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Liquid Chromatography–Mass Spectrometry Method for the Quantitative Determination of Residues of Selected Veterinary Hormones in Powdered Ingredients Derived from Bovine Milk

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Supporting Information

ABSTRACT: A rugged, quantitative liquid chromatography–tandem mass spectrometry method with modified QuEChERS (quick, easy, cheap, effective, rugged, and safe) sample preparation for 17 selected veterinary hormones in six different powdered ingredients derived from bovine milk was developed and comprehensively validated. A universal post-extraction spiked matrix-matching approach based on whole milk powder has been successfully implemented. Three validation runs based on four levels of pre-extraction spiked quality control (QC) samples have been conducted. Overall accuracy (86–117%), overall precision (<20% RSD), selectivity, absolute extraction recovery (62–82%), matrix effect (<15% for most compounds), limits of detection (0.1–0.8 μg/kg, except for diethylstilbestrol at 3.8 μg/kg), limits of quantitation (0.2–2.0 μg/kg, except for diethylstilbestrol at 10.0 μg/kg), and extract stability (48 h) have been determined. The method is proposed for the routine analysis of hormones potentially present in powdered ingredients derived from bovine milk.

KEYWORDS: *hormones, milk, QuEChERS, LC–ESI-MS/MS*

INTRODUCTION

Milk is traded globally mainly in the form of powdered ingredients.[1] In this context, quantitative determination of residues of veterinary hormones in powdered ingredients derived from milk is of paramount importance to the nutritional products industry to ensure ingredients safety.

While numerous methods have been reported in the scientific literature for the determination of selected hormones present in animal products, e.g., liquid milk,[2−8] muscle,[2,5,9−15] liver,[2] and eggs,[5,8] and biological fluids, e.g. serum[16,17] and urine,[18,19] no methods have been reported for powdered ingredients derived from milk, such as whole milk powder, nonfat dry milk, milk protein concentrate, whey protein concentrate, sodium caseinate, and lactose. The chemical composition of such ingredients derived from milk is variable and highly complex. The protein content ranges from 25% in whole milk powder to >80% in concentrated protein powders, i.e., milk protein concentrate, whey protein concentrate, and sodium caseinate. The lipid content is 26–40% in whole milk powder and minimal in all other ingredients. Apart from the ingredient lactose, which is pure carbohydrate (>99%), other ingredients derived from milk contain substantial amounts of lactose, e.g., whole milk powder at 37% and nonfat dry milk at 50%. All ingredients derived from milk contain certain amounts of inorganic matter (4–8%) and moisture (2–5%).[20]

The preferred instrumental approach for hormones analysis is liquid chromatography–tandem mass spectrometry as evidenced in the above referenced publications. The QuEChERS (quick, easy, cheap, effective, rugged, and safe) methodology, initially developed for pesticide analysis in agricultural products,[21] is rapidly being adapted and implemented for the analysis of a host of other food contaminants such as veterinary drugs,[22−25] mycotoxins,[26−29] persistent organic pollutants,[30−34] marine biotoxins,[35] and endocrine disruptors,[36] among others.

The hormones included in the scope of the present work (Table 1) are those that are regulated by the European Union[37] and those included on Japan's positive list,[38] plus a few others that are banned in veterinary practice but can be potentially abused or misused (nandrolone, its metabolite 19-norandrosterone, boldenone, and its ester form boldenone acetate, and diethylstilbestrol).[39] For hormones that are regulated as esters (e.g., melengestrol acetate[38]), the free form (i.e., melengestrol) was also included when commercially available. Even though chlormadinone is regulated as the free form,[37] it was difficult to obtain as a chemical standard; hence the acetate form was used. While maximum residue limits (MRLs) have been established for certain hormones in milk by regulatory bodies[37,38] (Table 1), it is important to note that these MRLs cannot be directly extended to powdered ingredients derived from milk because the partitioning behavior of individual compounds into different milk fractions and their chemical stability during processing are largely unknown.

The development of an analytical method for the analysis of hormones at trace levels in ingredients derived from milk is a challenging task, given the variability and complexity of matrices. A further constraint is imposed by the lack of availability of stable isotope-labeled internal standards for many of the analytes considered.

Herein a core analytical method for the analysis of hormones has been developed and comprehensively validated in whole

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Table 1. Veterinary Hormones Considered along with Certain Physical Properties, Regulated Concentration Levels in Milk, and Method Calibration Levels Used

						MRL[d] (μg/kg)			
no.	compound (class[a])	CAS no.	formula	FW[b]	clogP[c]	EU	Japan	MIASS[e] (μg/mL)	matrix-based calibration levels (μg/kg)
1	boldenone (A)	846-48-0	$C_{19}H_{26}O_2$	286.41	3.09	prohibited	prohibited	0.5	0.5, 1, 2.5, 5, 12.5, 25, 37.5, 50
2	boldenone acetate (A)	2363-59-9	$C_{21}H_{28}O_3$	328.45	4.05	prohibited	prohibited	0.5	0.5, 1, 2.5, 5, 12.5, 25, 37.5, 50
3	clostebol (A)	1093-58-9	$C_{19}H_{27}ClO_2$	322.87	3.64	prohibited	0.5	0.5	0.5, 1, 2.5, 5, 12.5, 25, 37.5, 50
4	nandrolone (A)	434-22-0	$C_{18}H_{26}O_2$	274.40	2.90	prohibited	prohibited	2.0	2, 4, 10, 20, 50, 100, 150, 200
5	19-norandrosterone (A)	1225-01-0	$C_{18}H_{28}O_2$	276.41	3.65	prohibited	prohibited	2.0	2, 4, 10, 20, 50, 100, 150, 200
6	trenbolone (A)	10161-33-8	$C_{18}H_{22}O_2$	270.37	3.17	prohibited	prohibited	1.0	1, 2, 5, 10, 25, 50, 75, 100
7	dexamethasone (C)	50-02-2	$C_{22}H_{29}FO_5$	392.46	2.03	0.3	20	1.0	1, 2, 5, 10, 25, 50, 75, 100
8	hydrocortisone (C)	50-23-7	$C_{21}H_{30}O_5$	362.46	1.76		10	10.0	10, 20, 50, 100, 250, 500, 750, 1000
9	methylprednisolone (C)	83-43-2	$C_{22}H_{30}O_5$	374.47	2.17	prohibited	10	2.0	2, 4, 10, 20, 50, 100, 150, 200
10	prednisolone (C)	50-24-8	$C_{21}H_{28}O_5$	360.44	1.64	6	0.7	1.0	1, 2, 5, 10, 25, 50, 75, 100
11	altrenogest (P)	850-52-2	$C_{21}H_{26}O_2$	310.44	4.18		3	0.5	0.5, 1, 2.5, 5, 12.5, 25, 37.5, 50
12	chlormadinone acetate (P)	302-22-7	$C_{23}H_{29}ClO_4$	404.93	3.80	2.5	3	0.5	0.5, 1, 2.5, 5, 12.5, 25, 37.5, 50
13	fluorogestone acetate (P)	2529-45-5	$C_{23}H_{31}FO_5$	406.49	2.82	1		1.0	1, 2, 5, 10, 25, 50, 75, 100
14	melengestrol (P)	5633-18-1	$C_{23}H_{30}O_3$	354.48	3.06			0.5	0.5, 1, 2.5, 5, 12.5, 25, 37.5, 50
15	melengestrol acetate (P)	2919-66-6	$C_{25}H_{32}O_4$	396.52	3.35			0.5	0.5, 1, 2.5, 5, 12.5, 25, 37.5, 50
16	norgestimate (P)	35189-28-7	$C_{23}H_{31}NO_3$	369.50	5.13	0.12	0.1	0.5	0.5, 1, 2.5, 5, 12.5, 25, 37.5, 50
17	diethylstilbestrol (E)	56-53-1	$C_{18}H_{20}O_2$	268.35	5.33	prohibited	prohibited	10.0	10, 20, 50, 100, 250, 500, 750, 1000

[a]A, androgens; C, corticosteroids; P, progestagens; E, estrogens. [b]Formula weight. [c]Partition coefficient. [d]Maximum residue limit. [e]Mixed intermediate analyte stock solution.

milk powder (the most complex matrix of all examined ingredients), followed by extension of the core method to the other ingredients derived from milk.

MATERIALS AND METHODS

Safety. Solvents (methanol, acetonitrile) and formic acid should be dispensed in a hood, and appropriate laboratory safety glasses, coat, and gloves should be worn.

Chemicals and Reagents. Betamethasone (≥98%), dexamethasone (≥97%), hydrocortisone (≥98%), methylprednisolone (≥98%), prednisolone (≥99%), altrenogest (≥99.9%), chlormadinone acetate (≥98%), diethylstilbestrol (≥99%), and melengestrol acetate (≥98.5%) were purchased from Sigma-Aldrich Corp. (St. Louis, MO). Melengestrol, fluorogestone acetate, 19-norandrosterone, and norgestimate (homogeneous by thin layer chromatography) were purchased from Steraloids Inc. (Newport, RI). Boldenone, nandrolone, and trenbolone (1 mg/mL in acetonitrile) were purchased from Cerilliant Corp. (Round Rock, TX). Boldenone acetate (100 μg/mL in acetonitrile) was purchased from EQ Laboratories Inc. (Atlanta, GA). Clostebol (1 mg/mL in acetonitrile) was purchased from Grace Davison Discovery Sciences (Deerfield, IL). Prednisolone-2,4,6,6,21,21-d_6 (98 atom % D), dexamethasone-4,6α,21,21-d_4 (95% purity, 96 atom % D), and chlormadinone acetate-d_3 (98 atom % D) were purchased from C/D/N Isotopes Inc. (Pointe-Claire, Quebec, Canada). Nandrolone-16,16,17-d_3 (98 atom % D) (1 mg/mL in methanol) was purchased from Cambridge Isotope Laboratories Inc. (Andover, MA). Methanol (LC/MS grade) was from Fisher Scientific (Fair Lawn, NJ). Acetonitrile (LC/MS grade) was from Honeywell Burdick & Jackson (Muskegon, MI). Formic acid ∼98% was from Sigma-Aldrich Corp. (St. Louis, MO). DisQuE QuEChERS extraction tubes (50 mL) containing 1.5 g trisodium citrate dihydrate, 0.5 g disodium hydrogencitrate sesquihydrate, 1 g sodium chloride, and 4 g magnesium sulfate (implementing method EN 15662[40]) and dispersive tubes (2 mL) containing 150 mg magnesium sulfate, 25 mg primary−secondary amine (PSA), and 25 mg C18 sorbent were purchased from Waters Corp. (Milford, MA).

Samples. Whole milk powder (28.5% fat) was purchased from The Great American Spice Co. (Fort Wayne, IN) and from Franklin Farms East Inc. (Asbury, NJ). Nonfat dry milk was from Dairy America Inc. (Fresno, CA). Whey protein concentrate (75% protein) was from Leprino Foods Co. (Denver, CO). Milk protein concentrate (80% protein) was from Idaho Milk Products (Jerome, ID). Sodium caseinate (low viscosity) was from The Tatua Co-operative Dairy Company Ltd. (Tatuanui, New Zealand). Lactose was from Brewster Dairy Inc. (Brewster, OH).

Stock Solutions. Individual analyte and internal standard stock solutions were prepared in methanol at concentrations of 100 μg/mL. Boldenone acetate (100 μg/mL) was used as supplied by the manufacturer. Stock solutions were stable for 6 months at +5 °C. A mixed intermediate analyte stock solution was prepared by diluting the individual analyte stock solutions in methanol at the concentrations listed in Table 1. A 1:20 dilution of the mixed intermediate analyte stock solution was also prepared in methanol. A mixed intermediate internal standard stock solution was prepared by diluting the individual

internal standard stock solutions in methanol at the following concentrations: prednisolone-d_6, 0.5 μg/mL; dexamethasone-d_4, 0.1 μg/mL; nandrolone-d_3, 0.1 μg/mL; chlormadinone acetate-d_3, 0.1 μg/mL.

Sample Preparation. Sample portions of 1.00 ± 0.01 g were weighed into 50-mL polypropylene centrifuge tubes. Quality control (QC) samples were spiked with appropriate aliquots of the mixed intermediate analyte stock solution and 100 μL of the mixed intermediate internal standard stock solutions at this stage. In the next step 10 mL of 90/10 water−methanol containing 1% formic acid, pH 2.3 was added to each tube (except for sodium caseinate where 10 mL of 1% formic acid in water was used). The tubes were vortexed to allow thorough mixing of contents. Acetonitrile (10 mL) was added to the tubes followed by shaking for 30 s. The contents of the tubes were poured into 50-mL DisQuE QuEChERS extraction tubes, followed by brief shaking. The tubes were next shaken on an orbital shaker at 1000 rpm for 2 min, followed by centrifugation at 1300 rcf for 5 min. The supernatant layer (8.5−10.5 mL) in each tube was transferred to a 15-mL glass centrifuge tube with a glass pipet. The extracts were concentrated to 1 mL under a gentle stream of nitrogen at 55 °C. Concentrated extracts (1 mL) were transferred to 2-mL DisQuE QuEChERS dispersive tubes with a glass pipet, followed by brief shaking. Dispersive tubes were next shaken vigorously by hand for 30 s, followed by centrifugation at 800 rcf for 5 min. The supernatant layer (0.5−0.7 mL) in each tube was transferred to a clean 15-mL glass centrifuge tube with a glass pipet and concentrated to 0.1 mL under a gentle stream of nitrogen at 55 °C.

Calibration standards were similarly prepared by spiking the extracted blank whole milk powder matrix (as described above), with appropriate aliquots of the mixed intermediate analyte stock solution and 100 μL of the mixed intermediate internal standard stock solution at this stage (in order to achieve the concentrations listed in Table 1), followed by addition of a small aliquot of methanol (up to a total volume of 0.3 mL).

QC sample extracts were reconstituted with 0.9 mL of 60/40 water−methanol containing 0.1% formic acid. Spiked calibration standard extracts were reconstituted with 0.7 mL of 77/23 water−methanol containing 0.13% formic acid. All samples were vortexed briefly. Whole milk powder extracts were filtered through stacked PTFE filters (0.45 μm and a 0.2 μm). All other sample extracts were filtered through a single 0.2 μm PTFE filter.

Instrumentation. Analysis was performed on a Waters ACQUITY Ultra Performance LC coupled to a Xevo-TQMS triple quadrupole mass spectrometer. Chromatographic separation was carried out on a Waters ACQUITY UPLC BEH C18 column (1.7 μm, 2.1 mm × 100 mm) maintained at 60 °C, according to the gradient described in Table 2. The injection volumes were 10 μL. The mass spectrometer was operated in the positive electrospray (ESI+) mode with both quadrupoles tuned for unit resolution. Selected operating parameters were capillary voltage (3 kV), desolvation temperature (350 °C), desolvation gas (900 L/h), cone gas (50 L/h), collision gas (0.15 mL/min). Two multiple reaction monitoring (MRM) transitions were monitored for each compound, with cone voltages and collision energies were optimized for each transition (Table 3). The solvent flow was diverted to waste between 0 and 3.5 min and 12 and 16 min of each run.

Table 2. Mobile-Phase Conditions Used for Chromatographic Separation

time (min)	flow (mL/min)	% A[a]	% B[b]	curve
0.00	0.30	60	40	6
1.00	0.30	60	40	6
8.00	0.30	40	60	6
10.00	0.30	20	80	6
11.00	0.30	0	100	6
13.50	0.30	0	100	6
13.51	0.30	60	40	6
16.00	0.30	60	40	6

[a]0.1% formic acid in water. [b]0.1% formic acid in methanol.

RESULTS AND DISCUSSION

Method Development. A modified QuEChERS (quick, easy, cheap, effective, rugged, and safe) procedure was implemented. Instead of dispersal of the powder in pure water, it was found that 90/10 water−methanol (containing 1% formic acid) offers substantial gains in terms of partitioning efficiency in the extraction step. The only exception was sodium caseinate, for which strong ion suppression was noticed in several areas of the chromatogram when 10% methanol was used in the extraction solvent. By omitting methanol and using 1% formic acid in water for sample dispersal this problem was eliminated. The raw extract was concentrated to 1 mL before dispersive cleanup in order to achieve maximum sensitivity. The overall dilution ratio during the entire procedure is 1:1, i.e., 1 mL of extract is obtained from 1 g of sample. The reconstitution solvents for calibration standards and QCs were chosen in such a way as to achieve equal concentrations of organic solvent and formic acid in the final extracts of both and achieve compatibility with the starting mobile phase composition.

In a previous report on hormones analysis in milk,[2] an enzymatic hydrolysis step has been used to release conjugated forms (glucuronide and sulfate) of hormones that could potentially be present. The usefulness of the enzymatic hydrolysis step remains controversial. There are multiple limitations and pitfalls associated with this procedure, such as incomplete hydrolysis of hormone conjugates and chemical conversion of one steroid into another.[39,41] No significant difference was reported for hydrocortisone in milk with or without enzymatic hydrolysis.[2] For all other hormones included in the scope of this method, it is unknown what fraction of each (if any) may be found in their conjugated forms in milk. Analytical standards for most conjugated hormones are presently not available commercially, and this makes the validation of the enzymatic hydrolysis procedure impossible. Furthermore, the effects of processing of milk into powdered ingredients on hormone conjugates are not known either. On the basis of the above, an enzymatic hydrolysis step was not included in the present method. However, the issue of enzymatic hydrolysis of conjugated hormones is relevant to powdered ingredients, although it is beyond the scope of the present work. Future work is needed to elucidate the fate of hormone conjugates during the processing of milk to powdered ingredients and to evaluate the efficacy of enzymatic hydrolysis of individual hormone conjugates (when they become commercially available).

The modified QuEChERS procedure was found to provide sufficient cleanup. In a relevant report[2] matrix effects in milk could not be totally overcome even after sequential cleanup using two different solid-phase extraction cartridges. Compared to such a laborious procedure, the modified QuEChERS-based methodology coupled with a matrix-matching approach offers obvious advantages.

The hormones included in the scope of this method cover a wide range of polarities with partition coefficients (clogP) between 1.6 and 5.3 (Table 1). Chromatographic separation was carried out on a sub-2 μm C18 stationary phase with an acidic water−methanol gradient in which the organic component was varied between 40% and 100%. The column temperature was maintained at 60° in order to reduce back

Table 3. Optimized Multiple Reaction Monitoring (MRM) Transitions for Considered Veterinary Hormones

no.	compound	rt[a] (min)	cone (V)	MRM1[b] (quantifier)	collision energy (eV)	MRM2[b] (qualifier)	collision energy (eV)	dwell time (ms)
1	prednisolone-d_6 (IS[c])	3.99	30, 15	367.4 > 150.2	15	349.4 > 151.1	20	50
2	prednisolone	4.05	30, 15	361.4 > 147.1	15	343.4 >147.1	25	50
3	hydrocortisone	4.07	30	363.4 > 121.1	15	363.4 > 309.3	15	50
4	dexamethasone-d_4 (IS[c])	5.33	15	397.4 > 359.3	10	397.4 > 341.3	10	25
5	dexamethasone	5.38	15	393.4 > 355.3	10	393.4 > 337.3	10	25
6	methylprednisolone	5.63	15	375.4 > 357.3	10	375.4 > 339.3	10	25
7	trenbolone	5.70	35	271.3 > 199.2	25	271.3 > 227.2	25	25
8	boldenone	6.26	25	287.4 > 121.1	20	287.4 > 147.1	15	25
9	fluorogestone acetate	6.45	35	407.5 > 267.2	20	407.5 > 309.3	20	25
10	nandrolone-d_3 (IS[c])	6.45	35	278.4 > 260.3	15	278.4 > 242.2	15	25
11	nandrolone	6.49	35	275.4 > 239.2	15	275.4 > 257.3	15	25
12	diethylstilbestrol	7.37	25	269.2 > 135.1	15	269.2 > 107.1	25	25
13	melengestrol	8.40	30	355.4 > 236.3	30	355.4 > 279.3	20	25
14	clostebol	8.98	30	323.4 > 143.0	20	323.4 > 131.0	20	25
15	altrenogest	9.04	30	311.4 > 227.2	25	311.4 > 269.2	15	25
16	19-norandrosterone	9.50	25	259.4 > 145.1	20	259.4 > 241.3	20	25
17	chlormadinone acetate-d_3 (IS[c])	9.66	25	408.4 > 309.3	15	408.4 > 345.3	15	25
18	chlormadinone acetate	9.68	25	405.4 > 309.3	15	405.4 > 345.3	15	25
19	melengestrol acetate	9.90	25	397.4 > 279.2	20	397.4 > 337.3	15	25
20	boldenone acetate	10.01	20	329.4 > 135.1	15	329.4 > 121.1	25	25
21	norgestimate	10.75	40	370.5 > 124.1	25	370.5 > 310.3	25	25

[a]Retention time. [b]Multiple reaction monitoring. [c]Internal standard.

pressure at the used flow rate (0.3 mL/min). Under these conditions analytes eluted at 4−11 min, with a total run time of 16 min (Figure 1). Three different mass acquisition windows were used (3.5−5, 5−8, and 8−12 min, respectively). The four stable isotope-labeled internal standards were assigned to one or more of the 17 analytes based on retention time proximity (Table 3).

Norgestimate is a mixture of stereoisomers and elicits two peaks of which the later eluting (10.75 min) and most intense peak was used for quantitation. Betamethasone (regulated in milk[37,38]) is a stereoisomer of dexamethasone and was not included in the present method because only partial resolution between the two could be achieved, and inclusion of both would have prevented satisfactory integration of either peak. However, since the two compounds have identical MRM transitions, similar sensitivities, and identical maximum residue limits (MRLs), betamethasone can be analyzed by the same method in place of dexamethasone. The method offers sufficient chromatographic resolution to distinguish between the two stereoisomers in an incurred sample.

In the method described all analytes were detected in the positive electrospray ion mode with an acidic water−methanol eluent. While others have described the analysis of corticosteroids in the negative ion mode due to enhanced sensitivity,[2,42−46] hereby excellent sensitivity was achieved in the positive ion mode for the $[M + H]^+$ ion of each compound. Diethylstilbestrol elicited lower sensitivity in the positive ion mode than all other compounds. Indeed, the negative ionization mode is preferred for this compound due to its phenolic structure.[47]

Method Validation. Core method validation was carried out in whole milk powder, which has the most complex composition among all ingredients derived from milk and was expected to be the most challenging from an analytical standpoint. The core method was then cross-validated on the other ingredient matrices.

Selectivity was verified by inspecting retention windows of all analytes for interfering matrix peaks. Hydrocortisone (a natural component of milk) was present in some ingredients at a low μg/kg level. Hence the lowest calibration level used for this compound was 10 μg/kg, which gave an instrumental response ≥5 times that of the background level. Monitoring residue levels of hydrocortisone below this threshold in powdered ingredients derived from milk is not justified given the high MRL for this compound established in liquid milk at 10 μg/kg.[38] For all other compounds no significant chromatographic interferences were found.

Post-extraction matrix-matched calibration curves were established in whole milk powder. Eight calibration levels were used spanning a 100-fold concentration range (Table 1), with the lowest level close to the limit of quantitation. Calibration standards were analyzed at the beginning and at the end of each run. A quadratic regression model ($1/x^2$-weighted) was used for all compounds. The coefficients of determination (R^2) were ≥0.99 for all compounds except for diethylstilbestrol and norgestimate, for which they were ≥0.98. Residuals were ≤20% at the lowest calibration level and ≤15% at all other levels. The slopes of calibration curves were reproducible over 3 days with relative standard deviations (RSDs) of <10% for most compounds. The only exceptions were diethylstilbestrol (13%) and norgestimate (23%), which were nevertheless successfully quantified.

Accuracy and precision was determined by carrying out three validation runs (on three separate days) consisting of 6 replicates each of 4 levels of pre-extraction matrix-spiked QC samples (24 samples/run), bracketed by the post-extraction

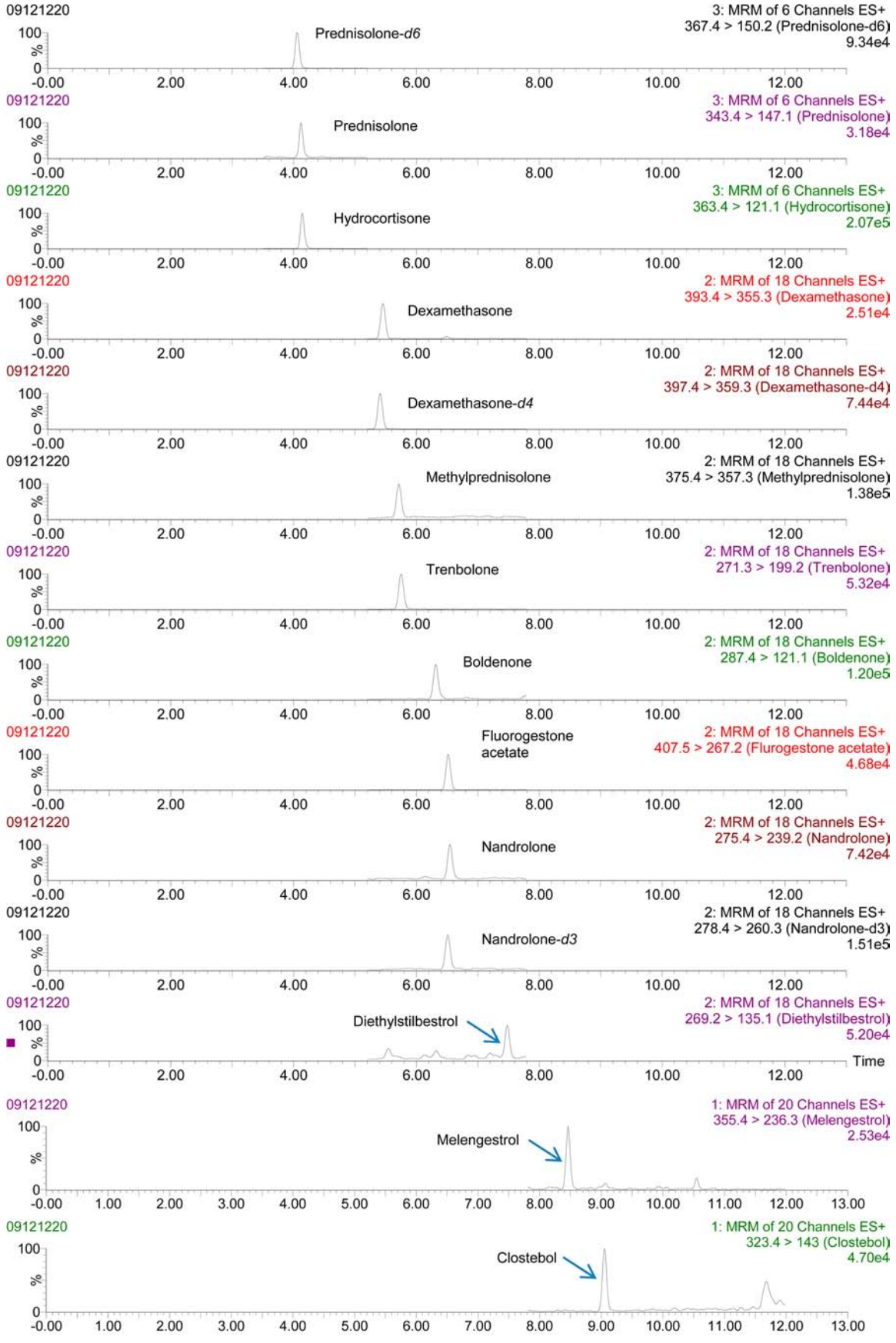

Figure 1. continued

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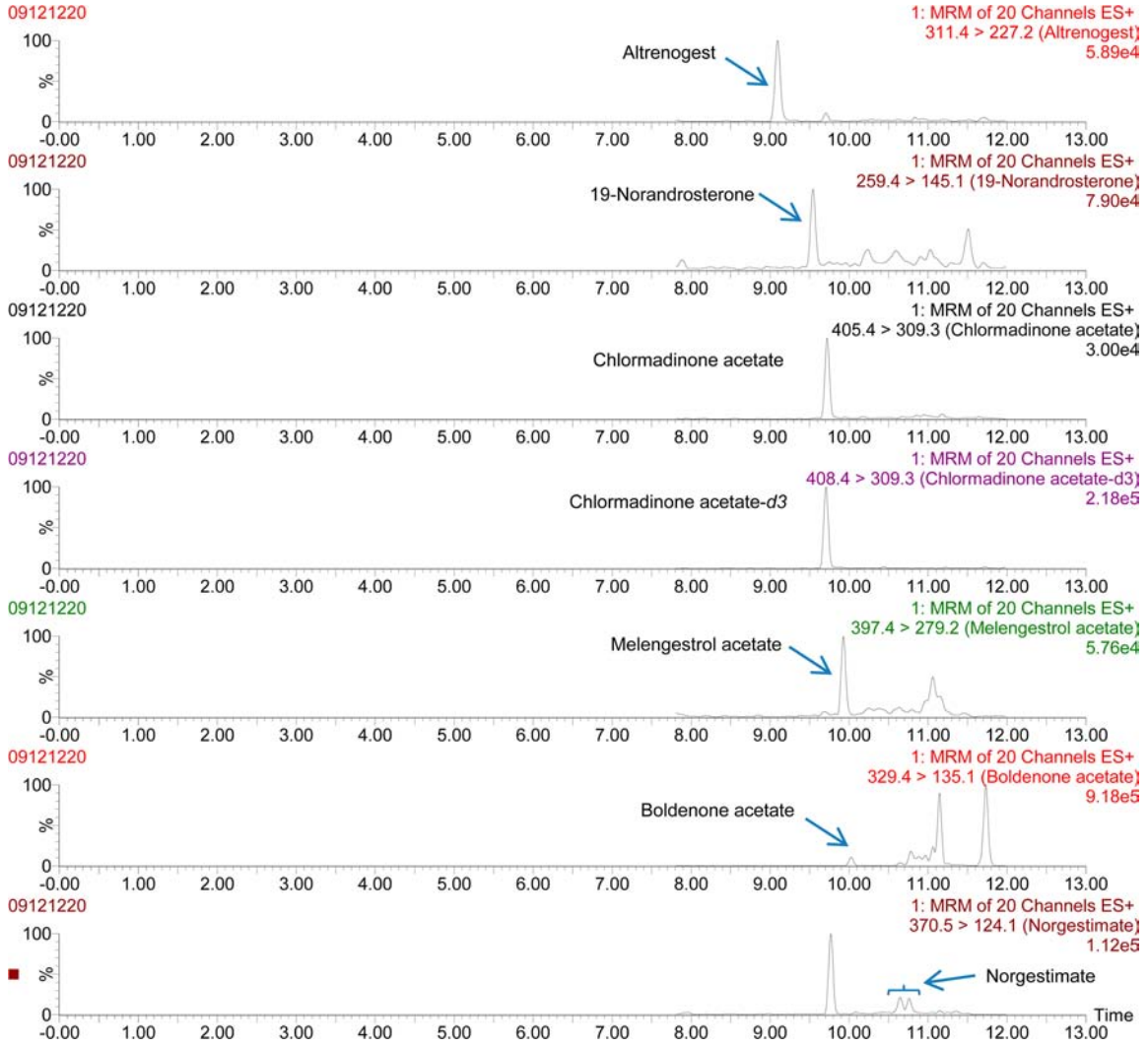

Figure 1. Multiple reaction monitoring (MRM) chromatograms of the transitions corresponding to the quantifier ions of considered veterinary hormones spiked at the QC2 level into a whole milk powder matrix.

spiked matrix-matched calibration standards (analyzed at the beginning and at the end of each run). The QC1 level was the same as the lowest calibration standard. The QC2, QC3, and QC4 levels corresponded to 3, 10, and 80 times the lowest calibration standard, respectively. A validation run was considered acceptable if the bias (deviation from true concentration) of ≥67% of all QC samples and ≥50% of QC samples at each level was between −30% and +20%. These criteria were derived from established guidelines.[48,49] Only those QCs that satisfied these acceptance criteria have been used in subsequent data analysis. Ion ratios (qualifier/quantifier) in all QC samples were within ±25% of average ion ratios in the matrix-matched calibration standards analyzed within the same run.[50]

The acceptance rates of QCs along with the overall biases (across all three validation runs) for all compounds are summarized in Table 4. Within-run precisions, between-run precisions, and overall precisions for all compounds were calculated according to established statistical procedures[51] and are summarized in Table 5. Overall biases across all compounds at the QC1, QC2, QC3, and QC4 levels were −10.6−16.9%, −9.8−11.2%, −10.7−11.5%, and −13.9−8.2%, respectively. Within-run precisions, between-run precisions, and overall precisions across all QCs and all compounds were 2.0−16.0%, 0.0−13.9%, and 1.7−18.1%, respectively. Precision tended to improve with increase of concentration (from QC1 through QC4). The consistent performance of the method across three validation runs established the ruggedness of the method.

Absolute extraction recovery has been determined at two concentration levels (QC2 and QC4) by comparing peak areas in pre-extraction matrix-spiked samples against peak areas in post-extraction matrix-spiked samples (6 replicates each). Absolute recoveries at the QC2 and QC4 levels across all compounds were 62−76% and 67−82%, respectively (Table 6).

Matrix effect (ionization efficiency) was determined by comparing the slopes of the linear terms of regression equations in matrix and in neat solvent, respectively. Calculated values are listed in Table 6. Signal suppression was <15% for most compounds, except for diethylstilbestrol, boldenone acetate, and norgestimate. In the case of diethylstilbestrol the significant signal suppression contributed to the relatively poor sensitivity achieved for this compound. In the case of boldenone acetate and norgestimate excellent sensitivity was achieved even with significant signal suppression.

Limits of detection were determined according to the procedure described by the U.S. Environmental Protection

Table 4. Accuracy (as % Bias) of Measurements of Considered Veterinary Hormones at Four QC levels (QC1−QC4) in a Whole Milk Powder Matrix Across Three Validation Runs

		QC1			QC2			QC3			QC4		
no.	compound	concn (μg/kg)	acceptance rate (%)	overall bias (%)	concn (μg/kg)	acceptance rate (%)	overall bias (%)	concn (μg/kg)	acceptance rate (%)	overall bias (%)	concn (μg/kg)	acceptance rate (%)	overall bias (%)
1	prednisolone	1.0	83	5.9	3.0	94	5.8	10	89	11.5	80	100	8.2
2	hydrocortisone	10.0	67	16.9	30.0	100	11.2	100	89	11.0	800	100	−12.6
3	dexamethasone	1.0	89	−6.6	3.0	94	−1.8	10	100	6.5	80	100	0.9
4	methylprednisolone	2.0	100	1.4	6.0	100	0.1	20	83	0.4	160	94	−8.4
5	trenbolone	1.0	100	−6.1	3.0	100	−7.2	10	94	−1.6	80	100	−2.0
6	boldenone	0.5	100	−4.3	1.5	100	−4.9	5	94	−0.2	40	100	−0.6
7	fluorogestone acetate	1.0	100	0.1	3.0	100	−0.1	10	89	4.0	80	100	4.8
8	nandrolone	2.0	94	4.1	6.0	100	−1.5	20	89	2.6	160	94	2.9
9	diethylstilbestrol	10.0	83	−8.8	30.0	83	−5.7	100	100	−10.7	800	89	−13.9
10	melengestrol	0.5	83	−2.8	1.5	83	7.2	5	78	5.8	40	100	0.2
11	clostebol	0.5	89	−5.0	1.5	100	−0.9	5	100	−0.9	40	89	−7.5
12	altrenogest	0.5	78	−10.1	1.5	100	−9.8	5	100	−6.3	40	100	−13.2
13	19-norandrosterone	2.0	94	−10.6	6.0	100	−0.6	20	100	1.0	160	100	−2.9
14	chlormadinone acetate	0.5	78	4.6	1.5	100	−0.5	5	100	3.4	40	100	−1.9
15	melengestrol acetate	0.5	78	5.9	1.5	94	5.6	5	89	7.3	40	100	3.5
16	boldenone acetate	0.5	94	4.0	1.5	100	0.8	5	94	7.6	40	100	4.4
17	norgestimate	0.5	78	0.8	1.5	83	−1.0	5	78	2.6	40	89	−1.8

Table 5. Precision of Measurements of Considered Veterinary Hormones at Four QC levels (QC1−QC4) in a Whole Milk Powder Matrix Across Three Validation Runs

		QC1				QC2				QC3				QC4			
			precision (%)				precision (%)				precision (%)				precision (%)		
no.	compound	concn (μg/kg)	WD[a]	BD[b]	TOT[c]	concn (μg/kg)	WD[a]	BD[b]	TOT[c]	concn (μg/kg)	WD[a]	BD[b]	TOT[c]	concn (μg/kg)	WD[a]	BD[b]	TOT[c]
1	prednisolone	1.0	8.1	2.3	8.4	3.0	5.8	3.5	6.7	10	3.6	2.4	4.3	80	3.0	0.0	2.9
2	hydrocortisone	10.0	2.0	0.0	1.7	30.0	3.3	3.7	5.0	100	3.0	2.9	4.1	800	3.6	1.2	3.8
3	dexamethasone	1.0	14.0	8.2	16.2	3.0	6.5	2.8	7.1	10	5.8	2.1	6.2	80	3.9	0.0	3.6
4	methylprednisolone	2.0	8.6	5.1	10.0	6.0	7.6	2.5	8.0	20	5.3	4.9	7.2	160	4.2	4.4	6.1
5	trenbolone	1.0	10.0	0.0	9.2	3.0	8.1	3.0	8.7	10	6.4	7.2	9.6	80	4.8	5.0	6.9
6	boldenone	0.5	9.9	6.0	11.6	1.5	4.6	1.5	4.8	5	6.0	6.5	8.8	40	4.9	9.8	11.0
7	fluorogestone acetate	1.0	8.5	4.5	9.6	3.0	7.0	4.0	8.1	10	6.6	2.8	7.2	80	4.8	2.6	5.5
8	nandrolone	2.0	9.4	0.0	8.6	6.0	7.6	0.0	7.0	20	4.6	8.1	9.3	160	4.0	4.7	6.2
9	diethylstilbestrol	10.0	9.5	11.2	14.7	30.0	11.8	11.3	16.4	100	11.3	1.6	11.4	800	13.0	0.0	12.3
10	melengestrol	0.5	12.3	10.0	15.9	1.5	6.0	2.5	6.5	5	6.2	2.9	6.8	40	5.2	8.9	10.3
11	clostebol	0.5	10.4	3.5	11.0	1.5	7.0	4.5	8.4	5	9.4	4.9	10.6	40	7.5	2.0	7.8
12	altrenogest	0.5	11.7	9.3	15.0	1.5	6.6	7.3	9.8	5	7.2	1.4	7.3	40	6.2	1.1	6.3
13	19-norandrosterone	2.0	16.0	8.6	18.1	6.0	7.8	6.4	10.1	20	7.7	0.0	7.7	160	4.6	4.2	6.2
14	chlormadinone acetate	0.5	8.0	13.9	16.0	1.5	6.4	2.1	6.8	5	6.3	0.0	5.8	40	2.0	1.5	2.4
15	melengestrol acetate	0.5	5.7	11.6	13.0	1.5	6.0	3.3	6.9	5	4.5	1.8	4.8	40	3.6	6.4	7.4
16	boldenone acetate	0.5	5.1	2.4	5.6	1.5	7.2	5.4	9.0	5	4.5	5.9	7.4	40	4.2	7.3	8.4
17	norgestimate	0.5	12.1	7.1	14.1	1.5	13.6	11.2	17.6	5	12.2	0.0	11.0	40	12.6	4.2	13.3

[a]Within-day. [b]Between days. [c]Total.

Agency.[52] This procedure defines the limit of detection as the minimum concentration of analyte that can be measured and reported with 99% confidence that it is greater than zero. This definition takes into account only the α (false positive or false noncompliant) error rate and not the β (false negative of false compliant) error rate. The limit of detection determined by this procedure is thus equivalent to the decision limit (CCα) as defined in ref 50 for substances with no established maximum residue limits (MRLs). However the former procedure is more objective since it does not involve extrapolation, the number of replicates and their concentration is clearly specified, calibration curves do not need to be linear, and it does not make use of a (subjective) minimum required performance level (MRPL). Briefly, limits of detection were first estimated as the matrix-based concentrations that gave signal-to-noise ratios of 4 for the qualifier (least intense) ions. Next, the matrix was spiked at concentrations equivalent to three times the estimated limits of detection, and $n = 7$ replicate analyses were carried out. The limits of detection were calculated as t·SD, where t is the Student's t-value (99% confidence level and $n - 1$ degrees of freedom), and SD is the standard deviation of the seven replicate analyses. Calculated limits of detection are listed in

Table 6. Absolute Extraction Recovery, Matrix Effect, Limit of Detection, and Limit of Quantitation of Considered Veterinary Hormones in a Whole Milk Powder Matrix

		recovery (%)				
no.	compound	QC2	QC4	matrix effect (%)	LOD[a] (μg/kg)	LOQ[b] (μg/kg)
1	prednisolone	70	76	2	0.1	0.4
2	hydrocortisone	71	78	2	N/A	10.0
3	dexamethasone	62	75	−13	0.6	1.0
4	methylprednisolone	70	75	−2	0.8	2.0
5	trenbolone	66	72	−8	0.2	0.5
6	boldenone	68	73	−14	0.1	0.4
7	fluorogestone acetate	71	77	−12	0.2	0.6
8	nandrolone	69	74	−2	0.7	2.0
9	diethylstilbestrol	69	67	−19	3.8	10.0
10	melengestrol	76	75	9	0.3	0.5
11	clostebol	66	74	13	0.2	0.5
12	altrenogest	63	71	7	0.1	0.3
13	19-norandrosterone	69	72	0	0.6	1.8
14	chlormadinone acetate	72	76	0	0.1	0.4
15	melengestrol acetate	70	74	−5	0.1	0.3
16	boldenone acetate	67	75	−20	0.1	0.2
17	norgestimate	76	82	−37	0.2	0.5

[a]Limit of detection. [b]Limit of quantitation.

Table 6. Limits of detection in whole milk powder were in the 0.1−0.8 μg/kg range for all compounds, except for diethylstilbestrol (3.8 μg/kg). A limit of detection was not determined for hydrocortisone since a blank matrix was not available. Limits of quantitation were established as three times the respective limits of detection or the lowest calibration level, whichever was smaller, and are also listed in Table 6.

Extract stability was determined by reanalyzing an entire validation run after keeping the samples on the autosampler tray for 48 h at ambient temperature. The run met the acceptance criteria after 48 h according to the criteria outlined above.

Extension of the Method to Other Ingredients Derived from Milk. The possible extension of the method to other ingredients derived from milk, such as nonfat dry milk, milk protein concentrate, whey protein concentrate, sodium caseinate, and lactose with a universal whole milk powder matrix-based calibration approach was investigated. This approach would allow the use of a core method for the analysis of hormones in five matrices (whole milk powder, nonfat dry milk, milk protein concentrate, whey protein concentrate, and lactose). In the case of sodium caseinate, a slight modification of the extraction procedure was needed, as described in the method development section. Hence sodium caseinate is not amenable to the universal whole milk powder matrix-based calibration approach.

A cross-validation run consisting of 6 replicates each of 2 levels of pre-extraction spiked QC samples (QC2 and QC4) was conducted for each ingredient matrix. These runs included bracketing post-extraction spiked matrix-matched calibration standards in a whole milk powder matrix (analyzed at the beginning and at the end of each run). For sodium caseinate a separate cross-validation run was conducted using the modified extraction procedure.

A cross-validation run was considered acceptable if the bias (deviation from true concentration) of ≥67% of all QC samples and ≥50% of QC samples at each level was between −30% and +20%.[48,49] Of 17 analytes studied, 14 satisfied these criteria in all matrices (Supplementary Table). For the remaining three analytes, i.e., diethylstilbestrol and norgestimate (in all matrices) and melengestrol (in nonfat dry milk only), the described approach can still serve a screening purpose. Accurate determination of these problematic compounds in positive samples necessitates the use of specific matrix-matched calibration standards for each ingredient derived from milk.

In conclusion, a rugged, quantitative liquid chromatography−tandem mass spectrometry method with modified QuEChERS-based sample preparation for 17 selected hormones in powdered ingredients derived from milk was developed and comprehensively validated. A universal matrix-matching approach based on whole milk powder has been successfully implemented. A slight modification of the extraction procedure was necessary for sodium caseinate in order to avoid ion suppression caused by matrix coextractives. The method is proposed for the routine analysis of hormones potentially present in powdered ingredients derived from bovine milk.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Supplementary table. Accuracy (as % bias) and precision of measurements of considered veterinary hormones at two QC levels (QC2 and QC4) in five different milk-based ingredient matrices in one cross-validation run. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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■ ABBREVIATIONS USED

CCα, decision limit; clogP, partition coefficient; MRL, maximum residue limit; MRM, multiple reaction monitoring; MRPL, minimum required performance level; PTFE, polytetrafluoroethylene; QC, quality control; QuEChERS, quick, easy, cheap, effective, rugged, and safe; RSD, relative standard deviation; SD, standard deviation

■ REFERENCES

(1) United States Department of Agriculture (USDA), *Dairy: World Markets and Trade*. Available at: http://usda01.library.cornell.edu/usda/fas/dairy-market//2010s/2012/dairy-market-12-14-2012.pdf. Retrieved August 30, 2013.

(2) Yang, Y.; Shao, B.; Zhang, J.; Wu, Y.; Duan, H. Determination of the residues of 50 anabolic hormones in muscle, milk and liver by very-high-pressure liquid chromatography−electrospray ionization tandem mass spectrometry. *J. Chromatogr., B* **2009**, *877*, 489−496.

(3) Ortelli, D.; Cognard, E.; Jan, P.; Edder, P. Comprehensive fast multiresidue screening of 150 veterinary drugs in milk by ultra-performance liquid chromatography coupled to time of flight mass spectrometry. *J.Chromatogr., B* **2009**, *877*, 2363−2374.
(4) Regal, P.; Cepeda, A.; Fente, C. Development of an LC-MS/MS method to quantify sex hormones in bovine milk and influence of pregnancy in their levels. *Food Addit. Contam., Part A* **2012**, *29*, 770−779.
(5) Courant, F.; Antignac, J. P.; Laille, L.; Monteau, F.; Andre, F.; Le Bizec, B. Exposure assessment of prepubertal children to steroid endocrine disruptors. 2. Determination of steroid hormones in milk, egg, and meat samples. *J. Agric. Food Chem.* **2008**, *56*, 3176−3184.
(6) Tso, J.; Aga, D. S. A systematic investigation to optimize simultaneous extraction and liquid chromatography tandem mass spectrometry analysis of estrogens and their conjugated metabolites in milk. *J. Chromatogr., A* **2010**, *1217*, 4784−4795.
(7) Yan, W.; Li, Y.; Lin, J. M. Determination of estrogens and bisphenol A in bovine milk by automated on-line C30 solid-phase extraction coupled with high-performance liquid chromatography-mass spectrometry. *J. Chromatogr., A* **2009**, *1216*, 7539−7545.
(8) Courant, F.; Antignac, J. P.; Maume, D.; Monteau, F.; Andre, F.; Le Bizec, B. Determination of naturally occurring oestrogens and androgens in retail samples of milk and eggs. *Food Addit. Contam., Part A* **2007**, *24*, 1358−1366.
(9) Chrusch, J.; Lee, S.; Fedeniuk, R.; Boison, J. O. Determination of the performance characteristics of a new multi-residue method for non-steroidal anti-inflammatory drugs, corticosteroids and anabolic steroids in food animal tissues. *Food Addit. Contam., Part A* **2008**, *25*, 1482−1496.
(10) Gentili, A.; Sergi, M.; Perret, D.; Marchese, S.; Curini, R.; Lisandrin, S. High- and low-resolution mass spectrometry coupled to liquid chromatography as confirmatory methods of anabolic residues in crude meat and infant foods. *Rapid Commun. Mass Spectrom.* **2006**, *20*, 1845−1854.
(11) Xu, C. L.; Chu, X. G.; Peng, C. F.; Yin, Z. Y.; Wang, L. Y. Development of a faster determination of 10 anabolic steroids residues in animal muscle tissues by liquid chromatography tandem mass spectrometry. *J. Pharm. Biomed. Anal.* **2006**, *41*, 616−621.
(12) Stolker, A. A.; Zoontjes, P. W.; van Ginkel, L. A. The use of supercritical fluid extraction for the determination of steroids in animal tissues. *Analyst.* **1998**, *123*, 2671−2676.
(13) Costain, R. M.; Fesser, A. C.; McKenzie, D.; Mizuno, M.; MacNeil, J. D. Identification of hormone esters in injection site in muscle tissues by LC/MS/MS. *Food Addit. Contam., Part A* **2008**, *25*, 1520−1529.
(14) Blasco, C.; Poucke, C. V.; Peteghem, C. V. Analysis of meat samples for anabolic steroids residues by liquid chromatography/tandem mass spectrometry. *J. Chromatogr., A* **2007**, *1154*, 230−239.
(15) Kaklamanos, G.; Theodoridis, G.; Papadoyannis, I. N.; Dabalis, T. Determination of anabolic steroids in muscle tissue by liquid chromatography-tandem mass spectrometry. *J. Agric. Food Chem.* **2007**, *55*, 8325−8330.
(16) McDonald, M.; Malone, E.; McBride, J. Confirmation of hormones in animal serum by liquid chromatography/tandem mass spectrometry according to European Commission Decision 2002/657. *J. AOAC Int.* **2010**, *93*, 343−349.
(17) Kaklamanos, G.; Theodoridis, G. A.; Dabalis, T.; Papadoyannis, I. Determination of anabolic steroids in bovine serum by liquid chromatography-tandem mass spectrometry. *J. Chromatogr., B* **2011**, *879*, 225−229.
(18) Rúbies, A.; Cabrera, A.; Centrich, F. Determination of synthetic hormones in animal urine by high-performance liquid chromatography/mass spectrometry. *J. AOAC Int.* **2007**, *90*, 626−632.
(19) León, N.; Roca, M.; Igualada, C.; Martins, C. P.; Pastor, A.; Yusá, V. Wide-range screening of banned veterinary drugs in urine by ultra high liquid chromatography coupled to high-resolution mass spectrometry. *J. Chromatogr., A* **2012**, *1258*, 55−65.
(20) Wisconsin Center for Dairy Research. *Dried Dairy Ingredients*. Available at: http://future.aae.wisc.edu/publications/dried_dairy_ingdients.pdf. Retrieved August 30, 2013.
(21) Anastassiades, M.; Lehotay, S. J.; Stajnbaher, D.; Schenck, F. J. Fast and easy multiresidue method employing acetonitrile extraction/partitioning and "dispersive solid-phase extraction" for the determination of pesticide residues in produce. *J. AOAC Int.* **2003**, *86*, 412−431.
(22) Wang, J.; Leung, D. The challenges of developing a generic extraction procedure to analyze multi-class veterinary drug residues in milk and honey using ultra-high pressure liquid chromatography quadrupole time-of-flight mass spectrometry. *Drug Test Anal.* **2012**, *Aug 4* (Suppl. 1), 103−111.
(23) Blasco, C.; Masia, A.; Morillas, F. G.; Picó, Y. Comparison of the effectiveness of recent extraction procedures for antibiotic residues in bovine muscle tissues. *J. AOAC Int.* **2011**, *94*, 991−1003.
(24) Aguilera-Luiz, M. M.; Vidal, J. L.; Romero-González, R.; Frenich, A. G. Multi-residue determination of veterinary drugs in milk by ultra-high-pressure liquid chromatography-tandem mass spectrometry. *J. Chromatogr., A* **2008**, *1205*, 10−16.
(25) Martínez-Vidal, J. L.; Frenich, A. G.; Aguilera-Luiz, M. M.; Romero-González, R. Development of fast screening methods for the analysis of veterinary drug residues in milk by liquid chromatography-triple quadrupole mass spectrometry. *Anal. Bioanal. Chem.* **2010**, *397*, 2777−2790.
(26) Rodriguez-Carrasco, Y.; Berrada, H.; Font, G.; Manes, J. Multi-mycotoxin analysis in wheat semolina using an acetonitrile-based extraction procedure and gas chromatography-tandem mass spectrometry. *J. Chromatogr., A* **2012**, *1270*, 28−40.
(27) Rubert, J.; Dzuman, Z.; Vaclavikova, M.; Zachariasova, M.; Soler, C.; Hajslova, J. Analysis of mycotoxins in barley using ultra high liquid chromatography high resolution mass spectrometry: comparison of efficiency and efficacy of different extraction procedures. *Talanta* **2012**, *99*, 712−719.
(28) Desmarchelier, A.; Oberson, J. M.; Tella, P.; Gremaud, E.; Seefelder, W.; Mottier, P. Development and comparison of two multiresidue methods for the analysis of 17 mycotoxins in cereals by liquid chromatography electrospray ionization tandem mass spectrometry. *J. Agric. Food Chem.* **2010**, *58*, 7510−7519.
(29) Frenich, A. G.; Romero-Gonzalez, R.; Gomez-Perez, M. L.; Vidal, J. L. M. Multi-mycotoxin analysis in eggs using a QuEChERS-based extraction procedure and ultra-high-pressure liquid chromatography coupled to triple quadrupole mass spectrometry. *J. Chromatogr., A* **2011**, *1218*, 4349−4356.
(30) Norli, H. R.; Christiansen, A.; Deribe, E. Application of QuEChERS method for extraction of selected persistent organic pollutants in fish tissue and analysis by gas chromatography mass spectrometry. *J. Chromatogr., A* **2011**, *1218*, 7234−7241.
(31) Sapozhnikova, Y.; Lehotay, S. J. Multi-class, multi-residue analysis of pesticides, polychlorinated biphenyls, polycyclic aromatic hydrocarbons, polybrominated diphenyl ethers and novel flame retardants in fish using fast, low-pressure gas chromatography−tandem mass spectrometry. *Anal. Chim. Acta* **2013**, *758*, 80−92.
(32) Johnson, Y. S. Determination of polycyclic aromatic hydrocarbons in edible seafood by QuEChERS-based extraction and gas chromatography-tandem mass spectrometry. *J. Food Sci.* **2012**, *77*, T131−T137.
(33) Ramalhosa, M. J.; Paiga, P.; Morais, S.; Delerue-Matos, C.; Pinto-Oliveira, M. B. P. Analysis of polycyclic aromatic hydrocarbons in fish: evaluation of a quick, easy, cheap, effective, rugged, and safe extraction method. *J. Sep. Sci.* **2009**, *32*, 3529−3538.
(34) Kalachova, K.; Cajka, T.; Sandy, C.; Hajslova, J.; Pulkrabova, J. High throughput sample preparation in combination with gas chromatography coupled to triple quadrupole tandem mass spectrometry (GC-MS/MS): a smart procedure for (ultra)trace analysis of brominated flame retardants in fish. *Talanta* **2013**, *105*, 109−116.
(35) Zhuo, L.; Yin, Y.; Fu, W.; Qiu, B.; Lin, Z.; Yang, Y.; Zheng, L.; Li, J.; Chen, G. Determination of paralytic shellfish poisoning toxins by

HILIC-MS/MS coupled with dispersive solid phase extraction. *Food Chem.* **2013**, *137*, 115−21.
(36) Pouech, C.; Tournier, M.; Quignot, N.; Kiss, A.; Wiest, L.; Lafay, F.; Flament-Waton, M. M.; Lemazurier, E.; Cren-Olivé, C. Multi-residue analysis of free and conjugated hormones and endocrine disruptors in rat testis by QuEChERS-based extraction and LC-MS/MS. *Anal. Bioanal. Chem.* **2012**, *402*, 2777−2788.
(37) Commission Regulation (EU) No 37/2010. *Off. J. Eur. Commun.* **2010**, *L15*, 1−72.
(38) *The Japanese Positive List System for Agricultural Chemical Residues in Foods*. Available at: http://www.ffcr.or.jp/zaidan/FFCRHOME.nsf/pages/MRLs-p. Retrieved August 30, 2013.
(39) Noppe, H.; Le Bizec, B.; Verheyden, K.; De Brabander, H. F. Novel analytical methods for the determination of steroid hormones in edible matrices. *Anal. Chim. Acta* **2008**, *611*, 1−16.
(40) European Committee for Standardization, EN 15662, 2008.
(41) Fabregat, A.; Pozo, O. J.; Marcos, J.; Segura, J.; Ventura, R. Use of LC-MS/MS for the open detection of steroid metabolites conjugated with glucuronic acid. *Anal. Chem.* **2013**, *85*, 5005−5014.
(42) Cui, X.; Shao, B.; Zhao, R.; Yang, Y.; Hu, J.; Tu, X. Simultaneous determination of seventeen glucocorticoids residues in milk and eggs by ultra-performance liquid chromatography/electrospray tandem mass spectrometry. *Rapid Commun. Mass Spectrom.* **2006**, *20*, 2355−2364.
(43) Malone, E. M.; Elliott, C. T.; Kennedy, D. G.; Regan, L. Screening and quantitative confirmatory method for the analysis of glucocorticoids in bovine milk using liquid chromatography-tandem mass spectrometry. *J. AOAC Int.* **2010**, *93*, 1656−1665.
(44) Tölgyesi, A.; Tölgyesi, L.; Sharma, V. K.; Sohn, M.; Fekete, J. Quantitative determination of corticosteroids in bovine milk using mixed-mode polymeric strong cation exchange solid-phase extraction and liquid chromatography-tandem mass spectrometry. *J. Pharm. Biomed. Anal.* **2010**, *53*, 919−928.
(45) Caretti, F.; Gentili, A.; Ambrosi, A.; Rocca, L. M.; Delfini, M.; Di Cocco, M. E.; D'Ascenzo, G. Residue analysis of glucocorticoids in bovine milk by liquid chromatography−tandem mass spectrometry. *Anal. Bioanal. Chem.* **2010**, *397*, 2477−2490.
(46) McDonald, M.; Granelli, K.; Sjöberg, P. Rapid multi-residue method for the quantitative determination and confirmation of glucocorticosteroids in bovine milk using liquid chromatography−electrospray ionization−tandem mass spectrometry. *Anal. Chim. Acta* **2007**, *588*, 20−25.
(47) Chen, X. B.; Wu, Y. L.; Yang, T. Simultaneous determination of clenbuterol, chloramphenicol and diethylstilbestrol in bovine milk by isotope dilution ultraperformance liquid chromatography-tandem mass spectrometry. *J. Chromatogr., B* **2011**, *879*, 799−803.
(48) United States Food and Drug Administration (US FDA). *Guidance for Industry−Bioanalytical Method Validation*. Available at: http://www.fda.gov/downloads/Drugs/Guidances/ucm070107.pdf. Retrieved August 30, 2013.
(49) European Medicines Agency (EMA). *Guideline on Bioanalytical Method Validation*. Available at: http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2011/08/WC500109686.pdf. Retrieved August 30, 2013.
(50) Commission Decision (EU) 2002/657/EC. *Off. J. Eur. Communities.* **2002**, *L221*, 8−36.
(51) *Watson LIMS 7.3 User Manual r1*; Thermo Electron Corporation: Waltham, MA, 2006; p 365.
(52) *Code of Federal Regulations*; U.S. Government Printing Office: Washington, DC, 2008; Title 40, Part 136, App. B.