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Quantitative Analysis of Nucleosides and Nucleobases in Deer Antler: Variation in Different Species

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Abstract

An HPLC-diode array detector analytical method was developed for the determination of 10 nucleosides and nucleobases, e.g. uracil, cytidine, uridine, hypoxanthine, inosine, guanine, guanosine, thymidine, adenosine and adenine, in 29 batches of deer antlers deriving from 11 species of deer: the official species of Cervi Cornu Pantotrichum recorded in Chinese Pharmacopoeia, i.e., *Cervus nippon* Temminck and *Cervus elaphus* Linnaeus, were included. This established HPLC–diode array detector method was validated to be sensitive, precise and accurate in determining the nucleosides and nucleobases of deer antlers. Quantitative analyses showed that most of deer antlers contained high amounts of nucleosides and nucleobases, except the amounts of cytidine, thymidine, adenosine and adenine; however, which varied greatly in different species and different regions of collection. The similarity of chemical fingerprints was determined, and the antlers from *C. nippon* and *C. elaphus* showed the highest similarity, suggesting the possible application in authentication. However, the species discrimination of deer antler could not be fully revealed by hierarchical cluster analysis (HCA) and/or principal component analysis (PCA).

Keywords: Deer antler; Nucleosides; Nucleobases; Quantitative analysis; HPLC–diode array detector

Introduction

Deer antler, Cervi Cornu Pantotrichum or called "Lurong" in China, is an valuable traditional Chinese medicine (TCM), recorded in the Compendium of Materia Medica (*Bencao Gangmu*) by Li Shi-Zhen in 1594 AD. Deer belongs to a family Cervidae having 10 genus and 17 species in China. Most of these species are commonly used in folk medicine in reinforcing kidney–yang, replenishing vital essence and blood, and strengthening the tendons and bones for thousands of years [1,2]. Additionally, deer antler has been widely sold as a dietary supplement in various Asian countries such as in China and Korea. According to Chinese Pharmacopoeia, the official source of Cervi Cornu Pantotrichum should be from the antlers of *Cervus nippon* Temminck and *Cervus elaphus* Linnaeus only [1]. In view of its expensive cost, the counterfeits of antler are commonly found in the market.

Deer antler is frequently consumed as a health food supplement in China. Pharmacological studies indicated that deer antler could be used to delay aging–related processes, to improve sexual function, to modulate immune responses and to lower blood pressure [3-5]. Deer antler contains various chemical compositions, including protein, amino acid, phospholipid, polysaccharide, steroid, nucleoside, nucleobase and trace element [6,7]; however, the actual quality and nutrient value of deer antler have never been well defined. Methods have been developed to identify the antler, but they are not quantitative and lack of specificity [8,9]. Moreover, there is no effective and accurate method to distinguish the ingredients of deer antler, as well as chemical markers for quality control, as that in Chinese Pharmacopoeia [1]. Therefore, it is important to establish a precise and sensitive method for quality evaluation of deer antler.

One of the major ingredients in deer antler is nucleoside, and indeed the nucleosides and their bases were shown to be involved in regulating and modulating various physiological processes [10,11], e.g. anti-oxidant, anti-convulsant, anti-platelet aggregation, inhibition of monoamine oxidase and anti-arrhythmic [12-14]. Furthermore, hypoxanthine, isolated from *n*-butanol extracts of *C. nippon* antler, was reported to inhibit monoamine oxidase B [15]. The qualification and quantification analyses of nucleosides and nucleobases in deer antler would be prerequisites for quality evaluation, which could be very helpful for improving its potential values. The quantitative methods have been previously developed to analyze nucleosides and nucleobases in deer antler; however, these methods focused on only a few of them [16-18]. Recently, nucleosides and nucleobases could be selected as the quality control markers in herbs and functional foods [19-21]. Here, 29 batches of deer antlers from 11 species of deer collected from different regions in China were simultaneously analyzed. An HPLC-diode array detector (DAD) method was applied to detect and quantify 10 nucleosides and nucleobases in deer antlers from different species.

Materials and Methods

Chemicals and reagents

HPLC grade acetonitrile and methanol were purchased from Merck (Darmstadt, Germany); ammonium acetate was purchased from Sigma (St. Louis, MO, USA); ultra–pure water was prepared from a Milli–Q purification system (Millipore S.A. Molsheim, France); chemical standards including uracil (**1**), cytidine (**2**), uridine (**3**), hypoxanthine (**4**), inosine (**5**), guanine (**6**), guanosine (**7**), thymidine (**8**), adenosine (**9**), and adenine (**10**) were purchased from Sigma (St. Louis, MO, USA). The purity of standard was higher than 98%, determined by HPLC–DAD analysis.

Deer antler materials

The antlers of *C. nippon* and *C. elaphus* were collected from Jilin, Shanxi, Liaoning, Beijing, Xinjiang provinces and Hong Kong market

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in China. The antlers of *Rangifer tarandus* Linnaeus, *C. timorensis* Linnaeus, *C. unicolor* Kerr, *C. eldi hainanus*, *Elaphurus davidianus* Milne–Edwards, *C. porcinus*, *C. albirostris* Pre–walski, *Dama dama* Linnaeus, *Capreolus capreolus* Linnaeus were obtained and authenticated by Dalian Institute for Drug Control. The antlers of *C. nippon* and *C. elaphus* were authenticated by one of the authors, Dr. Tina TX Dong, according to organoleptic characteristics. All voucher specimens with number (1–29) are deposited in the Centre for Chinese Medicine at the Hong Kong University of Science and Technology.

Preparation of solutions

All samples were ground into powder (50 meshes). 0.5 g of dried powder was accurately weighed and extracted with 25 mL of 10% aqueous methanol in an ultrasonic bath (240 W) for 45 min. After centrifugation (13,000 rpm, 10 min), the supernatant was stored at 4 °C and filtered through a 0.45 μm membrane filter before injection into the HPLC system for analysis. Stock standard solutions of individual nucleosides and nucleobases were prepared by weighing approximately 10 mg of each compound into a volumetric flask (10 mL). Standards were dissolved in 10% aqueous methanol, except for guanine, which required a few drops of 2 M HCl solution for dissolution. Working standard stock solutions for calibration curves were prepared by diluting the mixed standard stock solution with 10% aqueous methanol at different concentrations, and the concentration ranges for these 10 analytes were as follows: **1**, 0.61–61.08 μg/mL; **2**, 0.51–50.80 μg/mL; **3**, 0.60–60.24 μg/mL; **4**, 0.75–75.00 μg/mL, **5**, 0.70–70.42 μg/mL; **6**, 0.50–50.00 μg/mL; **7**, 0.52–51.90 μg/mL; **8**, 0.25–25.25 μg/mL; **9**, 0.25–25.45 μg/mL and **10**, 0.26–25.58 μg /mL. All solutions were stored at 4°C before analysis.

Chromatographic conditions and instrumentation

Analysis was performed on an Agilent 1200 series system consisting of a G1379B degasser, a G1312B binary pump, a G1367D autosampler and a G1315C DAD connected to an Agilent ChemStation running ChemStation software. Chromatographic separations were carried out on an Agilent ZORBAX SB–Aq C18 column (5 μm, 4.6 × 250 mm). The mobile phase was composed of acetonitrile (A) and 5 mM ammonium acetate solution (B) with a gradient program elution as follows: 1% A, at a flow-rate 0.4–1.0 mL/min in 0–18 min; 1% A–4% A, at a flow–rate 1.0 mL/min in 18–20 min; 4% A–5% A, at a flow–rate 1.0 mL/min in 20–25 min; 5% A–20% A, at a flow–rate 1.0 mL/min in 25–40 min. Re–equilibration duration was 15 min between individual runs. Chromatographic separation was performed at room temperature. The DAD detection wavelength was set between 200 and 400 nm, and the chromatographic peaks were measured at a wavelength of 260 nm to facilitate the detection of nucleosides and nucleobases. An aliquot of 10 μL was subjected for HPLC analysis.

HPLC method validation

Linear regression analysis for each analyte was performed at seven levels of concentrations, and triplicate injections were applied at each concentration. Calibration curves were constructed by plotting the peak area (y) against the corresponding concentration of the standard solutions (x, μg/mL). The limits of detection (LODs) and quantification (LOQs) were determined at signal-to-noise ratios (S/N) of about 3 and 10, respectively. The precision of the method was evaluated by analyzing the standard solutions containing the 10 standard compounds. The intra–day variation was determined by six consecutive injections on the same day, and the inter–day variation was determined by doing duplicates on three consecutive days. Then, the relative standard deviation (RSD) of peak area for each of the marker compound was calculated, respectively. To evaluate the stability of the solution, one of the sample solutions was stored at 22°C and analyzed at 0, 2, 4, 8, 12, and 24 h, respectively. Variation was expressed as RSD. In addition, to further evaluate the repeatability of the developed assay, the same sample (#13) was extracted and analyzed six times as described above. A recovery test was used to evaluate the accuracy of the developed method. An appropriate amount of deer antler (#13) was weighed and spiked with known amount of each reference compound, and then extracted and analyzed as described above. Three replicates were performed for the test. The percentage recoveries were calculated as follow formula: Recovery (%) = 100 × (amount found – original amount) / amount spiked.

Data analysis

Similarity analysis was performed by professional software named Similarity Evaluation System for Chromatographic Fingerprint of Traditional Chinese Medicine (Version 2004A), which was recommended by SFDA (State Food and Drug Administration of China). The software was to employ the correlative coefficient in evaluating the similarities of different chromatograms. HCA is a multivariate analysis technique that is used to sort samples into groups. Here, different samples of deer antlers were analyzed on the contents of 10 tested analytes by using SPSS 16.0 software, and the results were subjected to HCA. PCA was performed on the contents of 10 analytes by using software of SIMCA-P.

Results and Discussion

Optimization of analytical method

In order to develop an efficient sample preparation procedure of deer antler, the variables involved in extraction, such as solvent and method, were optimized. Due to high polarity of nucleosides and nucleobases, water, 10% methanol, 30% methanol and 50% methanol were employed as extraction solvents, and 10% methanol was the best solvent for extraction. After comparing ultrasonic extraction with refluxing extraction, the results indicated that ultrasonic extraction was more effective than refluxing extraction in extracting nucleosides and nucleobases by using 10% methanol as extract solvent. The extraction time was also investigated, in which the sample was ultrasonically extracted for 30, 45 and 60 min, respectively. The yield of target compounds did not increase after 45 min. The residue after the first extraction was further extracted with 10% methanol for additional 30 min, and no nucleosides and nucleobases were detected. Therefore, the 10 analytes were completely extracted within 45 min, and ultrasonic extraction with 10% aqueous methanol for 45 min in a single step was selected for routine usage.

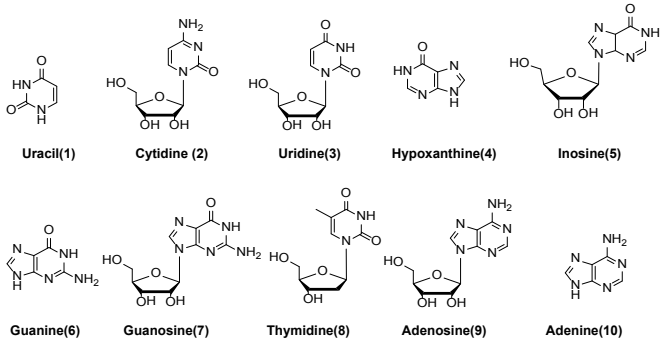
In order to obtain good separation and shorten analysis time of the experiment, the chromatographic conditions including the column, compositions of mobile phase, gradient elution program and flow rate were investigated and optimized. In our preliminary tests, different reversed-phase columns such as Zorbax Ecliopse XDB C18, Luna C18, Zorbax SB–Aq C18, Inertsil ODS–4 C18, and Alltima C18 were investigated. The results showed that the nucleosides and nucleobases were the compounds with high polarity, which were easily separated on Zorbax SB–Aq C18 column. For the mobile phase, acetonitrile performed better as an organic modifier than methanol and was thus

chosen to constitute the mobile phase with water and 5 mM ammonium acetate aqueous solution, it was found that 5 mM ammonium acetate aqueous solution could achieve the ideal separation. According to the UV absorption maxima of 10 reference compounds on UV spectra obtained by HPLC–DAD, the wavelength was set at 260 nm that provided an optimum *S/N* for simultaneous quantitative analysis of the 10 nucleosides and nucleobases (Figure 1). The HPLC chromatograms of standard analytes and deer antler samples were shown in Figure 2. Identification of investigated compounds was achieved by comparison of their retention times and their UV spectra with those of reference compounds under the same condition or by spiking the samples with stock standard solutions.

The calibration curves were constructed by plotting the peak area (y) against the corresponding concentration of the standard solutions (x, μg/mL). The calculated results were given in Supplementary Table 1. The good linearity (correlation coefficient value $r^2 \geq 0.9995$) was achieved in relatively wide concentration ranges for all the analytes. For 10 analytes, the LODs ranged from 0.031 to 0.141 μg/mL, and LOQs ranged from 0.097 to 0.423 μg/mL. The overall intra– and inter–day variations were less than 1.77% for the 10 analytes. Validation studies of this method showed a good repeatability with RSD less than 4.67% (n=6) for investigated compounds (Supplementary Table 2). As shown in Supplementary Table 3, the established method had an excellent accuracy with an overall recovery from 95.00% to 101.92% (n=3) for the analytes. Therefore, the developed HPLC–DAD method was precise, accurate and sensitive enough for the simultaneous quantitative determination of the 10 nucleosides and nucleobases in deer antler.

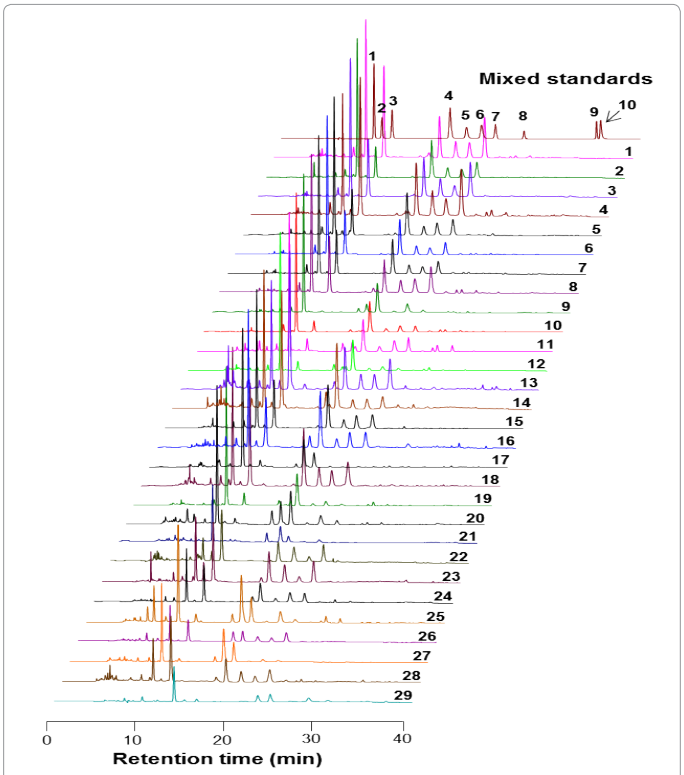
Nucleosides and nucleobases in deer antler

The established HPLC–DAD method was applied to simultaneous quantification of the 10 nucleosides and nucleobases in 29 batches of deer antlers from 11 species, including *C. nippon*, *C. elaphus*, *C. timorensis*, *C. unicolor*, *C. eldi hainanus*, *C. porcinus*, *C. albirostris*, *R. tarandus*, *E. davidianus*, *D. dama* and *C. capreolus*. The sample code and their places of collection were listed in Table 1. These antlers were extracted according to the established protocol as above. Thereafter, HPLC fingerprints were generated for the 29 samples (Figure 2). The fingerprint similarity of those antlers was calibrated, and *C. nippon* and *C. elaphus* showed the greatest value (Table 1). The worst similarity was revealed in *E. davidianus*. In addition, the contents of total nucleosides



The chemical structures of 10 marker chemicals, including uracil (1), cytidine (2), uridine (3), hypoxanthine (4), inosine (5), guanine (6), guanosine (7), thymidine (8), adenosine (9), and adenine (10), were shown. The notions were in accordance with the HPLC chromatograms in Figure 2.

Figure 1: Chemical structures of the 10 nucleosides and nucleobases analyzed in deer antler.



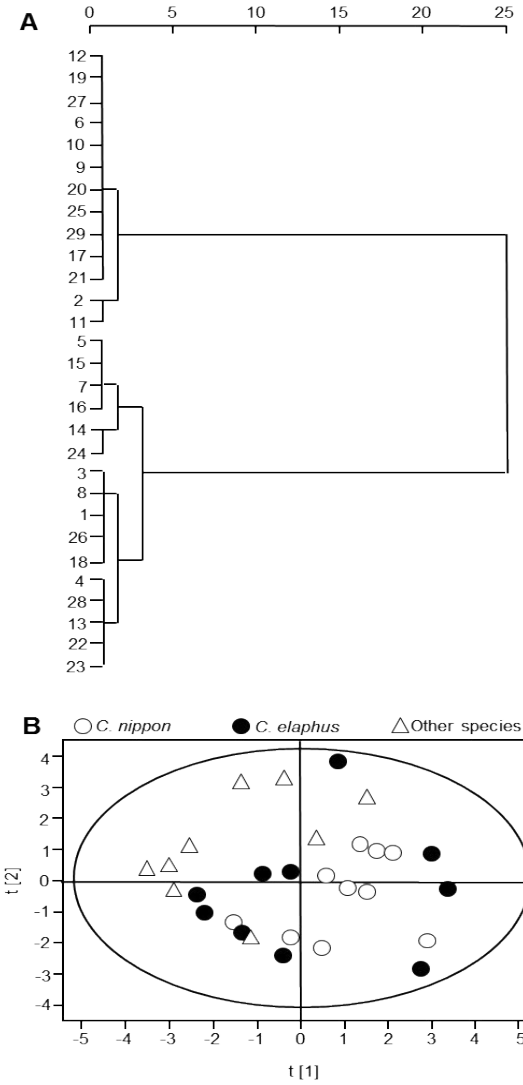
HPLC chromatograms of 29 samples (number 1 to 29 for different samples, shown in Table 1) at 260 nm were shown, n=5. The first profile showed the migration of marker chemicals, i.e. uracil (1), cytidine (2), uridine (3), hypoxanthine (4), inosine (5), guanine (6), guanosine (7), thymidine (8), adenosine (9), and adenine (10).

Figure 2: The representative HPLC chromatograms of mixed standards and 11 deer species.

and nucleobases in antlers were determined. The contents of nucleosides and nucleobases varied significantly in different species, as well as their habitats: the total content was ranged from 233.61 to 3,089.93 μg/g (Table 2). In general, these antlers contained high amounts of uracil, uridine, hypoxanthine, inosine and guanosine. In contrast, adenosine and adenine were hardly to be detected in the 29 samples. The contents of both total and individual nucleosides and nucleobases in *C. nippon* and *C. elaphus* varied greatly from different habitats (Table 2), which could be accounted for varying climate, local environment and feeding [22]. The contents of 10 tested analytes in 29 antler samples were input to HCA and PCA analyses. Both HCA and PCA could not discriminate deer antlers from different species (Figure 3).

To distinguish Cervi Cornu Pantotrichum from its counterfeits, HPLC fingerprint focusing on the amounts of nucleosides and nucleobases has been developed. Taking nucleosides and nucleobases as the only parameters for identification could not be possible: because these chemicals vary a lot in different collection areas as well as species origins. Nevertheless, the HPLC fingerprints of *C. nippon* and *C. elaphus* antlers shared a close similarity, regardless where they were collected, suggesting a possible application of this method.

Proteins have been proposed for identification purpose. From the extracts of deer antlers, specific protein bands were identified by SDS-PAGE [23,24]. Moreover, 5 proteins were identified from deer antler, e.g. β-3 subtype of hemoglobin, antimicrobial peptide, peptidoglycan recognition protein, β-c subtype of hemoglobin and pre-pro serum



(A): Dendrograms of hierarchical cluster analysis for the 29 samples of deer antlers. The loading plot was performed with the amounts of uracil, cytidine, uridine, hypoxanthine, inosine, guanine, guanosine, thymidine, adenosine, and adenine as shown in Table 2.
(B): Hierarchical clustering analysis for the 29 samples. The input data was as in **(A)**.

Figure 3: Analysis of 29 samples of deer antlers according to their content of nucleosides and nucleobases.

albumin [25]. Hormones have been identified in deer antlers, e.g. estradiol, testosterone, insulin-like growth factor-1 [26]. Nevertheless, the identified chemicals so far are not indicative for authentication of Cervi Cornu Pantotrichum from its counterfeits. Besides chemical analysis, DNA method has been used in authenticating Cervi Cornu Pantotrichum from its counterfeit antlers, which includes the sequencing of mitochondrial cytochrome b and cytochrome c oxidase subunit 1 genes [27], and PCR-RFLP analysis by using a set of primers flanking a conserved region of the mitochondrial 12S rRNA gene [28]. However, the molecular identification is not able to identify deer antlers of different quality. In addition, the employment of electronic nose has been reported in identifying fake antlers from Cervi Cornu Pantotrichum [29].

In conclusion, this is the first report using a simultaneous determination of 10 nucleosides and nucleobases in deer antlers from 11 species with HPLC–DAD method, which has been shown here to be a simple, rapid and accurate approach. The present results showed that most of deer antlers were rich in the content of nucleosides and nucleobases. The derived results showed similarity value in chemical fingerprints of *C. nippon* and *C. elaphus* was the highest. However, the investigated analytes could not fully discriminate deer antlers from different species. Therefore, specific chemical markers for quality control to discriminate deer antlers from different species would be studied in subsequent work.

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Sample	Species	Sources	Similarity[a,b]
1	*Cervus nippon*	Hongkong market	0.968 [a]
2	*Cervus nippon*	Hongkong market	0.994 [a]
3	*Cervus nippon*	Hongkong market	0.971 [a]
4	*Cervus nippon*	Hongkong market	0.836 [a]
5	*Cervus nippon*	Shanxi	0.997 [a]
6	*Cervus nippon*	Liaoning	0.969 [a]
7	*Cervus nippon*	Liaoning	0.998 [a]
8	*Cervus nippon*	Beijing	0.979 [a]
9	*Cervus nippon*	Jilin	0.936 [a]
10	*Cervus nippon*	Jilin	0.969 [a]
11	*Cervus elaphus*	Hongkong market	0.962 [a]
12	*Cervus elaphus*	Hongkong market	0.965 [a]
13	*Cervus elaphus*	Hongkong market	0.779 [a]
14	*Cervus elaphus*	Hongkong market	0.918 [a]
15	*Cervus elaphus*	Liaoning	0.986 [a]
16	*Cervus elaphus*	Jilin	0.974 [a]
17	*Cervus elaphus*	Xinjiang	0.887 [a]
18	*Cervus elaphus*	Jilin	0.909 [a]
19	*Cervus elaphus*	Jilin	0.965 [a]
20	*Cervus elaphus*	Jilin	0.927 [a]
21	*Rangifer tarandus*	Jilin	0.112 [b]
22	*Cervus timorensis*	Beijing	0.502 [b]
23	*Cervus unicolor*	Sichuan	0.548 [b]
24	*Cervus eldi hainanus*	Hainan	0.817 [b]
25	*Elaphurus davidianus*	Liaoning	0.852 [b]
26	*Cervus porcinus*	Beijing	0.749 [b]
27	*Cervus albirostris*	Sichuan	0.814 [b]
28	*Dada dama*	New Zealand	0.604 [b]
29	*Capreolus capreolus*	Jilin	0.935 [b]

[a] Similarities of each chromatogram to the corresponding representative standard fingerprint of each species by Similarity Evaluation System, *n*=4.
[b] Similarities of each species to the corresponding representative standard fingerprint of *C. nippon* by Similarity Evaluation system, *n*=4

Table 1: Similarities of HPLC fingerprints deriving from different species of deer antlers.

Sample	Uracil	Cytidine	Uridine	Hypoxanthine	Inosine	Guanine	Guanosine	Thymidine	Adenosine	Adenine	Total
1	409.08	10.32	706.43	331.7	362.74	201.75	617.12	30.22	nd[a]	nd	2669.36
2	720.61	23.58	385.66	554.86	401.68	196.6	454.87	170.9	nd	nd	2755.76
3	449.04	19.19	496.09	343.13	432.74	159.28	556.63	nd	tr[b]	nd	2456.1
4	230.36	36.93	711.29	289.1	356.11	142.13	471.32	39.81	tr	nd	2277.05
5	542.64	47.96	422.72	430.34	240.75	188.17	317.54	21.88	tr	nd	2212
6	770.77	nd	168.02	471.61	150.96	104.25	165.2	60.21	Nd	nd	1891.02
7	688.15	26.59	566.92	478.42	332.34	156.59	335.5	26.88	tr	nd	2611.39
8	405.03	1.95	443.41	270.25	277.85	191.61	414.77	31.33	tr	nd	2036.2
9	589.34	nd	5.38	343.82	22.97	167.79	51.94	8.45	tr	nd	1189.69
10	888.05	6.87	192.65	549.92	163.64	166.32	183.35	47.87	tr	nd	2198.67
11	907.84	25.44	196.33	587.92	241.54	305.42	393.22	148.2	nd	nd	2805.91
12	668.3	nd	109.5	405.94	72.29	92	62.83	59.34	nd	nd	1470.2
13	231.11	48.15	807.86	251.53	247.79	154.12	419.41	13.31	13.09	10.57	2196.94
14	256.67	24.38	536.97	330.66	110.66	67.36	111.55	24.17	tr	tr	1462.42
15	688.05	54.05	658.42	604.03	311.79	277.66	465.01	30.92	nd	nd	3089.93
16	340.63	48.18	375.37	377.44	156.74	154.1	220.17	nd	tr	tr	1672.63
17	847.27	36.69	86.34	234.14	35.89	nd	nd	22.31	tr	nd	1265.64
18	464.03	41.03	860.09	507.93	429.74	212.23	529.83	10.94	tr	tr	3055.82
19	859.98	nd	176.96	521.12	89.49	101.42	59.25	82.68	tr	tr	1890.9
20	326.2	10.68	24.27	199.7	16.36	89.43	47.82	16.39	tr	tr	730.85
21	150.93	nd	19.05	39.5	tr	24.13	Tr	nd	tr	tr	233.61
22	77.49	90.55	447.36	189.22	371.27	70.82	272.69	nd	tr	6.82	1526.22
23	218.37	177.74	539.75	310.72	447.41	85.49	375.8	nd	tr	nd	2155.28
24	176.58	17.74	319.26	188.69	86.87	137.93	163.66	29.33	17.71	9.89	1147.66
25	622.22	nd	90.7	265.72	21.14	177.44	70.88	87.62	nd	nd	1335.72
26	102.39	9.59	153.5	76.09	95.4	41.07	136.98	nd	nd	nd	615.02
27	273.95	7.86	32.62	186.98	16.55	28.14	42.82	nd	nd	nd	588.92
28	172.84	25.4	671.15	257.59	318.5	100.16	311.79	32.89	nd	nd	1890.32
29	115.39	15.9	22.74	70.07	14.77	60.27	30.8	14.62	nd	nd	344.56

The data in μg/g of dried antler, *n*=3 to 4
[a] nd: not detected
[b] tr: below the limit of quantification

Table 2: Contents of nucleosides and nucleobases in deer antlers

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