

Simultaneous quantification and splenocyte-proliferating activities of nucleosides and bases in *Cervi cornu Pantotrichum*

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ABSTRACT

Background: Cervi Cornu Pantotrichum has been a well known traditional Chinese medicine, which is young horn of *Cervus Nippon* Temminck (Hualurong: HLR). At present, the methods used for the quality control of Cervi Cornu Pantotrichum show low specificity. **Objective:** To describe a holistic method based on chemical characteristics and splenocyte-proliferating activities to evaluate the quality of HLR. **Materials and Methods:** The nucleosides and bases from HLR were identified by high performance liquid chromatography electrospray ionization mass spectrometry (HPLC-ESI-MS), and six of them were chosen to be used for simultaneous HPLC quantification according to the results of proliferation of mouse splenocytes *in vitro*. **Results:** In this study, eight nucleosides and bases have been identified. In addition, uracil, hypoxanthine, uridine, inosine, guanosine, and adenosine were chosen to be used for simultaneous HPLC quantification. Simultaneous quantification of these six substances was performed on ten groups of HLR under the condition of a TIANHE Kromasil C₁₈ column (5 μm, 4.6 mm × 250 mm i.d.) and a gradient elution of water and acetonitrile. Of the ten groups, HLR displayed the highest total nucleoside contents (TNC, sum of adenosine and uracil, 0.412 mg/g) with the strongest splenocyte-proliferating activities. **Conclusion:** These results suggest that TNC (such as particularly highly contained adenosine and uracil) in HLR has a certain correlation with the activity of splenocyte-proliferating, and it may be used as a quality control for HLR. This comprehensive method could be applied to other traditional Chinese medicines to ameliorate their quality control.

Key words: *Cervus Nippon* Temminck, electrospray ionization mass spectrometry, high performance liquid chromatography, immunomodulatory, nucleosides and bases

INTRODUCTION

Lurong (LR) or Cervi Cornu Pantotrichum has been a well-known traditional Chinese medicine and health food widely used in China for thousands years and also listed in the Chinese Pharmacopoeia,^[1] which is a young horn of *Cervus Nippon* Temminck (Hualurong: HLR).

HLR is commonly used in China for various activities, such as antioxidation, anti-aging, anti-tumor, immunomodulatory

and other biological activities, especially for improving sexual function.^[2-10] Traditional medical reports show that HLR is made of many components such as amino acids, fatty acids, lipids, polypeptides, polysaccharides, polyamines, inorganic elements, biological bases, and other chemical compositions.^[11] Recently, the analysis methods have been established for separately determining biological bases (i.e., uracil, hypoxanthine and uridine) in antler velvet.^[12] However, there has been no report regarding the quantification of biological bases and splenocyte-proliferating activity in HLR. A simultaneous quantification is required because several nucleosides and bases will individually promote spleen cell proliferation. Therefore, this study was aimed to evaluate the splenocyte-proliferating activity of HLR and to analyze the content of the active principles in HLR.

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MATERIALS AND METHODS

Chemical reagents, materials, and instruments

Acetonitrile used was of chromatographically grade and procured from Merck (Germany). The water used was treated with a Milli-Q water purification system (Millipore, Molsheim, France). Standards of uracil, hypoxanthine, uridine, inosine, guanosine, adenosine, 2'-deoxyguanosine, and guanine were procured from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China) [Figure 1]. Concanavalin A (ConA) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were procured from Sigma (St. Louis, MO, USA). RPMI1640 medium, penicillin–streptomycin and fetal bovine serum (FBS) were obtained from Hyclone (Utah, USA). Other reagents and solvents used were of analytical grade.

A total of 10 samples of HLR were collected from the cultivation areas in Jilin and Liaoning provinces of China, which four collected from Shuangyang (ShuangYang01, ShuangYang02, ShuangYang03, ShuangYang04), two collected from Dongfeng (DongFeng01, DongFeng02), two collected from Xifeng (XiFeng01, XiFeng02), and two collected from Aodong (AoDong01, AoDong02). All samples were verified by Prof. Ming-Lu Deng (Changchun University of Chinese Medicine, Changchun, China).

Agilent Technology 1100 Series high performance liquid chromatography (HPLC) system equipped with a quaternary pump, degasser, thermostatic auto-sampler, and photodiode array detector (DAD) was used for analysis. The MS instrument used to perform the studies was a 6320 ion trap LC/MS from Agilent.

Sample preparation

A 1-g sample of the fine-ground powder was accurately weighted and extracted with 20 mL of boiling H₂O for 60 min and centrifuged at 12000 rpm for 15 min,

and the supernatant was collected. The extraction was lyophilized, and the lyophilized powder was extracted with 60% methanol by ultrasonication for 15 min at room temperature. The mixture was then centrifuged at 12000 rpm for 15 min, and the supernatant was collected. The supernatant was dried in the rotary evaporator at 160 rpm at 30°C, which was named total nucleoside contents (TNC) for using on proliferation of mouse splenocytes *in vitro*. The powder of TNC was accurately weighed and dissolved in 60% methanol (1 mg/mL) for LC/MS analysis and determination. It was filtered through a membrane filter (0.45 μm pore size) prior to injection and analyzed three times.

Standard preparation

The reference standards of the eight nucleosides and bases were accurately weighed and dissolved in 60% methanol; six of them (uracil, hypoxanthine, uridine, inosine, guanosine, and adenosine) were then diluted to appropriate concentration ranges for the establishment of calibration curves. All stock and working standard solutions were stored in brown bottles at 4°C until used for analysis.

HPLC-DAD-electrospray ionization-mass spectrometry analysis of TNC

HPLC procedures. The analysis was performed on an Agilent 1100 liquid chromatography system, equipped with a diode array detector working in the range of 190–400 nm, a quaternary solvent delivery system, a column temperature controller, and an autosampler. The chromatographic data was recorded and processed with Agilent Chromatographic Work Station software.

Chromatographic separations were carried out on a C₁₈ analytical column (TIANHE Kromasil C₁₈, 4.6 mm × 250 mm, 5 μm) supplied by Dalian Elite Analytical Instruments, Dalian, China. The mobile phase consisted of water (A)–acetonitrile (B); A: B was as follows:

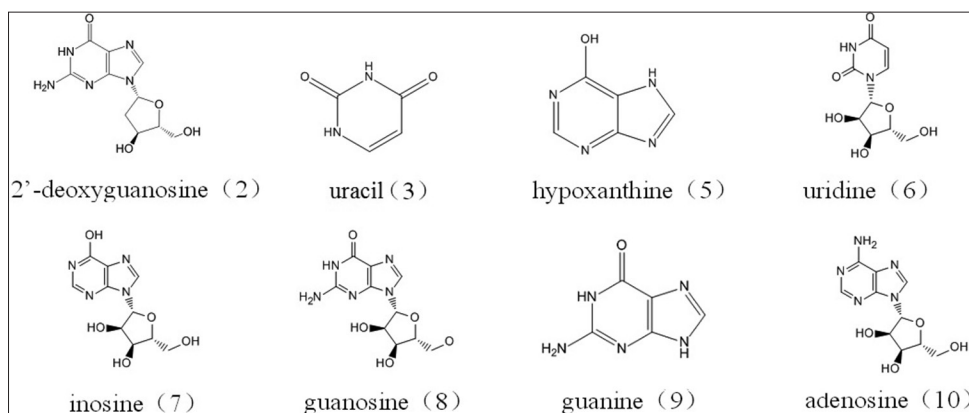


Figure 1: The structures of 8 standard compounds

0 min, 99:1; 5 min, 98:2; 10 min, 98:2; 20 min, 96:4; 40 min, 96:4; 42 min, 45:55; 55 min, 40:60; and 60 min, 0:100. The flow rate and column temperature were set constantly at 0.3 mL/min and 30°C, respectively. The detection wavelength was fixed at 260 nm. All solvents were filtered through a 0.45- μm pore size filter and then degassed by sonication in an ultrasonic bath before use.

ESI-MS parameter. The HPLC analytical conditions for liquid chromatography-mass spectrometry (LC-MS) were same as those used for the HPLC-DAD analysis, except that one-third of the elutant was introduced into the MS system by using split technique. Agilent 1100 HPLC/MSD Trap mass spectrometer (Agilent, Wilmington, Germany) equipped with an electrospray ionization source was used in positive ion mode. An HPLC system coupled with DAD was controlled by an HPLC-MSD ChemStation software system. Auto MS² mode of Mass spectrometer was chosen to analyze the sample. The following operation parameters were used: Capillary voltage: 4000V; nebulizer pressure: 35 psi; drying gas: 9.0 L/min; gas temperature: 350°C; fragmentor voltage: 200V; skimmer voltage: 60V. LC-ESI-MS accurate mass spectra were recorded across the range from 50 to 400 m/z . The data recorded was processed with the applied HPLC-MSD ChemStation software system.

Proliferation of mouse splenocytes *in vitro*

An assay method described by Kan F *et al.*^[13] was used to measure the splenocyte-proliferating activity of TNC and eight nucleosides and bases of HLR. Aliquots (100 μL) of the Splenocyte suspension and 100 μL of test samples were added to each well of a 96-well plate. PBS and ConA (5 $\mu\text{g}/\text{mL}$) served as the negative and positive controls, respectively. After incubation at 37°C in a 5% CO₂ atmosphere for 72 h, 20 μL MTT were added to each well, and the incubation continued for further 4 h. Extinction values at 570 nm were measured using an enzyme-linked immunosorbent assay (ELISA) reader (Model 680, Takara, Japan). The proliferation rate was calculated according to the following formula: Splenocyte proliferation rate (%) = $[A_{570}(\text{sample}) - A_{570}(\text{negative control})] / A_{570}(\text{negative control}) \times 100\%$.

Simultaneous determination of six nucleosides and bases in TNC of HLR

Chromatographic conditions. The HPLC conditions of determination coincide with those used for HPLC analysis in LC-MS.

LC method validation. The HPLC analysis method used in this study has been validated through several tests. Linearity was evaluated by the value of R² (correlation coefficient) in the calibration curve of serial concentrations. Sensitivities were determined by the value of limit of

detection (LOD) and limit of quantification (LOQ). They were calculated with corresponding standard solution on the basis of signal-to-noise ratio (S/N) of 3 and 10, respectively. The stability of the analysis method was assessed by measurements of the intra- and inter-day variability. To evaluate the accuracy, the recovery test was performed, spiking each standard compound to the sample solution.

RESULTS AND DISCUSSION

Optimization of HPLC conditions

DAD was used in HPLC analysis, and full scan runs were made initially to select the optimum wavelength that provided the best result in chromatographic analysis. Chromatogram at 260 nm showed the most abundant components information than at other wavelengths, in which Figure 2 demonstrates the separation obtained for a typical sample of the six nucleosides and bases; the six insets are DAD UV scan of uracil (3), hypoxanthine (5), uridine (6), inosine (7), guanosine (8), and adenosine (10) peak (190-400 nm). An optimum formula consisting of solvent A and solvent B was selected. Solvent A was H₂O and solvent B was acetonitrile. This composition was selected because it showed a better separation and the solvents were environment-friendly. A gradient elution method was employed for better chromatographic separation on a wide range of polarity in a shorter time.

HPLC-ESI-MS analysis

HPLC-ESI-MS was employed to analyze the components separated by HPLC. In ESI-MS experiment, molecular mass of the components can be obtained. Comparing MS results with the uracil, hypoxanthine, uridine, inosine, guanosine, adenosine, 2'-deoxyguanosine and guanine

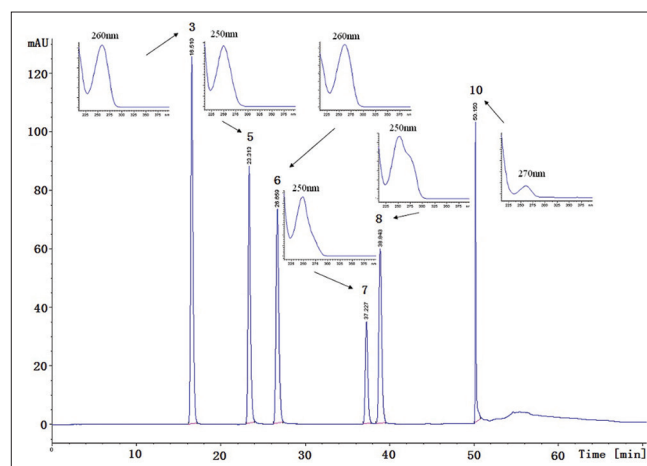


Figure 2: HPLC chromatogram of nucleosides and bases in HLR at 260 nm wavelength. The six insets are DAD UV scan of uracil (3), hypoxanthine (5), uridine (6), inosine (7), guanosine (8) and adenosine (10) peak (190-400 nm)

standard, and those in the literature,^[14,15] we have deduced eight structures of nucleosides and bases in the HPLC peaks. Since positive ionization ESI mode was used, all of the m/z data are $[M+H]^+$. The retention times and mass spectra of products exactly matched with the corresponding standard compounds, which are shown in Figure 3 and Table 1.

Effect of eight nucleosides and bases and the TNC of HLR on mouse splenocyte proliferation *in vitro*

Reports about immunomodulatory of protein and peptides in LR were very much in recent years. However, no specific studies have been carried out regarding water-soluble components of small molecular weight in HLR and their immunomodulatory activities. In the present study, the proliferation effect of eight nucleosides and bases and the TNC of HLR on mouse splenocytes was examined by MTT assay.

Effect of eight compounds. Exposure of mouse splenocytes to different concentrations (12.5, 25, 50, and 100 $\mu\text{mol/L}$) of eight compounds in HLR increased cell proliferation in each case [Table 2]. Proliferation rates in samples treated with uracil was dose dependently increased, the proliferation rates at 50 $\mu\text{mol/L}$ and 100 $\mu\text{mol/L}$ of uracil treatment was significantly higher than that of negative

control. Interestingly, the proliferation rates from adenosine treatment present significantly higher at lower dose such as 12.5 $\mu\text{mol/L}$ and 25 $\mu\text{mol/L}$ compared to negative control, the proliferation effect of adenosine was diminished following the increase of dose. In the case of other six compounds, the effect was marginal. Further study is still required to investigate the immunomodulatory effect of uracil and adenosine.

Effect of the TNC of HLR. Exposure of mouse splenocytes to TNC (25, 50, 100 and 200 $\mu\text{g/mL}$) of HLR increased cell proliferation in each case [Table 3]. Proliferation rates were much higher after the treatment of 25 $\mu\text{g/mL}$ and 50 $\mu\text{g/mL}$ of TNC compared to negative control. In case of higher concentrations, TNC resulted in decrease in the splenocyte viability. It indicated that TNC in higher concentrations (100 and 200 $\mu\text{g/mL}$) might have some cytotoxic effect on mouse splenocytes.

LC method validation

The optimized HPLC method was validated by examining the linearity, sensitivity, stability, and accuracy.

Linearity, LOD, and LOQ. The working solutions were all prepared as described above to construct calibration curves. Each calibration curve contained five different concentrations and was performed in triplicate. An aliquot (10 μL) of each standard working solution was subjected to LC analysis. The linearity for each compound was established by plotting the peak area (y) versus concentration (x) of each analyte. Every R^2 value was more than 0.9995, verifying the linearities of the calibration equations.

Stock solution containing six reference compounds was diluted to a series of appropriate concentrations, and an aliquot (20 μL) of the diluted solutions was injected into LC for analysis. LOD and LOQ for each analyte were calculated with corresponding standard solution on the

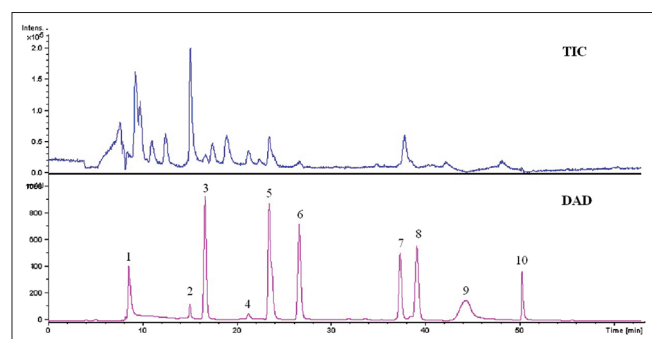


Figure 3: The HPLC/DAD chromatogram and total ion chromatogram in positive ion mode of HLR

Table 1: HPLC-ESI-MS data of nucleosides and bases in HLR

Retention time (min)	UV absorption characteristics λ_{max} (nm)	Mass data (m/z)	Compounds
8.4	265	243.3, 104.1	Unknown substance 1
14.9	235	268.0 $[M+H]^+$, 152.0 $[M+H\text{-ribose}]^+$	2'-deoxyguanosine
16.5	260	113.0 $[M+H]^+$, 71.1 $[M+H\text{-ethenone}]^+$	Uracil
21.3	275	181.2, 164.9	Unknown substance 2
23.6	250	137.1 $[M+H]^+$, 159.0 $[M+Na]^+$, 174.9 $[M+K]^+$	hypoxanthine
27.2	260	245.0 $[M+H]^+$, 113.0 $[M+H\text{-ribose}]^+$	Uridine
37.7	250	269.0 $[M+H]^+$, 136.9 $[M+H\text{-ribose}]^+$	Inosine
39.4	250	284.6 $[M+H]^+$, 151.8 $[M+H\text{-ribose}]^+$	Guanosine
44.2	260	152.0 $[M+H]^+$, 135.0 $[M+H\text{-NH}_3]^+$	Guanine
50.1	270	268.2 $[M+H]^+$, 135.9 $[M+H\text{-ribose}]^+$	Adenosine

HPLC-ESI-MS: High performance liquid chromatography electrospray ionization mass spectrometry; HLR: Hualurong

Table 2: Effect of eight nucleosides and bases on the proliferation of mouse splenocytes ($\bar{x} \pm s$, $n=3$)

Sample	The OD value (proliferation rate)					
	Negative control	Positive control (5 $\mu\text{g/mL}$)	Sample concentration ($\mu\text{mol/L}$) (%)			
			100	50	25	12.5
Uridine	0.706 \pm 0.033	1.097 \pm 0.057	0.697 \pm 0.006 (-1.27)	0.713 \pm 0.006 (0.99)	0.723 \pm 0.027 (2.41)	0.608 \pm 0.007** (-13.88)
Adenosine			0.665 \pm 0.006 (-5.81)	0.707 \pm 0.019 (0.14)	0.809 \pm 0.015* (14.60)	0.787 \pm 0.015* (11.47)
Inosine			0.776 \pm 0.039 (9.92)	0.712 \pm 0.003 (0.85)	0.692 \pm 0.005 (-1.98)	0.749 \pm 0.017 (6.09)
Uracil			0.867 \pm 0.014** (22.80)	0.879 \pm 0.025** (24.50)	0.91 \pm 0.067** (28.89)	0.742 \pm 0.011 (5.60)
Guanine			0.624 \pm 0.001* (-11.61)	0.768 \pm 0.020 (8.78)	0.690 \pm 0.007 (-2.27)	0.676 \pm 0.004 (-4.25)
Guanosine			0.850 \pm 0.014** (20.40)	0.704 \pm 0.007 (-0.28)	0.782 \pm 0.007* (10.76)	0.753 \pm 0.006 (6.56)
Hypoxanthine			0.698 \pm 0.007 (-1.13)	0.696 \pm 0.011 (-1.42)	0.728 \pm 0.014 (3.12)	0.747 \pm 0.008 (5.81)
2-deoxyguanosine			0.758 \pm 0.008 (7.36)	0.647 \pm 0.018 (-8.36)	0.694 \pm 0.006 (-1.70)	0.630 \pm 0.004* (-10.76)

OD: Optical density

Table 3: Effect of the TNC of HLR on the proliferation of mouse splenocytes ($\bar{x} \pm s$, $n=3$)

Sample	The OD value (proliferation rate)					
	Negative control	Positive control (5 $\mu\text{g/mL}$)	Sample concentration ($\mu\text{g/mL}$) (%)			
			200	100	50	25
TNC _(ShuangYang01)	0.704 \pm 0.045	1.091 \pm 0.066	0.595 \pm 0.011* (-15.48)	0.644 \pm 0.018 (-8.52)	0.912 \pm 0.021** (29.54)	0.876 \pm 0.021** (24.43)
TNC _(ShuangYang02)			0.602 \pm 0.045* (14.49)	0.637 \pm 0.009 (-9.52)	0.903 \pm 0.014** (28.27)	0.864 \pm 0.006** (22.73)
TNC _(ShuangYang03)			0.605 \pm 0.045* (-14.06)	0.619 \pm 0.009* (-12.07)	0.854 \pm 0.014** (21.31)	0.841 \pm 0.006** (19.46)
TNC _(DongFeng01)			0.587 \pm 0.045** (-16.62)	0.590 \pm 0.009** (-16.19)	0.786 \pm 0.014* (11.65)	0.767 \pm 0.006 (8.95)
TNC _(XiFeng01)			0.611 \pm 0.045* (-13.21)	0.625 \pm 0.009* (-11.22)	0.898 \pm 0.014** (27.56)	0.852 \pm 0.006** (21.02)
TNC _(XiFeng02)			0.643 \pm 0.011 (-8.66)	0.667 \pm 0.018 (-5.26)	0.905 \pm 0.021** (28.55)	0.871 \pm 0.021** (23.72)
TNC _(AoDong01)			0.609 \pm 0.045* (-13.49)	0.622 \pm 0.009* (-11.65)	0.859 \pm 0.014** (22.02)	0.844 \pm 0.006** (19.89)
TNC _(AoDong02)			0.595 \pm 0.045* (-15.48)	0.602 \pm 0.009* (-14.49)	0.819 \pm 0.014** (16.34)	0.802 \pm 0.006* (13.92)

TNC: Total nucleoside contents; HLR: Hualurong; OD: Optical density

basis of S/N of 3 and 10, respectively. Data for LOD and LOQ has been shown in Table 4.

Stability and accuracy. Intra-day and inter-day variations were utilized to determine the precision of the developed assay. The intra-day variation was determined by analyzing the six replicate samples (1.0 g, Shuangyang, Jilin province, ShuangYang-01) within 1 day and inter-day variation was determined on three consecutive days. To confirm the repeatability, five different working solutions prepared from the same sample (1.0 g, Shuangyang, Jilin

province, ShuangYang-01) were analyzed. Variations were expressed as relative standard deviations. The overall intra-day variations were < 2.83% and inter-day variations were < 3.55% for the analytes [Table 4].

The accuracy of the method was tested in terms of recovery percentage. The recovery test was determined by standard addition method. Six nucleosides and bases in a mixed standard solution were spiked into the samples (0.5 g, Shuangyang, Jilin province, ShuangYang-01), and then extracted, processed and quantified in accordance with

the established procedures, and finally the recovery rates were calculated. The average recovery with RSD values is presented in Table 4.

The results were considered to be satisfactory for subsequent analysis of all samples and the method proposed in our paper was accurate for the quantitative determination of the six nucleosides and bases in HLR.

Quantitative Determination of HLR

The developed analytical method was successfully applied to the simultaneous determination of uracil, hypoxanthine, uridine, inosine, guanosine, and adenosine in ten samples of HLR, which were obtained from various cities in Jilin province. Each sample was determined in triplicate. Peaks in the chromatograms were identified by comparing the retention times and online UV spectra with those of the standards. HPLC-DAD profiles are illustrated in Figure 2. Table 5 shows the content of the six nucleosides and bases in ten samples of HLR. It was found that the content of each analyte varied greatly among the different samples. In the majority of analytes, hypoxanthine was the main component whose content varied from 0.159 to 0.462 mg/g. Similar variation could also be found for the other nucleosides and bases. The variation in content of constituents could certainly lead to the variation of therapeutic effects. Hence, each procedure involved should be standardized.

CONCLUSION

In this study, a series of nucleosides and bases have been identified by HPLC-ESI-MS and adenosine, uracil, and TNC of HLR demonstrated immunomodulatory potential via stimulation of splenocyte proliferation. It is suggested that nucleosides and bases should act as the active principles of in HLR for its splenocyte-proliferating activity. Meanwhile, a validated rapid HPLC method for simultaneous rapid quantification of six nucleosides and bases in HLR samples for quality control was developed. This comprehensive strategy, providing reliable and adequate scientific evidence, could be applied to other traditional Chinese medicines to ameliorate their quality control.

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Table 4: Calibration curves, LOD, LOQ and recoveries of the analytes (n=5)

Analyte	Regression equation (liner model) ^a	Linear range (µg/mL)	R ^{2b}	LOD ^c (µg/mL)	LOQ ^d (µg/mL)	Recovery (%)	RSD (%)	
							Intra-day	Inter-day
Uracil	y=262.62x+107.82	0.8-40	0.9998	0.18	0.56	98.30	2.52	2.14
Hypoxanthine	y=210.49x+108	7-70	0.9995	0.82	2.46	97.50	2.36	2.59
Uridine	y=132.09x+13.675	4-100	0.9997	0.32	0.94	103.30	2.62	3.09
Inosine	y=196.33x+37.14	1-50	0.9999	0.11	0.33	97.30	2.41	2.04
Guanosine	y=144.45x+28.855	5-100	1	0.41	1.22	96.70	2.83	3.02
Adenosine	y=191.91x+230.75	2-50	0.9996	0.32	0.98	102.20	2.75	3.55

^ay: Peak area at 260 nm; x: concentration of each reference compound (µg/mL); ^bR²: correlation coefficient of regression equations; ^cLOD: Limit of detection (S/N=3); ^dLOQ: Limit of quantification (S/N=10)

Table 5: Content of each analyte in ten HLR samples and fingerprint similarity (n=3)

Samples	Content (mg/g)					
	Uracil	Hypoxanthine	Uridine	Inosine	Guanosine	Adenosine
ShuangYang01	0.229	0.389	0.176	0.149	0.236	0.183
ShuangYang02	0.154	0.328	0.216	0.087	0.154	0.188
ShuangYang03	0.135	0.286	0.087	0.049	0.104	0.080
ShuangYang04	0.099	0.159	0.091	0.051	0.104	0.058
AoDong01	0.129	0.263	0.222	0.080	0.264	0.088
AoDong02	0.097	0.240	0.223	0.062	0.215	0.075
DongFeng01	0.016	0.161	0.199	0.025	0.119	0.075
DongFeng02	0.027	0.225	0.291	0.032	0.179	0.117
XiFeng01	0.226	0.373	0.126	0.105	0.173	0.091
XiFeng02	0.282	0.462	0.169	0.161	0.242	0.099

HLR: Hualurong

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