

## **Supplementary Material**

**A simple, efficient, eco-friendly sample preparation procedure for the simultaneous determination of hormones in meat and fish products by gas chromatography–mass spectrometry**

Safae Chafi, Evaristo Ballesteros

Department of Physical and Analytical Chemistry, E.P.S of Linares, University of Jaén,  
23700 Linares, Jaén, Spain

**Table S1.** Comparison of the proposed and alternative methods for determining natural and synthetic hormones in meat and fish products from around the world.<sup>a</sup>

Analytes	Samples	Countries	Pretreatment methods	Analytical techniques	Analytical features	Concentration in real samples	Reference
4-Androstene-3,17-dione, stanozolol, 17 $\alpha$ -hydroxyprogesterone, prednisone, prednisolone, testosterone, hydrocortisone, megestrol acetate, medroxyprogesterone acetate, and progesterone	Chicken, pork, beef and sausages	China	MSPD	UPLC–MS/MS	LOD: 0.01–0.05 $\mu$ g/kg RSD: < 10% Recovery: 77–99%	Hydrocortisone: 2.4–3.4 $\mu$ g/kg Progesterone: 2.1 $\mu$ g/kg	[1]
17 $\beta$ -Estradiol, 17 $\alpha$ -ethinyl estradiol, diethylstilbestrol and others EDCs	Fish liver, brain and muscle	Spain	FUSLE, MSPD	LC–MS/MS	LOD: 3–20 $\mu$ g/kg RSD: < 26% Recovery: 62–132%	Diethylstilbestrol: 7–8 $\mu$ g/kg	[5]
Estrone, 17 $\alpha$ -ethinyl estradiol, diethylstilbestrol and others EDCs	Fish muscle, liver, gill and gonad	China	QuEChERS, derivatization (BSTFA–1% TMCS)	GC–MS	LOD : 0.03–1.95 $\mu$ g/kg RSD: 0.95–11.9 % Recovery: 47–128%	Estrone: 7.0–308 $\mu$ g/kg 17 $\alpha$ -Ethinylestradiol: 5.4–27.3 $\mu$ g/kg Diethylstilbestrol: 4.79–15.6 $\mu$ g/kg	[6]
Dienestrol, diethylstilbestrol, hexestrol, progesterone, 17 $\alpha$ -methyl testosterone, epitestosterone, 6 propyl-2-thiouracile, testosterone, melengestrol acetate, $\beta$ -estradiol-3-benzoate, 17 $\beta$ -estradiol, 17 $\alpha$ -ethinyl estradiol and estriol	Bovine matrices: muscle, kidney, liver and bile	Lebanon	QuEChERS	LC–MS/MS	LOD: 0.13–0.86 $\mu$ g/kg RSD: < 23 % Recovery: 52–107%	Progesterone: 0.11–11.7 $\mu$ g/kg Testosterone: 0.5–9.5 $\mu$ g/kg Epi-testosterone: 0.89–280 $\mu$ g/kg	[10]
Diethylstilbestrol, estrone, 17 $\beta$ -estradiol, 17 $\alpha$ -ethinylestradiol, estriol, levonorgestrel, norethisterone, megestrol acetate, progesterone, testosterone, boldenone, nandrolone, cortisone, prednisone and prednisolone	Fish: <i>Sphoeroides marmoratus</i> and <i>Boops boops</i> )	Spain	MAE, SPE	UHPLC–MS/MS	LOD: 0.14–49 $\mu$ g/kg RSD: < 20% Recovery: 20–130%	Norethisterone: 500–2710 $\mu$ g/kg Prednisolone: 90 $\mu$ g/kg Nandrolone: 70–520 $\mu$ g/kg Progesterone: 560–3950 $\mu$ g/kg	[11]
Progesterone, norethindrone, medroxyprogesterone, levonorgestrel, testosterone, 19-nortestosterone, methyltestosterone , stanozolol, androstenedione, dihydrotestosterone, estrone, 17 $\beta$ -estradiol, 17 $\alpha$ -ethinyl estradiol, dienestrol, hexestrol, diethylstilbestrol and others EDCs	Grass and silver carp, white bream and catfish	China	AALLME, QuEChERS	HPLC–MS/MS	LOD: 0.03–0.8 $\mu$ g/kg RSD: 1.1–14.5 % Recovery: 78–119%	Diethylstilbestrol: 2.2–3.6 $\mu$ g/kg	[13]
Estradiol, testosterone, progesterone, zeranol and diethylstilbestrol	Beef	South Korea	LLE, SPE, UAE, derivatization (MSTFA/ NH <sub>4</sub> I/DTE)	GC–MS	LOD: 0.1–0.4 $\mu$ g/kg RSD: 5–16% Recovery: 68–106%	Progesterone: 0.3–36.5 $\mu$ g/kg Testosterone: 0.2 $\mu$ g/kg	[19]
Testosterone, androstenedione, 17 $\beta$ -estradiol, estrone, pregnenolone, progesterone, dihydroandrostenedione, and dihydrotestosterone	Aquatic mollusks	France	MAE, SPE Derivatization (MSTFA/mercaptoethanol/ NH <sub>4</sub> I)	GC–MS	LOD: 0.1–0.4 $\mu$ g/kg RSD: < 16% Recovery: 85–114%	Progesterone/pregnenolone: <0.4–8.9 $\mu$ g/kg	[20]
Estrone, 17 $\beta$ -estradiol, estriol and anti-inflammatories.	Mussels: <i>Mytilus edulis trossulus</i>	Poland	ASE, SPE, derivatization (BSTFA–1% TMCS)	GC–MS	LOD: 1–7 $\mu$ g/kg RSD: 0.24–7.8% Recovery: 80–118%	Estrone: 256 $\mu$ g/kg	[22]
17 $\beta$ -Nortestosterone, fluoxymesterone, progesterone, estrone, estriol, 17 $\alpha$ -estradiol, 17 $\beta$ -estradiol, 17 $\alpha$ -hydroxyprogesterone, 17 $\alpha$ -ethinyl estradiol, diethylstilbestrol, dienestrol, $\alpha$ -zearalanol, $\beta$ -zearalanol, 17 $\alpha$ -testosterone, 17 $\beta$ -testosterone, hexoestrol, norgestrel, acetoxyprogesterone, medroxyprogesterone acetate and methyltestosterone and others hormones	Bovine muscle tissue	Belgium	LLE, SPE	UHPLC–Orbitrap–HRMS UHPLC–MS/MS	LOD: 0.04–2.50 $\mu$ g/kg RSD: < 20% Recovery: 81–119%	–	[24]
Hydrocortisone, cortisone, progesterone, prednisone, prednisolone, testosterone, melengesterol acetate, hydrocortisone-21-acetate, cortisone-21-acetate, testosteronepropionate, 17 $\alpha$ -methyltestosterone, 6 $\alpha$ -methylprednisolone, 17 $\alpha$ -hydroxyprogesterone and medroxyprogesterone	Meat (chicken, pork and beef)	Spain	QuEChERS	UHPLC–Orbitrap–HRMS	LOQ: 1–2 $\mu$ g/kg RSD: 1–20 % Recovery: 70–103%	Hydrocortisone: 2.8 $\mu$ g/kg Progesterone, 1.7 $\mu$ g/kg	[25]

Hydrocortisone, testosterone, dehydroepiandrosterone, epitestosterone, etiocholanolone, androsterone, progesterone, testosterone propionate, pregnanediol, pregnanetriol, estriol, 17 $\beta$ -estradiol, 17 $\alpha$ -estradiol and estrone	Beef	China	UAE, SPE	HPLC–MS/MS	LOQ: 0.004–0.24 $\mu$ g/kg RSD: 2.2–10.2 % Recovery: 66–115%	Hydrocortisone: 0.22–0.46 $\mu$ g/kg Progesterone: 0.22–1.00 $\mu$ g/kg 17 $\beta$ -Estradiol : 0.26–2.51 $\mu$ g/kg Etiocholanolone: 1.21–2.01 $\mu$ g/kg	[26]
Testosterone, progesterone, pregnenolone, cortisone, hydrocortisone, androsterone, trenbolone acetate and melengestrol acetate	Beef	Italy, Poland, Netherlands, France	LLE, SPE, derivatization (NH <sub>2</sub> OCH <sub>3</sub> -HCl)	UHPLC–MS/MS	LOD: 0.21–0.45 $\mu$ g/kg RSD: 3–13.8 % Recovery: 90–110%	Testosterone: 3.2–16.0 $\mu$ g/kg Progesterone: 1.9–5.4 $\mu$ g/kg Androsterone 4.1 $\mu$ g/kg Pregnenolone: 3.7–8.2 $\mu$ g/kg Cortisone: 2.0–24.8 $\mu$ g/kg Hydrocortisone: 0.73–1.8 $\mu$ g/kg Trenbolone acetate: 2.95 $\mu$ g/kg	[27]
Estrone, 17 $\beta$ -estradiol, estriol, 17 $\alpha$ -ethinyl estradiol, dienestrol, diethylstilbestrol and hexestrol	Eel, grass carp, goldfish, shrimp	China	DMI-MSPD	HPLC–MS/MS	LOD: 1.6–2.8 $\mu$ g/kg RSD: 2–5% Recovery: 89–103%	Diethylstilbestrol: 5.2 $\mu$ g/kg	[29]
Estrone, 17 $\beta$ -estradiol, estriol and diethylstilbestrol, 17 $\alpha$ -ethinyl estradiol, dienestrol, hexestrol, 2-methoxyestradiol, estradiol benzoate and estradiol-17-valerate and bisphenol A	Pork and chicken meat	China	QuEChERS-MSPE	UHPLC–MS/MS	LOD: 0.02–3 $\mu$ g/kg RSD: 0.48–15.1% Recovery: 81–115%	Estrone: 0.09–0.10 $\mu$ g/kg Diethylstilbestrol: 0.55–0.92 $\mu$ g/kg	[30]
Testosterone, mengestrol, methyltestosterone and others veterinary drugs	Pork, beef, mutton, chicken, pork liver, lamb liver and chicken liver	China	LLE, SPE	UPLC–MS/MS	LOD: 0.1–10 $\mu$ g/kg RSD: < 7.8% Recovery: 80–116%	–	[31]
Testosterone, progesterone, diethylstilbestrol and others EDCs	Maricultured fish	Malaysia	UAE, SPE	HPLC–MS/MS	LOD: 0.49–2.08 $\mu$ g/kg RSD: < 30% Recovery: 95–110 %	Testosterone: 0.57–4.25 $\mu$ g/kg Progesterone: 0.80–9.78 $\mu$ g/kg	[32]
Estrone, 17 $\beta$ -estradiol, estriol and others EDCs	Chicken meat, fish and pond water	China	LLE, DLLME, derivatization (BCEC-Cl)	HPLC–MS	LOD: 0.03–0.07 $\mu$ g/L RSD: 3.1–5.4% Recovery: 88–106%	Estriol: 3.94 $\mu$ g/L	[33]
Corticosterone, estrone, estriol, medroxyprogesterone, progesterone, 17 $\beta$ -estradiol, 17 $\alpha$ -hydroxyprogesterone, testosterone, and 19-nortestosterone	Fish tissue	China	DMAE, LLE	HPLC–MS/MS	LOD: 0.03–0.15 $\mu$ g/kg RSD: 3.3–5.6% Recovery: 75–95%	Testosterone: 0.45 $\mu$ g/kg 17 $\alpha$ -Estradiol: 0.39–0.44 $\mu$ g/kg 19-Nortestosterone: 0.30 $\mu$ g/kg 17 $\alpha$ -Hydroxyprogesterone: 0.26 $\mu$ g/kg Corticosterone: 0.57 $\mu$ g/kg	[34]
Progesterone, testosterone, estrone, 17 $\beta$ -estradiol, 17 $\alpha$ -ethinyl estradiol and others EDCs	Fish (sea catfish, croaker, marine gastropod mollusks)	Malaysia	QuEChERS	HPLC–MS/MS	LOD: 0.07–3.50 $\mu$ g/kg Recovery: 64–114%	17 $\beta$ -Estradiol: 0.14–0.91 $\mu$ g/kg Estrone: 0.04–0.38 $\mu$ g/kg Progesterone: 0.39–9.57 $\mu$ g/kg Testosterone: 0.16–0.7 $\mu$ g/kg	[38]
Hexestrol, diethylstilbestrol, estrone, 17 $\beta$ -estradiol, 17 $\alpha$ -ethinyl estradiol, estriol, testosterone, dihydrotestosterone, androstenedione, progesterone, norethindrone, levonorgestrel and pregnenolone	Fish and meat	Spain, Norway, Italy, Morocco, Greece, Portugal, Germany, USA	LLE, SPE derivatization (BSTFA–1% TMCS)	GC–MS	LOD: 0.0004–0.0150 $\mu$ g/kg RSD: 3.1–6.8 % Recovery: 90–105%	Hexestrol: 0.020 $\mu$ g/kg Estrone: 0.060–0.970 $\mu$ g/kg 17 $\beta$ -Estradiol: 0.170–0.550 $\mu$ g/kg Testosterone: 0.030–0.200 $\mu$ g/kg Dihydrotestosterone: 0.026–1.90 $\mu$ g/kg Androstenedione: 0.160–0.320 $\mu$ g/kg Progesterone: 0.0910.780 $\mu$ g/kg Levonorgestrel: 0.030–0.400 $\mu$ g/kg Pregnenolone: 0.380–0.810 $\mu$ g/kg	This work

<sup>a</sup> AALLME air-assisted liquid–liquid microextraction; ASE accelerated solvent extraction; BCEC-Cl 2-(11H-benzo[a]carbazol-11-yl) ethyl carbonochloridate; BSTFA–1% TMCS *N,O*-Bis(trimethylsilyl)trifluoroacetamide containing 1% trimethylchlorosilane; DLLME dispersive liquid–liquid microextraction; DMAE dynamic microwave-assisted extraction; DMI dummy molecularly imprinted; EDCs endocrine disrupting compounds; FUSLE focused ultrasound solid–liquid extraction; GC–MS gas chromatography–mass spectrometry; HPLC–MS/MS high-performance liquid chromatography–tandem mass spectrometry; LC-MS/MS liquid chromatography–tandem mass spectrometry; LLE liquid–liquid extraction; LOD limit of detection; LOQ limit of quantification; MAE microwave assisted extraction; MSPD matrix solid-phase dispersion; MSPE magnetic solid phase extraction; MSTFA/NH<sub>4</sub>I/DTE *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide/ammonium iodide/dithioerythritol; NH<sub>2</sub>OCH<sub>3</sub>-HCl *O*-methylhydroxylamine; QuEChERS Quick, Easy, Cheap, Effective, Rugged and Safe; RSD relative standard deviation; SPE solid-phase extraction; UAE ultrasonic assisted extraction; UHPLC–Orbitrap–HRMS ultra-high performance liquid chromatography–Orbitrap–high resolution mass spectrometry; UHPLC–MS/MS ultra-high performance liquid chromatography–tandem mass spectrometry; UPLC–MS/MS ultra-performance liquid chromatography–tandem mass spectrometry

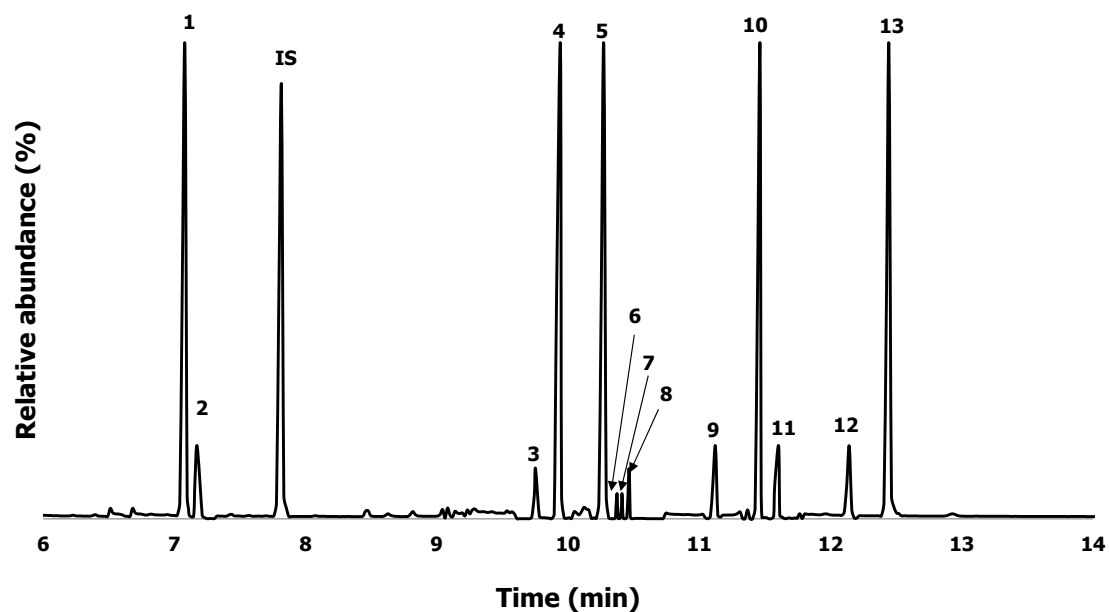
**Table S2.** Variables influencing the sample pretreatment, solid-phase extraction/clean-up process and derivatization of hormones.

Variable	Optimum range (selected value)
Sample pretreatment <sup>a</sup>	
Volume of water-acetonitrile mixture 9:1 (v/v) (mL)	> 8 (10)
Centrifugation rate (rpm)	3500-5000 (4000 rpm)
Centrifugation time (min)	11-16 (12)
Centrifugation temperature (°C)	0-10 (4 °C)
Continuous solid-phase extraction/clean-up <sup>b</sup>	
Sample pH	6.5-7.5 (7)
Amount of Oasis HLB sorbent (mg)	75-85 (80)
Volume of acetone (eluent) (μL)	550-650 (600)
Flow rate of sample (mL/min)	4-6 (5)
Flow rate of air (mL/min)	4-6 (5)
Breakthrough volume (mL)	1-200 (10)
Derivatization <sup>c</sup>	
Percentage of TMCS (catalyst) in BSTFA <sup>c</sup>	1-5 (1)
Microwave oven power (W)	180-220 (200)
Irradiation time (min)	3.5-4.5 (4)

<sup>a</sup> To 2 g of meat and fish samples.

<sup>b</sup> These variables have been previously optimized [35].

<sup>c</sup> TMCS, trimethylchlorosilane; BSTFA, *N,O*-bis(trimethylsilyl)trifluoroacetamide.



**Figure S1.** Typical chromatogram in the SIM mode for 1 g of chicken breast spiked with all hormones. Concentration: 250 ng/kg [(1) hexestrol, (4) estrone, (5) 17 $\beta$ -estradiol, (10) 17 $\alpha$ -ethinylestradiol, (11) levonorgestrel, (12) progesterone, (13) estriol] and 500 ng/kg [(2) diethylstilbestrol, (3) dihydrotestosterone, (6) androstenedione, (7) norethindrone, (8) testosterone and (9) pregnenolone]. IS: internal standard.