GEL PERMEATION CHROMATOGRAPHY CLEAN-UP FOR THE DETERMINATION OF GROWTH PROMOTERS IN KIDNEY FAT BY LIQUID CHROMATOGRAPHY–TANDEM MASS SPECTROMETRY

George Kaklamanos[1,2] and Georgios Theodoridis[2]

[1]Veterinary Laboratory of Serres, Terma Omonoias, 62110 Serres, Greece
[2]Laboratory of Analytical Chemistry, Department of Chemistry, Aristotle University, Thessaloniki, 54124 Greece

Introduction

The use of steroid hormones for food producing animals is prohibited in the European Union, because of their possible toxic effect.
Effective residue control plans is necessary to monitor illegal administration of growth promoters: different matrices at various stages in the food chain should be analysed: At farm level, urine, faeces and hair are monitored and at slaughterhouse level, injection sites, meat or fat tissue samples and organs (liver and kidney).
Steroid hormones are lipophilic compounds that accumulate in fat. Kidney fat is considered the tissue of choice for detection at slaughterhouse level mainly for gestagens and corticosteroids. Perinephric kidney fat is composed of adipose tissue: a collection of fat cells bound together by connective tissue supplied by blood vessels. Other steroid hormones can also diffuse through the membrane of target cells.
Analytical methods for the determination of corticosteroids and gestagens in kidney fat are mostly by liquid chromatography-tandem mass spectrometry (LC-MS).
Common sample preparation methods are labour intensive including steps such as: extensive extraction of fat with organic solvents, defatting of the extracts, solid phase extraction (SPE), liquid/liquid extraction (LLE), high performance liquid chromatography, accelerated solvent extraction (ASE) or supercritical fluid extraction (SFE).

Goal of the present study: to develop a highly sensitive and automated multi-analyte method for the analysis of growth promoters in kidney fat by combining Gel permeation chromatography (GPC) as clean-up and detection by LC-MS/MS.

Experimental

- **Sample preparation:** 2 g of fat
  Dissolved with 1ml n-hexane and 9 ml dichloromethane, clean-up with GPC, reconcentration and injection on the LC-MS/MS system (TSQ Quantum AM).
- **GPC conditions:**
  Glass column (1000 mm × 25mm ID) filled by Bio Beads (S-X3) 200–400mesh (Bio-Rad Laboratories, Richmond, CA), flow of 5 ml/min with dichloromethane, injection volume 5 ml, total run time 50 min and fraction collection from 36.5 to 50 min.
- **HPLC: Gradient RP-HPLC, mixture of Internal standards**
  Prevail C18, 150x4.6 mm, 3 μm column, column temperature: 20°C, mobile phase: MeOH and H_2O, flow rate 0.700 ml/min, injection volume 15 μl and total analysis time 13 min.
- **Atmospheric Pressure ionization: APCI**
  Discharge current : 7 μA, sheath gas: 40 arb, aux gas: 5 arb, capillary temperature: 300 ^oC and heated temperature 450 ^oC.
- **MS/MS conditions:**
  MRM mode acquiring 2 products ions per precursor ion.

Validation

- **Validation:** European Commission Decision 2002/657/EC, 3 different days at 1, 1.5 and 2 times the validation level (1 ng/g) and six replicates per concentration.
- **Linearity:** 9 calibration points at concentrations 0, 0.5, 1, 1.5, 2, 3, 4, 6,10 ng/g. A mixture of internal standards including methyltestosterone-d_3, testosterone-d_3, 17β-estradiol-d_3, diethylstilbestrol-d_6, triamcinolone acetonide-d_6, megestrol acetate-d_3 and α/β-zearalanol-d_4 set at 2ng/g. $\mathbf{R^2}$ was greater than 0.995.
- **Recoveries:** ranged between 80.9% and 121.2%.
- **Repeatability:** RSDs ranged from 0.7% to 12.0% .
- **Within-lab reproducibly:** RSDs ranged from 0.8% to 10.2%.
- **Specificity:** 20 different fat samples were analysed to evaluate for possible matrix interferences. No interfering peaks were detected.
- **Analytical limits:** CCα ranged from 0.10 to 0.30 ng/g while the detection capabilities CCβ ranged from 0.17 to 0.48 ng/g.
- **Measurement uncertainty *U:*** 2.33 was used for the factor k to cover a Gaussian distribution of 99% and ranged from 6.42% to 33.08%.

Results

Compound (Rt min)	Precursor ion	Product ions	CE
Flumethasone (5.23)	411.15(+)	**253.19**/235.08	25/31
Dexamethasone (5.61)	393.15(+)	**237.23**/147.16	30/25
Triamcinolone acetonide (5.71)	435.18(+)	**213.16**/321.20	34/19
α/β-trenbolone (6.47/6.23)	271.17(+)	**199.10**/253.12	28/22
α/β-zearalanol (6.57/5.53)	323.20(+)	**149.10/**189.16	36/32
Diethylstilbestrol (6.99)	273.15(-)	**222.12**/237.137	41/38
Ethynylestradiol (7.03)	295.19(-)	**145.30**/159.11	36/39
α/β-boldenone (7.06/6.36)	287.20(+)	**121.11**/135.25	30/15
Dienestrol (7.15)	265.10(-)	**93.03**/236.17	35/27
β-estradiol (7.16)	271.18(-)	**145.17**/183.20	39/49
Hexestrol (7.25)	269.15(-)	**119.01**/133.02	39/20
α/β-nortestosterone (7.25/6.68)	275.20(+)	**109.12**/145.16	21/28
Methyltestosterone (7.65)	303.00(+)	**109.11**/97.08	22/32
Chlormadinone acetate (8.04)	405.11(+)	**309.17**/345.08	18/12
Megestrol acetate (8.08)	385.24(+)	**224.32**/267.35	30/26
Medroxyprogesterone acetate (8.18)	387.24(+)	**123.20**/285.47	30/23
Melengestrol acetate (8.27)	397.24(+)	**279.32**/236.26	23/37

Compound	CCα (ng/g)	CCβ (ng/g)	*U* (%)
Flumethasone	0.20	0.34	33.08
Dexamethasone	0.13	0.22	15.89
Triamcinolone acetonide	0.18	0.30	10.29
α/β-trenbolone	0.10/0.13	0.17/0.23	6.72/9.49
α/β-zearalanol	0.17/0.16	0.28/0.28	6.42/11.58
Diethylstilbestrol	0.17	0.28	17.85
Ethynylestradiol	0.24	0.40	13.38
α/β-boldenone	0.12/0.11	0.20/0.18	14.01/11.62
Dienestrol	0.18	0.31	14.80
β-estradiol	0.12	0.21	8.59
Hexestrol	0.17	0.28	16.71
α/β-nortestosterone	0.15/0.11	0.25/0.18	12.58/8.15
Methyltestosterone	0.10	0.17	7.79
Chlormadinone acetate	0.20	0.33	12.26
Megestrol acetate	0.26	0.44	11.41
Medroxyprogesterone acetate	0.30	0.48	12.59
Melengestrol acetate	0.20	0.34	11.66

Conclusions

- A simple, rapid and automated method was developed for the determination and confirmation of 21 growth promoters in kidney fat covering stilbenes, steroids, RALs, corticosteroids and gestagens.
- GPC clean-up procedure proved efficient providing clear extract of the analytes completely separated from the fat extract.
- The developed method can be considered rapid achieving satisfactory chromatographic separation in 8.5 min for all analytes.
- The validation of the method was based on the EC Decision 2002/657/EC proving sufficient analytical performance, satisfactory accuracy and precision.
- The method is therefore suitable for laboratories involved in official residue control analyses and animal administration studies with growth promoters.

References

1. Tao, Y., Paula, R., Stolker, A.A.M., Chen, D. and Yuan, Z., 2015. Simultaneous determination of seven gestagens in kidney fats by Ultra Performance Convergence Chromatography tandem mass spectrometry. Journal of Chromatogr. B, 988: 143-48.

Contact:
Dr George Kaklamanos
Veterinary Laboratory of Serres
Email: vetlabs@otenet.gr