Comparative analysis of nucleoside concentrations in water extracts of natural *Ophiocordyceps sinensis* at 30°C and 100°C.

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Abstract

Sample preparation is an important aspect in the extraction of potentially pharmaceutical compounds found in traditional Chinese medicines (TCMs). It is valuable to understand which preparation method is ideal for each compound of interest. Cordyceps are among the most sought after TCM products today, with a market value of 32,000 USD per kilogram in 2006. Increasing the efficiency of extraction could potentially reduce the cost to consumers and the ecological impact of their harvesting in Tibet. An accurate, precise, and sensitive reverse-phase high performance liquid chromatography method was developed for the simultaneous separation and quantitation of uridine, inosine, and adenosine in *Ophiocordyceps sinensis* for boiling (100°C) and ambient temperature (30°C) water extractions. The optimum separation was achieved with a gradient elution of methanol and ammonium acetate through a 2.0 x 250mm Phenomenex Luna 5u C18 column. Linearity of the apparatus was significant with values of R^2 = 0.9994985, 0.9998885, and 0.9999775 for uridine, inosine, and adenosine respectively. The percent relative standard deviation for intra-day and inter-day precision ranged from 0.88-2.82 %RSD, and 1.88-7.57 %RSD respectively. Accuracy determined through percent recovery tests yielded an accuracy range of 99.32 - 99.91%. Limit of detection and limit of quantification for nucleosides were determined to have ranges of 0.042-0.2240.042µg/mL and 0.128-0.678µg/mL respectively. Similar results were obtained for cordyceps obtained from both Qinghai and Xizang at each temperature treatment. Boiling water was more effective for adenosine, while ambient temperature yielded more inosine and uridine. Mean concentration differences between temperature treatments were calculated for adenosine, uridine, and inosine of 9.79, 32.38, and 28.35µg/mL respectively. One-way ANOVA for the two temperature conditions of uridine, inosine, and adenosine were [F=788.89, df =11, p<0.001], [F=3352.46, df= 11, p<0.001], and [F=1211.17, df=11, p<0.001] respectively. The results suggest that sample preparation has a significant effect on the expression of nucleosides in *Cordyceps*.

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Table of Contents

List of Tables

1. Introduction

1.1 Traditional Chinese Medicine in a Global Economy

Traditional Chinese Medicine (TCM) is thought to date as far back as 1,100BC (Unschuld 2010). TCM is a primary source of health care in China, but interest in its potential health benefits has been popularized around the world (Cassileth, et al. 2001). Many TCM products have seen a steep increase in price due to an upsurge in demand on a local and global scale (Alves and Rosa 2005). Since many of these products have an ecological component, a high demand can have detrimental effects on their local ecology (Alves and Rosa 2005, Sharma 2004, Winkler, Caterpillar Fungus (Ophiocordyceps sinensis) Production and Sustainability on the Tibetan Plateau and in the Himalayas. 2009). Overharvesting, population decline, and ecosystem degradation are common for many of the products available in TCM, particularly for those species that have not yet been successfully grown outside of their natural habitats (Eisen 2007, X. Zhou, et al. 2010).

These issues are especially apparent with the product 冬虫夏草 (Chinese: DMng chóng xià cÎo), which literally means •summer grass, winter worm", but is more commonly known in English by the name cordyceps. Huge consumer interest began after it was discovered that three female runners, who broke five world records during the 1993 National Games in Beijing, were taking cordyceps as a supplement (Chen, et al. 2013). Since this time cordyceps have become a large contributor to the regional GDP of Tibet, making up 40% of the annual household income (Winkler, The mushrooming fungi market in Tibet exemplified by Cordyceps sinensis and Tricholoma matsutake

2008a, Winkler, Yartsa Gunbu (Cordyceps sinensis) and the fungal commodification of the rural economy in Tibet Autonomous Region 2008b). In 2006 the retail price for top quality cordyceps reached a high of 32,000 USD per kilogram (Winkler 2008b). The recommended daily dose for cordyceps is reported to be 3-9 grams (Bensky, et al. 2004), which would be 96 to 288 USD a day.

1.2 Description of Ophiocordyceps sinensis

Cordyceps is a genus of ascomycete endoparasitic fungi, which primarily infects insects and other arthropods. The first record of a cordyceps species being described was made in Tibet during the 15th century as *yartsa gunbu*, which is known today as *Ophiocordyceps sinensis* (Boesi and Cardi 2009). Use of this fungus in medicine has a history dating back to that time and was typically considered a cure for all ailments (Boesi and Cardi 2009). In Chinese medicine it is referred to as DMng chóng xià cÎo. This name is also used to describe another species by the name *Cordyceps militaris* because of their historically identical genus nomenclature (Shresthaa, et al. 2010), which had to be considered when sourcing the materials. The species used to be referred to by its previous synonym *Cordyceps sinensis*, though recent molecular analysis was used to revise the classification of this species into a new family Ophiocordycipitaceae (Sung, et al. 2007). The study of *Cordyceps* has been over-whelmed with problems of loose taxonomic regulation and misidentification which stresses the need to ensure true *O. sinensis* is collected for study (Chen, et al. 2013).

Its current distribution is limited to the Tibetan Plateau making it endemic to the region (Winkler 2008a). The limited geographic range and large-scale harvesting of *Cordyceps* has caused this species to be listed as an endangered species by CITES

(Convention on International Trade in Endangered Species). Recent methods have been developed to culture some species of the fungus in the laboratory in response to the increase in price and decrease in population (Yu, et al. 2006). These species include *O. sinensis* and *C. militaris*.

1.3 Bioactivity in *Cordyceps*

In recent years a great deal of research has been conducted to alleviate strain on demand and to better understand the pharmacological properties of *O. sinensis*, which has been claimed to have anti-cancer and anti-viral properties among its list of potential treatments (Ali 2012, Chen, et al. 2013). At the heart of this research is the inventory of biologically active compounds and their proposed links to human health. The primary chemical constituents that are widely considered biologically active and have potential pharmacological uses are saccharides, nucleosides, sterols, with other active components including fatty acids, cyclic dipeptides, polyamines, volatile components, vitamins, and metals (Chen, et al. 2013, Zhao, et al. 2014). Of these groups of compounds, nucleosides have been identified as the most likely source for the proposed benefits derived from using *Cordyceps* medicinally (Ali 2012, Chen, et al. 2013, Fan, et al. 2006, Li, Yang and & Tsim 2006).

1.4 Nucleosides

1.4.1 Proposed Mechanisms

Nucleosides and their analogs are glycosylamines. They differ from nucleotides in that they are bound to a ribose or deoxyribose sugar. Most cells are capable of *de novo* biosynthesizing nucleosides since they are a fundamental component of internal cellular

processes (Dueregger, et al. 2013). However, their availability for therapeutic processes is limited because nucleoside stocks within the cell are preferentially maintained by more energetically efficient salvage pathways (Molina-Arcas, Casado and Pastor-Anglada 2009). Nucleoside transporters, a type of integral membrane protein located on the cell membrane, are in charge of recycling and managing the reuptake of extracellular nucleosides (Molina-Arcas, Casado and Pastor-Anglada 2009). Research suggests that inhibition of these nucleoside transport proteins is capable of redirecting the flow of nucleosides towards other pathways, yet the mechanisms for their attenuation are still under investigation (Kanekoa, et al. 2014, Yawata, et al. 2009). Novel nucleosides like cordycepin are polyadenylation inhibitors with a broad range of biological pathways, which include protein synthesis inhibition as well as anti-inflammatory, pro-apoptotic, and anti-proliferative effects (Wong, et al. 2010).

1.4.2 Nucleosides in Cordyceps

Although more research needs to be conducted to confirm their proposed health claims, nucleosides are suitable as an indicator for the comparison. Many review articles have been produced to outline the nucleosides identified in *Cordyceps*, along with their suggested health benefits (Chen, et al. 2013, Xie, et al. 2010, X. Zhou, et al. 2010). Current inventory of nucleosides include cytidine, hypoxanthine, uridine, inosine, guanosine, thymidine, adenosine, 2-deoxyuridine and cordycepin. Due to the availability of standards, uridine, inosine, and adenosine were used for the experiment (Table 1).

Table 1. List of nucleosides in study and their proposed health benefits

Nucleoside	Therapeutic Properties	Reference
Adenosine	Treatment for cerebral and cardiac ischemic diseases, sleep disorders, cancer, glaucoma,	(Gessi, et al. 2011) (Jacobson and Gao 2006)

	asthma, arthritis, and other inflammation related disorders.	
Uridine	Regulating the maturation and differentiation of HL-60 leukemia cells, and the inhibition of astrocyte death caused by glucose deprivation. Possible antidepressant in combination with omega-3.	(Choi, et al. 2006) (Sokoloski, et al. 1991) (Carlezon Jr., et al. 2005)
Inosine	Regenerative treatments for heart failure, and retinal disease. Treatment of cerebral ischemia.	(Czarnecki and Czarnecki 1989), (Wu, et al. 2003), (Wu, et al. 2010)

1.5 Sample Preparation Rational

Many of these of the products used in Chinese medicine are typically ground and prepared in the form of a decoction in water (Yang and Li 2008, Yuan and Lin 2000). In the case of cordyceps they are generally ground and boiled, or added to soup whole (X. Zhou, et al. 2009). While there are many solvents that are used in chemical extraction, water has long been used in the study of food chemistry largely due to its lack of toxicity in humans. Typically, the chemical constituents of a product will have different solubility based on the physical and/or chemical properties of the solutes and solvent (Petrucci, et al. 2010). Under normal atmospheric pressure in water, the primary factor affecting solubility in water is temperature (Petrucci, et al. 2010). However, high temperature can have the added effect of breaking down compounds into their derivatives (Petrucci, et al. 2010, Yang and Li 2008).

The optimal nucleoside extraction times for both temperature treatments were determined based on previous research, where HPLC analysis of concentration changes over time was performed (Yang and Li 2008). Optimization for boiling water nucleoside extraction time determined that concentrations plateau after 30 minutes, with no significant change appearing in the following 8 hours sampled. Additionally, ambient

temperature analysis determined that all three of the nucleosides being investigated in this study would have the optimal peak area at 18 hours.

1.6 Reverse Phase High Performance Liquid Chromatography

The analytical procedure being used to determine the concentrations of each nucleoside is reverse phase high precision liquid chromatography (RP-HPLC). This instrumental method was chosen because of its high precision, accuracy, and sensitivity, while allowing for all components within a solution to separate in a column at high pressure and then exit in “plates” to be detected individually (Gerber, et al. 2004). Each analyte in the sample interacts differently with the adsorbent material in the column, varying the flow speed of the analytes (Magaña, et al. 2008). If the interaction is weak, the analyte is retained in the column for a short amount of time. Inversely, if the interaction is strong, then the retention time increases.

Standards of each nucleoside at known concentrations were used to determine retention times and create calibration curves. These calibration curves in turn allow the concentration of nucleosides to be determined by measuring the peak area under chromatogram curve. Identifying retention times helped to locate the peaks associated with nucleosides within the chromatogram. The column, detector wavelength, and solvents used must be appropriate for the compounds being studied (Magaña, et al. 2008). The solvents used in the elution gradient and the method that determines their percent concentration must be suited to the nucleosides (Guo, et al. 2005). Furthermore, the method inputted into the HPLC machine must be suited to all of the environmental variables and hardware used. Otherwise the peaks can widen, appear too closely together, or fail to pass through the column in the allotted time.

1.7 The Effects of Preparation Method on Nucleoside Content

The inherent variability in the solubility of compounds raises the question of whether or not a single preparation method is efficient for a broad range of nucleosides. Cordyceps contain a multitude of biologically active compounds, each with their own set of proposed health benefits (Chen, et al. 2013). If a consumer is looking for health benefits from a specific component of cordyceps, then it would be advantageous to optimize extraction using an equally specific method. In order to test whether certain compounds are more readily extracted in boiling water, an HPLC analysis of the key nucleoside concentrations in *Ophiocordyceps sinensis* extracts at 30°C and 100°C was performed.

2. Materials & Methods

2.1 Sample & Standard Materials

HPLC Grade Methanol and crystalline Ammonium Acetate were purchased from Chengdu Mansite Pharmaceutical Co., Ltd. Adenosine, inosine, and uridine standards were ordered through Shaanxi Normal University from Chengdu Mansite Pharmaceutical Co., Ltd in Xi'an. All triplicated water used in the experiment was processed through a Millipore Q-Gard® 1.

Ophiocordyceps sinensis samples were collected from two provinces in China, one from Yushu county, Qinghai, and the other from Naqu county, Xizang (Tibet). The cordyceps were purchased by the Good Agricultural Practices Group (GAP Group) and their identification established by a member of GAP.

2.2 Sample Preparation

Samples were dried in an oven for three hours and then ground in a mortar and pestle until finely powdered (~Mesh 60). A single sample of *Cordyceps* weighed approximately 0.7g. Therefore, each of the treatments for both locations was allotted 0.3 grams of *Cordyceps* material. All prepared samples were refrigerated at 4°C until they were used for analysis.

2.2.1 Ambient Temperature Water Extraction

Using a milligram scale, 0.3g of accurately weighed cordyceps material was mixed with 5ml of Milli-Q triplicated water at in a plastic centrifuge tube. The container was placed in an incubator carefully maintained at a temperature of 30°C for 18 hours. The extracted solution was then centrifuged at $2.0x10^4$ rpm for 30 minutes, the supernatant filtered for HPLC analysis using 0.45µm syringe filters, and placed in 1.5 mL HPLC bottles.

2.2.2 Boiling Water Extraction

Using a milligram scale, 0.3g of accurately weighed cordyceps material was mixed with 5 ml of boiling Milli-Q triplicated water at in a plastic centrifuge tube. The container was quickly placed back in a boiling water bath for 30 minutes. The extract was then cooled to ambient temperature and centrifuged for 30 minutes at $2.0 x10^4$ rpm. The supernatant was then filtered using 0.45µm syringe filters and placed into 1.5 mL HPLC filters

2.3 Reference Standard Preparation

A stock solution was prepared with the standard reference nucleosides at a concentration of 100µg/mL in triplicated water. Measurements of the standards were made using a milligram scale. From this stock solution, seven standard solutions at different concentrations were prepared (Table 2). Each standard solution was placed in multiple 1.5mL HPLC bottles to be analyzed to determine retention times for each nucleoside. These standards were also essential for the statistical validation of the analytical procedure (Section 2.5).

Table 2. Ratio of stock to water required to dilute to required concentration

Goal (µg/mL)	Ratio	Stock (mL)	Water (mL)
1	1:99	0.04	3.96
10	1:9	1	9
20	1:4	1	4
40	1:1.5	2	3
60	1:0.6667	5	3.334
80	1:0.25	4	1
100	1:0	3	0
	Total:	**16.04**	**24.29**

2.3.1 Reference Standard Identification

In order to identify which peak signifies the presence and concentration of each nucleoside, standards for each were prepared and run through the HPLC apparatus. With the HPLC method and gradient elution established, each compound was represented at a specific and consistent retention time during each injection procedure. These retention times were used to identify the peaks of interest in the chromatograms of cordyceps samples. A second test to confirm the retention time of nucleosides was also created. Each sample treatment contained one HPLC bottle which was spiked with the reference

stock solution, which allowed for the side by side visual confirmation of chromatograms within the software.

2.4 HPLC Description

The RP-HPLC unit used in this experiment was a Shimadzu LC-2010C(HT). The unit is equipped with the original pumping system and injector. The column chosen for the stationary phase was a Phenomenex Luna 5u C18(2) 100A, which has a 4.6mm diameter, 250mm length, and contains 5 micron silica. The detector installed with this machine is a SPD-M20A Diode Array Detector (DAD), capable of attaining the 260nm UV wavelength necessary for detection of the nucleosides. Injection volume was set to 20µL and the oven maintained the temperature of the column at 30°C

The mobile phase consists of an elution gradient containing Ammonium Acetate 40mM 5.2pH (Pump A) and Methanol HPLC Grade (Pump C). Solvents were regularly degassed using a Kunshan Shu Mei KQ-600DB ultrasonic degasser to remove any potential micro-bubbles. In order to ensure no bubbles entered the system, degassing was performed for 30 minutes at 30°C. All solvents were also passed through a 0.22µm vacuum filter to ensure all significant contaminants were removed.

2.4.1 HPLC Controller Method

The HPLC software was programed to perform large batches for this experiment. This method can be broken down into four essential components. A low flow washing phase to clear any debris left in the column (Table 3), a balancing phase that brings the ambient initial concentration to 5% Methanol (Table 4), intermittent washing phases between samples (Table 5), and finally a gradient elution phase which contains the

injected sample material. Batch processing was used to create daily tasks for the HPLC machine to perform automatically; routine inspections were done every two hours to ensure reliability and continuity.

Table 3. Initial column washing process

Step	Time (min)	Methanol Conc. (%)	Flow Rate (mL/min)
1	0	100	1.0
2	30	100	0.8
3	60	100	0.5
4	90	100	0.2
5	120	100	0.5
6	150	100	0.8
7	180	100	1.0

Table 4. Intermediate washing process

Step	Time (min)	Methanol Conc. (%)	Flow Rate (mL/min)
1	0	5	1.0
2	15	100	1.0
3	30	100	1.0
4	45	5	1.0
5	60	5	1.0

2.4.2 Elution Gradient

After careful adjustments to an elution gradient in the literature (Guo, et al. 2005), a gradient method was determined for the eventual experiment (Table 5). The final elution gradient method created reproducible retention times, peak separations, and peak widths over the course of each test batch. This method was entered into the system and applied to each of the replicates for every sample.

Table 5: Elution gradient of Ammonium Acetate (A) and Methanol (C)

Step	Time (Min)	Pump A (% Conc.)	Pump C (% Conc.)
1	0	95	5
2	6	85	15
3	12	80	20
4	17	80	20
5	22	95	5

2.5 HPLC Statistics

A proper HPLC method must be thoroughly tested to ensure not only that statistical rigor has been met, but also that the apparatus itself is performing within acceptable ranges. All analytical procedures were designed around the document created by the *International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use* in order to maintain a high level of method validation (ICH 2005). This ensured that analytical limits for linearity, precision, accuracy, and sensitivity could be defined.

2.5.1 Linearity

The calibration of the instrument was evaluated to ensure that the detector is capable of producing acceptable data before any samples are analyzed. Standard solutions with a concentration range of 1 to 100µg/mL (1, 10, 20, 40, 60, 80, and 100) were selected to prepare for the potential range of sample concentrations. Three calibration curves were prepared in the same day with the aforementioned concentrations. The number of standards used in the initial calibration was determined to be at least four in order to ensure statistical significance for a linear calibration range of three orders of magnitude. However, seven concentrations were used to ensure the degrees of freedom and uncertainty met the specified tolerance.

The variation in instrumental response of the detector, measured using the peak area (sum of detector signals), defines a straight line that can be tested using linear regression analysis. The coefficient of determination (R^2) for chemical analysis of organic compounds is suggested to be greater than 0.998, while the percent relative standard deviation (%RSD) for the mean response factors should be less than 15% (ICH 2005). Significance was tested using a least-squares approach.

2.5.2 Precision

Repeatability or intra-day precision of the HPLC apparatus was also tested to ensure that the equipment was capable of producing consistent results. Six replicates of 40µg/mL concentration standard solution were tested on the same day to determine variation in the detector. Percent relative standard deviation (%RSD) of the six replicates was determined for each nucleoside. Inter-day precision was determined to test for variation in the HPLC apparatus from one day to the next. This required six replicates of a standard solution repeated daily for 2 days (%RSD).

2.5.3 Accuracy

The recovery of adenosine in an assay was determined to assess the detector's capability to obtain the accurate concentration of the nucleosides. By adding a known amount of nucleoside to a standard solution of known concentration, a comparison was made between the amount of standard added and response obtained from the detector. In doing so, the extraction efficiency of the HPLC method was statistically tested to ensure the variability is within suggested limits. A recovery experiment was conducted for initial concentrations of 40µg/mL. Three additions were performed at +80%, +100%, and

+120% and were prepared by adding a known amount of the 100µg/mL stock solution to each of the standard solutions (Equation 1, Table 6).

$$Stock\ Volume = \frac{Std.Vol.\ (Initial\ Conc. - Desired\ Conc.)}{(Desired\ Conc. - Stock\ Conc.)} \qquad \textbf{Eq. 1}$$

Table 6. Recovery test dilution calculations

Initial Conc. (µg/mL)	Desired Conc. (µg/mL)	Standard to Stock Ratio Needed	Amount Added (ml)
40	72	1:1.143	1.143
40	80	1:2	2
40	88	1:4	4

2.5.4 Sensitivity

Instrumental Limits of HPLC Machine were determined by calculating Limit of Detection (LOD) and Limit of Quantitation (LOQ) using the calibration curve method according to ICH Q2(R1) recommendations. The detection limit can be expressed in terms of the standard deviation (Ã) of the response factor and the slope (S) of the calibration curve (Equation 2). Concentrations below the LOD cannot be differentiated from signal noise. The quantitation limit can be expressed similarly using the same factors (Equation 3). Although a concentration value may be higher than the LOD it is not necessarily true that the difference between two values can be measured until the value is greater than the LOQ.

$$LOD = \frac{3.3\ \sigma}{S} \qquad \textbf{Eq. 2}$$

$$LOD = \frac{10\ \sigma}{S} \qquad \textbf{Eq. 3}$$

3. Results

3.1 Method Validation

3.1.1 Linearity

The standard solution with a prepared concentration of 60µg/mL was found to be contaminated when processed using the HPLC apparatus. Source of the contamination is unknown; however multiple peaks with unfamiliar retention times were detected during their analysis. This calibration standard was therefore appropriately removed from the data used in the production of the calibration curves.

Linear regression analysis performed by the HPLC proprietary LabSolutions software resulted in the following values (Table 7). The curve was not forced or weighted towards the origin for any of the nucleosides. The calibration curves were plotted with the area under the curve represented on the X-axis and concentration of injected material on the Y-axis. The slope and intercepts for uridine (Equation 4), inosine (Equation 5), and adenosine (Equation 6) were calculated.

Table 7. Least-squares linear regression results for nucleoside standard detections

Nucleoside	RF Mean	RF Std. Dev.	RF %RSD	df	R^2
Adenosine	1.690023e-05	2.159513e-07	1.277801	4	0.9999775
Inosine	1.763279e-05	2.439489e-07	1.383496	3	0.9998885
Uridine	2.508452e-05	1.764443e-06	7.033993	4	0.9994985

* RF denotes Response Factor

$$Y = 2.604112 * 10^{-5}X - 0.8199956 \quad \textbf{Eq. 4}$$

$$Y = 1.77223 * 10^{-5}X - 0.1343861 \quad \textbf{Eq. 5}$$

$$Y = 1.691329 * 10^{-5}X - 1.314897 * 10^{-2} \quad \textbf{Eq. 6}$$

3.1.2 Precision

An intra-day study was carried out for 40µg/mL concentration injections to determine the ruggedness of the proposed method. Six replicates of the standard solution were studied in the same day to define the variation in concentration readings of the HPLC apparatus (Table 8). Results for the intra-day precision test provided % RSD values for inosine, uridine, and adenosine being 0.8781, 2.8214, and 1.3738 respectively. Inter-day precision was also tested for 40µg/mL concentration injections to determine the variation over a two day period (Table 9). Mean, standard deviation, and percent relative standard deviation were calculated with inosine, uridine, and adenosine returning %RSD values of 4.0572, 1.8794, and 7.5703 respectively.

Table 8. Intra-day precision of nucleoside detection using HPLC apparatus

Expected Conc. (µg/mL)	Actual Inosine Conc. (µg/mL)	Actual Uridine Conc. (µg/mL)	Actual Adenosine Conc. (µg/mL)
40	39.9701	39.3408	39.9254
40	40.4439	41.0452	39.5419
40	39.9902	40.9271	39.6363
40	39.6662	39.1076	40.2618
40	40.4080	41.0367	40.8605
40	40.5758	41.1000	40.7163
Mean	40.1757	40.5929	40.1570
Std. Dev.	0.3528	1.1453	0.5517
%RSD	0.8781	2.8214	1.3738

Table 9. Inter-day precission of nucleoside detection using HPLC apparatus

Nucleoside	Conc. (µg/mL)	Day 1 (µg/mL)	Day 2 (µg/mL)	Mean Conc. (µg/mL)	Std. Dev. Conc. (µg/mL)	%RSD
Inosine	40	40.1757	42.5490	41.3624	1.6782	4.0572
Uridine	40	40.5929	41.6863	41.1396	0.7732	1.8794
Adenosine	40	40.1570	44.6994	42.4282	3.2119	7.5703

3.1.3 Accuracy

A recovery test was performed using adenosine to determine the accuracy of the detector by spiking the standard samples with known amounts of extra stock solution (Table 10). Percent recovery values were collected for three samples, with values of 99.32%, 99.91%, and 99.89% for sample with +80%, +100%, and +120% additions respectively.

Table 10. Recovery test for adenosine concentrations

Sample Number	Concentration (µg/mL)	Amount Added (µg/mL)	Amount Found (µg/mL)	Amount Recovered (µg/mL)	% Recovery
1	40	32	71.7831	31.7831	99.32
2	40	40	79.9245	39.9245	99.91
3	40	48	87.9467	47.9467	99.89

3.1.4 Sensitivity

Based on the data collected during the linearity test, values for the Limit of Detection and Limit of Quantitation were calculated (Table 11). Limit of Detection values were determined to be 0.045µg/mL, 0.224µg/mL, and 0.042µg/mL for inosine, uridine, and adenosine respectively.

Table 11. Limit of Detection (LOD) and Limit of Quantitation (LOQ) for nucleosides

Nucleoside	RF Std. Dev.	Slope of CC	LOD (µg/mL)	LOQ (µg/mL)
Inosine	2.439489E-07	1.77223E-05	4.54248E-02	1.37651E-01
Uridine	1.764443E-06	2.60412E-05	2.23595E-01	6.77559E-01
Adenosine	2.159513E-07	1.69133E-05	4.21349E-02	1.27681E-01

* RF denotes Response Factor, CC denotes Calibration Curve

3.2 Sample Analysis

3.2.1 Chromatograms

HPLC chromatograms were produced by comparing retention time within the stationary phase in the X-axis and the measure of intensity in the light observed in the detector. Chromatograms of cordyceps samples under the same treatment, along with a spiked sample containing stock solution of the nucleosides were placed together within the same graph for side-by-side comparison (Figure 1). Spiked samples (denoted by -S) clearly indicate the locations for each of the nucleosides. Concerning the visualizations of the boiling water treatment of the *Cordyceps* harvested in Qinghai, injection 1 (A-BW-1) and injection 2 (A-BW-2) were almost identical and cannot be distinguished within the figure at this scale. Some variation in peak retention time is visibly present for A-BW-3 and A-RT-3. However, the %RSD values calculated (Table 12-13) indicate that they are well within acceptable displacement ranges.

3.2.2 Quantitation of Retention Times

The separation of the three nucleosides was achieved within the first 15 minutes, with retention times for Qinghai (Table 12) and Xizang (Table 13) remaining similar across the entire analysis. Mean retention time for each nucleoside was determined using the individual retention times calculated from all chromatograms produced. The mean

retention time for uridine, inosine, and adenosine were found to be 7.287 (SD=0.098), 9.320 (SD=00.102), and 14.098 (SD=0.158) respectively. All retention times received %RSD values < 3%.

3.2.3 Quantitation of Concentrations

The composition of nucleosides in cordyceps extractions from Qinghai and Xizang under two temperature treatments was determined. Three replicates were analyzed for each treatment, as well as a spiked sample to confirm peak identification. The concentration readings for all nucleosides maintained a %RSD value <5%. All concentrations were well within the limits of detection and limits of quantitation determined in the method validation phase.

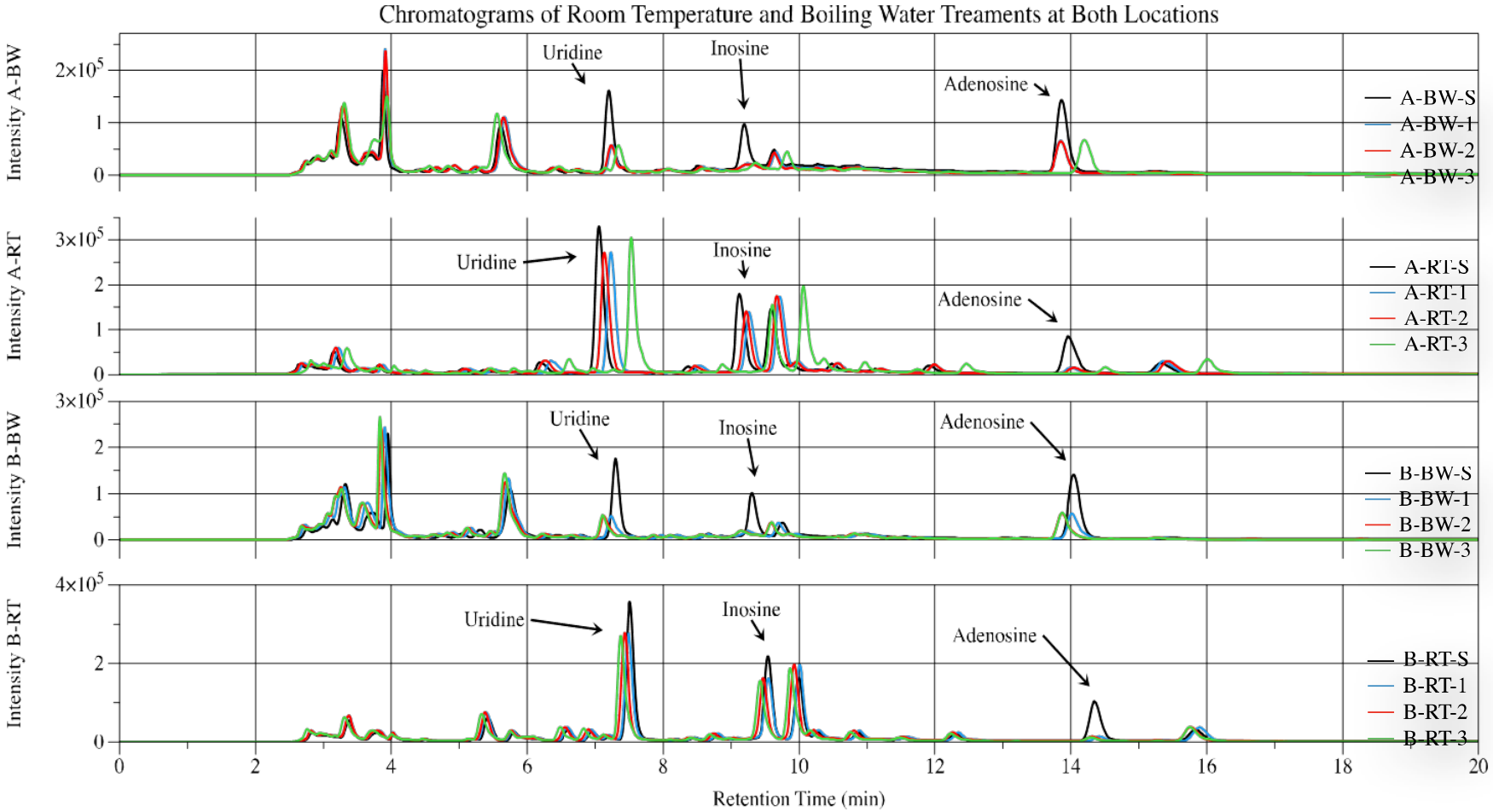

Figure 1. HPLC chromatograms for boiling water (BW-) and ambient temperature (RT-) treatment replicates of Qinghai (A-) and Xizang (B-) *Cordyceps*

Table 12. Nucleoside content in Qinghai *Cordyceps* for boiling water (BW) and ambient temperature water (RT)

	Uridine		Inosine		Adenosine	
Treatment	Conc. (μg/mL)	Retention Time (min)	Conc. (μg/mL)	Retention Time (min)	Conc. (μg/mL)	Retention Time (min)
BW-Spike	22.6723	7.195	12.1257	9.186	26.8718	13.857
BW-1	6.7096	7.245	3.6692	9.237	12.0836	13.844
BW-2	6.4382	7.229	3.4959	9.209	12.1983	13.848
BW-3	6.6177	7.339	3.8503	9.359	13.2047	14.194
Mean	6.5885	7.271	3.6718	9.268333	12.49553	13.962
Std. Dev.	0.138036	0.059431	0.177214	0.079758	0.616828	0.200928
%RSD	2.095107	0.817365	4.826361	0.860543	4.93639	1.439105
RT-Spike	52.0073	7.048	41.1126	9.118	18.9963	13.955
RT-1	40.777	7.224	30.861	9.261	2.567	13.997
RT-2	41.5311	7.132	30.2853	9.215	2.6084	14.043
RT-3	42.4748	7.529	32.0587	9.602	2.6187	14.501
Mean	41.5943	7.295	31.06833	9.359333	2.598033	14.18033
Std. Dev.	0.850663	0.207805	0.904697	0.21141	0.027365	0.278656
%RSD	2.045142	2.848598	2.91196	2.258818	1.053283	1.96509

Table 13. Nucleoside content in Xizang *Cordyceps* for boiling water (BW) and ambient temperature water (RT)

	Uridine		Inosine		Adenosine	
Treatment	Conc. (μg/mL)	Retention Time (min)	Conc. (μg/mL)			Conc. (μg/mL)
BW-Spike	25.0281	7.295	20.8436	9.304	28.2517	14.035
BW-1	6.7934	7.104	3.6309	9.141	11.7613	13.868
BW-2	6.8457	7.121	3.5311	9.147	11.9793	13.865
BW-3	6.9029	7.228	3.5295	9.242	11.3517	14.011
Mean	6.847333	7.151	3.563833	9.176667	11.69743	13.91467
Std. Dev.	0.054768	0.067224	0.058087	0.05666	0.318637	0.083441
%RSD	0.799848	0.940057	1.629901	0.617433	2.723992	0.599659
RT-Spike	48.9385	7.505	44.3043	9.537	20.6941	14.345
RT-1	37.3146	7.486	33.1242	9.543	2.074	14.414
RT-2	36.2702	7.43	32.2524	9.469	2.0809	14.308
RT-3	36.1998	7.373	33.2423	9.42	2.0138	14.282
Mean	36.59487	7.429667	32.87297	9.477333	2.056233	14.33467
Std. Dev.	0.6243	0.056501	0.540661	0.061922	0.03691	0.069924
%RSD	1.705978	0.760475	1.644697	0.653369	1.795027	0.487795

A comparison of the nucleoside concentrations, and their associated standard deviations, for each extraction method and location was generated (Figure 2). This side-by-side comparison illustrates a clear difference between temperature treatments. It also show a strong similarity between cordyceps from both locations.

One-way ANOVA tests were conducted using Minitab 16 to compare the effects of temperature on concentration for each of the nucleosides in ambient temperature and boiling water conditions. The independent variable in this case was temperature, with two conditions of 30°C and 100°C. The concentrations of each nucleoside for both temperature treatments and both locations were grouped together and entered as the response variable. There was a significant effect on the amount of uridine, inosine, and adenosine expressed at the $p<0.05$ confidence level for the two conditions, with values of [$F(1,10) = 788.89$, $p<0.001$], [$F(1,10) = 3352.46$, $p<0.001$], and [$F(1,10) = 1211.17$, $p<0.001$] respectively (Table 14-16).

Table 14. Summary of One-way ANOVA Minitab statistics for Uridine

Source	Degrees of Freedom	Sum of Squares	Mean Square	F-value	p-value
Temperature	1	3144.75	3144.75	788.89	0.000
Error	10	39.86	3.99		
Total	11	3184.61			

* S = 1.997; R-Sq = 98.75%

Table 15. Summary of One-way ANOVA Minitab statistics for Inosine

Source	Degrees of Freedom	Sum of Squares	Mean Square	F-value	p-value
Temperature	1	2411.649	2411.649	3352.46	0.000
Error	10	7.194	0.719		
Total	11	2418.843			

* S = 0.8482; R-Sq = 99.70%

Table 16. Summary of One-way ANOVA Minitab statistics for Adenosine

Source	Degrees of Freedom	Sum of Squares	Mean Square	F-value	p-value
Temperature	1	286.321	286.321	1211.17	0.000
Error	10	2.364	0.236		
Total	11	288.685			

* S = 0.4862; R-Sq = 99.18%

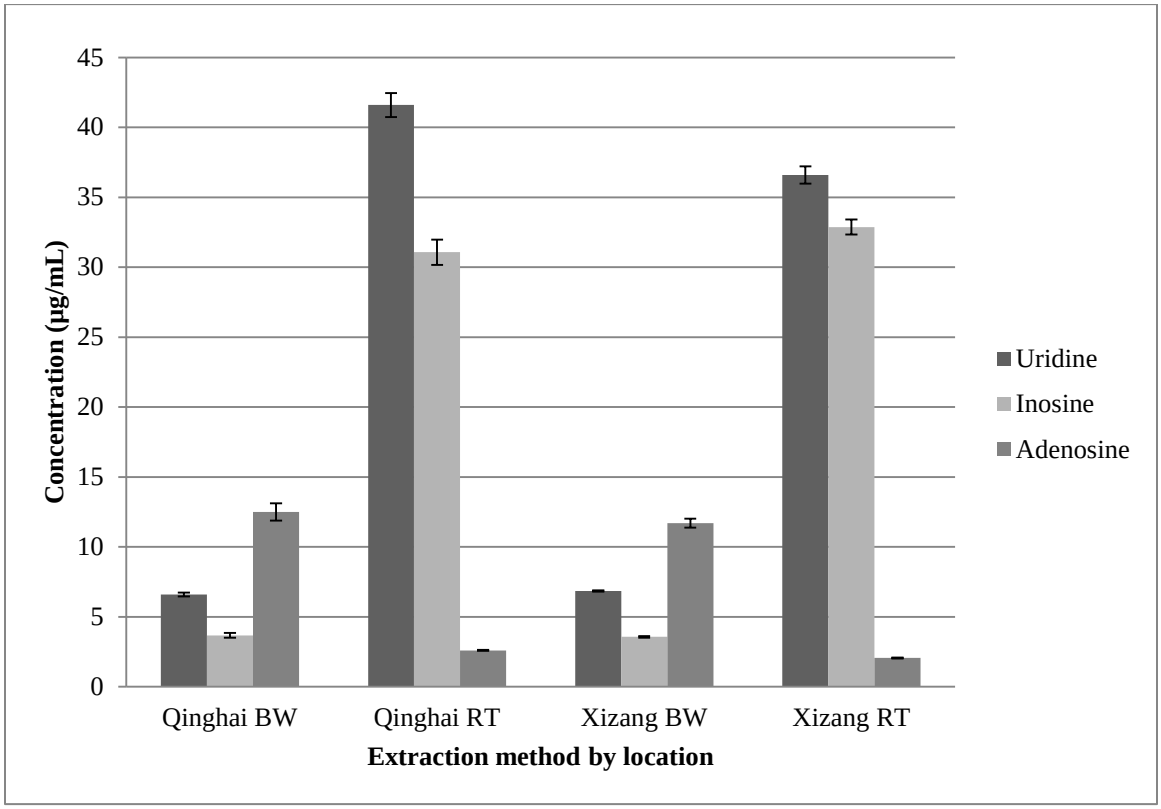

Figure 2. Nucleoside profiles of Qinghai and Xizang *Cordyceps* for boiling water (BW) and ambient temperature water (RT) extraction with standard deviations

The variation between Qinghai and Xizang in response to temperature treatment was also measured. This was achieved by separating the values for both locations into two temperature determined groups. Standard deviation and the coefficient of determination for grouped concentration of uridine, inosine, and adenosine were

[S = 1.997, R^2 = 98.75%], [S = 0.8482, R^2 = 99.70%], and [S = 0.4862, R^2 = 99.18%] respectively.

Table 17. Comparison of temperature treatment on concentration for both locations pooled

	Uridine			Inosine			Adenosine		
Temperature	Mean	St.Dev	%RSD	Mean	St.Dev	%RSD	Mean	St.Dev	%RSD
30	39.095	2.818	7.208	31.971	1.192	3.728	2.327	0.298	12.806
100	6.718	0.17	2.5313	3.618	0.132	3.648	12.096	0.62	5.1261

5. Discussion

Method validation for the HPLC apparatus performed well within expected analytical limits for linearity, precision, accuracy, and sensitivity. This shows that the HPLC apparatus, selected column, and standards were all in excellent condition and well suited to this experiment design. Calibration curves provided tight linear regression to identify the nucleoside concentrations found within the *Cordyceps* samples. The calculated coefficient of determination for nucleosides ranged from 0.9994985 to 0.9999775%. Reference standards were successful in indicating the retention times for each nucleoside. The solvents used in the elution gradient, as well as their timing and concentration, were successful in providing good separation of the peaks in order to measure peak area. The intra-day precision test resulted in excellent %RSD values of ranging from 0.88 to 2.82%, while inter-day precision tests resulted in values of 1.88 to 7.57%. Precision was therefore within acceptable limits. Accuracy of the detector using the percent recovery test provided was well within the appropriate limits for accuracy. However, it would be good practice to run this test on all nucleosides in future studies. Limits of Detection value were significantly small enough to allow detection of concentration greater than 0.045, 0.224, and 0.042µg/mL for inosine, uridine, and

adenosine respectively. Limits of Quantification were well outside the range of the experimental results and did not interfere with the resolution of concentrations measured. LOQ values of 0.138, 0.678, and 0.128µg/mL were calculated for uridine, inosine and adenosine respectively. Together these tests for method validation ensured that the comparison of extraction methods could be performed within recommended guidelines.

Similarly repeatable results were produced for the sample injected into the HPLC apparatus. The chromatograms of replicates were significantly similar with regards to retention time and concentration for each treatment. Relative variance in retention times for nucleosides in A-BW, A-RT, B-BW, and B-RT treatments were < 1.44, < 2.84, < 0.94, and < 0.76 %RSD respectively. This suggests that the pump, mobile phase, and column were performing within acceptable ranges. The relative variance in concentration of nucleosides in A-BW, A-RT, B-BW, and B-RT treatments were < 4.93, < 2.91, < 2.72, and <1.80 %RSD. The retention times for nucleosides between treatments and locations were also significantly similar, with relative variance value between 1.10 and 1.34 %RSD.

Mean concentration for each nucleoside varied significantly depending on which treatment was applied for cordyceps from both locations. The difference between temperature treatments for adenosine was significant, with a value of 9.79µg/mL. It was determined that boiling water was the more effective by 5.2 times that of ambient temperature. The null hypothesis for this nucleoside is therefore rejected and confirms the prediction made. The other two nucleosides analyzed in this experiment produced the opposite response. The difference between temperature treatments for Uridine and Inosine were significantly different, with values of 32.38µg/mL and 28.35µg/mL

respectively. However, in this case the larger concentrations were produced in the ambient temperature extraction.

Concentration values between locations yielded similar results. The concentration data collected for both locations were pooled and temperature treatments were determined to have the same effect on both cordyceps. %RSD calculated for boiling water concentration values for both locations were 2.53, 3.65, and 5.13% for uridine, inosine, and adenosine respectively. The same calculation for ambient temperature water yielded %RSD values of 7.21, 3.73, and 12.81% respectively. This suggests that the method could be applied to other specimens of this species with similar effect, although a larger sample size is recommended to confirm this.

The trend for both sites is clear. Boiling water extraction resulted in higher adenosine concentrations, but lower concentrations of uridine and inosine than the ambient temperature treatment. This effect was similar for both cordyceps, which suggests that one or more temperature based mechanisms is causing nucleoside concentration to vary.

Adenosine solubility increased with temperature and the explanation could simply be that this temperature range has a direct positive effect on solubility. This is certainly the case for a vast majority of compounds when other factors of pH and pressure are not involved (Petrucci, et al. 2010). Another study that looked at the effects of extraction method on nucleoside concentration using a different HPLC apparatus and method yielded similar results (Yang and Li 2008). In that case boiling water extraction did provide a higher concentration of adenosine. However, no adenosine was detected in any

of the ambient temperature treatments for natural *O. sinensis*. A comparison of the sample preparation method of this study and Yang & Li 2008 for ambient temperature found only a variation in the centrifuge speed and duration. This could indicate that concentration is influenced by the centrifuge method, although further study would be needed.

Inosine and uridine concentration were significantly lower in boiling water, which point toward there being additional mechanisms acting on these compounds. Degradation of inosine and uridine into their bases could account for the loss of their expression. Inosine degrades into hypoxanthine and a ribose ring in the presence of an enzyme called purine nucleoside phosphorylase (Canduri, et al. 2004). Similarly, uridine is degraded into uracil by either uridine kinase or other pathways (Prior and Santi 1984). In fact the pathways to and from nucleotides, nucleosides, and nucleobases are numerous and would require the analysis of all compounds found in these pathways to determine how temperature affects these transitions. Regardless, it is apparent that the proposed degradation pathway is not active in ambient temperature water.

From these results it is clear that the preparation method is an important factor in how nucleosides are expressed. If an individual is using cordyceps to derive a specific health benefit from one of the nucleosides, then it would be beneficial to know the best way to prepared them. For example, in a Harvard study Carlezon Jr. et el., (2003) suggest that uridine may be used in combination with omega-3 as an antidepressant. Based on the concentration data collected it would require 5.8 times more *Cordyceps* material to attain the same concentration if the preparation method involved boiling water. The increase in efficiency that would be derived from considering the temperatures used in preparation

could significantly reduce the cost to consumers and has the potential of easing the burden on the local ecology in Tibet.

6. Conclusion

Extraction methods have a significant influence in the expression of nucleosides in decoctions of *Ophiocordyceps sinensis*. By comparing the extraction of *O. sinensis* in ambient and boiling water treatments it was determined that adenosine is more prevalent in boiling water, while inosine and uridine are more commonly found in ambient temperature extractions. However, in order to describe the degradation mechanisms likely at work there will need to be a broader analysis and identification of nucleotides, nucleosides, and nucleobases under these conditions.

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