Bundesamt für Verbraucherschutz und Lebensmittelsicherheit

Validation and multivariate effect analysis of an LC-MS/MS method for the determination of steroids in bovine muscle

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hormonally active substances banned for use as growth promoters in animal production

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B2f Corticosteroids

(Council Directive 96/22/EC)

USA, Canada, Australia, New Zealand

Testosterone, 17β-Estradiol, Progesterone, Trenbolone, Zeranol, Melengestrolacetate legally used

Registered trade name	Active ingredient					To be used for
	E2	T	P	TB	Z	
Synovex S	+		+			steer, bull
Synovex H	+	+				heifer
Synovex Plus	+			+		calf, bull, steer
Implix BM	+		+			bull
Implix BF	+	+				heifer
Implus S	+		+			steer
Implus H	+	+				heifer
Compudose*	+					calf, steer
Revalor	+			+		calf, bull, steer
Torrevex S	+		+			bull, steer
Torrelor	+			+		bull, steer
Finaplix H				+		heifer, cow
Ralgro					+	steer, bull, calf sheep, lamb
Magnum					+	cattle
Steer-oid	+		+			steer
Heifer-oid	+	+				heifer
Calf-oid	+		+			calf
Component E-C	+		+			bull
Forplix				+	+	calf
Proferm					+	cattle, sheep

E2: 17β-estradiol or its benzoate; T: testosterone or its propionater; P: progesterone; TB: Trenbolone acetate® and Z: Zeranol®.

Introduction

European Union (EU)

- **hormonally active substances banned for use as growth promoters in animal production** **(Council Directive 96/22/EC)**

- **residue testing mandatory** **(Council Directive 96/23/EC)**

  (Regulation 882/2004/EC)

- **validation of analytical methods required** **(Commission Decision 2002/657/EC)**

  ⇨ **CCα (decision limit), CCβ (detection capability), repeatability, reproducibility**

  ⇨ **uncertainty of measurement** **(SANCO 2726/2004 rev. 1)**

- **for application in routine residue analysis:**

  ⇨ **information on a method´s ruggedness to define its scope of applicability and fitness for purpose**

Introduction

A3 Steroids		
Estrogens	**Androgens**	**Gestagens**
Estradiol	α + β –Testosterone	Progesterone
	α + β – Nortestosterone	Hydroxy-progesterone
	α + β – Boldenone	
Ethinyl-estradiol	Methyltestosterone	Medroxy-progesterone
	α + β – Trenbolone	Chlor-madinone
	Norethandrolone	Melengestrol
	Methylboldenone	Megestrol
	Stanozolol	
Urine, Kidney, Liver, Bile, Plasma		Kidney fat

endogeneous - exogeneous

import samples, meat samples at retail level:

matrix muscle

cattle treated with steroid implants:
low levels of steroids in muscle

Testosterone: ≈ 0.1 - 0.3 ppb (heifer, calves)

Trenbolone: ≈ 0.01 - 0.3 ppb

⇓

for determination of steroids in muscle:

need for an analytical method with appropriate sensitivity !

Method description (1)

Method

Analytes	Internal Standards (IS)
17α-Nortestosterone	17β-Nortstosterone-D3
17β-Nortestosterone	17β-Nortstosterone-D3
17α-Testosterone	Methylboldenone-D3
17β-Testosterone	Methylboldenone-D3
17β-Boldenone	17β-Boldenone-D3
17β-Trenbolone	17β-Trenbolone-D3
Ethinylestradiol	Methylboldenone-D3
Methylboldenone	Methylboldenone-D3
Methyltestosterone	Methylboldenone-D3
Stanozolol	Stanozolol-D3

Method description (2)

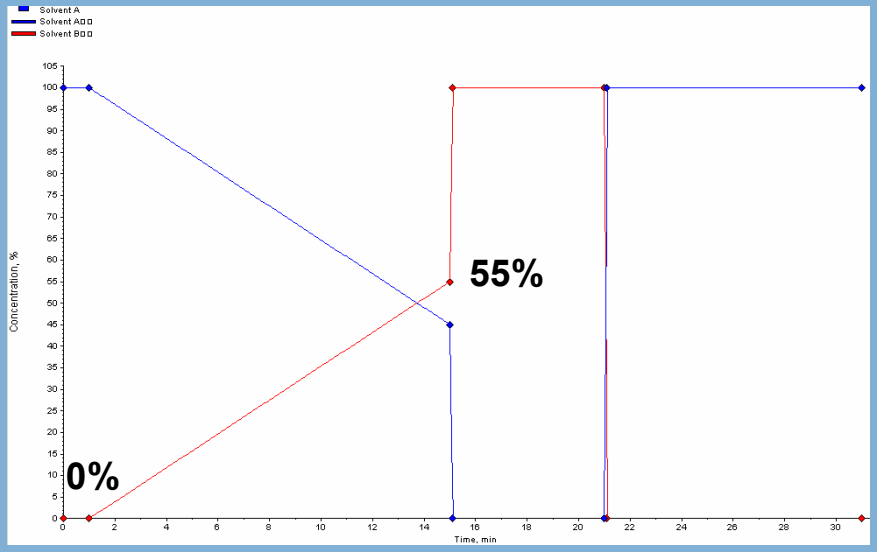
Sample preparation

1. Fortification of samples (10 g bovine muscle)	- spike homog. samples, add 10 ml of Tris-Puffer (pH 8)
2. Hydrolysis with Protease XIV	- add 100 µl aqueous Protease XIV solution (50mg/ml) - hydrolyse for 16h at 37°C - centrifuge samples (15 min, 3000 rpm)
3. Liquid/liquid extraction	- extract twice with 30 ml of t-Butylmethylether - evaporate organic layer to dryness - dissolve in 2.0 ml of Methanol/Water (80/20, v/v)
4. Defattening	- wash samples 3x with 3 ml of n-Hexane
5. SPE using Oasis HLB cartridges	-precondition Oasis HLB (6cc, 200 mg) with 5 ml of Methanol and 5 ml of Water - apply samples to columns - wash with 5 ml of H_2O and dry for 1 min - wash with 5 ml 40% Methanol and dry for 3 min - elute with 5 ml of Acetone
6. SPE using NH_2 cartridges	- precondition aminocartridge (Isolute, 500 mg, 3 ml) with 5 ml of Methanol and with 5 ml Acetone - apply samples of 6) to column and collect the eluate - evaporate to dryness (55°C, nitrogen stream) - dissolve in 100 µl ACN / Wasser (50/50, v/v)
7. LC MS/MS measurement (API 4000, APCI (+))	

Method description (3)

HPLC conditions

- Agilent 1100 HPLC system
- column: Luna C18, 5 µ, 2.1 x 150 mm
- Eluent A: Water /Acetonitrile (65/35, v/v)
- Eluent B: Acetonitrile
- Flow: 500 µl /min
- HPLC-Gradient:



MS/MS conditions

- API 4000 QTrap (Applied Biosystems)
- Ionensource: APCI(+), heated nebulizer
- Source temperature: 550 °C
- Scan type: MRM, resolution: Q1 / Q3: unit
- GS1: 40 psi
- Curtain gas (Nitrogen) : 20 psi
- CAD gas (Nitrogen): 10 psi
- Nebulizer current: 3000 V

Method description (4)

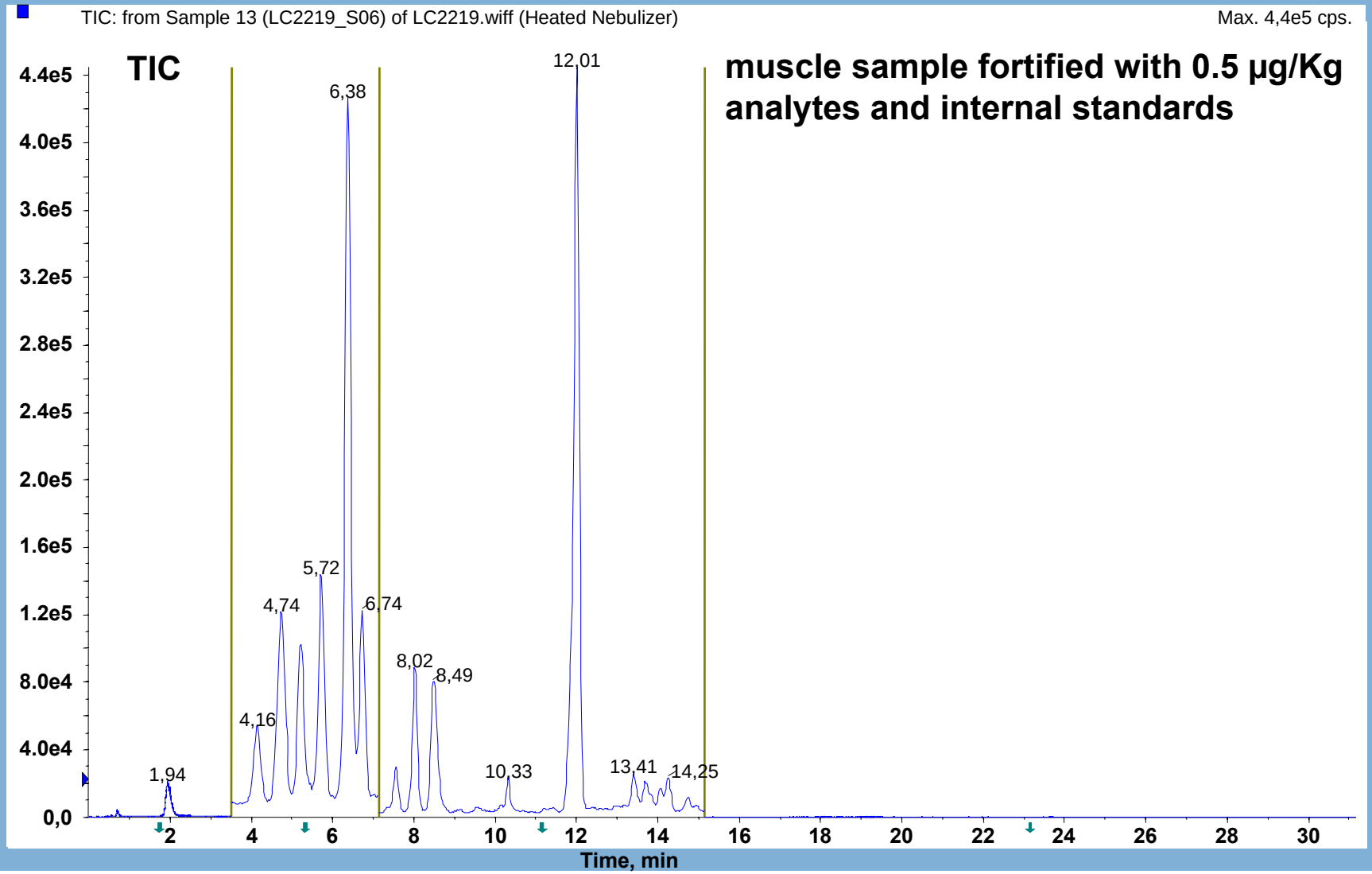
MS/MS conditions

Analyte	Q1 Mass m/z	Q3 Mass m/z	IS
17α-Nortestosterone	275	109 145 239	278 / 260
17β-Nortestosterone	275	109 145 239	278 / 260
17α-Testosterone	291	109 97	304/121
17β-Testosterone	291	109 97	304/121
17β-Boldenone	287	121 135 77	290 / 121

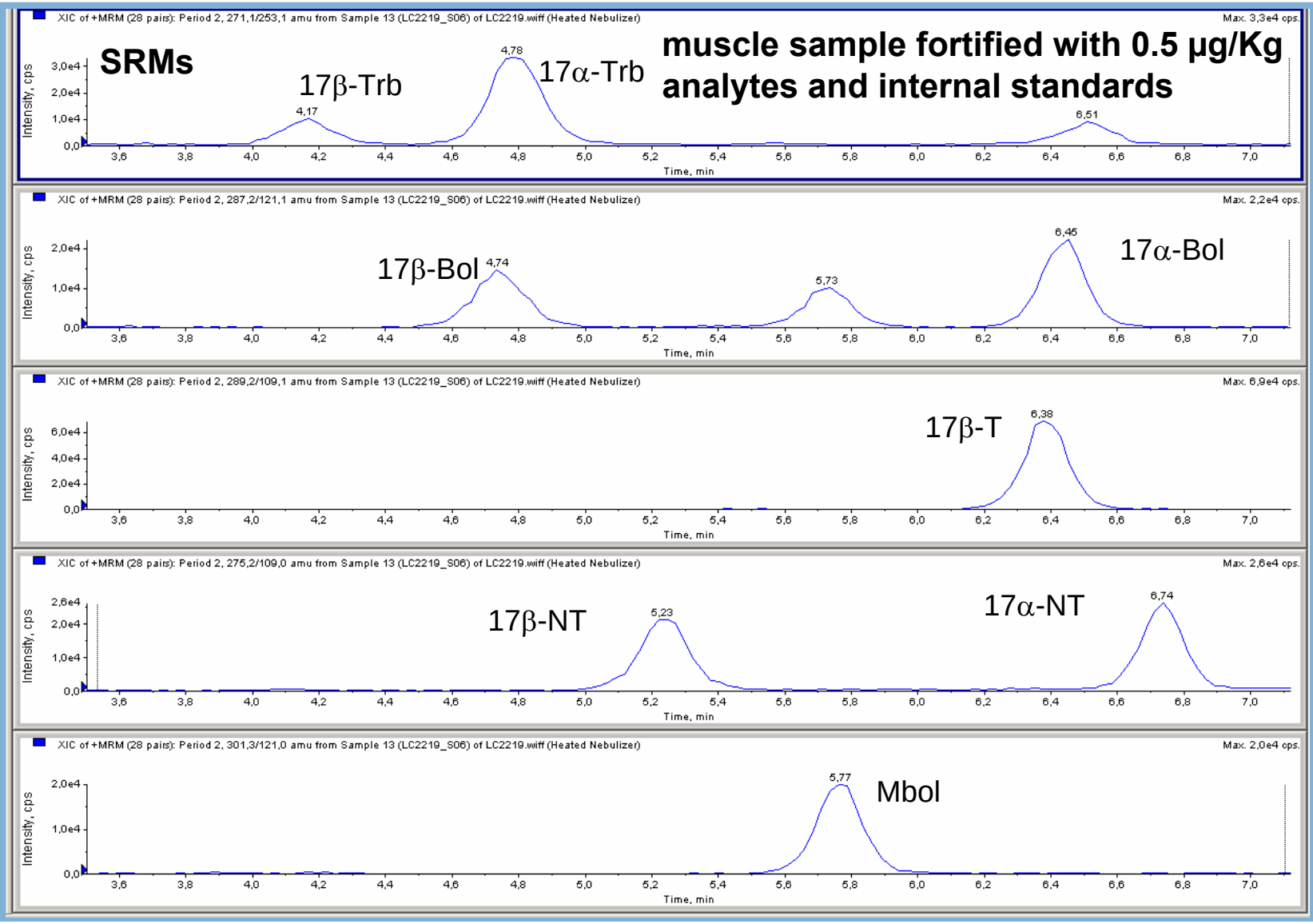
Analyte	Q1 Mass m/z	Q3 Mass m/z	IS
Ethinylestradiol			304/121
Methylboldenone	301	121 149	304/121
Methyltestosterone	303	109 97	304/121
Stanozolol	329	81 93 95	332 / 81
17β-Trenbolone	271	253 199 114	273/199

Method description (5)

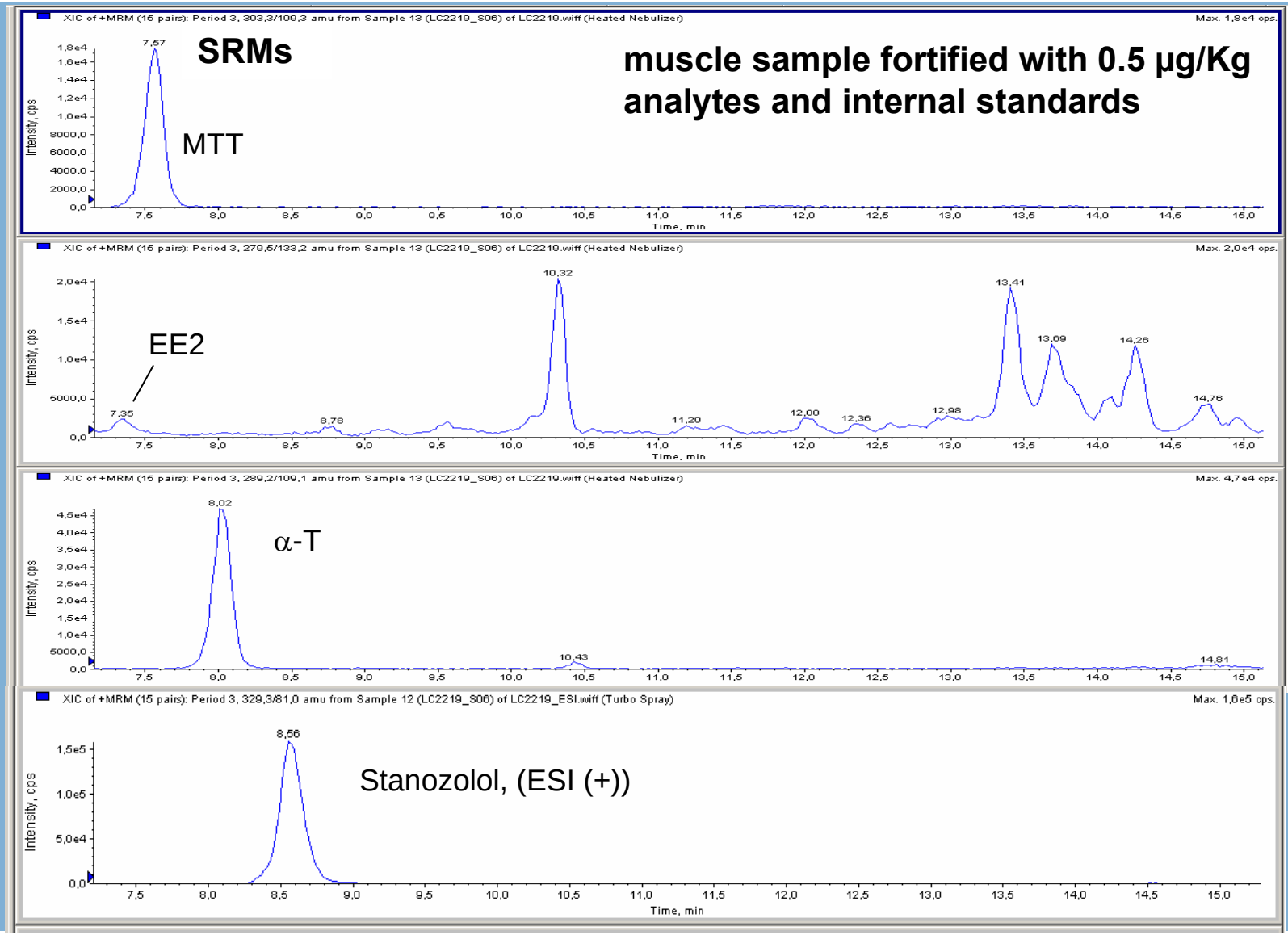
HPLC chromatogram



Method description (6)



Method description (7)



Validation plan (1)

Validation concept

Matrix-comprehensive in-house validation concept

- **developed on the basis of a variance component model**
- **based on fractional factorial design**

InterVal Software (*quo data GmbH*)

- **decision limit CCα, detection capability CCβ**
- **repeatability std. dev., in-house reproducibility std. dev.**
- **uncertainty of measurement**
- **Validation according to Commission Decision 2002/657/EC !**

References:

1) B. Jülicher, P. Gowik, S. Uhlig, Analyst 123 (1998) 173-179.

2) B. Jülicher, P. Gowik, S. Uhlig, Analyst 124 (1999) 537-545.

Validation plan (2)

Choice of the validation factors → ***"noise factors"*** → **+ level, - level**

"Factors that cannot be controlled in routine analysis but that have potential influence on the result"

Sample preparation	**Operator**	**OP `G`, OP `Z`**
1. Fortification of samples (5 ml bovine urine)		
2. Hydrolysis with Protease XIV		
3. Liquid / liquid extraction		
4. Defattening		
5. SPE using Oasis HLB cartridges	**Lot Nr.**	**A , B**
6. SPE using NH_2 cartridges	**Lot Nr.**	**C, D**
7. LC MS/MS measurement (API 4000, APCI(+))	**Storage of extracts**	**0 d, 3 d**

Validation plan (3)

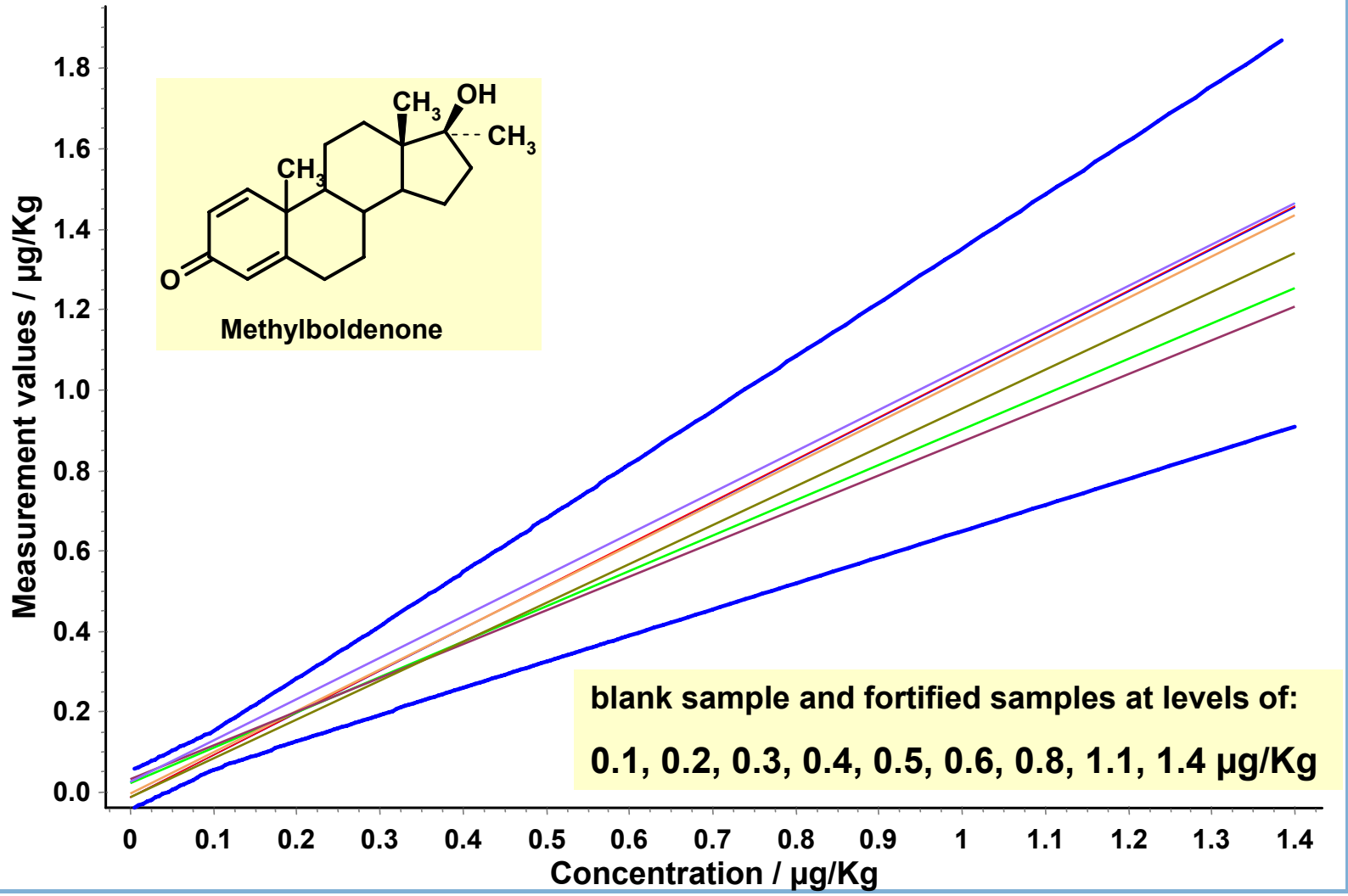
Design of the validation experiment (InterVal Plus)

Factor Run	1 operator	2 lot of HLB cartridge	3 lot of NH_2 cartridge	4 storage of extracts
S01	Z	B	D	3 d
S02	Z	B	D	0 d
S03	G	B	C	3 d
S04	G	B	C	0 d
S05	Z	A	C	3 d
S06	Z	A	C	0 d
S07	G	A	D	3 d
S08	G	A	D	0 d

- Variation of 4 factors on two different levels
- 8 different samples of bovine muscle, sample of bovine muscle for matrix calibration
- Fortification levels: 0.1 (0.3, 0.5) - 1.4 µg/Kg
- Validation study performed within 5 weeks

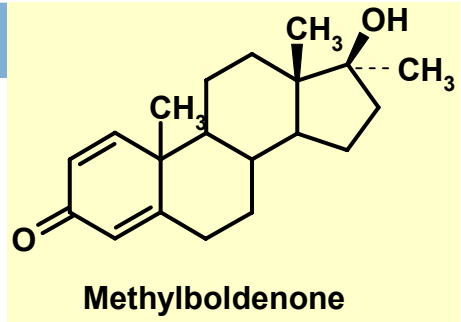
Validation results (1)

Validation calibration curves and prediction interval

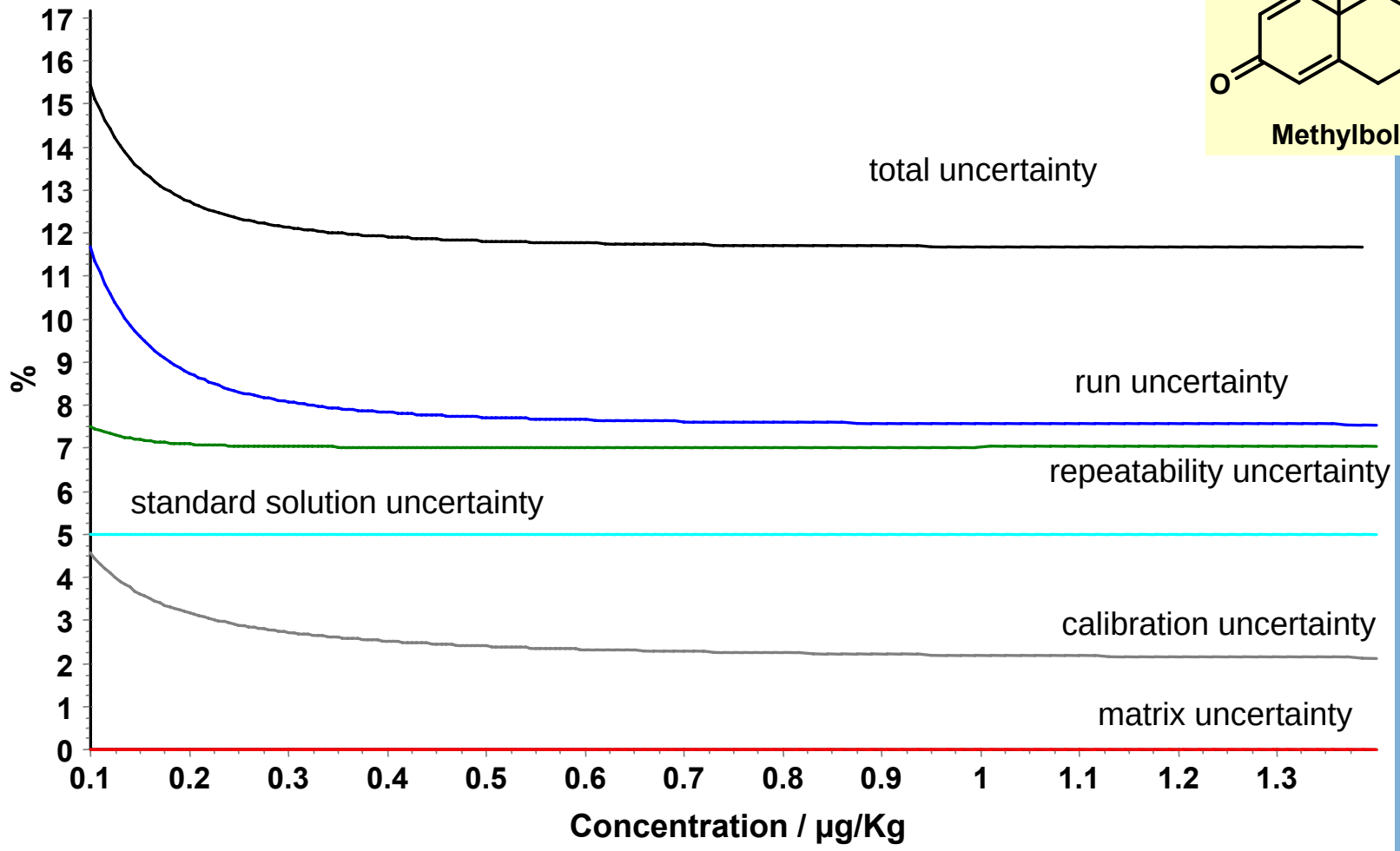


Validation results (2)

Uncertainty of measurement components

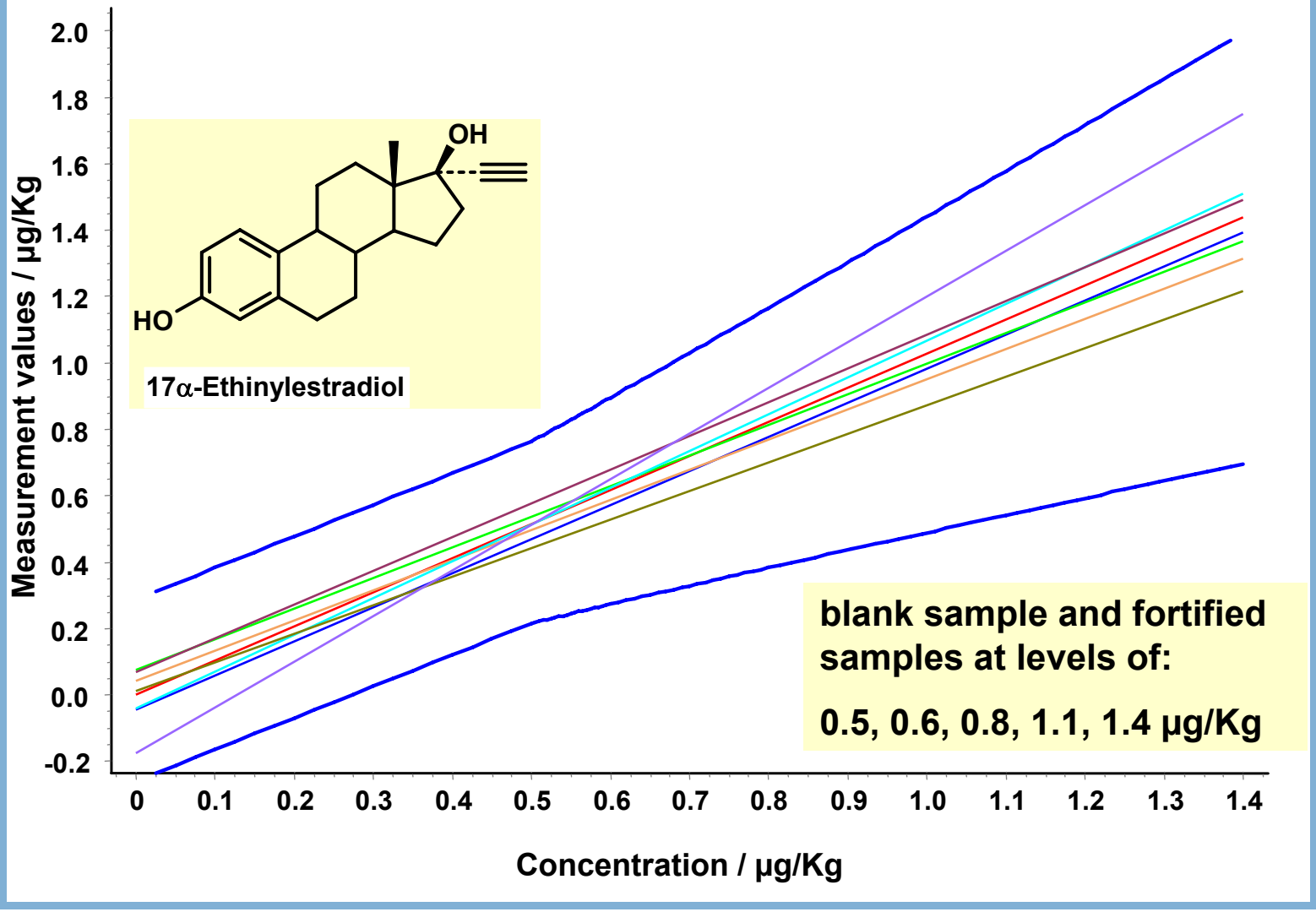


Methylboldenone



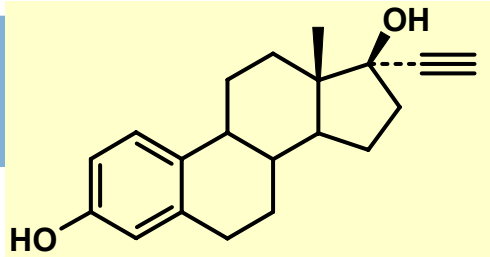
Validation results (3)

Validation calibration curves and prediction interval

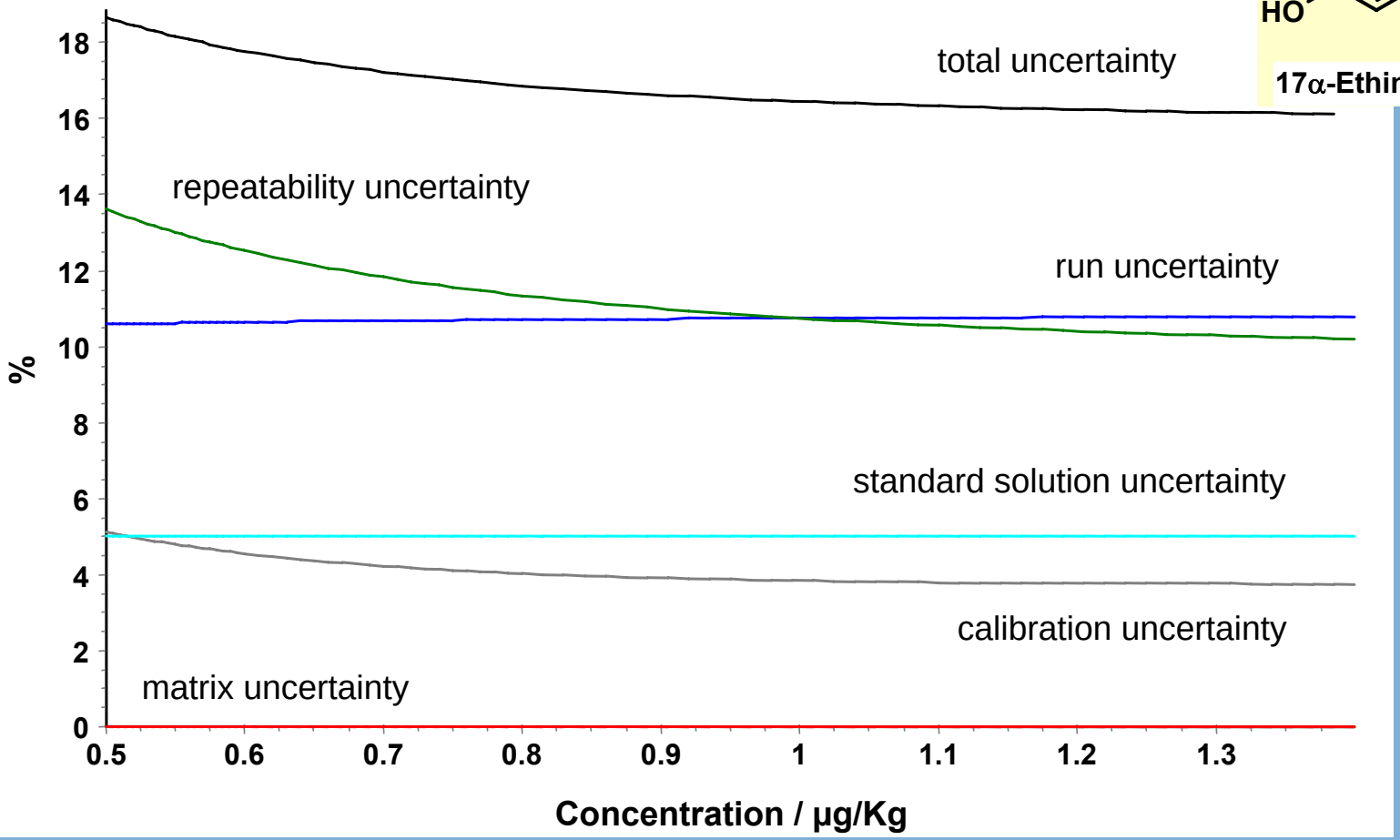


Validation results (4)

Uncertainty of measurement components

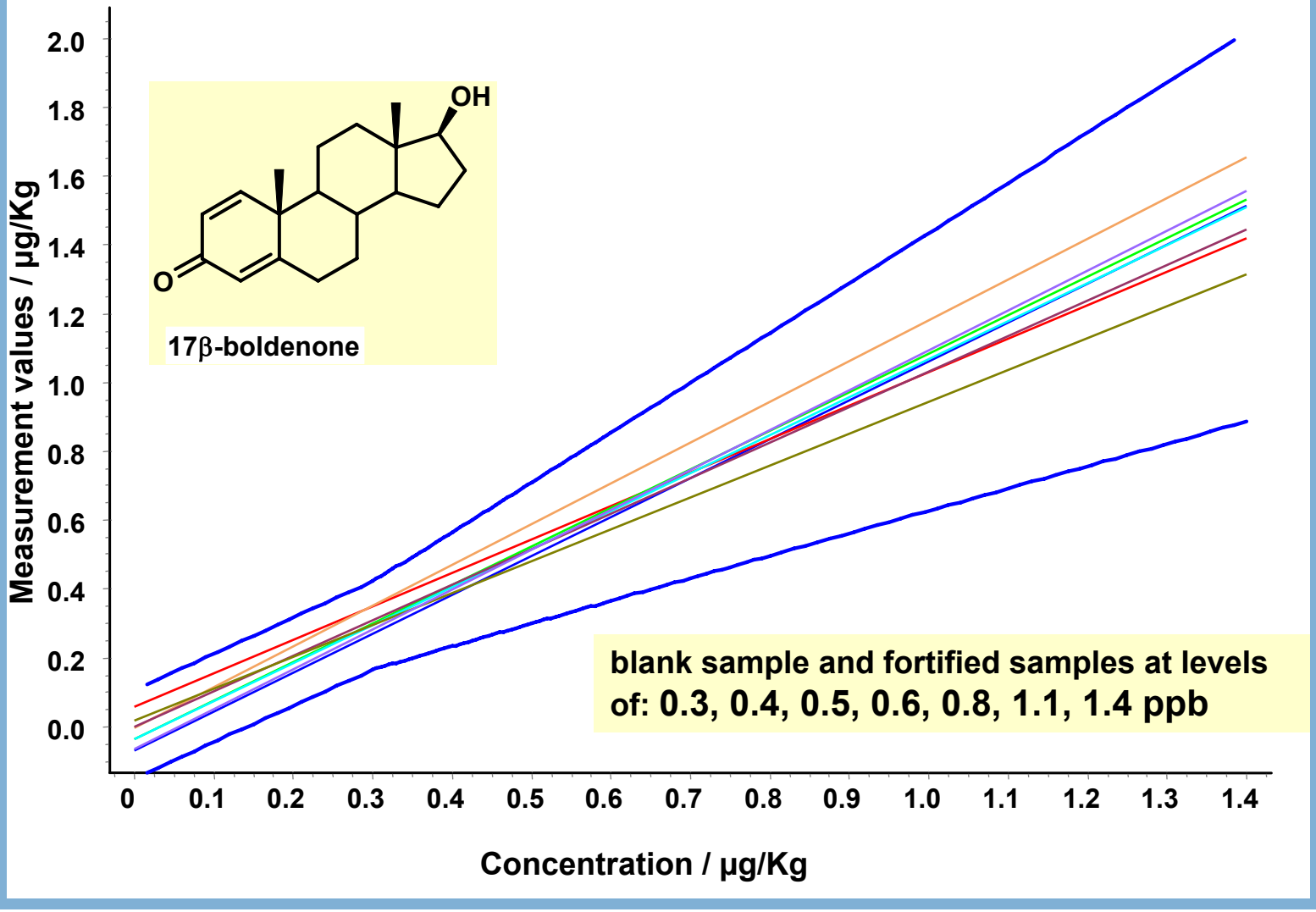


17α-Ethinylestradiol



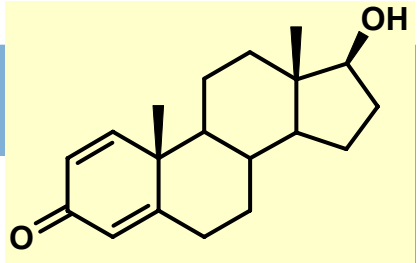
Validation results (5)

Validation calibration curves and prediction interval

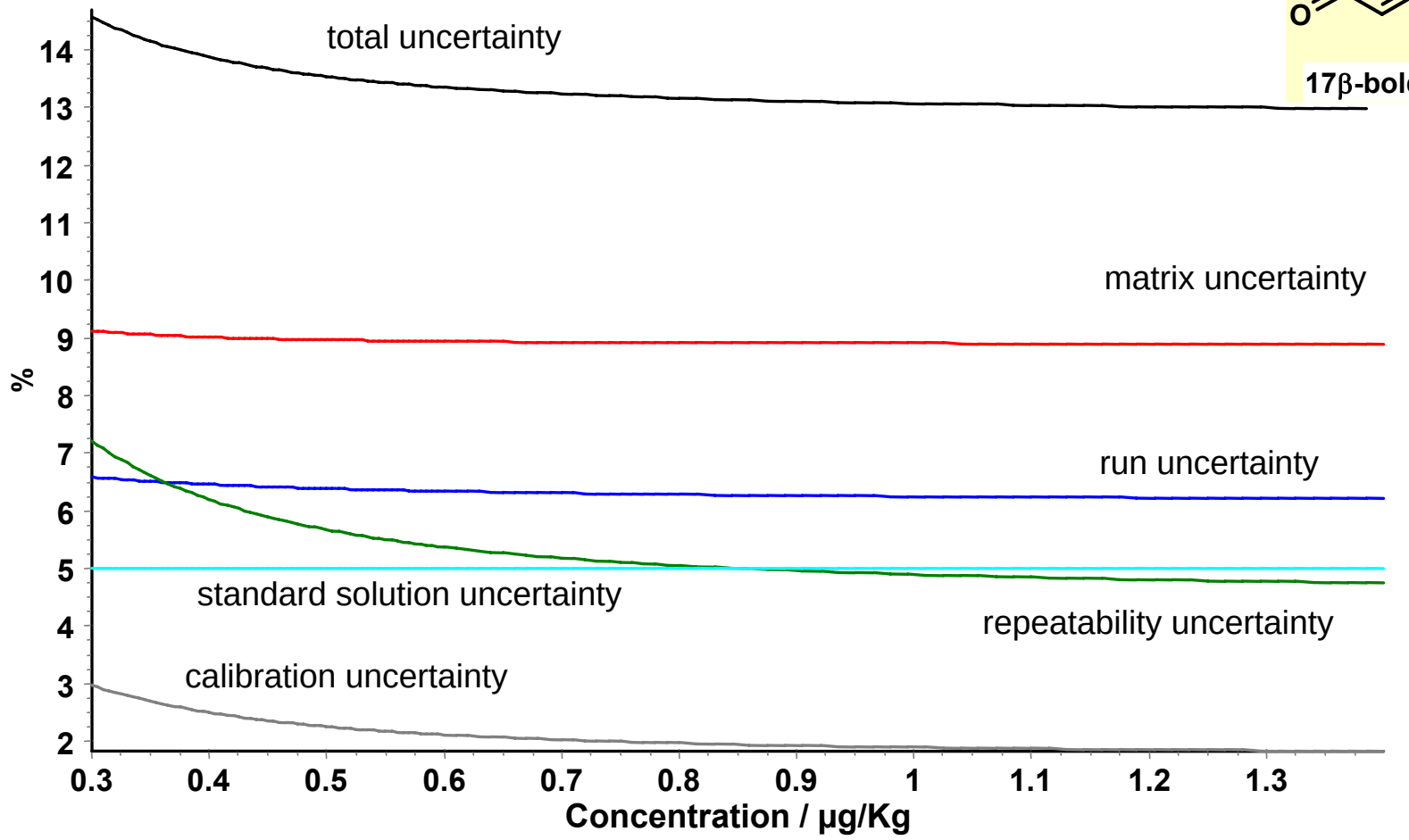


Validation results (6)

Uncertainty of measurement components



17β-boldenone



Validation results (7)

Summary

Analyte	CCα (µg/Kg)	CCβ (µg/Kg)	CV s_{wR} at CCα (%)	CV s_{wR} at 0.95 µg/Kg (%)	recovery at CCα (%)
17α-Nortestosterone	0.481	0.612	13.9	9.0	98.1
17α-Testosterone	0.430	0.537	12.3	9.9	101.9
17β-Boldenone	0.422	0.544	13.8	13.5	100.1
17β-Nortestosterone	0.404	0.499	10.7	10.2	97.3
17β-Testosterone	0.581	0.837	19.5	16.9	100.3
17β-Trenbolone	0.467	0.631	15.6	13.6	98.8
Ethinylestradiol	0.788	1.101	16.4	16.0	96.8
Methylboldenone	0.148	0.193	13.9	11.7	102.7
Methyltestosterone	0.551	0.686	12.1	11.1	104.4
Stanozolol	0.300	0.383	13.2	9.8	99.1

Multivariate effect analysis (1)

Multivariate effect analysis

- aim: 1) systematic identification of factors that influence the measurement signal
  2) optimisation of recovery with concomitant minimisation of dispersion

- put InterVal data into

↪ OptiVal (*quo data GmbH*)

- based on factorial experiments
- allows multivariate effect analysis

↪

- estimation of the effect of each factor/factor level on each analyte

Multivariate effect analysis (2)

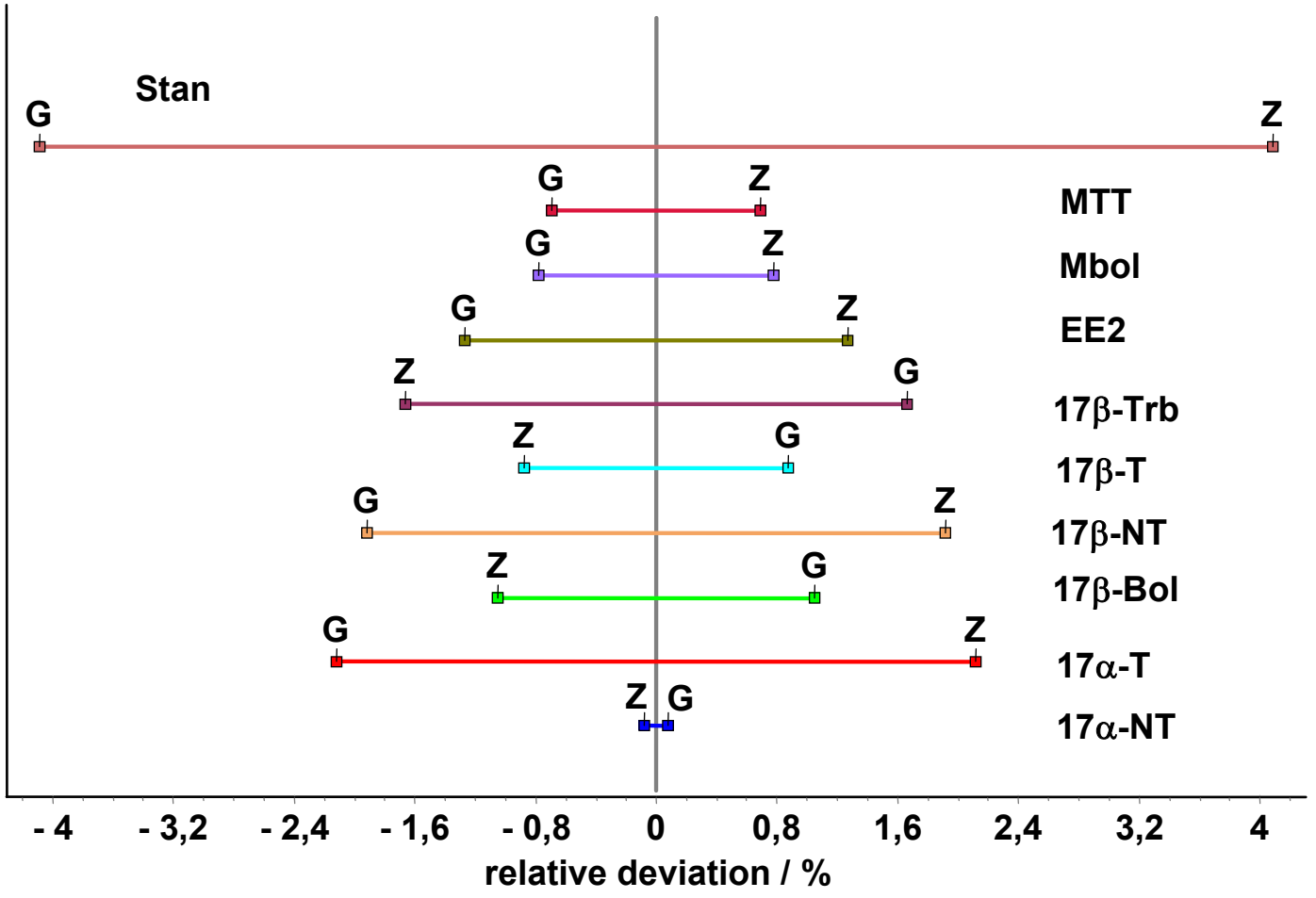
Choice of the validation factors	***"noise factors"***	**+ level, - level**

"Factors that cannot be controlled in routine analysis but that have potential influence on the result"

Sample preparation	**Operator**	**OP `G`, OP `Z`**
1. Fortification of samples (10g bovine muscle)		
2. Hydrolysis with Protease XIV		
3. Liquid / liquid extraction		
4. Defattening		
5. SPE using Oasis HLB cartridges	**Lot Nr.**	**A, B**
6. SPE using NH_2 cartridges	**Lot Nr.**	**C, D**
7. LC MS/MS measurement (API 4000, APCI(+))	**Storage of extracts**	**0 d, 3 d**

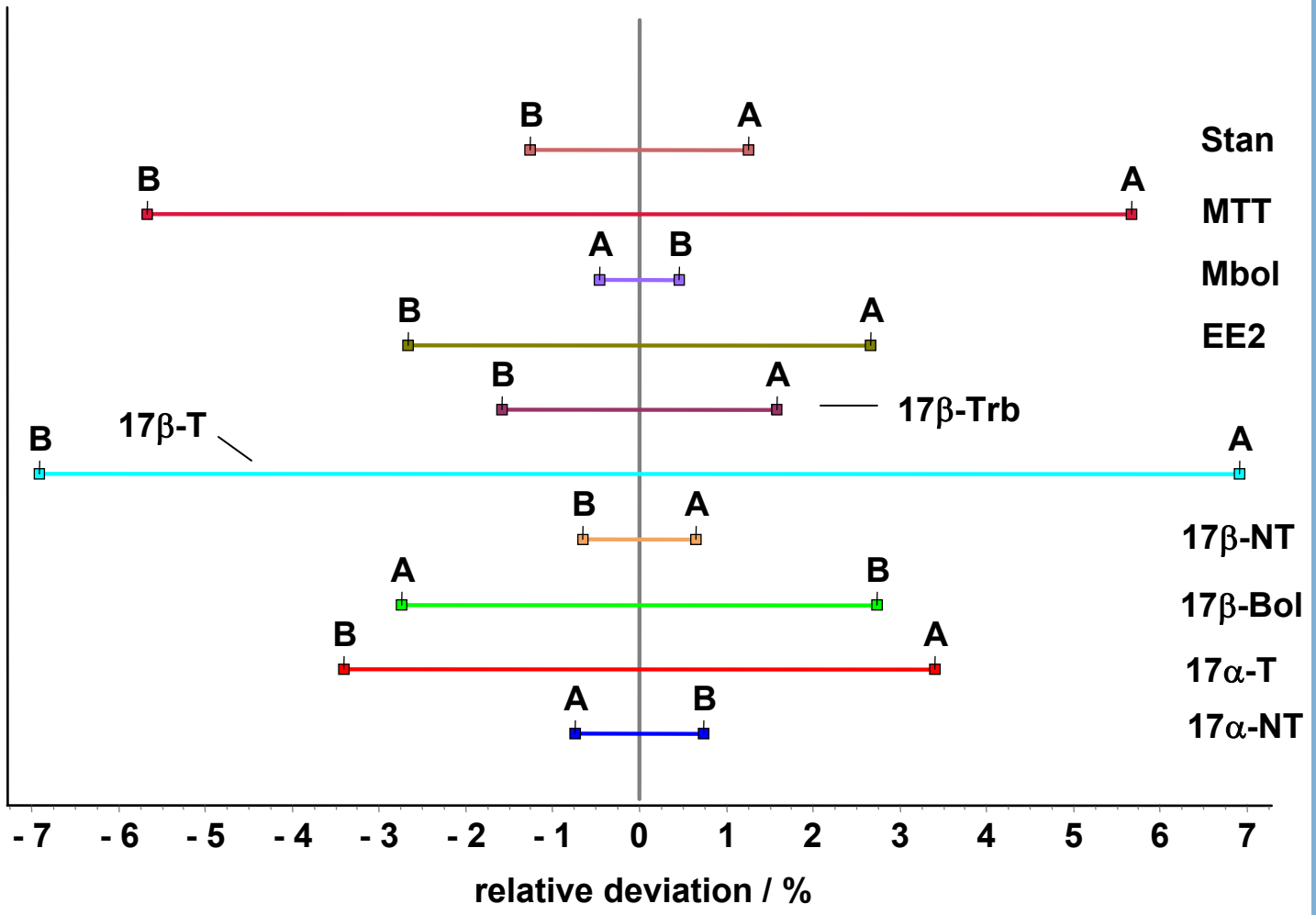
Multivariate effect analysis (3)

Operator



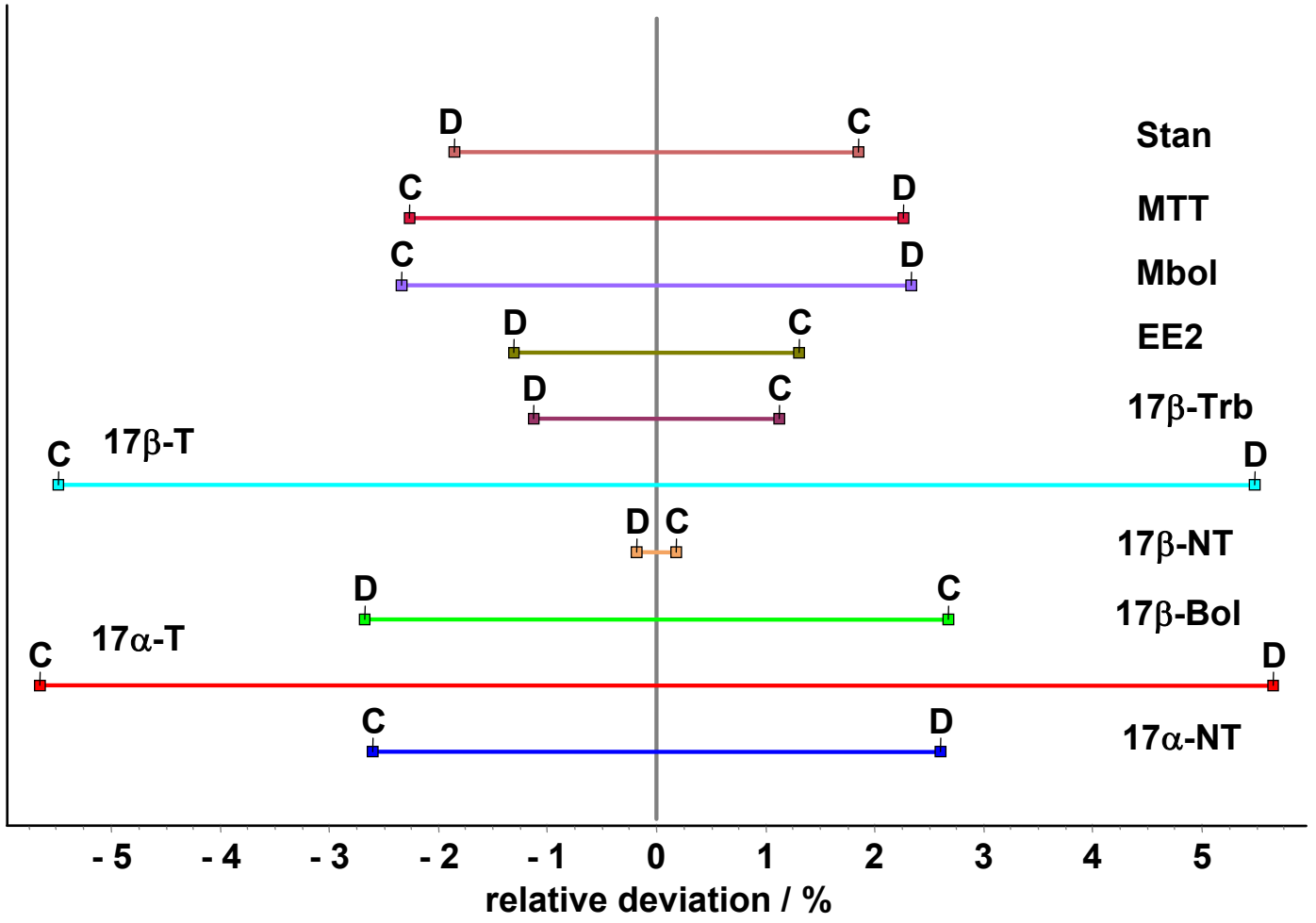
Multivariate effect analysis (4)

Lot HLB cartridge



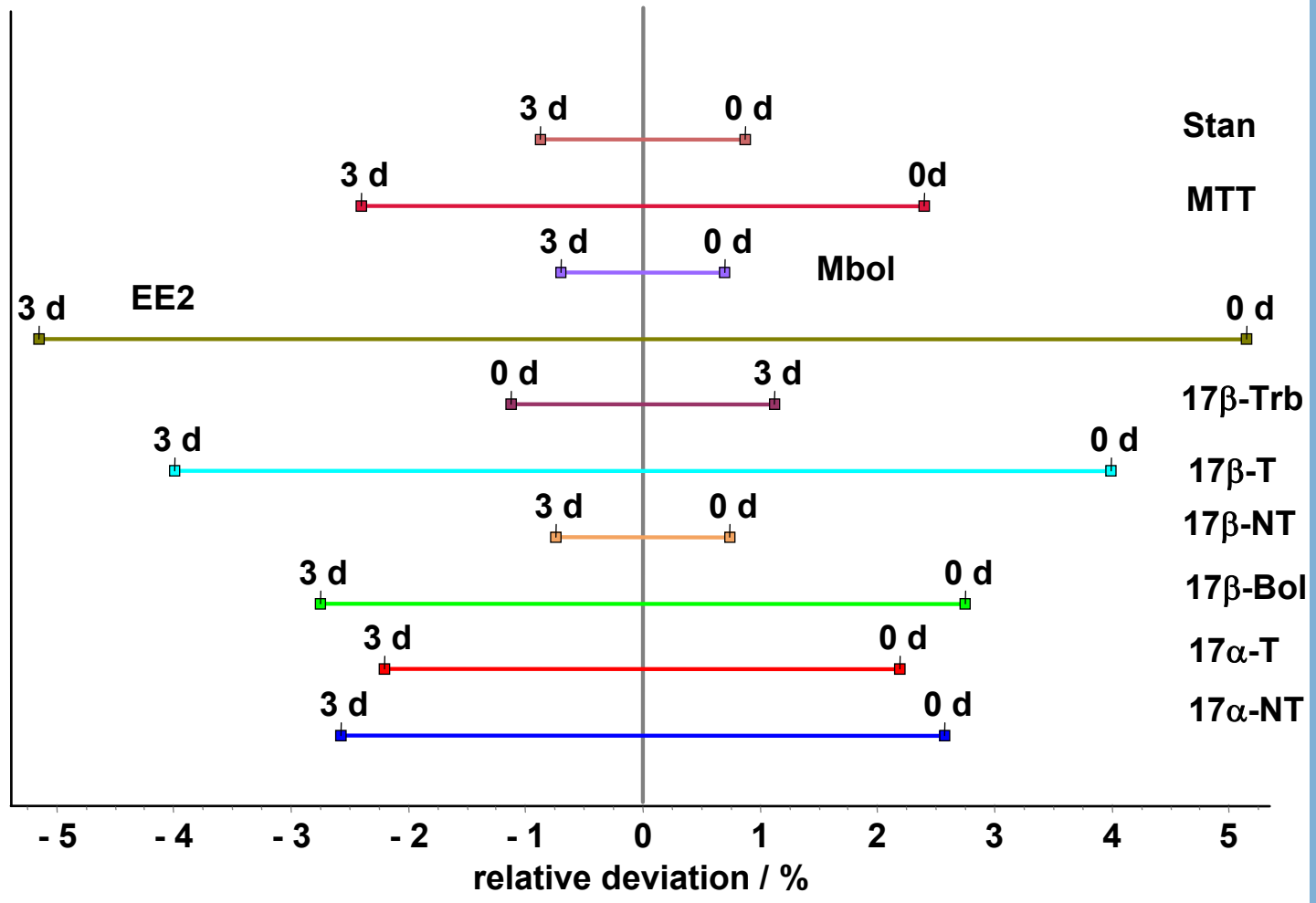
Multivariate effect analysis (5)

Lot NH2 cartridge



Multivariate effect analysis (6)

Storage of extracts



Multivariate effect analysis (7)

Factor ranking

Factor	17α-NT	17α-T	17β-Bol	17β-NT	17β-T	17β-Trb	EE2	Mbol	MTT	Stan
lot HLB cartridge	3	2	2	3	1	2	2	2	1	3
operator	4	3	4	1	4	1	3	3	4	1
storage of extracts	2	4	3	2	3	3	4	4	3	4
lot NH2 cartridge	1	1	1	4	2	4	1	1	2	2

1: highest influence

4: lowest influence

Summary (1)

Summary

Analyte	recomm. conc.* (µg/Kg)	CCα (µg/Kg)	CCβ (µg/Kg)
17α-Nortestosterone	1	0.481	0.612 ✓
17α-Testosterone	1	0.430	0.537 ✓
17β-Boldenone	1	0.422	0.544 ✓
17β-Nortestosterone	1	0.404	0.499 ✓
17β-Testosterone	1	0.581	0.837 ✓
17β-Trenbolone	1	0.467	0.631 ✓
Ethinylestradiol	1	0.788	1.101 (✓)
Methylboldenone	1	0.148	0.193 ✓
Methyltestosterone	1	0.551	0.686 ✓
Stanozolol	1	0.300	0.383 ✓

***CRL Guidance paper (7 December 2007): CRLs VIEW ON STATE OF THE ART ANALYTICAL METHODS FOR NATIONAL RESIDUE PLANS**

Summary (2)

Method for analysis of steroids in bovine muscle

- Validation according to Commission Decision 2002/657/EC
  - ⇨ decision limit (CCα) and detection capability (CCβ) of the anaytes below "recommended concentrations"
  - ⇨ ruggedness of method sufficiently proven by systematic variation of the validation factors on two levels

- Multivariate effect analysis
  - ⇨ identification of factors that have potential influence on the result
  - ⇨ estimation of the magnitude of influence of the factor
  - ⇨ influence of validation factors on the measurement result was acceptable

⇨ **Method: fit for purpose and applicable in routine analysis**

Acknowledgements

Thanks to

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for doing the practical work

Thank you for your attention !