor activities usually contain large amounts of adenosine (Ikumoto et al., 1991). Furthermore, many well-known medicinal mushrooms are rich in nucleosides, especially adenosine. For example, the concentrations of adenosine in *Lentinula edodes*, *Auricularia auricula-judae*, *Cordyceps sinensis*, and *Ganoderma* '*lucidum*' were reported as 2.33, 0.32, 1.71 and 0.40 mg/g dry weight, respectively (Shiao, Wang, Lin, Lien, & Wang, 1994). The health effects of adenosine could be expected, as it is involved in inhibition of platelet aggregation, even when moderate amounts of these mushrooms are consumed (Hammerschmidt, 1980). The concentration of adenosine in *Hericium* sp. is 3.01 mg/g dry weight, which is higher than the figures mentioned above. It was found that nucleosides from food can enter an organism, which indicates the human body is able to absorb nucleosides and take advantage of them (Van Buren & Rudolph, 1997). It is important to note that even though healthy persons can make and reprocess nucleosides in their bodies, nucleosides from nucleoside-rich foods can be useful to meet the need for nucleosides in specific situations, such as during recovery from illness or when there is immune system disorder. The high concentration of adenosine, as well as presence of cytidine, uridine and guanosine, justifies further attention as the total concentration of these nucleosides in *Hericium* sp. was as high as 1% dry weight, and this predicts potential pharmacological effects.

4. Conclusion

As medicinal mushroom is regarded as a natural and healthy food source and has received increasing attention from consumers, there is a need to reveal its active ingredients and set up reliable testing methods for quantification. This is the first scientific report of four nucleoside compounds, cytidine, uridine, adenosine and guanosine from a New Zealand native edible *Hericium* sp. Extraction was optimized & nucleosides determination was conducted using an HPLC–DAD–MS method. The validation data showed that the method had good precision, accuracy, and robustness, and was replicable in research laboratories. The concentration of four nucleosides was measured as 10.7 mg/g, more than 1% dry weight of the mushroom. This is an exciting finding, indicating that *Hericium* sp. is a rich source of nucleosides with potential health benefits. Only four nucleosides were identified in this study; some other nucleoside compounds may exist in this species, waiting to be discovered. By developing a reliable and robust method, this study is a step closer to the future work of determining the nucleoside profiles in *Hericium* sp. The findings in this study also led to our further research on the potential health benefits of *Hericium* sp., which is currently in progress.

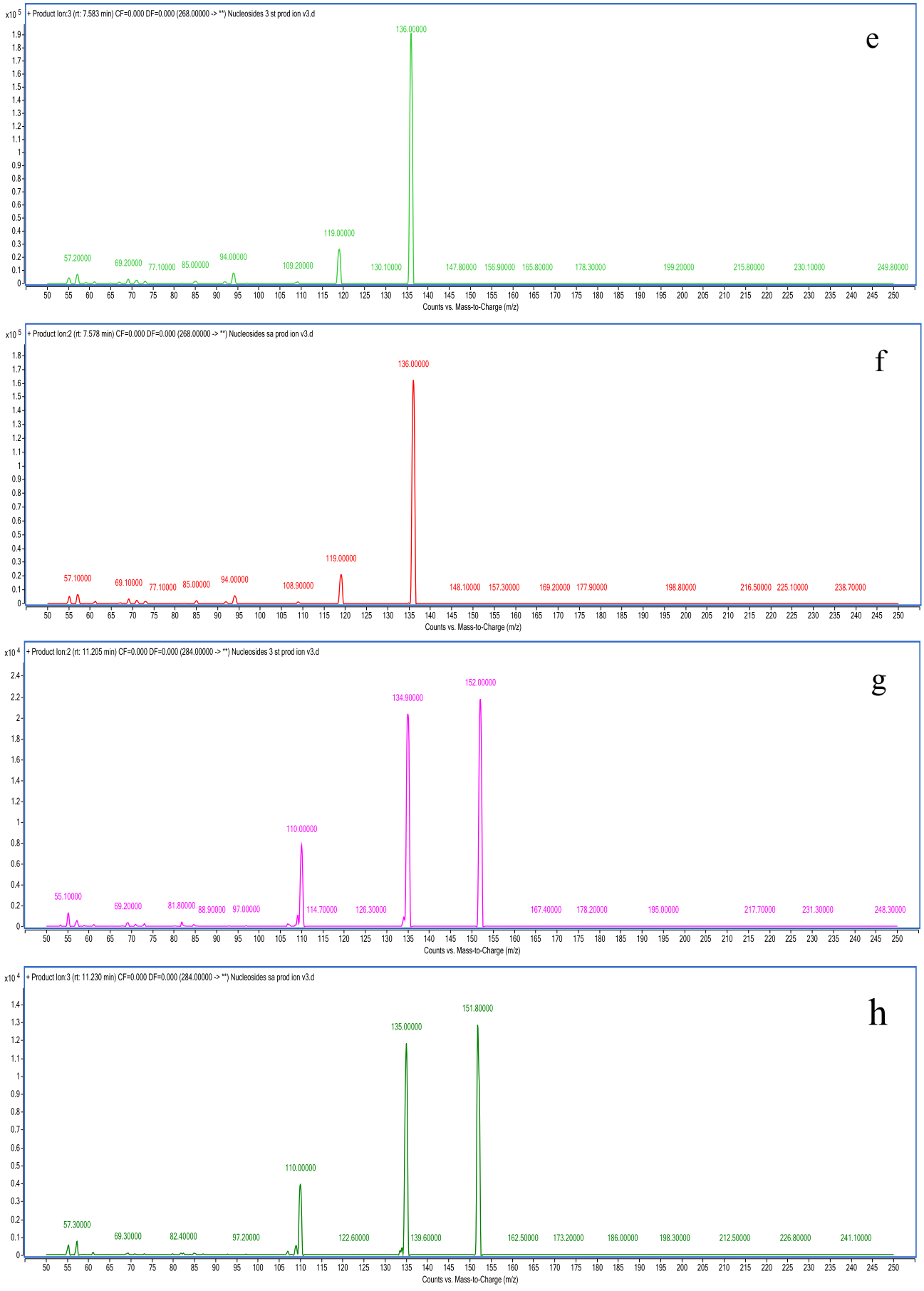

Fig. 2. (*continued*)

Table 2
The regression analysis for examining the linearity, LOQ and LOD, precision and accuracy, for the four nucleoside compounds.

Parameter	Cytidine	Uridine	Adenosine	Guanosine
Linearity range of standards (μg/mL)	6.504–48.78	6.44–48.30	9.6–72.0	6.488–48.66
Correlation coefficient	0.9999	0.9999	0.9999	0.9999
Confidence limit of slope	7.625–7.768	11.008–11.089	14.753–14.848	11.488–11.648
p for the slope	7.86E–17	4.79E–20	1.53E–20	7.53E–18
SE of slope	0.0311	0.0176	0.0205	0.0348
Confidence limit of intercept	−3.784 to 0.184	−1.982 to 0.254	−2.373 to 1.499	−3.251 to 1.187
SE of intercept	0.860	0.485	0.839	0.962
p for the intercept	0.069	0.112	0.616	0.314
LOQ (μg/mL)	0.84	0.60	0.36	0.60
LOD (μg/mL)	0.28	0.20	0.12	0.20

Table 3
Precision and recoveries (%) of the four nucleoside compounds spiked at three concentration levels (high, medium, and low).

Compound	Precision-RSD (%) intra-day	Precision-RSD (%) inter-day	Spiked amount (mg/g)	Recovery replicates (%) (n = 3)	RSD (%)
Cytidine	2.77	2.58	0.9	100.46 ± 2.02	2.01%
			1.8	100.37 ± 2.30	2.79%
			2.7	100.11 ± 2.20	2.20%
Uridine	2.26	1.58	1.5	99.91 ± 1.53	1.53%
			3.0	99.16 ± 2.98	3.00%
			4.5	99.39 ± 1.68	1.69%
Adenosine	2.25	2.06	1.5	100.30 ± 2.65	2.64%
			3.0	101.12 ± 1.51	1.50%
			4.5	100.41 ± 1.88	1.87%
Guanosine	1.86	1.96	1.4	99.76 ± 2.57	2.57%
			2.8	99.10 ± 1.53	1.55%
			4.2	101.26 ± 2.24	2.21%

Table 4
Content of the nucleoside compounds in *Hericium* sp.

Nucleoside	Working range (mg/g)	Content (mg/g) (n = 6)	RSD (%)
Cytidine	0.81–6.10	1.796 ± 0.050	2.77
Uridine	0.81–6.04	3.051 ± 0.069	2.26
Adenosine	1.20–6.00	3.006 ± 0.069	2.25
Guanosine	0.81–6.08	2.820 ± 0.053	1.86
TNC (mg/g)		10.67	

Conflict of interest

The authors declare no conflict interest.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

This work was financially supported by the Faculty of Science Research Development Fund (project no. 3714970), University of Auckland. The authors acknowledge Bruce Mackinnon for generously providing fresh samples of *Hericium* sp. for this research.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2018.11.115.

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