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Determination of the residues of 50 anabolic hormones in muscle, milk and liver by very-high-pressure liquid chromatography–electrospray ionization tandem mass spectrometry

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ABSTRACT

A sensitive and specific method for determination of the residues of 50 anabolic hormones in muscle (pork, beef, shrimp), milk and pig liver was developed. Analytes were separated and acquired by liquid chromatography coupled with an electrospray ionization tandem mass spectrometer (LC–ESI–MS/MS). Target compounds were simultaneously extracted with methanol after enzyme hydrolysis, and purified using a graphitized carbon-black solid-phase extraction (SPE) and followed by NH_2 SPE cartridge. Limits of quantification were 0.04–2.0 μg kg^{-1}; average recoveries were 76.9–121.3%; and the relative standard deviation was 2.4–21.2%. This method has been successfully applied in real samples.

1. Introduction

Anabolic promoters include anabolic hormones (androgens (AS), estrogens (ES), glucocorticoids (GS) and progestogens (PS)) and related synthetic compounds. Many natural hormones have important roles in the organism (e.g., reproduction). GS have metabolic and anti-inflammatory properties, and help to protect against stress and shock. Many hormones are used for therapy. AS are generally used as therapeutic agents for the restoration of the size and strength of muscle [1]. Ethinylestradiol, norgestrel and other PS or ES are oral contraceptives. ES combined with PS are often used to treat climacteric syndrome.

During the past decade, the residue of anabolic growth promoters in foods has been concerned issues for their impact of endocrine disruptors on human health and wildlife [2–5]. Anabolic promoters were initially used as growth promoters, and improved the efficacy of feeding conversion in animals through increasing in bone density, muscular mass and red cells. Administration of anabolic growth promoters in animals is now prohibited by the EU [6] and China [7] because of their potential risk to human beings [8–11]. These drugs have been banned in the Olympic Games since 1974 because of their acute effects on the health of athletes [12]. Regulations may be ignored for the pursuit of economic benefits. Drugs may be converted from one compound to another; or a low level of "cocktail compounds" can be used to escape surveillance, which may result in multiple residues of these banned substances. There is, therefore, a need to develop a wide range of, multi-residue, highly sensitive and specifically analytic method for the determination of hormones of animal origin in foods.

An effective sample preparation procedure is required to determine the trace residue (ng kg^{-1} to μg kg^{-1}) of these compounds in animal food. In recent years, several sample preparation procedures for purification have been developed for concentration and cleanup of multi-residue hormones in different matrices. These include liquid–solid extraction (LSE) [13]; liquid–liquid extraction (LLE) [14]; multi-step SPE extraction (MSPEE) [15]; accelerated solvent extraction (ASE) [16]; microwave-assisted extraction (MAE) [17] and supercritical fluid extraction (SFE) [18]; gel permeation chromatography (GPC) [19], liquid-phase microextraction (LPME)[20]; and immunoaffinity techniques [21]. Conventional SPE techniques are commonly used because of their easy operation and low cost. Equipment expense and the time-consuming optimizations of ASE, MAE, SFE, and GPC techniques prevent their wide usage. Immunoaffinity and molecularly imprinted techniques are potentially promising for complex biosamples, but their application is limited because of high specificity and low column capability.

The commonest methods for determination of hormones are gas chromatography (GC) and liquid chromatography (LC) coupled to different mass spectrometry (MS) detectors (e.g., ion trap [22–24], quadrupole [25–27] and time-of-flight [28]). There is a

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Table 1
Instrument conditions for target compounds analysis.

Compounds		AS and PS	GS						ES					
LC condition	Mobile phase	A: water containing 0.1% (v/v) formic acid B: methanol							A: water B: acetonitrile					
	Gradient list	Time (min)	0	8	11	12	15	15.5	Time (min)	0	4	4.5	7	7.5
		A (%):	50	36	16	0	0	50	A (%):	65	50	0	0	50
		B (%):	50	64	84	100	100	50	B (%):	35	50	100	100	50
	Total flow	0.3 mL min^{-1}												
	Column	Acquity UPLC™ BEH C18 column (100 mm × 2.1 mm, 1.7 μm)												
MS condition	Ionization mode	ESI positive	ESI negative						ESI negative					
	Capillary voltage	3.5 kV	3.0 kV						3.3 kV					
	Desolvation gas		600 L h^{-1}											
	Cone gas		50 L h^{-1}											
	Source temperature		100 °C											
	Desolvation gas temperature		400 °C											
	Ion energy		1.0											
	Entrance voltage		0											
	Exit voltage		2											
	Collision gas		Ultrahigh-purity argon											
	Pressure of collision chamber		3.8×10^{-3} mbar											

trend from GC–MS [29] or GC–MS/MS [30] to LC–MS/MS [22] because LC–MS techniques are more straightforward without a tedious and time-consuming derivation procedure. Lack of a universal derivatization reagent for different classifications of hormones is a serious disadvantage of GC–MS. Multi-residue methods mainly focus on hormones in meat [22,28,30]; few methods are described for milk and shrimp [15]. Recently, Noppe et al. reviewed the analytic method for the determination of hormones in edible matrices [31].

In this study, a very-high-pressure liquid chromatographic method coupled to electrospray ionization tandem mass spectrometry (LC–ESI–MS/MS) was developed to analyze the wide range of polarity in 50 anabolic hormones in pork, liver, milk, beef and shrimp considering the need of monitoring illicit usage and food safety for athletes in Games. The method is comprehensively validated.

2. Experimental

2.1. Chemicals and materials

Organic solvents such as methanol, dichloromethane (DCM) and acetonitrile were all liquid chromatographic grade; they were purchased from Scharlau Chemic S.A. (Barcelona, Spain). Formic acid (99%) was from Acros organics (New Jersey, NY, USA). Ultrapure water was made by the Milli-Q ultrapure system (Millipore, Bedford, MA, USA). Standards (listed in Table 1; analytic grade >98% purity) were obtained from Sigma–Aldrich (St. Louis, MO, USA) and Dr. Ehrenstorfer Gmbh (Augsburg, Germany). Internal standards, including norgestrel-d_6, progesterone-d_9, northindrone-ethynyl-$^{13}C_2$, 16β-OH-stanozolol-d_3, cortisol-d_3, testosterone-3,4-$^{13}C_2$, estrone-2,4-d_2, estradiol-3,4-$^{13}C_2$ with concentration of 0.1 mg mL^{-1}, were obtained from Cambridge Isotope Laboratories (Andover, MA, USA). Diethylstilbestrol-d_6, dienestril-d_2, hexestrol-d_4 methyltestosterone-d_3, medroxyprogesterone-d_3, megestrol-d_3 acetate, chlortestosterone-d_3 and 17β-boldenone-d_3 at 0.1 mg mL^{-1} were available from Community Reference Laboratory RIVM (Bilthoven, Netherlands). Standards were stored at −18 °C. ENVI-carb cartridge (500 mg, 6 mL; GCB cartridge) was purchased from Supelco Company (Bellefonte, PA, USA). Sep-Pak amino-propyl (500 mg, 6 mL) (NH_2 SPE) and C18 (500 mg, 6 mL) and Oasis HLB (150 mg, 6 mL) solid-phase extraction cartridges were purchased from Waters Company (Milford, MA, USA).

Stock solutions were prepared for all standard substances at 0.1 mg mL^{-1} in methanol. Spiking and calibration mixtures at various concentrations were obtained by combining aliquots of stock solutions and internal standard with methanol. The concentration of internal standard in all calibration mixtures and final sample solutions was 10 ng mL^{-1}. Tuning solutions (0.5 μg mL^{-1}) were freshly prepared in methanol containing 0.1% formic acid for AS, GS and PS; and in methanol for ES.

2.2. Sample preparation

Five grams of sample were transferred into a polypropylene centrifuge tube (50 mL) and spiked with internal standard (100 μg L^{-1} mixed standard, 100 μL). Ten milliliters of 0.2 mol L^{-1} acetate buffer (pH 5.2) was added, and samples homogenized with an ultra turrax machine for about 1 min. One hundred microliters glucuronidase/arylsulfatase from *Helix Pomatia* (Roche Diagnostics GmbH, Mannhein, Germany) was added and incubated overnight at 37 °C. The sample was cooled to room temperature and then 25 mL methanol was added. After mixing by a vortex stirrer for 2 min, the mixture was centrifuged at 10,000 rpm for 10 min at 4 °C. The supernatant was decanted into a 250-mL beaker, and was diluted to 125 mL with ultrapure water. The pooled solution was subjected to preconditioned SPE cartridges at the flow of 3–5 mL min^{-1}. The GCB cartridge was preconditioned sequentially with 6 mL dichloromethane–methanol (70/30, v/v), 6 mL methanol, and 6 mL water. After loading sample, the GCB cartridge was washed with 1 mL methanol and dried by a vacuum pump. A NH_2 SPE cartridge was preconditioned with 4 mL DCM–methanol (70/30, v/v) and placed directly below the GCB cartridge. Analytes were eluted using 8 mL of DCM–methanol (70/30, v/v). The eluent was dried under a gentle nitrogen stream, and the residue reconstituted with 1.0 mL methanol/water (1/1, v/v). As a comparison, the processes for C18 and HLB were as follows: the C18 and HLB were preconditioned with 6 mL methanol and 6 mL water. After sample loading, C18 and HLB were washed with 6 mL water and dried by a vacuum pump. An NH_2 SPE cartridge preconditioned with 6 mL of methanol was connected below the C18 and HLB, and the target analytes eluted with 8 mL of

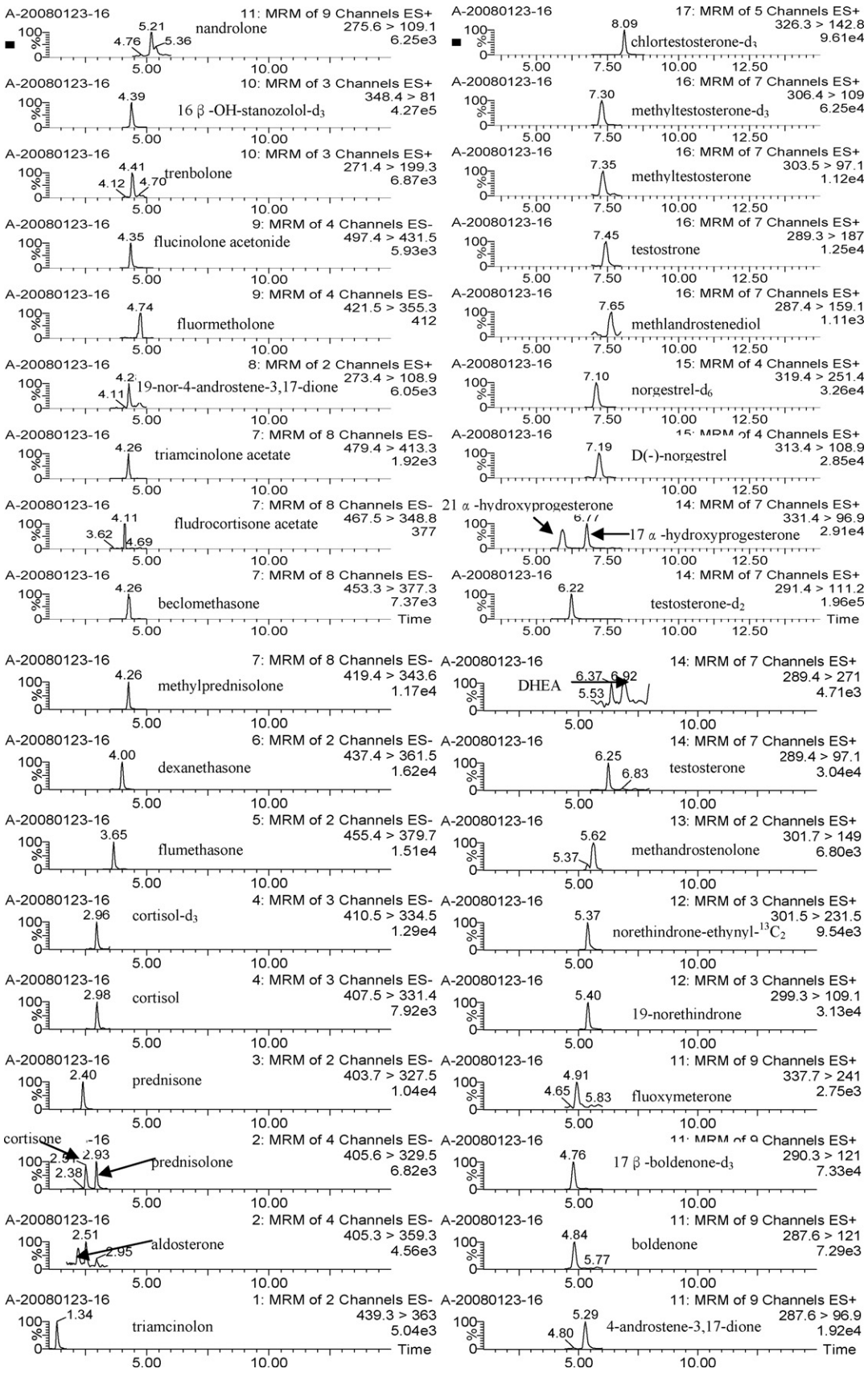

Fig. 1. MRM chromatograms of all analytes in a spiked milk sample (1 and 5 $\mu g\,kg^{-1}$ for epiandrosterone, fluorometholone, budesonide, fluoxymesterone and 5α-androstan-17β-ol-3-one).

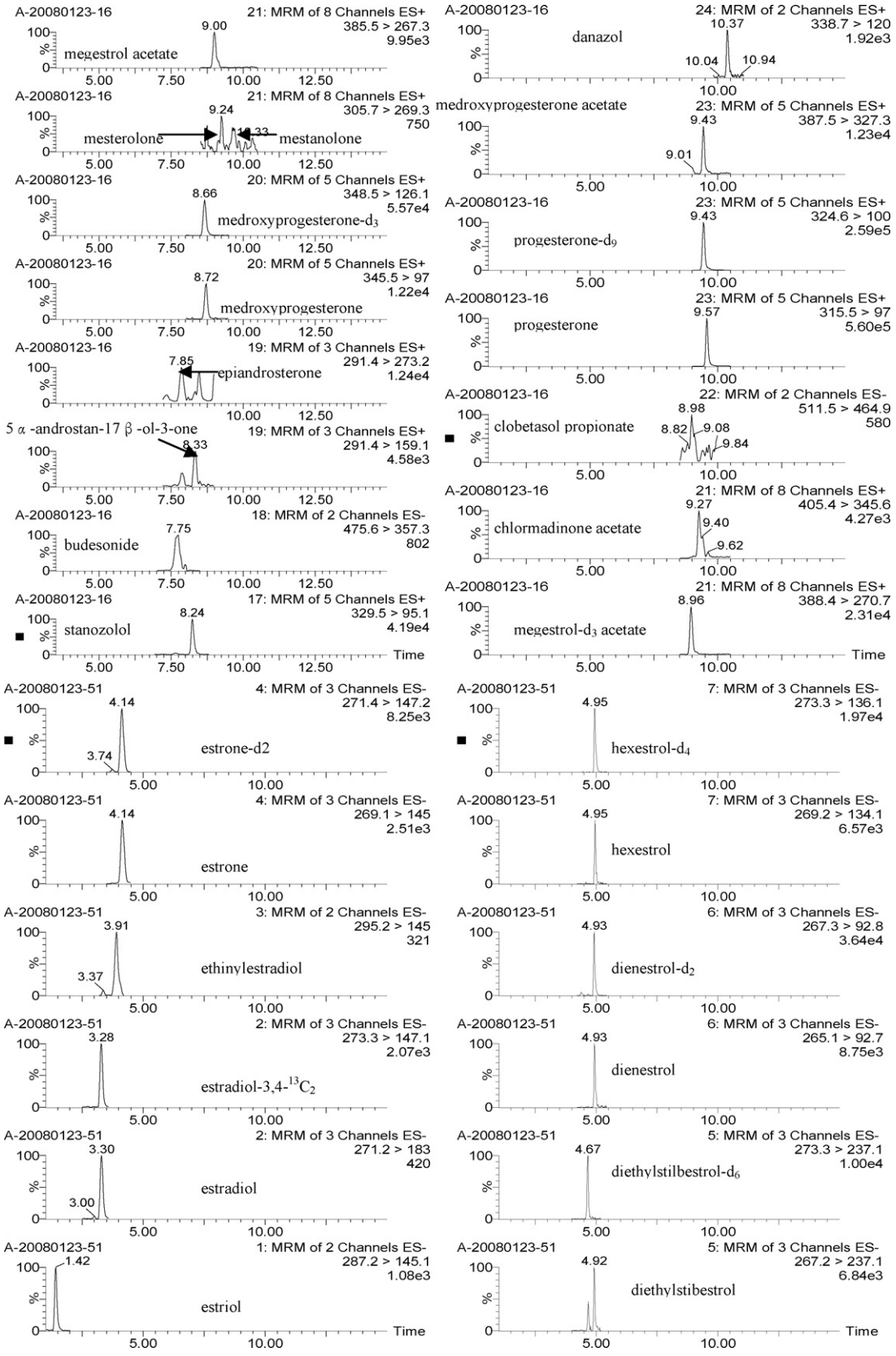

Fig. 1. (*Continued*).

methanol. The eluent was dried under a gentle stream of nitrogen, and the residue reconstituted with 1.0 mL of methanol/water (1/1, v/v).

2.3. LC–MS/MS

Analysis was done on a Waters Acquity UPLC™ system coupled with a Micromass-Quattro Premier XE Mass spectrometer (Waters Corp., Milford, MA, USA). The column oven was set at 40 °C, and the injection volume was 10 μL. Optimized instrumental conditions were shown in Table 1.

2.4. Method validation

Calibration curves for the target compounds exclusive of 5α-androstan-17β-ol-3-one, mesterolone, danazol and 4-androstene-3,17-dione were obtained by carrying out a linear regression analysis on the ratio of standard solution areas to internal standard areas versus analyte concentrations from 0.5 to 100 μg L^{-1} (concentrations of D(−)-norgestrel, budesonide, fluormetholone and fluoxymesterone were five times higher than those of other analytes) with 10 ng of internal standard spiked in samples. Quantification of 5α-androstan-17β-ol-3-one, mesterolone, danazol and 4-androstene-3,17-dione were based on matrix-fortified curves of 0.1–20 μg kg^{-1} (0.5–100 μg kg^{-1} for 5α-androstan-17β-ol-3-one).The recovery was evaluated by 5 g samples spiked with 2, 5 and 10 ng of each standard analyte (concentrations of D(−)-norgestrel, epiandrosterone, budesonide, 5α-androstan-17β-ol-3-one, fluormetholone and fluoxymesterone were five times higher than those of other analytes) and 10 ng of internal standard in six replicates. The precision expressed as percentages of relative standard deviation (R.S.D.%) was determined for each compound from six replicates of spiked samples. The limit of detection (LOD) and limit of quantification (LOQ) for each compound was calculated by determining the signal-to-noise (S/N) ratio of the lowest measured concentration and extrapolating to S/N ratios of 3 and 10 for the diagnostic transition ions, respectively.

Table 2
LC–MS/MS acquisition parameters and corresponding internal standards for the analytes.

Compound	Parent ion (*m*/*z*)	Daughter ion (*m*/*z*)[a]	Collision energy (eV)	Cone voltage (V)	Internal standard
19-Nor-4-androstene-3,17-dione	273.4	*108.9*, 197.3	25, 18	42	16β-OH-stanozolol-d_3
Trenbolone	271.4	*253.3*, 199.3	18, 24	33	16β-OH-stanozolol-d_3
Boldenone	287.6	*121.0*, 135.0	22, 15	22	17β-Boldenone-d_3
Fluoxymeterone	337.7	*241.0*, 131.0	22, 30	33	17β-Boldenone-d_3
Nandrolone	275.6	*109.1*, 257.4	26, 15	35	17β-Boldenone-d_3
4-Androstene-3,17-dione	287.6	*96.9*, 108.9	20, 23	25	–
Methandrostenolone	301.2	*149.0*, 121.0	15, 26	22	17β-Boldenone-d_3
Testosterone	289.4	*97.1*, 109.1	22, 20	38	Testosterone-3,4-$^{13}C_2$
DHEA	289.5	*271.0*, 253.1	7, 11	13	Testosterone-3,4-$^{13}C_2$
Methyltestosterone	303.5	*109.1*, 97.1	27, 25	20	Methyltestosterone-d_3
Testostrone	289.3	*187.0*, 205.1	18, 15	30	Methyltestosterone-d_3
Methlandrostenediol	287.4	*159.1*, 269.1	11, 21	16	Chlortestosterone-d_3
Epiandrosterone	291.4	*273.5*, 255.2	16, 8	15	Chlortestosterone-d_3
Stanozolol	329.5	*81.1*, 91.1	42, 40	60	16β-OH-stanozolol-d_3
5α-Androstan-17β-ol-3-one	291.5	*159.1*, 255.1	20, 15	25	–
Mesterolone	305.7	*269.3*, 173.1	16, 20	33	–
Danazol	338.7	*120.0*, 148.0	29, 25	35	–
Mestanolone	305.6	*269.0*, 229.2	16, 19	33	Medroxyprogesterone-d_3
19-Norethindrone	299.3	*109.1*, 231.4	27, 17	35	Northindrone-ethynyl-$^{13}C_2$
21α-Hydroxyprogesterone	331.5	*96.9*, 108.9	21, 21	35	Norgestrel-d_6
17α-Hydroxyprogesterone	331.5	*96.9*, 108.9	22, 22	35	Norgestrel-d_6
D(−)-Norgestrel	313.4	*108.9*, 245.4	26, 20	38	Norgestrel-d_6
Medroxyprogesterone	345.5	*123.0*, 97.0	24, 24	35	Medroxyprogesterone-d_3
Megestrol acetate	385.5	*267.3*, 325.6	16, 16	30	Megestrol-d_3 acetate
Chlormadinone acetate	405.4	*345.6*, 309.6	12, 16	28	Megestrol-d_3 acetate
Progesterone	315.5	*97.0*, 297.5	20, 35	35	Progesterone-d_9
Medroxyprogesterone acetate	387.5	*327.3*, 285.4	16, 16	30	Progesterone-d_9
Triamcinolone	439.3	*363.0*, 329.9	14, 10	25	Cortisol-d_3
Aldosterone	405.3	*359.3*, 331.4	10, 22	26	Cortisol-d_3
Prednisone	403.7	*327.5*, 357.2	14, 9	18	Cortisol-d_3
Cortisone	405.6	*329.5*, 359.4	16, 16	19	Cortisol-d_3
Cortisol	407.5	*331.5*, 361.7	16, 13	25	Cortisol-d_3
Prednisolone	405.4	*329.5*, 359.4	26, 16	26	Cortisol-d_3
Flumethasone	455.4	*379.7*, 409.2	18, 12	22	Cortisol-d_3
Dexanethasone	437.4	*361.5*, 391.3	16, 12	30	Cortisol-d_3
Fludrocortisone acetate	467.4	*421.2*, 349.0	12, 24	20	Cortisol-d_3
Methylprednisolone	419.7	*343.6*, 373.3	19, 12	20	Cortisol-d_3
Beclomethasone	453.3	*377.3*, 407.3	15, 12	20	Cortisol-d_3
Triamcinolone acetonide	479.4	*413.3*, 337.6	25, 19	25	Cortisol-d_3
Fluocinolone acetonide	497.4	*431.5*, 355.4	20, 20	25	Cortisol-d_3
Fluormetholone	421.4	*355.4*, 254.6	16, 25	20	Cortisol-d_3
Budesonide	475.1	*357.3*, 339.3	14, 18	22	Cortisol-d_3
Clobetasol propionate	511.4	*465.4*, 429.4	13, 18	25	Cortisol-d_3
Estriol	287.3	*145.2*, 171.1	44, 47	56	Estradiol-3,4-$^{13}C_2$
Estradiol	271.4	*183.1*, 145.2	40, 45	45	Estradiol-3,4-$^{13}C_2$
Ethinylestradiol	295.4	*145.2*, 159.2	41, 35	45	Estradiol-3,4-$^{13}C_2$
Estrone	269.4	*145.2*, 159.2	41, 41	49	estrone-2,4-d_2
Diethylstibestrol	267.3	*237.3*, 251.3	25, 28	43	Diethylstilbestrol-d_6
Hexestrol	269.5	*133.9*, 119.1	16, 40	30	Hexestrol-d_4
Dienestrol	265.2	*92.9*, 171.2	25, 25	40	Dienestrol-d_2

[a] The italicized product ions were used for quantitative analysis.

3. Results and discussion

3.1. Optimization of LC–MS/MS

Each tuning solutions was introduced into the electrospray source by direct infusion (10 μL min^{-1}). The main ions produced in MS and MS/MS were identified in positive and negative ionization modes. The diagnostic fragment ions were selected and all mass spectrometer parameters were optimized to increase sensitivity. The retention time of analytes and MRM transition conditions used for quantification and confirmation are listed in Table 2. The commonly used mobile-phase compositions such as acetonitrile/water and methanol/water were optimized. The results were identical to our previous reports [15], i.e., methanol/water containing 0.1% formic acid was suitable for AS, GS and PS; acetonitrile/water was preferable for ES. Fig. 1 shows the multiple reaction monitoring (MRM) chromatograms of target compounds in a spiked milk sample, while MRM chromatograms of a blank milk sample are shown in Fig. S1.

3.2. Optimization of sample preparation

3.2.1. Enzymatic hydrolysis

Many hormones in plasma and urine were presented in the conjugated state; enzymatic hydrolysis was necessary in the sample preparation procedure to obtain free hormones. The necessity of enzymatic hydrolysis of hormone glucuronides or sulfates in tissues is controversial [31]. A recent study indicated that the conjugated portion could be omitted because the proportion of cleavable conjugated testosterone in muscle tissue was <20%, and that of conjugated 17β-estradiol was <5% [22]; whereas enzymatic hydrolysis was required in another recent study [32]. In our study, the levels of endogenous hormones in meat, milk and shrimp were detected using an enzymatic hydrolysis procedure and without enzymatic hydrolysis. There were no significant differences for cortisol and progesterone between the two procedures except in the liver matrix (Table 3). It seemed that enzymatic hydrolysis could be neglected for muscle and milk. The present study covered several compounds but there were no data for other analytes. Enzymatic hydrolysis was used in this study to ensure the accuracy of results.

3.2.2. Extraction

The compounds studied in this contribution had a wide range of polarities, with octanol–water partition coefficients (log K_{ow}) ranging from 0.63 to 4.31 (calculated by EPI K_{ow}win software, EPI suite 3.1). Methanol was selected as a solvent and added to extracted analytes with high log K_{ow} (e.g., progesterone, danazol).

3.2.3. Concentration and purification

Three conventional SPE cartridges (GCB, C18, HLB) were evaluated with respect to recovery and matrix effect. Firstly, extraction efficiencies of all target compounds using GCB, C18 and HLB were investigated by spiking standard solution into pure water at 10 μg L^{-1}. Irrespective of cartridge used, satisfactory recoveries (85–105%) for most analytes were achieved (data not shown). For the GCB cartridge, absolute recoveries of diethylstilbestrol, hexestrol and dienestrol were <50%, which was much lower than that for HLB and C18 (70–87%). Similar to our previous study [15], this may be attributed to the strong adsorbance between analytes and GCB sorbent. GCB is considered to be a reversed-phase sorbent and an anion-exchanger due to positively charged chemical heterogeneities on its surface [33]. Phenolic hydroxyls in diethylstilbestrol, hexestrol and dienestrol lead to their tight bonding with cartridge material.

The effect of co-eluting residual matrix components may result in suppression (or less frequently) of the enhancement of the analyte response. The liver matrix was selected as the typical case for the matrix effect experiment because of its complexity. The experiment was carried out as described in Section 2. The matrix effect was defined by subtracting the ratio between the slope of matrix-matched standard curves and the slope of standard solution curves, and then multiplied by 100%. As a result, high

Table 3
Effect of enzymatic hydrolysis for cortisol and progesterone (n = 3).

Matrix	Cortisol (μg kg^{-1})		Progesterone (μg kg^{-1})	
	Enzymatic hydrolysis	Without enzymatic hydrolysis	Enzymatic hydrolysis	Without enzymatic hydrolysis
Pork	16.2	15.9	0.35	0.37
Liver	1.29	0.97	ND	ND
Beef	0.56	0.53	8.62	7.93
Milk	0.85	0.80	7.38	7.42
Shrimp	0.55	0.57	ND	ND

Table 4
Matrix effect of target compounds in liver samples upon different SPE cartridges.

Compound	Matrix suppression ratio (%)		
	HLB–NH_2	C18–NH_2	GCB–NH_2
19-Nor-4-androstene-3,17-dione	87	89	0
Trenbolone	25	40	0
Boldenone	30	36	0
Fluoxymeterone	37	42	12
Nandrolone	10	20	10
4-Androstene-3,17-dione	42	46	27
Methandrostenolone	33	32	16
Testosterone	34	34	20
DHEA	51	69	22
Methyltestosterone	36	33	20
Testostrone	12	14	15
Methlandrostenediol	0	0	0
Epiandrosterone	13	35	0
Stanozolol	0	0	0
5α-Androstan-17β-ol-3-one	0	32	10
Mesterolone	0	0	0
Danazol	52	57	30
Mestanolone	20	48	12
19-Norethindrone	23	33	0
21α-Hydroxyprogesterone	44	49	19
17α-Hydroxyprogesterone	31	37	15
D(−)-Norgestrel	36	42	18
Medroxyprogesterone	10	20	0
Megestrol acetate	45	52	35
Chlormadinone acetate	41	51	30
Progesterone	0	0	0
Medroxyprogesterone acetate	43	50	10
Triamcinolone	83	76	15
Aldosterone	60	70	12
Prednisone	48	51	0
Cortisone	34	36	0
Cortisol	16	14	0
Prednisolone	20	30	0
Flumethasone	0	0	0
Dexanethasone	0	0	0
Fludrocortisone acetate	63	74	0
Methylprednisolone	0	0	0
Beclomethasone	32	30	0
Triamcinolone acetonide	0	0	0
Fluocinolone acetonide	35	0	0
Fluormetholone	0	0	0
Budesonide	25	10	0
Clobetasol propionate	21	17	0
Estriol	47	41	80
Estradiol	20	20	55
Ethinylestradiol	46	49	32
Estrone	50	56	35
Diethylstibestrol	88	77	29
Hexestrol	70	82	36
Dienestrol	58	61	28

Table 5
The slopes of different matrix-fortified calibration for target compounds ($n = 5$).

Compound	Standard	Pork	Liver	Milk	Beef	Shrimp	R.S.D. (%)
19-Nor-4-androstene-3,17-dione	0.07	0.09	0.07	0.10	0.07	0.08	16.80
Trenbolone	0.14	0.11	0.11	0.13	0.13	0.13	9.30
Boldenone	0.13	0.13	0.10	0.12	0.13	0.10	13.90
Fluoxymeterone	0.05	0.03	0.03	0.04	0.03	0.04	19.40
Nandrolone	0.07	0.06	0.05	0.08	0.06	0.07	14.20
4-Androstene-3,17-dione	0.99	0.27	0.35	0.70	0.27	1.03	59.00
Methandrostenolone	3.01	3.68	2.38	2.67	2.97	3.97	19.40
Testosterone	0.60	0.52	0.53	0.52	0.60	0.68	11.40
DHEA	0.05	0.07	0.05	0.06	0.06	0.06	14.00
Methyltestosterone	0.87	1.01	0.80	1.02	1.05	1.12	12.20
Testostrone	0.75	0.78	0.72	0.89	0.81	1.01	13.20
Methlandrostenediol	0.21	0.23	0.16	0.21	0.17	0.20	14.10
Epiandrosterone	0.75	1.05	0.65	0.88	0.90	0.96	16.50
Stanozolol	1.29	1.48	1.17	1.25	1.54	1.37	10.40
5α-Androstan-17β-ol-3-one	0.48	0.75	0.40	0.65	0.58	1.12	38.50
Mesterolone	0.07	0.11	0.06	0.12	0.11	0.10	25.60
Danazol	0.05	0.16	0.05	0.04	0.15	0.18	63.20
Mestanolone	0.01	0.01	0.01	0.01	0.02	0.01	13.80
19-Norethindrone	13.10	11.30	10.70	11.50	13.10	15.30	13.50
21α-Hydroxyprogesterone	0.56	0.45	0.40	0.50	0.49	0.43	12.50
17α-Hydroxyprogesterone	0.39	0.40	0.32	0.40	0.38	0.39	8.20
D(−)-Norgestrel	5.11	6.55	4.77	5.37	5.50	5.20	11.20
Medroxyprogesterone	1.80	1.69	1.09	1.57	1.85	1.97	18.80
Megestrol acetate	3.08	2.28	2.23	2.73	2.44	3.56	19.10
Chlormadinone acetate	1.13	0.95	0.86	1.12	0.90	0.94	11.80
Progesterone	1.04	0.91	0.79	1.11	0.91	1.03	12.10
Medroxyprogesterone acetate	0.40	0.28	0.27	0.31	0.41	0.36	17.90
Triamcinolone	2.67	2.37	2.05	2.81	2.25	2.89	13.30
Aldosterone	1.99	1.86	1.47	1.93	1.65	2.01	11.80
Prednisone	5.24	5.99	4.96	5.53	6.03	5.72	7.60
Cortisone	2.79	2.53	1.88	2.77	2.03	2.49	15.70
Cortisol	1.82	1.76	1.64	1.94	2.04	1.74	8.00
Prednisolone	2.81	3.21	2.53	2.75	3.15	3.01	8.90
Flumethasone	5.86	6.15	5.16	6.04	6.50	5.92	7.50
Dexanethasone	6.03	6.78	5.93	6.55	7.07	6.31	6.80
Fludrocortisone acetate	1.09	0.87	0.81	1.12	0.95	0.93	12.90
Methylprednisolone	4.58	5.03	4.24	4.91	4.99	4.78	6.30
Beclomethasone	3.71	4.05	3.48	4.11	4.64	4.05	9.90
Triamcinolone acetonide	0.43	0.45	0.39	0.48	0.39	0.40	8.70
Fluocinolone acetonide	2.98	2.77	2.54	2.79	2.83	2.65	5.50
Fluormetholone	0.17	0.16	0.15	0.16	0.16	0.19	9.00
Budesonide	0.99	0.77	0.69	0.82	0.61	0.72	16.80
Clobetasol propionate	0.50	0.43	0.41	0.47	0.40	0.41	9.30
Estriol	1.64	1.57	1.47	1.64	1.69	1.48	5.80
Estradiol	4.74	4.55	4.09	4.31	4.96	4.66	6.80
Ethinylestradiol	0.93	1.02	0.89	0.90	0.92	1.05	7.10
Estrone	2.09	1.89	1.81	2.14	1.96	1.79	7.40
Diethylstibestrol	3.02	3.27	2.89	3.19	2.95	3.15	4.80
Hexestrol	1.63	1.74	1.63	1.79	1.60	1.77	4.90
Dienestrol	2.00	1.91	1.86	1.85	2.11	1.94	5.00

suppression (>50%) was observed for most analytes and some of them (ES and GS) were suppressed completely in the elution of C18 or HLB cartridges. Although the thing upon GCB is much better, signal suppression >30% was still occurred for 21 compounds. Therefore, a further purification step by means of NH_2 cartridge was added. As shown in Table 4, the matrix suppression for most target compounds by GCB–NH_2 was significantly lower than that by C18–NH_2 and HLB–NH_2, except for estriol, estradiol. This indicates that GCB is more effective in sample purification.

Taking it into account that (i) GCB–NH_2 exhibited high recovery and excellent matrix cleanup for most analytes; (ii) the high sensitivities of diethylstilbestrol, hexestrol and dienestrol during LC–MS/MS analysis; and (iii) the convenience of operation of the GCB cartridge over C18 and HLB cartridges, GCB cartridge was used in sample preparation.

3.3. Method validation

In general, isotopic-dilution methods are favorable techniques to compensate for the loss of target analytes in the sample preparation procedure, and for ion suppression of the mass spectrometer in trace-level analysis. There may not be sufficient commercial isotopic standards for multi-residue analysis, so several compounds were analyzed with one isotopic standard in our study. The standard curves in methanol and in different matrices after cleanup were compared (Table 5). The slopes of most analytes in methanol and cleanup matrix were comparable (R.S.D. of slopes in methanol and different matrixes ranged from 4.8% to 19.4% for 46 compounds), suggesting that the selection of internal standards was appropriate. Internal standards for 5α-androstan-17β-ol-3-one, mesterolone, danazol and 4-androstene-3,17-dione were not available because the R.S.D.s of slopes were >25% thus, matrix-fortified curves were used to quantify these four analytes. Acceptable linearities for all target compounds were obtained with correlation coefficients of $r > 0.98$. Average recoveries (Tables S1 and S2) of each compound at three spiking levels ranged from 76.9% to 121.3%. Within-day reproducibility was represented by R.S.D. percentages, and ranged from 2.4% to 21.2% at three spiked levels on a day. The between-day repeatability during five consecutive days ranged from 7.9% to 23.2%. The LODs and LOQs (defined as the concentration that yields

Table 6
Hormone concentrations in pork, milk, beef, and shrimp samples.

Sample	Compound	Mean concentration ($\mu g\,kg^{-1}$)	Minimum concentration ($\mu g\,kg^{-1}$)	Maximum concentration ($\mu g\,kg^{-1}$)
Pork, $n=10$	Cortisol	15.65	5.89	24.80
	Cortisone	0.15	0.10	0.22
	Testosterone	0.32	0.06	1.42
	Progesterone	0.38	0.22	0.61
Milk, $n=20$	Cortisol	0.43	0.05	1.25
	17α-Hydroxyprogesterone	0.14	0.10	0.19
	Testosterone	0.11	0.06	0.52
	Progesterone	6.10	1.39	11.28
	4-Androstene-3,17-dione	0.15	0.10	0.47
	Estradiol	1.25	N.D.	3.52
Beef, $n=6$	Cortisol	5.18	2.68	10.82
	Cortisone	1.06	0.10	2.76
	Testosterone	0.13	0.05	0.26
	Progesterone	3.84	0.52	9.27
Shrimp, $n=10$	Cortisol	1.40	0.51	6.78

S/N ratio of 3 and 10) were 0.01–0.70 and 0.04–2.0 $\mu g\,kg^{-1}$, respectively. The LOD and LOQ of pork liver were significantly higher than those of other matrix, which could be attributed to the complexity of liver matrix.

3.4. Real sample analysis

This method has been successfully applied to detect anabolic hormones in animal food and seafood (including pork, beef, shrimp and whole milk) available from local supermarkets in Beijing, China. The endogenous hormone of cortisol was found in all samples ranging from 0.05 $\mu g\,kg^{-1}$ (milk) to 24.80 $\mu g\,kg^{-1}$ (pork) (Table 6). Cortisone was observed in only pork and beef samples at 0.10–0.22 and 0.10–2.76 $\mu g\,kg^{-1}$, respectively. Testosterone and progesterone presented at 0.05–0.52 and 0.22–11.28 $\mu g\,kg^{-1}$, respectively, in pork, milk and beef samples. 17α-Hydroxyprogesterone and 4-androstene-3,17-dione were detected in milk at $\leq$0.5 $\mu g\,kg^{-1}$; estradiol was observed in 8 milk samples of 20. These findings were in accordance with previous reports [34,35].

4. Conclusion

We presented a simple and sensitive analytic method for the simultaneous determination of 50 anabolic hormones with a wide range of polarity and classification in samples of beef, pork, milk and shrimp. This study is the first report on the simultaneous analysis of >30 anabolic hormones in foods. This method is also applicable for the effective routine surveillance of large-scale samples.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jchromb.2008.12.054.

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