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Excellence in Science

Application Note

LCMS-8050

Low level quantitation of steroids in milk using LC/MS/MS

No. LC-11-ADI-032

Introduction

Increase in incidences of some hormone-related cancers throughout the world is of great concern[1]. Dairy cows continue to lactate during the later half of pregnancy, when the concentration of estrogens in blood is high, and hence increases in milk. Main concern is about cows' milk, which contains considerable quantity of estrogens. Increased consumption of such animal-derived food may have adverse effects[2][3][4].
Hormones are chemicals that are naturally produced in the body of animals and human beings and have a number of important functions in life, such as reproduction or growth. However, due to the important role of these chemicals in several body functions, they also have been exogenously applied to animals and humans in order to obtain some kind of benefit in health or even to improve physical growth and performance. As a matter of fact, anabolic steroid hormones have played a key role among veterinary products in farming history and they have been one the most used and controversial components among veterinary drugs.

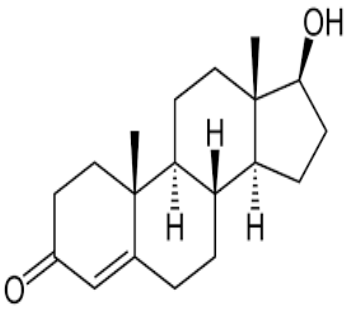

Figure 1A. Testosterone

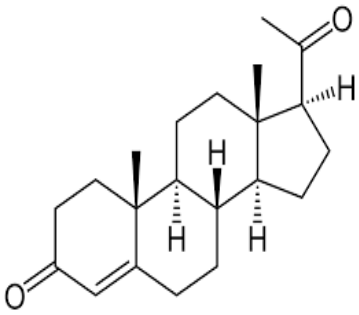

Figure 1B. Progesterone

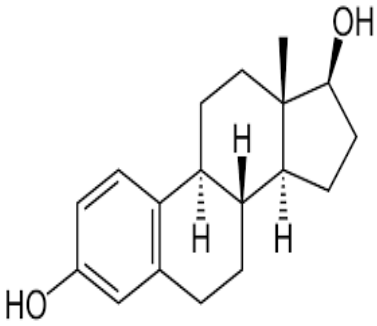

Figure 1C. Estradiol

The February 2010 article in the journal Pediatric International (February 2010. Vol. 52 #1) showed a potentially disturbing issue related to milk and its impact on the hormonal system.

The modern dairy cow has been converted to a full time milk factory. It is not often talked about, but modern industrialized dairy cattle continue to produce milk throughout their pregnancy. This milk goes directly into the food supply and contains varying amounts of bovine estrogen and progesterone. These hormones are then directly absorbed by consumers.

A highly specific LC/MS/MS method has been developed for trace level quantitation of steroids in milk using LCMS-8050, a triple quadrupole mass spectrometer from Shimadzu Corporation, Japan. Ultra high sensitivity of LCMS-8050 due to heated ESI source, enabled development of ppt level quantitation for testosterone, progesterone and estradiol.
(shown in Figures 1A, 1B and 1C) with good repeatability and specificity even in presence of complex matrix.

Experimental

Simple and straightforward sample preparation technique of QuEChERS was used which involves liquid-liquid extraction followed by dispersive Solid Phase Extraction (d-SPE). Sample preparation using QuEChERS allows for fast throughput and high sensitivity in food analysis. Various trials were taken for optimization of pre-treatment method. Such simple methodology can be used by small dairy farms and corporate milk producers.

Sample Preparation

Extraction (QuEChERS)

Place 10 mL whole milk into a 50 mL centrifuge tube. Add 10 mL acetonitrile and shake the tube vigorously for 1 minute. Add the contents of Q-Sep pouch salts for European Committee for Standardization (CEN) QuEChERS (cat # 26236) and shake vigorously for 1 minute. Centrifuge for 5 minutes at 4000 rpm and take a 1 mL aliquot of the supernatant (top layer) for d-SPE cleanup.

d-SPE Cleanup

Transfer 1 mL aliquot of supernatant to a 2 mL d-SPE cleanup tube that contains 150 mg of magnesium sulfate, 50 mg PSA sorbent and 50 mg C18 sorbent (cat # 26243) and shake vigorously for 1 minute. Centrifuge for 3 minutes at 4000 rpm and take a 0.5 mL aliquot as a sample for LCMS/MS analysis.

LC/MS/MS analysis

LCMS-8050 triple quadrupole mass spectrometer by Shimadzu (shown in Figure 2A), sets a new benchmark in triple quadrupole technology with an unsurpassed sensitivity (UF sensitivity) with scanning speed of 30,000 Da/Sec (UF scanning) and polarity switching speed of 5 msec (UF switching). This system ensures highest quality of data, with very high degree of reliability. In order to improve ionization efficiency, the newly developed heated ESI probe combines high-temperature gas with the nebulizer spray, assisting in the desolvation of large droplets and enhancing ionization. This development allows high-sensitivity analysis of a wide range of target compounds with considerable reduction in background. Presence of heated electro spray interface in LCMS-8050 (shown in Figure 2B) ensured good quantitative sensitivity for steroid seven in presence of a complex matrix like milk.MS voltages and collision energies were optimized to achieve maximum transmission of precursor and product ion. Gas flow rates and source temperature conditions were optimized, and linearity graph was plotted for three steroids.

Table 1. MRM conditions

Compound name	MRM transition (m/z)	Collision Energy (CE)	Dwell time (msec)
Testosterone	289.40>109.20	27	10
Progesterone	315.10>97.15	23	10
Estradiol	255.10>159.20	22	40

Figure 2A. LCMS-8050 triple quadrupole mass spectrometer by Shimadzu

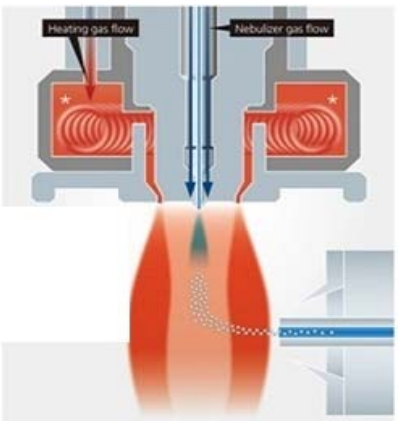

Figure 2B. Heated ionization probe

Table 2. LC conditions

<table>
<tr><td>Column</td><td>Shim-pack XR-ODS (75 mm L x 2.0 mm ID; 2.2 µm)</td></tr>
<tr><td>Mobile Phase</td><td>A : 0.1 % formic acid in water
B : 0.1 % formic acid in acetonitrile</td></tr>
<tr><td>Gradient Program</td><td>
<table>
<tr><th>Time (min)</th><th>A conc. (%)</th><th>B conc. (%)</th></tr>
<tr><td>0.01</td><td>70</td><td>30</td></tr>
<tr><td>3.5</td><td>10</td><td>90</td></tr>
<tr><td>4.0</td><td>10</td><td>90</td></tr>
<tr><td>4.3</td><td>70</td><td>30</td></tr>
<tr><td>6.0</td><td colspan="2">Stop</td></tr>
</table>
</td></tr>
<tr><td>Flow Rate</td><td>0.4 mL/min</td></tr>
<tr><td>Oven Temperature</td><td>40 ^{0}C</td></tr>
<tr><td>Injection Volume</td><td>20 µL</td></tr>
</table>

Table 3. LCMS conditions

Interface	Electro Spray Ionization (ESI)
Polarity	Positive
Nebulizing Gas Flow	2 L / min
Drying Gas Flow	10 L / min
Heating Gas Flow	10 L / min (zero air)
Interface Temp.	350 ^{0}C
Desolvation Line (DL) Temp.	250 ^{0}C
Heating Block Temp.	500 ^{o}C

Results and Discussion

LCMS/MS Analysis

Nexera UHPLC coupled with LCMS-8050 system was used for quantitation of progesterone, testosterone and estradiol in cow milk. The analysis was carried out using Shim-pack XR-ODS column with mobile phase containing 0.1 % formic acid in water & acetonitrile. The optimized gradient method was preferred to ensure complete elution of the matrix components (shown in Table 2).

MRM was optimised for all three steroids and following transitions were used for quantitation; testosterone, progesterone and estradiol in ESI positive mode (shown in Table 1). MS voltages and collision energy were optimised to achieve maximum transmission of mentioned precursor and product ions using the automatic MRM optimization feature of LCMS-8050. In addition, gas flow rates (Heating gas, Nebulizing gas, Drying gas), source temperature conditions (Interface temperature, DL temperature, Heat block temperature) and collision gas were also optimised (shown in Table 3).
Ultralow carryover is required with very high sensitive LC/MS/MS system. Nexera system coupled with fast autosampler demonstrates exceptional carry over performance for this analysis.

Under the optimized MRM conditions no interference was found in blank at the retention time of the steroids (shown in Figures 3A, 3B and 3C). Linearity solutions were prepared in methanol from 0.05 to 10 ppb for testosterone and estradiol and 0.25 to 50 ppb for progesterone. The linearity standards show correlation coefficient >0.999 for all three steroids, demonstrating the low level quantitation sensitivity of the instrument (shown in Figures 4A, 4B and 4C). Accuracy was found within acceptance criteria as shown in Table 4.

Specificity and interference

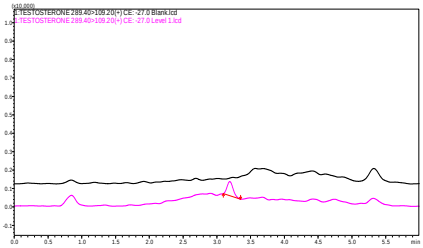

Figure 3A. Testosterone

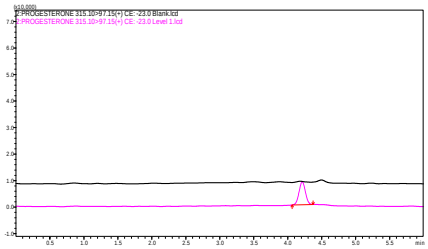

Figure 3B. Progesterone

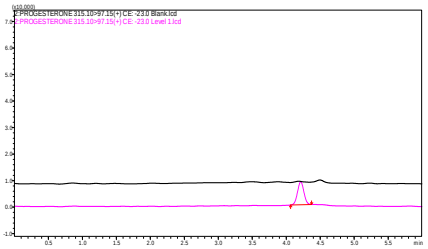

Figure 3C. Estradiol

Linearity

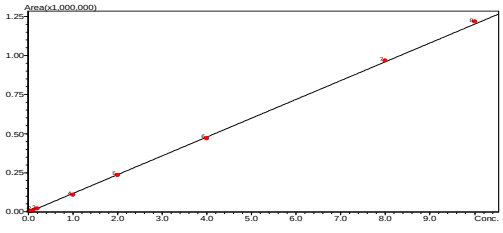

Figure 4A. Testosterone

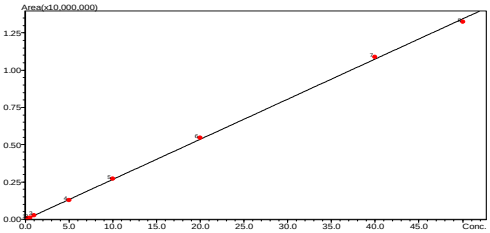

Figure 4B. Progesterone

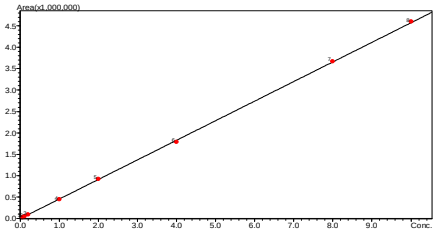

Figure 4C. Estradiol

Table 4. Result table

Level	Testosterone			Progesterone			Estradiol		
	Nominal conc. (ppb)	Measured conc. (ppb)	Accuracy (%)	Nominal conc.	Measured conc. (ppb)	Accuracy (%)	Nominal conc. (ppb)	Measured conc. (ppb)	Accuracy (%)
Std 1	0.05	0.059	118.0	0.25	0.273	109.1	0.05	0.052	103.9
Std 2	0.10	0.094	93.7	0.5	0.474	94.8	0.10	0.099	99.2
Std 3	0.20	0.197	98.3	1.0	0.979	97.9	0.20	0.199	99.5
Std 4	1.0	0.920	91.9	5.0	4.767	95.3	1.0	0.973	97.3
Std 5	2.0	1.960	98.1	10.0	10.131	101.3	2.0	2.028	101.4
Std 6	4.0	3.910	97.8	20.0	20.351	101.8	4.0	3.913	97.8
Std 7	8.0	8.070	100.9	40.0	40.500	101.2	8.0	8.021	100.3
Std 8	10.0	10.140	101.4	50.0	49.274	98.5	10.0	10.065	100.7

Recovery

Recovery was checked for each steroid hormone at low, mid and high concentration levels. Recovery was calculated by comparing the MRM peak area of samples spiked prior to QuEChERS extraction with calibrations standards linearity prepared in methanol. Progesterone was found around 5 ppb in sample and the same was taken for recovery study. Testosterone and estradiol of 0.1 ppb each and 0.5 ppb of progesterone was spiked in milk sample as a low spiked sample. For high spike sample 10 ppb of testosterone and estradiol and 50 ppb of progesterone was spiked in milk. The QuEChERS extraction procedure was followed to prepare sample and recovery was calculated which was found within the acceptance criteria (shown in Figures 5A, 5B and 5C).

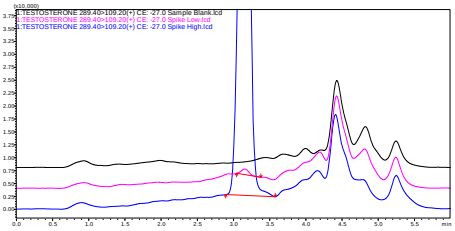

Figure 5A.
Spiked sample Testosterone

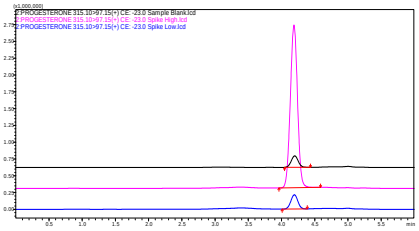

Figure 5B.
Spiked sample Progesterone

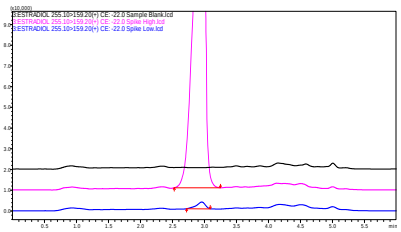

Figure 5C.
Spiked sample Estradiol

Conclusion

The QuEChERS is effective method for extraction of steroid hormones from milk.

D-Sep dispersive sample preparation is an easy and straightforward technique that requires minimal user training, resulting in high throughput sample preparation of complex milk matrix.

Nexera- LCMS-8050 enables sensitive quantitation of steroids in milk at low ppt concentrations.

References

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2. Farlow DW, Xu X, Veenstra TD, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci, 877, (2009), 1327-1334.

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