



## APPLICATION NOTE

### Liquid Chromatography / Mass Spectrometry

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## Simultaneous Detection of Estrogenic and Progestogenic Hormones in Edible Bird's Nest by Ultra-Performance Liquid Chromatography Tandem Mass Spectrometry

### Introduction

Edible bird's nests are created by swiftlets using solidified saliva and feathers. The nests are commonly used as an ingredient in soups, beverages and other foods. Besides other nutrients, the naturally occurring hormones in the edible bird's nests are considered to contribute to their nutritional and cosmetic value. To further enhance the cosmetic value, the nests can be

spiked with illegally high concentrations of hormones which can lead to deleterious health effects including metabolic disorders. In order to monitor occurrences of such illegal adulteration, it is necessary to develop a robust method for detection and quantification of hormones in edible bird's nests. This application note presents a sensitive and reliable LC/MS/MS method, with simple sample preparation, to determine if prosterogenic and estrogenic hormones are present in edible birds nest.

## Materials and Methods

### Sample Preparation

Sample was prepared for analysis using protocols described previously in the literature.<sup>1,2,3</sup> The test sample (0.5 g) was accurately weighed into a centrifuge tube. After adding 4 mL of water, the sample was blended. Having been allowed to stand for 30 minutes, 5 mL of acetonitrile was added. The sample was extracted with ultrasonication for 10 minutes and centrifuged for 5 minutes at 6000. The supernatant was transferred into a second glass tube and evaporated to approximately 1 mL using nitrogen flow at 45 °C. The solution was diluted with 5 mL water for C18 solid phase extraction (SPE). Firstly, the SPE cartridges were conditioned with 3 mL of methanol, followed by 3 mL of water. Then, the extract solution was loaded to the cartridges and washed with 5 mL of 10% methanol in water solution and 5 mL of water to remove interfering components. This was followed by elution with 3 mL methanol. The eluent was evaporated to dryness, and the residue was reconstituted with 0.5 mL acetonitrile/water (V/V, 1:2).

### LC/MS/MS Conditions

Analytes were separated on a PerkinElmer Altus® A-30 UPLC® system using a PerkinElmer SPP C18 column (2.1x100 mm, 2.7 µm). The LC method parameters are shown in Table 1. Mobile phase A was acetonitrile, and mobile phase B was water containing 0.1% formic acid (for positive mode) or 0.01% ammonia (for negative mode).

Table 1. LC method parameters.

Time (min)	Flow rate (mL/min)	%A	%B	Curve
Initial	0.3	25	75	
2.5	0.3	60	40	6
4.0	0.3	98	2	6
5.5	0.3	98	2	6
5.6	0.3	25	75	6
7.0	0.3	25	75	6

The the Altus UPLC® system was coupled to a PerkinElmer QSight™ 210 triple quadrupole mass spectrometer equipped with an electrospray ionization source operating in either positive or negative ion mode. The mass spectrometer operating conditions are summarized in Tables 2 through 4:

Table 2. MS Source conditions.

Drying Gas (nitrogen)	Nebulizer Gas (nitrogen)	Heating Gass Temp	HSID Temp	Electrospray Voltage
50	160	500 °C	320 °C	5000 V

Table 3. Optimized LC/MS/MS method parameters (positive mode).

Compound	CAS	RT/min	Transitions m/z	EV /V	CE /eV
Positive Mode: Progestogenic Steroid Hormone					
19-norethindrone	68-22-4	3.76	299.1/108.9	37	-40
			299.1/231		-25
21α-hydroxyprogesterone	64-85-7	3.83	331/109.1	39	-38
			331/97.1		-31
17α-hydroxyprogesterone	68-96-2	4.06	331/109.1	32	-41
			331/97.1		-24
D-(-)-Norgestrel	797-63-7	4.26	313/245.1	35	-24
			313/108.9		-41
Medroxyprogesterone	520-85-4	4.44	345/123.1	38	-36
			345/97.2		-41
Chlormadinone Acetate	302-22-7	4.83	405/345	34	-18
			405/308.9		-21
Progesterone	57-83-0	4.85	315/109.1	39	-40
			315/97		-33
Medroxyprogesterone-17-acetate	71-58-9	4.87	387/327	30	-20
			387/285.1		-25

Table 4. Optimized LC/MS/MS method parameters (negative mode).

Negative Mode: Estrogenic Hormone					
Compound	CAS	RT/min	Transitions m/z	EV /V	CE /eV
Estradiol	50-27-1	1.90	286.8/170.8	-20	48
			286.8/144.9		60
Estriol	50-28-2	3.52	270.8/144.8	-20	57
			270.8/182.8		54
Ethinylestradiol	57-63-6	3.80	294.8/144.9	-20	57
			294.8/158.9		48
Estrone	53-16-7	3.93	268.8/144.6	-27	50
			268.8/158.7		50
Diethylstilbestrol	6898-97-1	4.05	266.8/236.8	-20	37
			266.8/250.9		35
Dienestrol	84-17-3	4.13	264.7/92.7	-23	36
			264.7/170.8		27
Hexestrol	84-16-2	4.15	268.8/118.9	-20	65
			268.8/134		21

## Standards Calibration Solutions

The standard solutions were prepared in acetonitrile/water (V/V, 1:2). A concentration range of estradiol standards were prepared at 0.5, 1.0, 5.0, 10.0, 50.0 and 100.0 ng/mL respectively. The standards of 19-norethindrone, 21 $\alpha$ -hydroxyprogesterone, 17 $\alpha$ -hydroxyprogesterone, D-(-)-Norgestrel, Medroxyprogesterone, Chlormadinone acetate, Progesterone, Medroxyprogesterone-17-acetate, Estriol, Ethinylestradiol, Estrone, lethystilbestrol, Dienestrol and, Hexestrol were prepared at 0.1, 0.5, 1.0, 5.0, 10.0, 50.0 and 100.0 ng/mL respectively. All samples are prepared fresh prior to analysis.

## Data Acquisition and Processing

Data were acquired using PerkinElmer Simplicity Acquisition™ software and processed using Simplicity Quant™ software.

## Results and Discussion

### Sample Preparation Method Optimization

The effect of sample preparation conditions from the edible bird's nests was studied. This included parameters such as the volume of extraction solvent, ultrasound extraction time, type of rinse solutions, and the volume of elution solvents on recovery of analytes. The results showed that only varying the type of rinse solutions had a significant impact on recovery of analytes. The effect of rinse solution on recovery results is shown in Figure 1. A rinse solution comprising of methanol/water (10:90) gave the best recovery results.

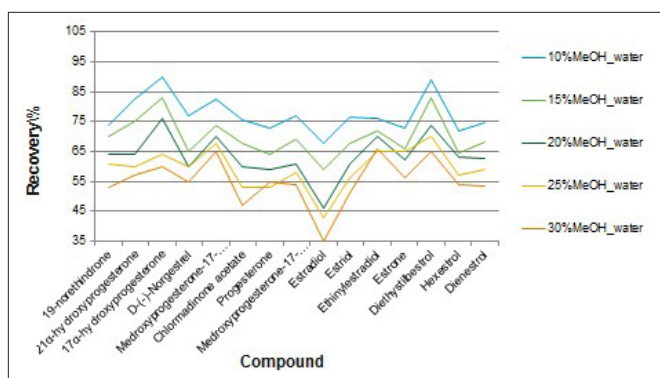


Figure 1. Effect of rinse solution on recovery.

### Calibration and Recovery Experiments

Figure 2 shows MRM chromatograms for progestogenic hormones at 0.05 ng/ml and estrogenic hormones at 0.2 ng/ml with excellent signal to noise ratio (shown in Table 5). Calibration curves were generated by using the calibration standards prepared according to the protocol described in the

experimental section. Figure 3 shows an example of the calibration curve for 21 $\alpha$ -hydroxyprogesterone over three orders of magnitude. Table 6 summarizes the linear dynamic range, LOQ, and RSD of the method. The LOQs of all hormones ranged from 0.01 to 0.2  $\mu$ g/Kg. The calibration curves showed good linearity with regression coefficients ( $R^2$ ) values greater than 0.993. Table 6 shows excellent precision of response for each analyte at low concentration levels. The data showed that the RSD of the response for the analytes was between 2.05 and 7.88% at low concentration levels.

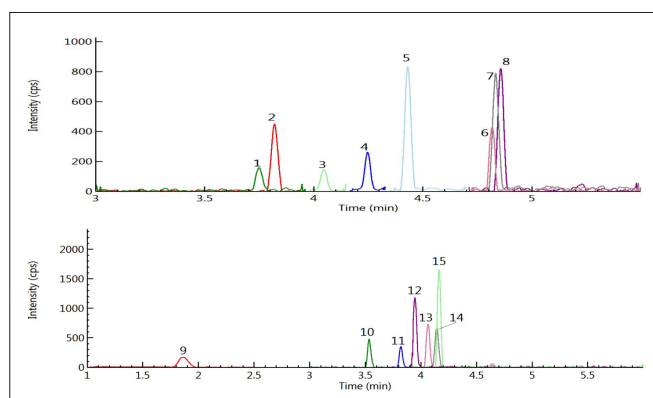


Figure 2. Typical LC/MS/MS chromatograms of hormones. (A) MRMs of progestogenic hormone standards at 0.05 ng/mL (B) MRMs of estrogenic hormone standards at 0.2 ng/mL.

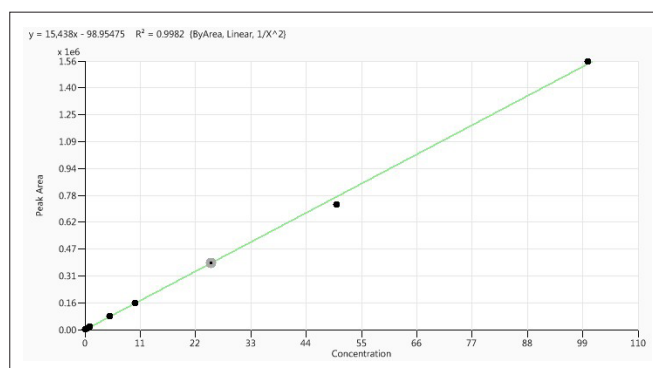


Figure 3. Calibration curves for 21 $\alpha$ -hydroxyprogesterone.

Recovery of the analytes was evaluated at concentrations of 0.5, 2, and 10  $\mu$ g/kg. All analyte recoveries were quite good, ranging from 60.5% to 99.5% (Table 7).

Table 5. Signal to noise ratio (S/N) of the analytes at low concentration level.

Compound	S/N
<b>Progestogenic Hormone</b>	
S/N at 0.05 ng/mL	
19-norethindrone	30
21 $\alpha$ -hydroxyprogesterone	100
17 $\alpha$ -hydroxyprogesterone	60
D-(-)-Norgestrel	50
Medroxyprogesterone	200
Chlormadinone Acetate	55
Progesterone	200
Medroxyprogesterone-17-acetate	97
<b>Estrogenic Hormone</b>	
S/N at 0.2 ng/mL	
Estradiol	40
Estriol	100
Ethinylestradiol	90
Estrone	120
Diethylstilbestrol	100
Dienestrol	83
Hexestro	190

Table 6. Calibration working range, Regression coefficient, LOQ and RSD%.

Compound	Working Range ng/mL	Regression Coefficients (R <sup>2</sup> )	LOQ ( $\mu$ g/kg)	RSD in 0.1 $\mu$ g/kg n=6
19-norethindrone	0.1-100	0.999	0.05	5.53%
21 $\alpha$ -hydroxyprogesterone	0.1-100	0.998	0.02	3.87%
17 $\alpha$ -hydroxyprogesterone	0.1-100	0.999	0.05	3.06%
D-(-)-Norgestrel	0.1-100	0.999	0.05	2.05%
Medroxyprogesterone	0.1-100	0.999	0.02	2.52%
Chlormadinone Acetate	0.1-100	0.993	0.05	6.02%
Progesterone	0.1-100	0.996	0.01	3.75%
Medroxyprogesterone-17-acetate	0.1-100	0.994	0.02	6.67%
Estradiol	0.5-100	0.998	0.2	7.88% (0.5 $\mu$ g/kg)
Estriol	0.1-100	0.999	0.05	6.27%
Ethinylestradiol	0.1-100	0.999	0.1	4.26%
Estrone	0.1-100	0.999	0.05	5.81%
Diethylstilbestrol	0.1-100	0.997	0.05	4.79%
Dienestrol	0.1-100	0.998	0.05	2.86%
Hexestrol	0.1-100	0.999	0.02	4.11%

Table 7. Recovery results at different spiked levels (n=6).

Compound	Spiked Level (0.5 $\mu$ g/kg) (%)	Spiked level (2 $\mu$ g/kg)(%)	Spiked Level (10 $\mu$ g/kg)(%)
19-norethindrone	75.2	79.3	73.9
21 $\alpha$ -hydroxyprogesterone	72.4	75.6	82.6
17 $\alpha$ -hydroxyprogesterone	99.5	91.7	90.1
D-(-)-Norgestrel	75.4	73.2	76.8
Medroxyprogesterone	71.1	80.2	82.7
Chlormadinone acetate	79.1	76.2	75.8
Progesterone	81.1	75.2	72.9
Medroxyprogesterone-17-acetate	71.5	73.3	76.8
Estradiol	60.5	68.2	67.8
Estriol	77.6	77.8	76.4
Ethinylestradiol	73.4	76.4	76.0
Estrone	74.1	74.2	72.8
Diethylstilbestrol	89.9	84.2	89.1
Dienestrol	77.6	76.4	74.5
Hexestrol	76.4	70.2	71.9

## Conclusions

In this study, we developed a rapid, sensitive and selective method for determination of fifteen hormones in edible bird's nests. The method has the advantage of detecting progestogenic and estrogenic hormones in a single extraction. The confirmation of the presence of analytes in edible bird's nests was achieved by ion ratio and retention time matching with their standards. The results demonstrated that the accuracy and precision was acceptable for routine monitoring purpose.

## References

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