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Bovine Colostrum: Determination of Naturally Occurring Steroid Hormones by Liquid Chromatography−Tandem Mass Spectrometry (LC−MS/MS)

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ABSTRACT: The aim of this study was to collect further data about levels of endogenous hormones in foodstuffs derived from animal production. Because of expected higher concentrations of especially estrogens in colostrum compared to other foodstuffs, our investigation focused on this matrix. For evaluation of endogenous steroid hormones in separated colostrum (fat and defatted fraction) and colostrum powder, the relevant free and conjugated estrogens (estradiol-17β, estradiol-17α, estrone, and estriol) androgens (androstendione, 19*nor*-androstendione, 19*nor*-testosterone-17β, 19*nor*-testosterone-17α, testosterone-17β, and testosterone-17α), and progesterone were determined by means of liquid chromatography−tandem mass spectrometry (LC−MS/MS). Upmost determined concentrations were found in the fat fraction, with 25.56 and 7.59 μg/L for estrone and androstendione, respectively. In defatted milk and colostrum powder, conjugated estrogens dominated, whereas total (free and conjugated) estrone (5.51 μg/L; 15.0 μg/kg) exceeded estradiol-17α (2.66 μg/L; 7.5 μg/kg) and estradiol-17β (2.28 μg/L; 3.3 μg/kg). Neither 19*nor*-steroids nor estriol were detected in colostrum fractions or processed colostrum.

KEYWORDS: Androgens, colostrum, estrogens, estradiol, LC−MS/MS, steroids

INTRODUCTION

In recent years, there have been concerns about the presence of steroid hormones in edible matrices, covering a wide range of physical types of matrices, from tissue to fat and milk.[1] Estrogens are considered to be risk factors for cancer, especially of breasts, ovaries, and prostate.[2−5] Numerous investigations for the development of breast cancer bring the natural estradiol-17β (E2) in focus of possible causes. E2 is evaluated as a so-called "full carcinogen"; it is suspected to not only promote the tumor but also initiate the tumor. This implies a serious shift in the evaluation of endogenous compounds, with E2 belonged to the first endogenous compounds that raise suspicion to exhibit a tumor-initiating effect,[1,5] and leads to the situation that food derived from animals is discussed from a completely different perspective.

The trend in milk production, to prolong the lactation period (shorten the "dry off" period), is discussed as a possibility for increased estrogen residues in milk, especially at the time prior to parturition. Hoffmann et al.[6] has already demonstrated a further distinct increase of estrogens in the last month of gestation, culminating especially for estradiol 1−2 days prior to parturition. Within the discussion about possible risks of endogenous hormones in edible matrices, it is absolutely necessary to complete the data spectrum as well as to confirm or amend existing data by specific, current methods.

Natural steroid hormones are generally derived from cholesterol and have lipophilic properties. In biological fluids, e.g., milk, they are usually found in either a conjugated form, as sulfate or glucuronide derivatives, or bound to proteins.

Colostrum means, according to commission regulation (EC) number 1662/2006, the fluid secreted by the mammary glands of milk-producing animals up to 3−5 days post-parturition that is rich in antibodies and minerals and precedes the production of raw milk. Bovine calves are born without a fully developed immune system. After parturition, the cow transfers immune globulins to her calf via the colostrum.[7] Colostrum is rich in immunoglobulins (Ig), which provide a general disease resistance for the calf. An early (during the first 24 h following parturition) and adequate consumption of colostrum by the calf is the key factor that controls death loss and early calfhood diseases.[8]

Colostrum has been allowed to be distributed on the European market as a food of its own kind, such as dietary nutrition, since November 2006.[9] It has been considered a food substance and, therefore, has not been prohibited under the World Anti-Doping Code 2007 Prohibited List (effective from January 1, 2007). Rising scientific evidence suggests that human consumption of bovine colostrum is beneficial to general health.[10,11] Colostrum as a dietary supplement or as a "medical food" is suggested to raise general immunity and physical strength.[8,12,13]

The determination of residues in matrices of animal origin requires the development of extraction and cleanup methods prior to detection. This is due to the matrix complexity and the low concentrations (nanograms up to micrograms per kilogram) that would be expected.

The purpose of this study was to establish a specific and sensitive method for analysis of steroids at trace levels in colostrum by means

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of mass spectrometry.[14,15] To evaluate the steroid hormones in colostrum and colostrum products, the steroid profile in colostrum at different points of time was analyzed and the relevant free and conjugated steroids (progesterone, androgens, and estrogens) were determined.

MATERIALS AND METHODS

This study was performed according to the requirements of the Bavarian state animal welfare committee (Germany).

Reagents and Chemicals. All steroid standards, besides (IS) clostebol-D3 from RIVM (Bilthoven, The Netherlands), were purchased from Sigma-Aldrich (St. Louis, MO) and Makaira, Ltd., London, formerly Steraloids, Inc. (Newport, RI). Methanol for liquid chromatography−mass spectrometry (LC−MS) was LiChrosolv hypergrade for LC−MS, and water was Lichrosolv for chromatography from Merck (Darmstadt, Germany). Solvents for extraction and purification were high-performance liquid chromatography (HPLC)-grade.

β-Glucuronidase/arylsulfatase from *Helix pomatia* was obtained from (Merck), and β-glucuronidase from *Escherichia coli* was purchased from Roche Applied Science (Mannheim, Germany).

Animals and Samples. Colostrum samples from eight healthy Simmental cows, in their first or second lactation, were collected postpartum at five different points in time at intervals of around 12 h. Two different colostrum powders, Immunmilch 20% and Immunmilch 30%, were purchased from Phytobiotics Futterzusatzstoffe GmbH (Eltville, Germany). Plasma samples were taken within the last weeks of gestation (>250 days), and a urine sample was collected immediately after birth.

Hydrolysis and Sample Preparation. Frozen samples were thawed at room temperature. Colostrum samples were separated into the fat fraction (butter oil) and the skim milk fraction by centrifugation at 2000 rpm for 20 min. Butter oil was obtained by repeated shock freezing (liquid N_2) and heating (80 °C water bath). In default of a hormone-free matrix, quality-control samples were prepared by fortifying water with a standard mixture of estrogens and androgens + progesterone to final concentrations of 0.1, 0.2, 0.5, and 1.0 μg/L. All samples (1 mL of defatted milk/50 μL of butter oil) were fortified with 2 ng of internal standard (17β-estradiol-d_3 or chlortestosterone-d_3).

To determine the total concentration, free and conjugated forms, of estrogens in colostrum, 1 mL of skim milk fractions were enzymatically hydrolyzed at 37 °C for 3 h with β-glucuronidase/aryl sulfatase from *H. pomatia* in 1.5 mL of 50 mM acetate buffer (pH 4.8). To determine the total concentration of androgens, 1 mL of skim milk fraction was enzymatically hydrolyzed at 37 °C for 3 h with 1.5 mL of 40 mM phosphate buffer (pH 7.2) containing β-glucuronidase from *E. coli*.

Cleanup Procedures. A volume of 3 mL of a mixture of *tert*-butyl methyl ether (MTBE) with petroleum ether (PE; 30:70 MTBE/PE, v/v) was added to all (besides butter oil) samples, homogenized by overhead rotation for 20 min and centrifuged at 4000 rpm for 15 min at 18 °C. Organic phases were transferred to new glass tubes and evaporated. The extraction was repeated, and the organic phases were combined and evaporated. The extracts were then resuspended in 3 mL of PE (100%) and 1 mL of 80% methanol in water and homogenized for 20 min. After centrifugation at 4000 rpm for 15 min, the PE phases were discarded and the remaining 80% methanol was transferred to new glass tubes and evaporated. The residues were redissolved in 1.5 mL of water, and extraction procedures were performed as described with 3 mL of MTBE. Separation of the two fractions was performed by freezing the aqueous phase and decanting the organic phase into new glass tubes. After evaporation of the organic phase, extracts were redissolved in 100 μL of sample diluent, vortexed, agitated in an ultrasonic bath for 5 min, and vortexed again. The sample diluent for estrogens was composed of water/methanol (70:30, v/v) with 0.1% ammonium hydroxide. The diluent composite for androgens and gestagens was water/methanol (35:65, v/v) with 0.007% formic acid. After a short centrifugation step, the samples were transferred into MS sample tubes.

Table 1. Composition and Flow Rate of the Mobile Phase Gradient for Androgens + Progesterone[a]

time (min)	flow (μL/min)	eluent A (%)	eluent B (%)
0.0	200	35	65
20.0	200	0	100
30.0	200	0	100
30.1	200	35	65
45.0	200	35	65

[a] Eluent A, water with 0.007% formic acid; eluent B, methanol with 0.007% formic acid; injection volume, 20 μL.

Table 2. Composition and Flow Rate of the Mobile Phase Gradient for Estrogens[a]

run time (min)	flow rate (μL/min)	eluent A (%)	eluent B (%)
0.0	400	70	30
0.5	400	70	30
6.0	400	20	80
7.0	400	5	95
8.0	400	5	95
8.1	400	70	30
10.0	400	70	30

[a] Eluent A, water with 0.1% ammonium hydroxide; eluent B, methanol with 0.1% ammonium hydroxide; injection volume, 10 μL.

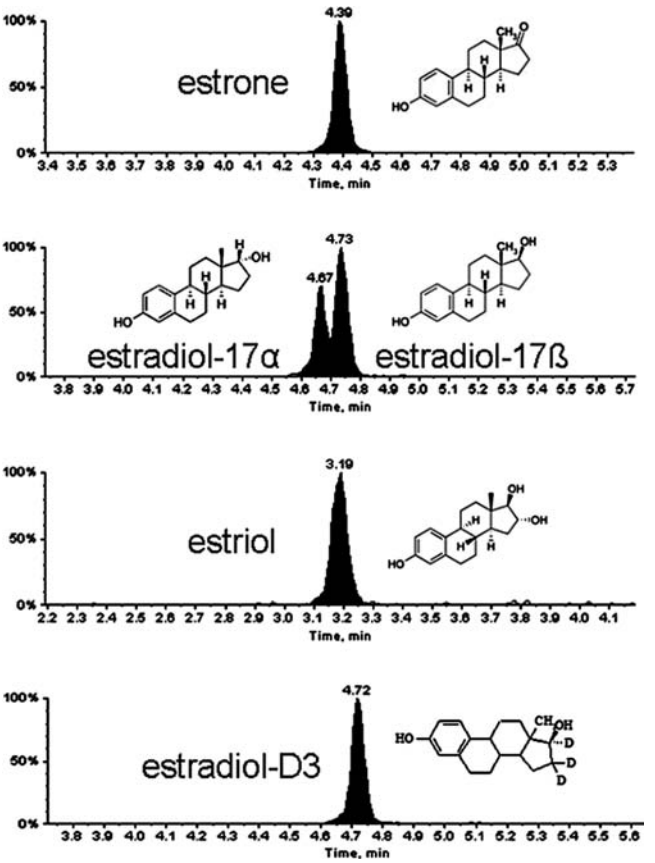

Figure 1. LC−MS/MS spectra of the diagnostic estrogen ions at 1 ng/mL.

A total of 50 μL of colostrum butter oil was transferred by a replacement pipet into 3 mL of PE, succeeded by rinsing the tip. Prior to the addition of 80% methanol in water, the PE was warmed up and vortexed. The two solvents were homogenized by overhead rotation for 20 min, succeeded by a centrifugation at 4000 rpm for 15 min. The PE phases were discarded, and the degreasing procedure was repeated by the addition of a further 3 mL of PE. The PE phases were again discarded. The remaining 80% methanol in water was transferred into new glass tubes and evaporated. The residues were reconstituted in 3 mL of MTBE, followed by vortex mixing and adding of 1.5 mL of water. After homogenization for 30 min and centrifugation for 15 min at 4000 rpm, the fractions were separated by freezing the aqueous phase and decanting the MTBE phases into new glass tubes for evaporation. Extracts were then redissolved in 100 μL of sample diluent (estrogens and androgens + progesterone), vortexed, agitated in an ultrasonic bath for 5 min, and vortexed again. After a short centrifugation step, the samples were transferred into MS sample tubes.

LC−MS/MS Measurements. The LC system for measurement of androgens and progesterone, a Perkin-Elmer series 200, was equipped with an autosampler, two micropumps, a mixer, and a vacuum degaser. The HPLC column was a Luna 18(2), 150 × 3 mm, 3 μm particle size (Phenomenex, Torrance, CA) and a C_{18} security guard column, 4 × 3 mm (Phenomenex), connected upstream, in a Jasco column oven with a temperature set at 25 ± 2 °C. Tandem MS monitoring of the LC separating androgens and progesterone was performed on an API 2000 (Applied Biosystems, Foster City, CA) using positive electrospray ionization (ESI). The composition and flow rate of the mobile phase are shown in Table 1.

LC for estrogens was performed on a Waters Acquity ultra-performance liquid chromatography (UPLC) consisting of a binary high-pressure-gradient management system, a sample manager, as well as a column manager. The column was an Acquity UPLC BEH Shield RP18, 2.1 × 100 mm, with a particle size of 1.7 μm (Waters Corporation, Milford, MA). The temperature was set at 60 °C. Tandem MS monitoring of the UPLC separated estrogens was performed on an API 4000 (Applied Biosystems, Foster City, CA) using negative ESI. The composition and flow rate of the mobile phase are shown in Table 2. Both LC−MS/MS systems (API 2000 and API 4000) were controlled, and data were visualized (Figure 1) by Analyst software (version 1.4.1, Applied Biosystems).

Quantification Procedure. Confirmation of results was in accordance with decision 2002/657/EC, beside the uncertainty in analytical quantification. Because of missing steroid-free colostrum, matrix effects could not be fully excluded, despite extraction and purification.

Statistics are given as means and standard deviation (Excel, MS Office).

Table 3. Steroid Concentrations (μg/L) in the First Sampling of Colostrum Postpartum (n = 8 Cows)

	skim milk (μg/L)		fat fraction (μg/L)
	free	total	free
progesterone	0.10 ± 0.10		2.52 ± 2.49
androstendione	0.77 ± 0.53	0.77 ± 0.53	7.59 ± 6.9
testosterone-17β	0.02 ± 0.01	0.032 ± 0.01	<0.4
testosterone-17α	0.13 ± 0.12	0.14 ± 0.11	1.18 ± 0.95
19*nor*-androstendione	<0.15	<0.15	<3
19*nor*-testosterone-17β	<0.05	<0.05	<1
19*nor*-testosterone-17α	<0.05	<0.05	<1
estrone	1.69 ± 2.05	5.51 ± 6.16	25.56 ± 35.79
estradiol-17α	0.84 ± 0.68	2.66 ± 2.74	5.94 ± 4.51
estradiol-17β	0.54 ± 0.52	2.28 ± 2.11	4.73 ± 2.37
estriol	<0.15	<0.15	<3

RESULTS AND DISCUSSION

For all analyzed samples, the sum of conjugated and free hormones was determined. The results obtained for the estrogens, androgens, and progesterone are presented in Tables 3 and 4. The maximum contents of estrone, estradiol-17β, and estradiol-17α (15, 3.3, and 7.5 μg/kg, respectively) in colostrum powder (Table 4) exceeded by far the concentrations published for products, such as meat, milk, milk products, and the highest measured concentrations in fish roe and domestic fowl eggs (1.0 and 0.9 μg/kg of estradiol-17β, respectively).[1,16,17] The progress of the measurable designated hormones plotted against the sampling interval followed the same pattern as demonstrated

Table 4. Concentrations (μg/kg and μg/L) of Different Steroids in Two Different Colostrum Powders, Plasma Samples at >256th Day of Pregnancy, and Urine Directly Postpartum

	colostrum powder (μg/kg)		plasma (μg/L) (n = 6)	urine (μg/L)
	"Immunmilch 30%" (free and conjugated if so)	"Immunmilch 20%" (free and conjugated if so)	pregnant cows (>256 days) (free and conjugated if so)	postpartum (free and conjugated if so)
progesterone	0.5	0.36	0.78 ± 0.49	0.4
androstendione	3.7	4.0	0.05 ± 0.01	1.4
testosterone-17β	0.2	<LOD	0.08 ± 0.06	1.2
testosterone-17α	1.4	<LOD	2.78 ± 1.14	>100
19*nor*-androstendione	<LOD	<LOD	<LOD	0.04
19*nor*-testosterone-17β	<LOD	<LOD	<LOD	0.23
19*nor*-testosterone-17α	<LOD	<LOD	<LOD	15.8
estrone	15.0	3.4	7.58 ± 2.52	>100
estradiol-17α	7.5	4.5	11.95 ± 7.94	>100
estradiol-17β	3.3	1.4	0.53 ± 0.33	81.0
estriol	<LOD	<LOD	<LOD	4.0

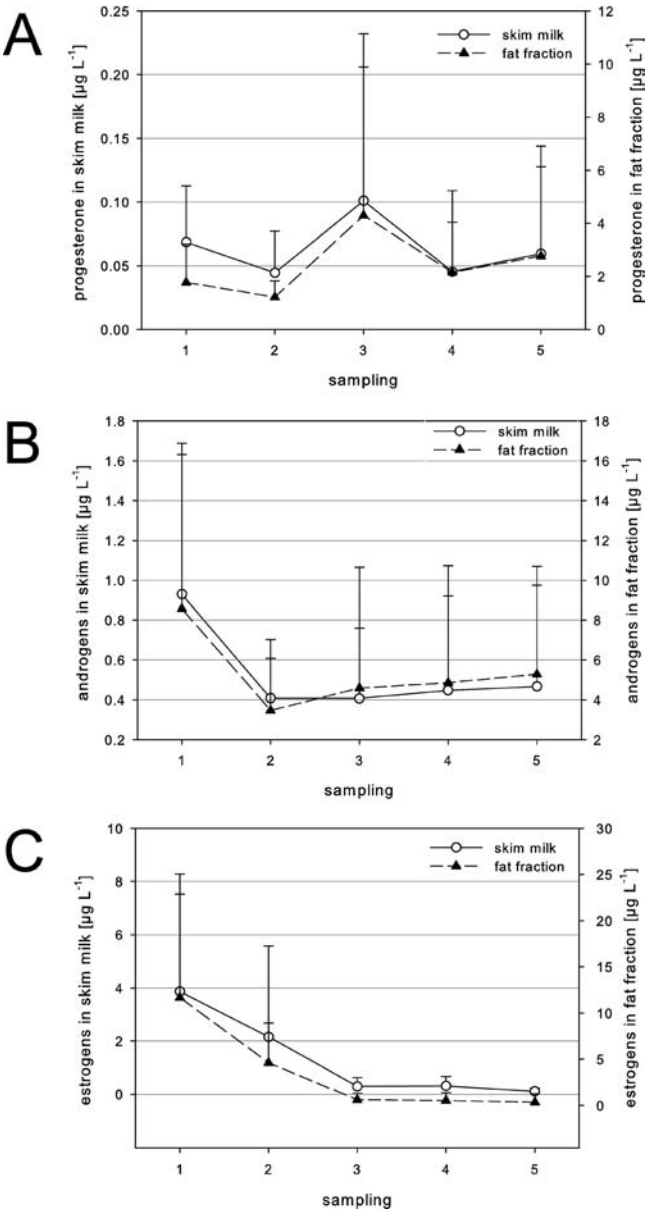

Figure 2. Progress of (A) progesterone, (B) total androgen, and (C) total estrogen concentrations in the colostrum skim milk and colostrum fat fraction, plotted against the sampling interval (12 h).

in Figure 2 for total estrogens (Figure 2C) and androgens (Figure 2B). Only progesterone (Figure 2A) and androstendione (not shown) remained on an equal level. The high deviations for the first interval reflect the differences in sampling time postpartum (hours). Within the colostrum sample, highest concentrations were found in the fat fraction. In the defatted fraction, the conjugated (sulfate or glucuronide) forms exceeded the free form (Table 3). The published[6] increased concentrations of estradiol at the end of gestation could be confirmed by the measured mean value of 12 μg/L estradiol-17α in the plasma of cows >256th day of pregnancy (Table 4). Only in the urine of a cow, taken next to parturition, could all designated hormones be detected.

It was evident that hormone contents in colostrum were highest when sampling took place next to parturition, with the exception of progesterone, remaining at a low level. Unconjugated steroid concentrations dominated in the fat fraction and

Table 5. LC−MS/MS Acquisition and LOQs for Estrogens

compound ESI (negative)	precursor ion (m/z)	product ions (m/z)	LOQ (μg/L) (μg/L oil)
estrone	269	145, 159, 183	0.03 (0.6)
estradiol-17α	271	145, 183, 143	0.08 (1.5)
estradiol-17β	271	145, 183, 143	0.08 (1.5)
estriol	287	171, 145, 183	0.15 (3.0)
estradiol-17β-D3	274	145, 185, 242	

Table 6. LC−MS/MS Acquisition and LOQs for Androgens + Progesterone

compound ESI (positive)	precursor ion (m/z)	product ions (m/z)	LOQ (μg/L) (μg/L oil)
progesterone	315	109, 97	0.01 (0.2)
androstendione	287	97, 109, 269	0.05 (1.0)
testosterone-17β	289	97, 109	0.01 (0.2)
testosterone-17α	289	97, 109	0.01 (0.2)
19*nor*-androstendione	273	197, 109	0.15 (3.0)
19*nor*-testosterone-17β	275	109, 239	0.05 (1.0)
19*nor*-testosterone-17α	275	109, 239	0.05 (1.0)
Cl-testosterone-D3	326	143	

the conjugated forms exceeded the free forms in skim milk. Predominant estrogen was estrone (26 μg/kg of fat), whereas estradiol-17α and estradiol-17β were present at lower levels. The major androgen was androstendione (8 μg/kg of fat), followed by testosterone-17α and testosterone-17β. Colostrum powder and plasma exhibited similar patterns, just differing in the range. Only urine comprised the whole spectrum of the designated steroids (Table 4).

The limit of detection (LOD) and limit of quantification (LOQ) for each analyte were determined on the basis of the concentration, inducing signal-to-noise ratios of 3 and 6, respectively. The LOQs for estrogens were found to be between 0.03 and 0.15 and between 0.6 and 3.0 μg/L in skim milk and fat, respectively (Table 5). For androgens, the LOQs were between 0.01 and 0.15 and between 0.2 and 3.0 μg/L in skim milk and fat, respectively (Table 6). The LOQs were found to be twice the LODs.

In conclusion, the used UPLC−MS/MS method could be validated to quantify the estrogens, androgens, and progestins in colostrum and colostrum products. The levels that we found were clearly beyond levels of steroid hormones in other foodstuffs.[1,16,17] The high concentrations of estrogens in colostrum are reflecting the unique situation of placental production at the end of gestation.

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