Study on the Marker Steroids of New Zealand Deer (*Cervus elaphus* var. *scoticus*) Velvet Antler by UPLC-MS/MS and HPLC-PDA Methods - (II)

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Abstract – Eleven steroid hormones (SHs: androstene-3,17-dione, estrone, β-estradiol, α-estradiol, testosterone, dehydroepiandrosterone, 17á-hydroxyprogesterone, medroxyprogesterone, megestrol acetate, progesterone, and androsterone) were detected from New Zealand deer (*Cervus elaphus* var. *scoticus*) velvet antler (NZA, 鹿茸). A method for the quantification of eleven SHs was established by using ultraperformance liquid chromatography (UPLC)-MS/MS. The linearities ($R^2 > 0.991$), limits of quantification (LOQ values, 0.3 ng/mL to 23.1 ng/mL), intraday and interday precisions (relative standard deviation: RSD < 2.43%), and recovery rates (97.3% to 104.6%) for all eleven SHs were determined. In addition, a method for the quantification of three 7-oxycholesterols (7-*O*-CSs: 7-ketocholesterol, 7α-hydroxycholesterol, and 7β-hydroxycholesterol) in the NZA was established by using an HPLC-photodiode array (PDA) method. The linearities ($R^2 > 0.999$), LOQ values (30 ng/mL to 350 ng/mL), intraday and interday precisions (RSD < 1.93%), and recovery rates (97.2% to 103.5%) for the three 7-*O*-CSs were determined. These quantitative methods are accurate, precise, and reproducible. As a result, it is suggested that the five steroid compounds of androstene-3,17-dione, androsterone, 7-ketocholesterol, 7α-hydroxycholesterol, and 7β-hydroxycholesterol could be marker steroids of NZA. These methods can be applied to quantify or standardize the marker steroids present in NZA.

Keywords – New Zealand deer antler, marker steroid, UPLC-MS/MS, HPLC-PDA, steroid hormone, 7-oxycholesterol

Introduction

Deer velvet antler (鹿茸) is among the most popular oriental herbal medicines. New Zealand deer (*Cervus elaphus* var. *scoticus*) velvet antler (NZA) is by-product of the deer species. There have been many studies on deer antler,[1,2] and 53 components including steroids have been detected and isolated previously from NZA.[3] Velvet antler is used as a growth tonic for children as well as for body strengthening, to increase blood cell production, to improve the immune system and cardiovascular health and as a geriatric therapy.[4] In addition, since the deer antler has a structure in which bones are reproduced at a very high speed, the genes related thereto are confirmed, and their proliferation and osteogenesis functions are also confirmed.[5]

NZA has steroid hormones (SHs) and 7-oxycholesterols (7-*O*-CSs).[3,6] SHs were detected from the raw material (RM) of deer antler by GC-MS/MS.[6] The SHs were synthesized from CSs. When the CS residues were cut off, the intermediates and androstene-3,7-dione were formed. This compound was converted to testosterone, and estrone, which was produced in an alternative pathway, was converted to estradiol.[7] Testosterone was expected to directly improve andropausal symptoms.[8] SHs help control the metabolism, inflammation, immune functions, salt and water balance, and development of sexual characteristics.[6,9-13] It is presumed that the tonic activities of deer antler is due to the biological activity of the corresponding SHs. Three 7-*O*-CSs (7-ketocholesterol, 7α-hydroxycholesterol, and 7β-hydroxycholesterol) were isolated from NZA.[3] Oxysteroids were detected in human plasma by ultra (U)PLC-MS/MS.[14] 7-*O*-CSs, oxygenated forms of cholesterols or their precursors, are intermediates of SHs in their biosynthetic pathways and show cytotoxicity.[15]

Additionally, the levels of steroids differ between the four parts (tip, upper, middle and base) of NZA. However, until now, no steroids have been assigned as chemical markers for the standardization of NZA. This can be a major grievance factor for the quality assessment of

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NZA-containing health food products. Therefore, it is very important to quantify marker steroids for standardization and quality control. Preferably, all steroids including SHs and 7-*O*-CSs can be candidate marker steroids for the standardization of NZA. However, minimizing the number of marker steroids is preferable for standardization. Therefore, it is considered appropriate to select suitable SHs and 7-*O*-CSs present in NZA as marker steroids.

The aim of the present study was to develop and validate two suitable UPLC-MS/MS and HPLC-PDA methods for the qualitative and quantitative determination of the marker SHs and 7-*O*-CSs in NZA following the International Conference on Harmonization (ICH) Guidelines.[16] Therefore, we analyzed the levels of various SHs and 7-*O*-CSs in NZA, and compared their data in detail to identify appropriate marker steroids for NZA.

Experimental

Reagents – HPLC solvents were from J.T. Baker (Phillipsburg, NJ, USA). Ultra-pure water was prepared using a Milli-Q Plus system at 18.2 MΩ (Millipore, Bedford, MA, USA). Androstene-3,17-dione, estrone, β-estradiol, α-estradiol, testosterone, DHEA, 17α-hydroxy-progesterone, medroxyprogesterone, megestrol acetate, progesterone, androsterone, cholesterol (CS), 7-ketocholesterol, 7α-hydroxycholesterol, and 7β-hydroxycholesterol were purchased from Sigma Chemical (St. Louis, MO, USA). All eluents were filtered through 0.45 μm filters (Millipore Filter Corp., Bedford, MA, USA) or Sep-Pak C_{18} cartridges (Waters Associates Inc., Milford, MA, USA) prior to injection.

Sample collection & extraction – Dried NZA (4-months-old) from *Cervus elaphus* var. *scoticus* was imported from New Zealand in 2015 and used as the RM. The materials were confirmed taxonomically by KGC raw materials headquarters, Korea Ginseng Corp., Daejeon, Korea, and a voucher specimen (NZA-2015) was deposited at the R&D headquarters of Korea Ginseng Corp., Daejeon, Korea. NZA (550 - 735 g/antler: tip 5.5%; upper 23.2%; middle 34.3%; base 36.9%) was sliced into 3-mm thick pieces and dried in the shade for one week. The pieces were ground into fine powders using a mill and used as a five kinds of NZA-RM samples (mixture of tip, upper, middle, and base; tip; upper; middle; base), respectively.

NZA-RM (mixture of tip, upper, middle, and base, 1 kg) was refluxed 4 times with 70% ethanol, concentrated to dryness, and lyophilized to yield the alcohol extract (NZA-AE: mixture of tip, upper, middle, and base, 131 g, 13.1%). and used as the samples of NZA-AE (mixture of tip, upper, middle, and base).

NZA-RM (mixture of tip, upper, middle, and base, 1 kg) was refluxed 4 times with water, concentrated to dryness, and lyophilized to yield the water extract (NZA-WE: mixture of tip, upper, middle, and base, 259 g, 25.9%) and used as the samples of NZA-WE (mixture of tip, upper, middle, and base).

Instrumentation & LC – The levels of eleven SHs in NZA were analyzed by a UPLC–MS/MS method. The chromatographic separations were carried out using a Waters Acquity UPLC®H-Class Quaternary solvent manager, a Waters Acquity™ Triple quadrupole (TQ) mass spectrometer, and an Acquity UPLC sample manager FTN. Waters Acquity Instrument control and data acquisition were performed with MassLynx™ V 4.1 software (Waters MS Technologies, Manchester, UK). A UPLC BEH shield RP_{18} column (2.1 mm ID × 50 mm, 1.7 μm) was used. The standard stock solution was separated using a gradient mobile phase consisting of water containing 0.1% formic acid (mobile phase A) and acetonitrile containing 0.1% formic acid (mobile phase B). Gradient elution was carried out as follows: 0→10 min, 90% A; 10→30 min, linear gradient to 0% A. The flow rate was 0.3 mL/min. The mass spectrometer was equipped with an ESI interface running in multiple reaction monitoring (MRM), and the ionization conditions are shown in Table 1. The sample was identified by comparing peak RTs, molecular ions [M+H]$^+$, and fragmentation patterns (MRM) with those of authentic steroid hormones.

The levels of three 7-*O*-CSs in NZA was analyzed by an HPLC–PDA method. The HPLC equipment used in this study included a Waters 1525 binary HPLC pump coupled with a Waters 2998 PDA. Data acquisition and processing were performed using Empower 3 software (Waters MS Technologies, Manchester, UK). The separations were carried out with a Chiralpak® AD-H column, 5 μm 4.6 mm ID × 250 mm column (Daicel Co, Japan). The mobile phase consisted of n-hexane/isopropanol (20/1, v/v) for 35 min. The flow rate was 0.5 mL/min at 30 ℃. The eluate was monitored at 205 nm. The sample was identified by comparing the peak retention times (RTs) and the absorbance in the PDA chromatogram (λ) to the data of authentic samples of the three 7-*O*-CSs.

Preparation of the standard solutions – Eleven SHs (androstene-3,17-dione, estrone, β-estradiol, α-estradiol, testosterone, DHEA, 17α-hydroxyprogesterone, medroxy-progesterone, megestrol acetate, progesterone, and andro-

Table 1. UPLC-MS/MS detection conditions for the eleven SHs

Compounds (SHs)	Ret. time (min)	Parent ion (*m/z*)	Adduct	Cone (voltage)	Collision (voltage)	Daughter ions (*m/z*)
Androstene-3,17-dione	14.8	287.41	$[M+H]^+$	36	22	109.04, 97.01
Estrone	15.1	271.37	$[M+H]^+$	28	26	133.05
β-Estradiol	15.1	273.38	$[M+H]^+$	24	26	107.03
α-Estradiol	15.4	273.37	$[M+H]^+$	30	20	135.07
Testosterone	15.8	289.43	$[M+H]^+$	36	26	109.04
Dehydroepiandrosterone	16.5	289.43	$[M+H]^+$	20	20	213.17
17α-Hydroxyprogesterone	16.7	331.46	$[M+H]^+$	36	26	109.04, 96.95
Medroxyprogesterone	19.3	345.49	$[M+H]^+$	38	26	123.02, 97.01
Megestrol acetate	19.5	385.51	$[M+H]^+$	30	16	325.19
Progesterone	20.3	315.47	$[M+H]^+$	22	24	108.98, 96.95
Androsterone	21.4	291.45	$[M+H]^+$	22	26	147.10

SH: steroid hormone

sterone) were accurately weighed and dissolved in methanol to prepare mixed stock solutions at 200 μg/mL. Three 7-*O*-CSs (7-ketocholesterol, 7α-hydroxycholesterol, and 7β-hydroxycholesterol) were accurately weighed and dissolved in methanol to prepare mixed stock solutions at 2.0 mg/mL. To construct each calibration curve (CC), the standard solutions (SHs: 0.1, 1.0, 5.0, 10.0, 50.0, 100.0, and 200 μg/mL; 7-*O*-CSs: 10.0, 50.0, 100.0, 500, 1000.0, and 2000 μg/mL) were prepared by diluting the stock solutions. The standard solutions were filtered through a membrane filter (0.45 μm) before analysis by UPLC-MS/MS or HPLC-PDA.

Preparation of sample solutions – The five powders (each 5.0 g) of NZA-RM (mixture of tip, upper, middle, and base; tip; upper; middle; base) were extracted by sonication in chloroform/methanol (2/1, v/v, 200 mL) at room temperature for 1 h. The suspensions were centrifuged, and the solutions were concentrated to afford the fractions. Subsequently, the fractions were partitioned between water (100 mL) and ethyl acetate (100 mL), the ethyl acetate layers were concentrated to afford the fractions. The ethyl acetate fractions were redissolved in methanol (20 mL), and the suspensions were centrifuged. The supernatants were passed through a Sep-Pak C_{18} cartridge, and the methanol eluates (each 2 μL) were analyzed by UPLC-MS/MS, respectively.

The fractions (each 500 mg) of NZA-AE and NZA-WE (each mixture of tip, upper, middle, and base) were partitioned between water (50 mL) and ethyl acetate (50 mL), and the ethyl acetate layers were concentrated to afford the fractions. The ethyl acetate fractions were redissolved in methanol (10 mL), the suspensions were centrifuged, and the supernatants were passed through a Sep-Pak C_{18} cartridge. The methanol eluates (each 2 μL)

were analyzed by UPLC-MS/MS, respectively.

The five powders (each 5.0 g) of NZA-RM (mixture of tip, upper, middle, and base; tip; upper; middle; base) were extracted by sonication in chloroform/methanol (2/1, v/v, 100 mL) at room temperature for 1 h. The suspensions were centrifuged, and the solutions were concentrated to afford the fractions. Subsequently, the fractions were partitioned between n-hexane (50 mL) and methanol (50 mL), and the methanol layers were concentrated to afford the fractions. The methanol fractions were partitioned between ethyl acetate (50 mL) and water (50 mL), and the ethyl acetate layers were concentrated to afford the fractions. The ethyl acetate fractions were redissolved with n-hexane/iso-propanol (20/1, v/v) (2 mL), and the solutions were filtered through a membrane filter (0.45 μm). The eluates (each 2 μL) were analyzed by HPLC-PDA analysis, respectively.

The fractions (each 100 mg) of NZA-AE and NZA-WE (each mixture of tip, upper, middle, and base) were partitioned between n-hexane (50 mL) and methanol (50 mL), and the methanol layers were concentrated to afford the fractions. The methanol fractions were partitioned between ethyl acetate (50 mL) and water (50 mL), and the ethyl acetate layers were concentrated to afford the fractions. The ethyl acetate fractions were redissolved with in n-hexane/iso-propanol (20/1, v/v) (2 mL), and the solution were filtered through a membrane filter (0.45 μm). The eluates (each 2 μL) were analyzed by HPLC-PDA analysis, respectively.

Method validation – The UPLC-MS/MS and HPLC-PDA methods were validated in terms of specificity, linearity, sensitivity, precision, and accuracy.

Specificity – Eleven SHs and three 7-*O*-CSs were well-separated from the other compounds (Figs. 1 and 2). The
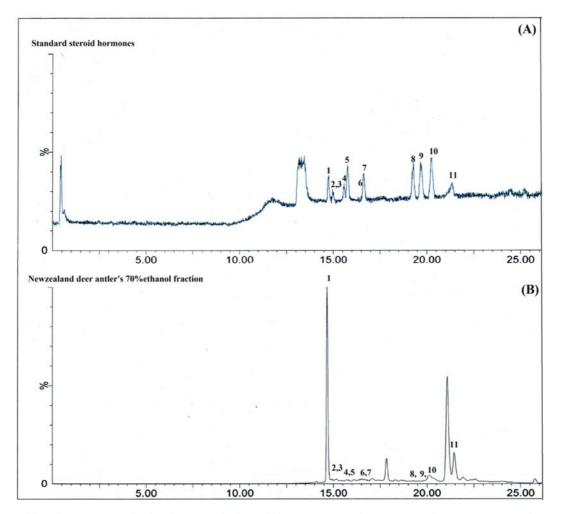
Fig. 1. The total ion chromatograms for the eleven standard steroid hormones (1. androstene-3, 17-dione; 2. estrone; 3. β-estradiol; 4. α-estradiol; 5. testosterone; 6. dehydroepiandrosterone; 7. 17α-hydroxyprogesterone; 8. medroxyprogesterone; 9. megestrol acetate; 10. progesterone; 11. androsterone) (A) and New Zealand deer antler's 70% ethanol fraction (B) by UPLC-MS/MS in multiple reaction monitoring mode.

amount of each of the eleven SHs and three 7-*O*-CSs in the sample was determined using a calibration curve (CC) generated with the corresponding authentic standard. The quantity of each analyte was subsequently determined from the corresponding CC.

Linearity – The standard curves of the eleven SHs and three 7-*O*-CSs were constructed by plotting the peak area ratio of the SHs and three 7-*O*-CSs over their concentrations, which ranged from 0.01 to 200 μg/mL or 0.5 to 2,000 μg/mL, respectively. The eleven SHs and three 7-*O*-CSs were dissolved in methanol and diluted to generate solutions of 6 different concentrations (n=3), which were used to calculate the linear regression equation (LRE) and correlation coefficient for the corresponding analyte (Table 2).

Sensitivity – The limit of detection (LOD) and the limit of quantitation (LOQ) were calculated with 3.3 σ/s

and 10 σ/s, respectively, where σ is the standard deviation (SD), and s is the slope of the regression equation (RE).

Precision – The precision was determined by analyzing the same sample 3 times by UPLC-MS/MS or HPLC-PDA for intraday and interday, respectively. The solutions of the eleven standard SHs (10 μg/mL) and three 7-*O*-CSs (100 μg/mL) were analyzed in triplicate.

Accuracy – The accuracy of this method was evaluated by a recovery test (standard addition method). The recovery tests were performed by adding three different levels [eleven SHs standard solutions: androstene-3,17-dione (3, 15, and 30 μg/mL), estrone (1.0, 10.0, and 20.0 μg/mL), β-estradiol (0.3, 3.0, and 30.0 μg/mL), α-estradiol (0.1, 0.5, and 1.0 μg/mL), testosterone (0.3, 3.0, and 30.0 μg/mL), DHEA (0.5, 5.0, and 30.0 μg/mL), 17α-hydroxyprogesterone (0.2, 0.5, and 5.0 μg/mL), medroxy-progesterone (0.2, 1.0, and 2.0 μg/mL), megestrol acetate
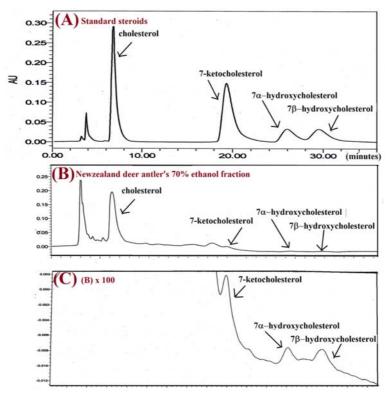
Fig. 2. The chromatograms for (A): the standard of three 7-oxycholesterols (7-ketocholesterol, 7α-hydroxycholesterol, and 7β-hydroxycholesterol); (B): the 70% ethanol fraction (mixture of tip, upper, middle, and base) of New Zealand deer antler; (C): the chromatograph of 100 times the height of (B) by HPLC-PDA.

Table 2. Regression equation, correlation coefficients (R^2), linear range, the LODs, and the LOQs for eleven SHs and three 7-*O*-CSs

Compounds	Range (μg/mL)	Regression equation (n=3)	R^2	LOD (ng/mL)	LOQ (ng/mL)
Androstene-3,17-dione (A)	0.1 – 200	y = 825x – 4750	0.999	3.3	10
Estrone	0.1 – 200	y = 722000x – 96700	0.997	0.26	0.78
β-Estradiol	0.1 – 200	y = 6430 x – 33600	0.999	7.13	23.1
α-Estradiol	0.1 – 200	y = 83645x + 5823.7	0.996	0.32	0.96
Testosterone	0.1– 200	y = 753x – 4390	0.999	3.67	11.0
Dehydroepiandrosterone	0.1 – 200	y = 1310x – 2180	0.999	7.0	21.0
17α-Hydroxyprogesterone	0.1 – 200	y = 121921x + 37587	0.999	0.1	0.3
Medroxyprogesterone	0.1 – 200	y = 45393x + 9989.7	0.999	0.38	1.14
Megestrol acetate	0.1 – 200	y = 34299x + 30807.8	0.999	1.1	3.3
Progesterone	0.1 – 200	y = 184045 x + 118121	0.999	0.5	1.50
Androsterone (A)	0.1 – 200	y = 5636.2 x + 1061.8	0.992	3.6	10.8
7-Ketocholesterol (A)	10.0 – 2000	y = 621000x – 28700	0.999	10	30
7α-Hydroxycholesterol (A)	10.0 – 2000	y = 659000x – 29200	0.999	95	285
7β-Hydroxycholesterol (A)	10.0 – 2000	y = 547000x – 31700	0.999	116	348

A: marker steroid of New Zealand deer antler; mixture: mixture of 4 part (tip, upper, middle, and base); LOD: the limit of detection; LOQ: the limit of quantitation; SH: steroid hormone; 7-*O*-CS: 7-oxycholesterol

(0.1, 0.5, and 2.0 μg/mL), progesterone (0.2, 1.0, and 2.00 μg/mL), and androsterone (0.5, 5.0, and 100.0 μg/mL), three 7-*O*-CSs standard solutions: 7-ketocholesterol (50.0, 100.0, and 1000.0 μg/mL), 7α-hydroxycholesterol (20.0, 50.0, and 500.0 μg/mL), and 7β-hydroxycholesterol (50.0, 100.0, and 1000.0 μg/mL)] to the sample of NZA-AE.

Statistical analysis – The data (mean ± SD) are based on the triplicate determinations. The statistical comparisons were made by one-way ANOVA and were considered statistically significant at p<0.05 by Tukey's test.

Results and Discussion

Eleven SHs were well-resolved by the reversed-phase UPLC-MS/MS conditions. However, isomeric 7α-hydroxycholesterol and 7β-hydroxycholesterol coeluted and showed the same pattern of daughter fragment ions in UPLC-MS/MS. Isomeric 7α-hydroxycholesterol and 7β-hydroxycholesterol could only be separated by normal-phase HPLC-PDA. However, the SHs and 7-O-CSs cannot be simultaneously detected using the normal-phase HPLC-PDA conditions because the LOQ is low for SHs. Therefore, the two types of steroids (SHs and 7-O-CSs) were independently analyzed by UPLC-MS/MS and HPLC-PDA methods, respectively.

Eleven SHs of androstene-3,17-dione, estrone, β-estradiol, α-estradiol, testosterone, DHEA, 17α-hydroxyprogesterone, medroxyprogesterone, megestrol acetate, progesterone, and androsterone were detected in the NZA by UPLC-MS/MS.

The molecular ion peaks of each of the above eleven SHs were acquired by UPLC-MS/MS (ESI$^+$): androstene-3,17-dione m/z: 287.41 [M+H]$^+$, 309.42 [M+Na]$^+$; estrone m/z: 271.37 [M+H]$^+$, 293.37 [M+Na]$^+$; β-estradiol m/z: 273.38 [M+H]$^+$, 256.38[M-H$_2$O]$^+$, 295.38 [M+Na]$^+$; α-estradiol m/z: 273.38 [M+H]$^+$, 256.38[M-H$_2$O]$^+$, 295.39 [M+Na]$^+$; testosterone m/z: 289.43 [M+H]$^+$, 311.43 [M+Na]$^+$; DHEA m/z: 289.43 [M+H]$^+$, 311.43 [M+Na]$^+$, 272.43 [M-H$_2$O]$^+$; 17α-hydroxyprogesterone m/z: 331.46 [M+H]$^+$, 353.43 [M+Na]$^+$; medroxyprogesterone m/z: 345.49 [M+H]$^+$, 367.49 [M+Na]$^+$; megestrol acetate m/z: 385.51 [M+H]$^+$, 407.51 [M+Na]$^+$; progesterone m/z: 315.47 [M+H]$^+$, 337.47 [M+Na]$^+$; and androsterone m/z: 291.45 [M+H]$^+$, 312.45 [M+Na]$^+$, 274.45 [M-H$_2$O]$^+$.

The selectivity of the detection of SHs was determined based on their retention times (RTs). The RTs of the SHs, except those of β-estradiol and estrone, were identical, and coeluting peaks were not observed. In the MRM method, even if the different SHs (for example, estrone and β-estradiol) coeluted and only differed from each other in their patterns of daughter fragment ions, the two SHs can be extracted separately and quantified. Fortunately, the isomers β-estradiol and α-estradiol eluted with different RTs. Therefore, eleven SHs in the NZA could be quantified using UPLC-MS/MS. As a result of a preliminary examination using various columns, a BEH shield RP$_{18}$ column was selected. The mobile phase based on a gradient of acetonitrile and water under various conditions was optimized, and formic acid buffer was selected to improve reproducibility. As a result, eleven SHs were simultaneously detected, as shown in Fig. 1.

The UPLC-MS/MS method for evaluating the SHs in NZA was validated with respect to parameters including linearity, LOD, LOQ, precision, accuracy and specificity. Tables 2 – 4 showed the results of the method validation. The calibration curves of the eleven SHs were constructed in the range of 0.1 – 200 µg/mL to display the linear relationship. As shown in Table 2, all the calibration

Table 3. Intraday and interday precisions of eleven SHs and three 7-O-CSs (n=3)

Compound	Intraday RSD (%)			Interday RSD (%)
	Day 1	Day 2	Day 3	
Androstene-3,17-dione (A)	1.32	1.64	1.39	1.54
Estrone	1.46	1.95	2.12	1.54
β-Estradiol	1.85	2.08	2.43	2.31
α-Estradiol	1.90	2.37	2.28	2.25
Testosterone	1.53	1.85	1.78	1.77
Dehydroepiandrosterone	1.64	1.94	1.85	1.73
17α-Hydroxyprogesterone	1.76	1.72	1.97	2.08
Medroxyprogesterone	1.69	1.77	2.26	1.95
Megestrol acetate	1.82	2.17	2.17	2.04
Progesterone	1.54	1.42	1.78	1.63
Androsterone (A)	1.57	1.65	1.68	1.45
7-Ketocholesterol (A)	1.63	1.88	1.90	1.67
7α-Hydroxycholesterol (A)	1.38	1.93	1.85	1.63
7β-Hydroxycholesterol (A)	1.54	1.43	1.89	1.78

A: marker steroid of New Zealand deer antler; SH: steroid hormone; 7-O-CS: 7-oxycholesterol; RSD: relative standard deviation

Table 4. Recovery of eleven SHs and three 7-*O*-CSs

Compound	Recovery (%)		
	low	middle	high
Androstene-3,17-dione (A)	98.9	100.7	102.3
Estrone	97.3	98.7	101.8
β-Estradiol	97.4	98.1	99.2
α-Estradiol	97.5	98.9	103.6
Testosterone	98.7	100.8	103.4
Dehydroepiandrosterone	97.7	100.3	102.3
17α-Hydroxyprogesterone	97.7	99.4	102.9
Medroxyprogesterone	98.9	99.8	99.6
Megestrol acetate	98.4	99.6	101.4
Progesterone	98.9	101.7	103.6
Androsterone (A)	100.5	102.5	104.6
7-Ketocholesterol (A)	99.8	101.6	103.5
7α-Hydroxycholesterol (A)	97.2	99.2	101.5
7β-Hydroxycholesterol (A)	98.7	99.5	100.7

A: marker steroid of New Zealand deer antler; SH: steroid hormone; 7-*O*-CS: 7-oxycholesterol

curves show high correction coefficients ($R^2 > 0.991$) within the relevant range for the eleven SHs, indicating good linearity. The LODs and LOQs were determined to be in the ranges from 0.1 – 7.13 ng/mL to 0.3 – 23.1 ng/mL, respectively (Table 2). For all SHs, the relative standard deviations (RSD values) of the intraday and interday precision were found to be within the ranges of 1.32 – 2.43 and 1.45 – 2.31%, respectively (Table 3). The average recoveries were calculated, and the observed SHs exhibited good recovery rates of 97.3 – 104.6% (Table 4). Based on these results, the developed UPLC-MS/MS method for analyzing the SHs in NZA is accurate.[16]

As shown in Table 5, eleven SHs (androstene-3, 17-dione, estrone, β-estradiol, α-estradiol, testosterone, DHEA, 17α-hydroxyprogesterone, medroxyprogesterone, megestrol acetate, progesterone, and androsterone) were determined in NZA.

The levels of the SHs in NZA-AE (mixture of tip, upper, middle, and base) were approximately 3 - 20 times higher than the levels in NZA-RM (mixture of tip, upper, middle, and base). However, the SHs were not present in the NZA-WE (mixture of tip, upper, middle, and base).

The order of the NZA-AE-SH (mixture of tip, upper, middle, and base) levels was androsterone > androstene-3,17-dione > estrone > DHEA > 17α-hydroxyprogesterone > testosterone > progesterone > medroxyprogesterone > megestrol acetate > α-estradiol > β-estradiol. As described above, since NZA was a male deer's antler, the level of androgenic SH was estimated to be much higher than that of estrogenic SH. In particular, the level of androsterone

was the highest among the eleven SHs in NZA. Androsterone is produced in the body as a metabolite of testosterone.[17] It is a weak neurosteroid that can cross into the brain and can affect brain function.[17] On the other hand, α-estradiol was not detected in the NZA-RM (mixture of tip, upper, middle, and base) but was detected in the NZA-AE (mixture of tip, upper, middle, and base). In addition, β-estradiol was not detected in either the NZA-RM (mixture of tip, upper, middle, and base) or NZA-AE (mixture of tip, upper, middle, and base), but it was detected in the NZA-RM-tip and NZA-RM-upper.

In addition, as a rule, the order of the NZA-RM-SH levels according to the antler region was tip > upper > middle > base.

The levels of testosterone in pork, milk, and beef were 0.32, 0.11 and 0.13 ng/g, and the levels of progesterone in pork and beef were 0.38 and 3.84 ng/g, respectively, which were very low compared to those of NZA.[18]

As described above, the level of androsterone was the highest among the eleven SHs in NZA. The highest contents of androsterone and androstene-3,17-dione were 111.1 and 14.91 μg/g, respectively, in NZA-AE (mixture of tip, upper, middle, and base). In addition, these are distinct active ingredients, and they are easy to detect and separate. Therefore, androstene-3,17-dione and androsterone are presumed to be valid marker SHs of NZA.

The NZA-AE (mixture of tip, upper, middle, and base: 10 g) was suspended in water and partitioned with ethyl acetate. The resulting fractions were concentrated to give the ethyl acetate fraction (6.8 g) and water fraction (2.6 g),

respectively. The ethyl acetate fraction (6.8 g) was chromatographed on a silica gel column chromatography and eluted with a gradient of n-hexane n-hexane : ethyl acetate = 50 : 1→20 : 1→10 : 1→5 : 1→2 : 1→1 : 1→ 1 : 2→1 : 5→ethyl acetate →methanol in 4 fractions (AE-1: 890 mg, mixture of fats & cholesterol fatty acid ester; AE-2:2.67 g, mixture of fats & cholesterol; AE-3: 242 mg, mixture of cholesterol & 7-O-CSs, and AE-4: 2.4 g, mixture of nucleosides & amino acids). AE-3 (242 mg) was subjected to normal phase HPLC eluting with a silica gel column (n-hexane: ethyl acetate = 5 : 1, RI detector) to create 7-ketocholesterol (17 mg), 7β-hydr-oxycholesterol (8 mg), 7α-hydroxycholesterol (12 mg), respectively. These compounds were identified through comparing their FAB-MS, and ^{1}H- and ^{13}C-NMR data with related data in the cited literature.[3] Three 7-O-CSs of 7-ketocholesterol, 7α-hydroxycholesterol, and 7β-hydro-xycholesterol were detected in the NZA by HPLC-PDA (Fig. 2).

Various kinds of columns were tested for the separation of the three 7-O-CSs of 7-ketocholesterol, 7α-hydro-xycholesterol, and 7β-hydroxycholesterol. First, a normal-phase silica gel column was able to separate the isomeric compounds which are 7α-hydroxycholesterol and 7β-hydroxycholesterol, but it was not suitable for quantitative analysis due to the limited options for other conditions such as the mobile phase solvent (n-hexane, ethyl acetate, chloroform, etc.) and detector (refractive index detector).

Second, the separation of the isomeric compounds which are 7α-hydroxycholesterol and 7β-hydroxycholesterol with a normal-phase solvent (n-hexane, isopropanol, methanol, acetonitrile, etc.) using an aminopropyl (NH$_2$) column and a cyanopropyl (CN) column and HPLC-PDA detector was tested. The three 7-O-CSs including the isomers could be separated on these columns. However, since the two isomeric compounds eluted with similar RTs, the peaks of the two isomeric compounds commonly over-lapped during the quantitative analysis of several analytical samples, making the separation impossible. Third, in the MRM-UPLC-MS/MS method for the analysis of sterols in human plasma with a reversed-phase solvent,[14] quantitative analysis of the two compounds was not possible because 7α-hydroxycholesterol and its isomer which is 7β-hydroxycholesterol had the same RT and the same pattern of daughter ions. When a Chiralpak® AD-H column using normal-phase solvent was used, the three 7-O-CSs including the isomers were obtained (Fig. 2), and all compounds were sufficiently well resolved without any overlap of their peaks. Finally, the combination of a normal-phase solvent (n-hexane/isopropanol, 20/1, v/v) and a PDA detector was found to be beneficial.

The HPLC-PDA method for the evaluation of 7-O-CSs in NZA was validated with respect to parameters including linearity, LOD, LOQ, precision, accuracy and specificity. Tables 2–4 show the results of the method validation. The calibration curvs of the three 7-O-CSs

Table 5. The levels of eleven SHs and three 7-O-CSs in NZA

Mean ± SD (µg/g) Compound	New Zealand deer antler						
	Tip	Upper	Middle	Base	Mixture		
	RM	RM	RM	RM	RM	AE	WE
Androstene-3,17-dione (A)	10.1±0.64	5.72±0.23	1.38±0.43	0.29±0.01	2.94±0.11	14.91±0.63	0
Estrone	17.25±0.59	8.61±0.49	0.72±0.03	0	0.91±0.04	10.05±0.60	0
β-Estradiol	0.33±0.01	0.15±0.01	0	0	0	0	0
α-Estradiol	0.11±0.01	0	0	0	0	0.96±0.04	0
Testosterone	0.27±0.05	0.27±0.01	0.27±0.01	0.27±0.01	0.27±0.01	2.46±0.17	0
Dehydroepiandrosterone	1.22±0.08	0.47±0.02	0.41±0.02	0.12±0.01	0.61±0.04	4.42±0.18	0
17α-Hydroxyprogesterone	0.36±0.02	0.33±0.01	0.32±0.01	0	0.14±0.01	2.97±0.12	0
Medroxyprogesterone	0.25±0.01	0.25±0.01	0	0	0.125±0.01	2.31±0.17	0
Megestrol acetate	0.22±0.01	0.22±0.01	0.22±0.01	0.17±0.01	0.21±0.01	2.08±0.12	0
Progesterone	0.31±0.01	0.27±0.01	0.31±0.01	0.23±0.01	0.31±0.02	2.45±0.11	0
Androsterone (A)	78.57±4.43	7.54±0.35	5.86±0.17	0.52±0.02	8.44±0.36	111.01±3.23	0
7-Ketocholesterol (A)	113.82±5.78	81.42±6.13	72.12±2.76	51.01±2.36	71.33±4.29	1114.47±63.15	0
7α-Hydroxycholesterol (A)	71.61±4.66	44.76±1.14	21.27±1.29	23.57±1.53	35.51±1.85	460.16±16.23	0
7β-Hydroxycholesterol (A)	123.11±5.91	72.38±3.75	48.59±2.85	46.56±1.98	53.36±2.32	805.19±38.21	0

The data (mean±standard deviation) are based on the triplicate determinations; A: New Zealand deer antler marker steroid; Mixture: mix-ture of 4 part (tip, upper, middle, and base); RM: raw material; AE: alcohol extract.; WE: water extract; SH: steroid hormone; 7-O-CS: 7-oxycholesterol; NZA: New Zealand deer antler

were constructed in the range of 0.01 to 2.0 mg/mL to display the linear relationships. As shown in Table 2, all the CCs ($R^2 \geq 0.999$) showed good linearity within the range of the three 7-*O*-CSs. The LODs and LOQs were shown to range from $10 – 116$ ng/mL to $30 – 348$ ng/mL, respectively (Table 2). For all three 7-*O*-CSs, the RSD values of the intraday and interday precision were found to be within the scope of $1.38 – 1.93$ and $1.63 – 1.78$%, respectively (Table 3). The average recoveries were calculated, and the three 7-*O*-CSs showed good recovery rates of $97.2 – 103.5$% (Table 4). The selectivity of the detection of each 7-*O*-CS was determined by the RT. The RTs of the three 7-*O*-CSs were identical without any shift, and coeluting peaks were not observed. As a result, the HPLC-PDA method is highly accurate.[16]

As shown in Table 5, 7-ketocholesterol, 7α-hydroxycholesterol, and 7β-hydroxycholesterol were all detected in the NZA-RM (mixture of tip, upper, middle, and base; tip; upper; middle; base) and NZA-AE (mixture of tip, upper, middle, and base). The order of the levels of the three 7-*O*-CSs were all AE > RM. However, the three 7-*O*-CSs were not detected in NZA-WE (mixture of tip, upper, middle, and base).

By comparing the data of the above RM and AE in detail, the three 7-*O*-CSs were all present in NZA. Therefore, the three 7-*O*-CSs of 7-ketocholesterol, 7α-hydroxycholesterol and 7β-hydroxycholesterol are presumed to be valid marker 7-*O*-CSs for NZA.

In conclusion, eleven SHs (androstene-3,17-dione, estrone, β-estradiol, α-estradiol, testosterone, DHEA, 17α-hydroxyprogesterone, medroxyprogesterone, megestrol acetate, progesterone, and androsterone) and three 7-*O*-CSs (7-ketocholesterol, 7α-hydroxycholesterol, and 7β-hydroxycholesterol) were detected in NZA by a UPLC-MS/MS method and an HPLC–PDA method.

However, since NZA is a male deer's antler, the level of the androgenic SH was estimated to be much higher than that of the estrogenic SH.

In addition, the order of the NZA-RM-SH levels according to the antler region was tip > upper > middle > base. It is estimated that the contents of the SHs at the tip of the deer antler, where the SHs are generated, are much higher than in the other parts where the synthesis of these compounds has stopped.

The fourteen steroidal components (eleven SHs & three 7-*O*-CSs) were identified from NZA. However, the five compounds (two SHs: androstene-3,17-dione, androsterone; three 7-*O*-CSs: 7-ketocholesterol, 7α-hydroxycholesterol, and 7β-hydroxycholesterol) could be used as a marker substance for NZA.

Simple, rapid, specific, and accurate UPLC-MS/MS & HPLC-PDA methods for the quantification of the marker steroids (SHs & 7-*O*-CSs) in NZA were developed and validated. These methods were successfully applied for the analysis of the maker steroids in NZA.

These methods provide a rapid analytical process for the evaluation of the marker steroids in NZA and therefore may contribute to fingerprint analysis of SHs & 7-*O*-CSs and the quality evaluation of marker steroids in NZA. This is an important advance for quality control in the health food industry manufacturing oriental herbal medicinal products derived from NZA, various deer co-products and animal products containing the target SHs and 7-*O*-CSs.

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Conflict of interest   The authors declare no conflict of interest.

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