VET RESIDUES DETECTION

European Experience

2024 GFoRSS Webinar Series

15 October 2024

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Introduction

- Doctor of Veterinary Medicine: 1974
- Director of the CER Health Department: 1977 - 2015
  - Residue detection in foodstuffs by immunological and physico-chemical methods
  - National Reference Laboratory (NRL) for Food Allergens, for Forbidden Substances and Veterinary Medicines – Mycotoxins and Marine Biotoxins
- Vice President of the Scientific Committee of the Belgian Federal Agency for Safety of the Food Chain (FASFC)

- Member of the Committee for medicinal products for veterinary use safety working party of EMA .

Outline

Introduction

Legislative aspect

Developments in analytical methods

General considerations

Natural hormones

Androgens

Corticosteroids

β *agonists*

Conclusion

Why Anabolic Agents in Animal Production?

- Most anabolic agents are hormones
- Better conversion of dietary nitrogen
- Especially interesting for cattle, less so for poultry and pigs
- Estimated weight gain of 10-20 %

- In 1938, DES was the 1st synthetic hormone to be used
- All growth promoters are currently banned in Europe
- In other parts of the world they are partly legalized

Legislative Background (1)

Council Directive 96/22/EC of 29 April 1996 concerning the prohibition on the use in stockfarming of certain substances having a hormonal or thyrostatic action and of ß-agonists, ….

- **Ban**

for substances having a hormonal effect

Beta agonists

Thyreostatic

- **Authorisation for therapeutic reasons under controlled conditions**

Legislative Background (2)

***Commission Delegated Regulation (EU) 2022/1644 of 7 July 2022…. requirements for the performance of official controls on the use of pharmacologically active substances authorised as veterinary medicinal products or as feed additives and of prohibited or unauthorised pharmacologically active substances and residues thereo*Council Directive 96/23/EC of 29 April 1996 on measures to monitor certain substances and residues thereof in live animals and animal products**

ANNEX I

- GROUP A – Prohibited or unauthorized pharmacological active substancesin food producing animals
  1. Substances with hormonal and thyrostacic action and beta-agonists…
     - (a) Stilbenes
     - (b) Antithyroid agents
     - (c) Steroids
     - (d) Resorcylic acid lactones including zeranol
     - (e) Beta-agonists
  2. Prohibited substances listed in Table 2 of the regulation 37/2010 (Chloramphenicol, dimetridazole, …)
  3. …..

Legislative Background (3)

- COMMISSION IMPLEMENTING REGULATION (EU) 2022/1646 of 23 September 2022 on uniform practical arrangements for the performance of official controls….

There are now 3 types of plans:

- Plan 1: risk-based national
- Plan 2: EU monitoring (195 for BE)
- Plan 3: imports

Legislative Background (4)

- A Maximum Residue Limit (MRL) is the maximum acceptable concentration of a substance that may be found in a food product obtained from an animal that has received a veterinary medicine.
  - Or authorized substances
  - Value based on toxicological data

- REGULATION (EC) NO 470/2009 OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL of 6 May 2009 laying down Community procedures for the establishment of residue limits of pharmacologically active substances in foodstuffs of animal origin, repealing Council Regulation (EEC) No 2377/90 and amending Directive 2001/82/EC of the European Parliament and of the Council and Regulation (EC) No 726/2004 of the European Parliament and of the Council

- COMMISSION REGULATION 37/2010 OF 22 DECEMBER 2009 on pharmacologically active substances and their classification with respect to maximum residue limits in foods of animal origin.

Maximum Residue Limit of Florfenicol

Florfenicol	Sum of florfenicol and its metabolites measured as florfenicol-amine	Bovine, ovine, caprine	200 µg/kg 3 000 µg/kg 300 µg/kg	Muscle Liver Kidney	Not for animals from which milk is produced for human consumption. Not for animals from which eggs are produced for human consumption.	Anti-infectious agents/Antibiotics
		Porcine	300 µg/kg 500 µg/kg 2 000 µg/kg 500 µg/kg	Muscle Skin and fat Liver Kidney		
		Poultry	100 µg/kg 200 µg/kg 2 500 µg/kg 750 µg/kg	Muscle Skin and fat Liver Kidney		
		Fin fish	1 000 µg/kg	Muscle and skin in natural proportions.		
		All other food producing species	100 µg/kg 200 µg/kg 2 000 µg/kg 300 µg/kg	Muscle Fat Liver Kidney		
Fluazuron	Fluazuron	Bovine	200 µg/kg 7 000 µg/kg 500 µg/kg 500 µg/kg	Muscle Fat Liver Kidney	Not for animals from which milk is produced for human consumption.	Antiparasitic agents/ Agents against ectoparasites

Legislative Background (5)

- COMMISSION DECISION of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results

  - SCREENING METHODS: ELISA

    False positive / No False negative

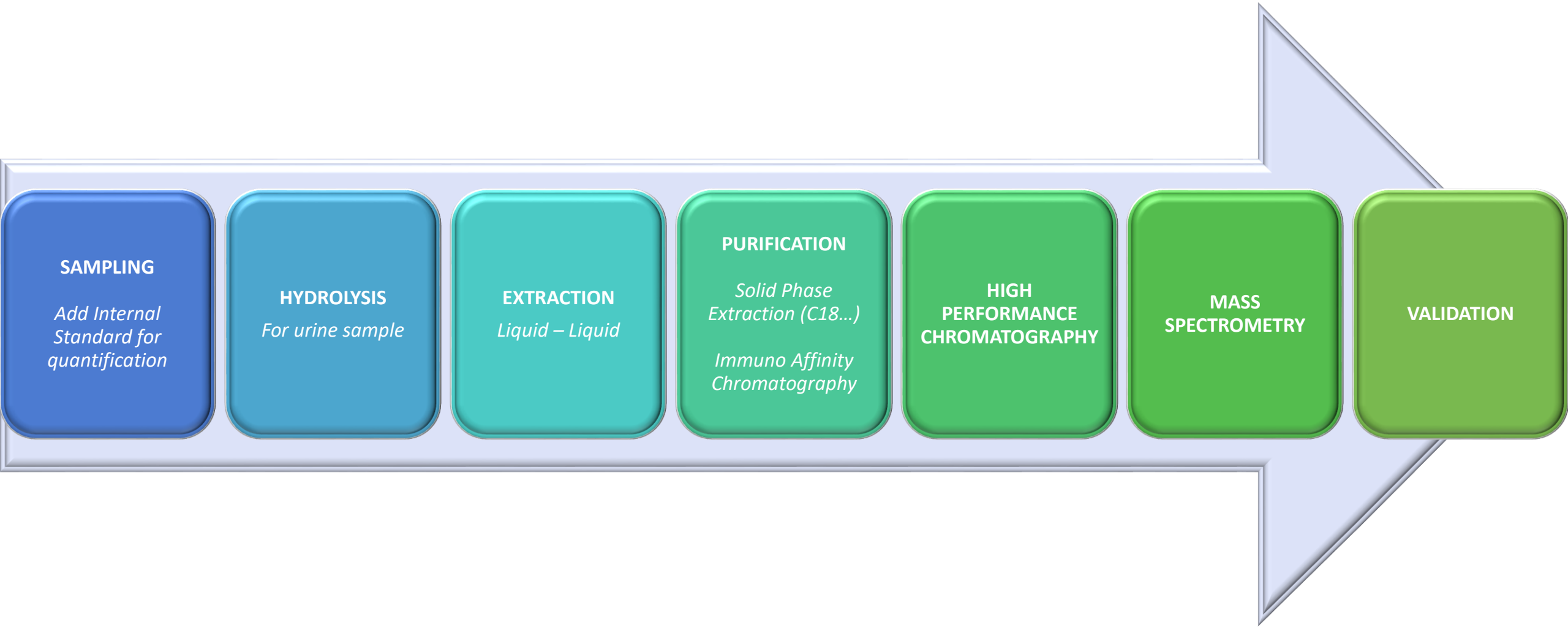
  - CONFIRMATORY METHODS FOR ORGANIC RESIDUES AND CONTAMINANTS:

    Provide information on the chemical structure of the analyte: LC/MS-MS

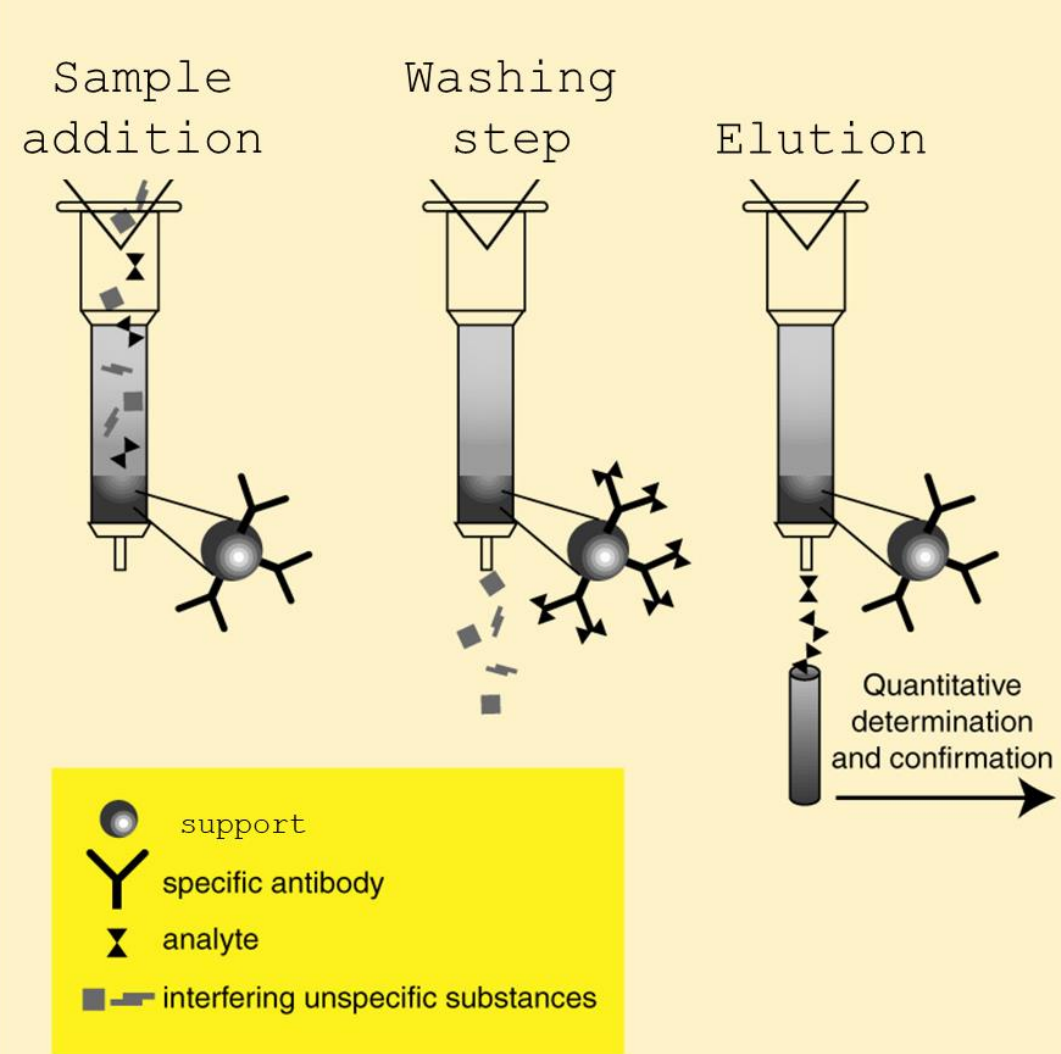
Analytical Strategy

- Target molecule and metabolites
- Matrix of interest for controls
  - Not necessary animal products (milk, meat, offal, …)
  - Majority of strategies are based on urine
    - main route of excretion of most drugs
    - easy to collect at the slaughterhouse and at the farm
  - For substances with MRL → edible tissues (milk, meat, ...)
  - Other matrices (steroid esters in hair, progestagens in fat, …)

Analysis Steps

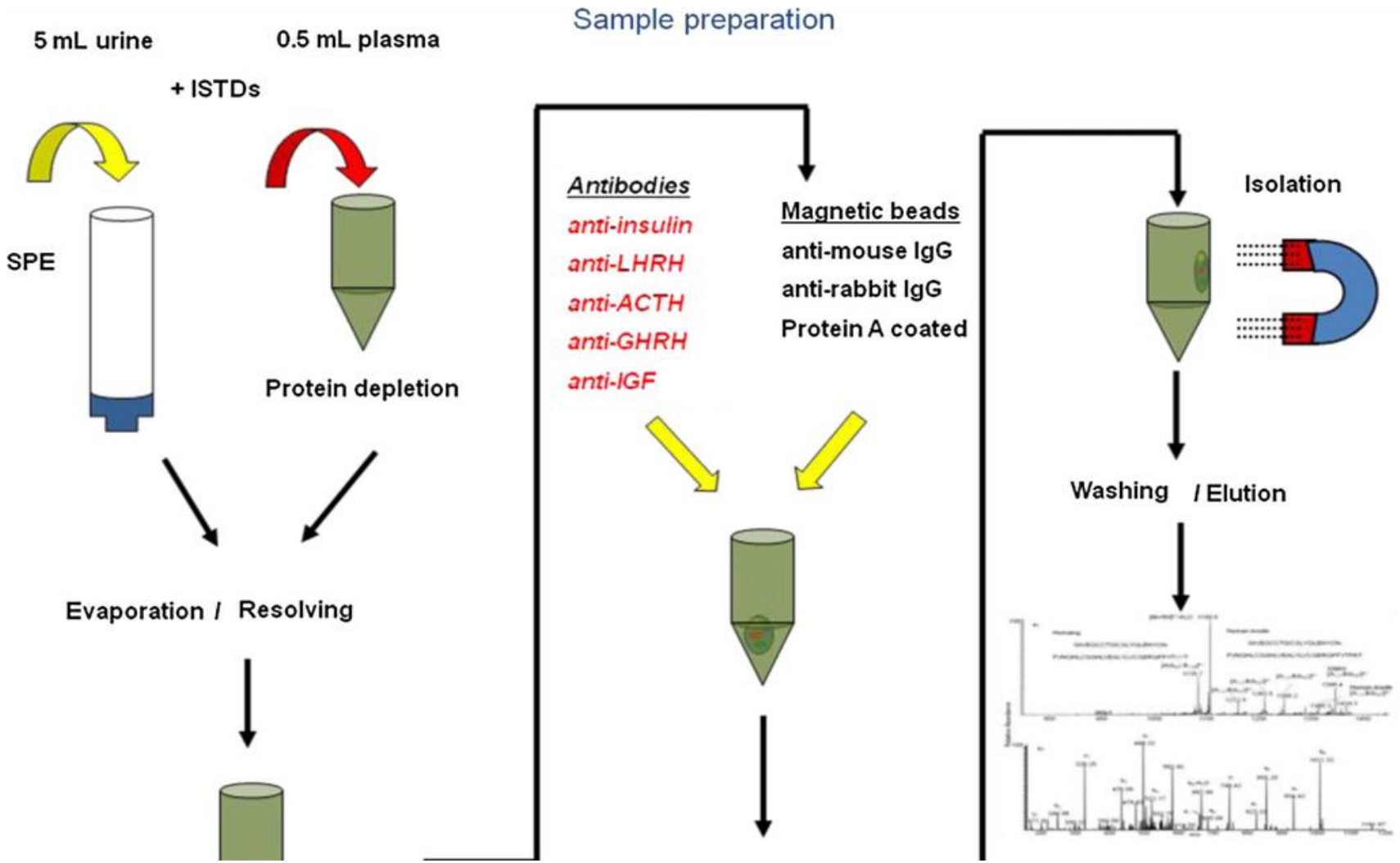


IAC Principle



- Suitable in different areas such as environmental monitoring, food safety, clinical diagnostics, pharmaceutical analysis, doping controls,…
- Suitable for a wide range of substances ranging from proteins to low molecular mass molecules

Sample Preparation Procedure for Urine & Plasma by Immunoaffinity



Analytical Methods

Characteristics of the Methods

Immunoassay ELISA or LFD

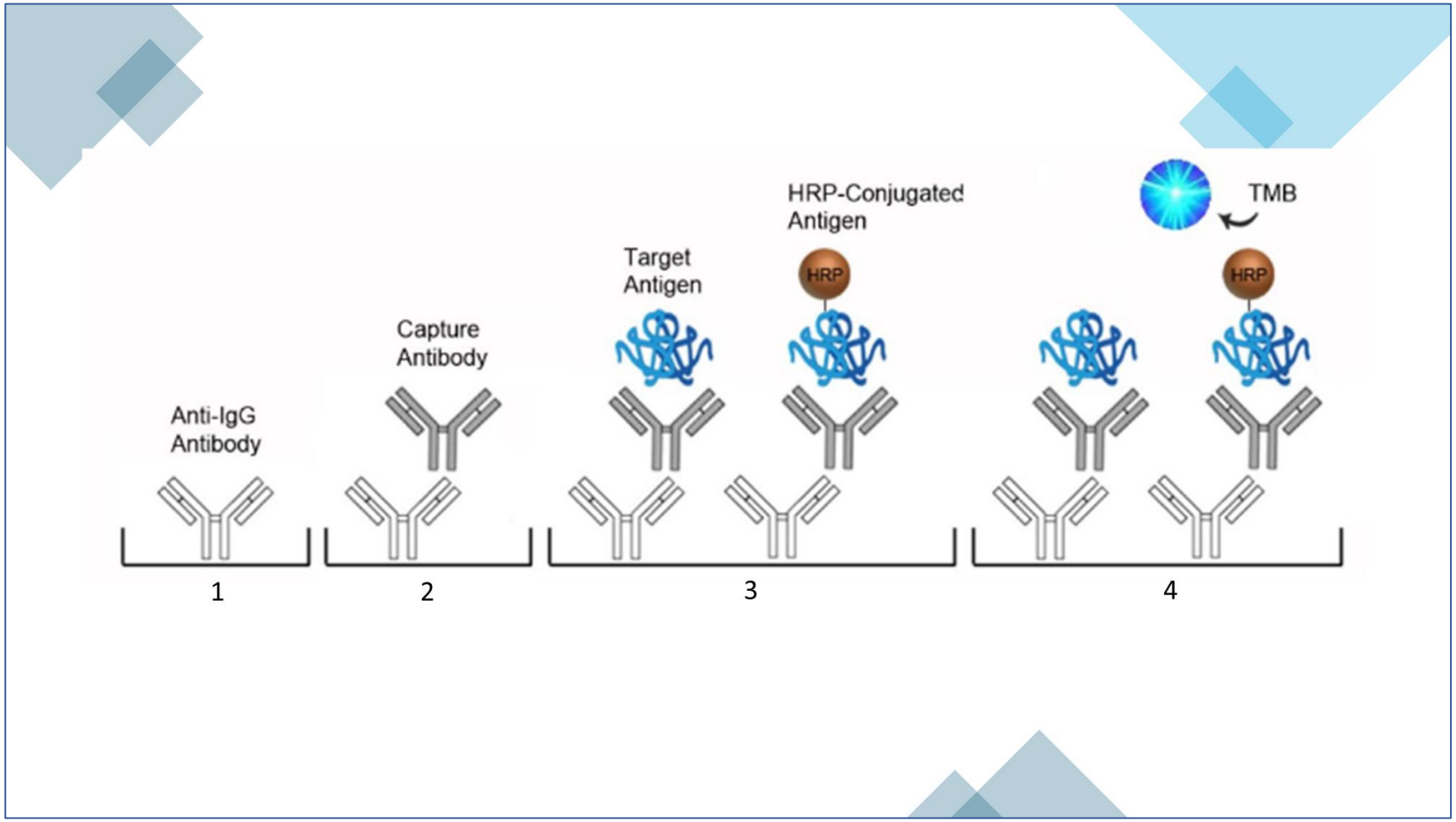
- Available in the field
- Rapid
- No false negatives
- No extraction step
- Cheap

Mass Spectrometry LC-MS/MS

- Laboratory technique
- Specific
- No false positives
- Extraction step
- Expensive

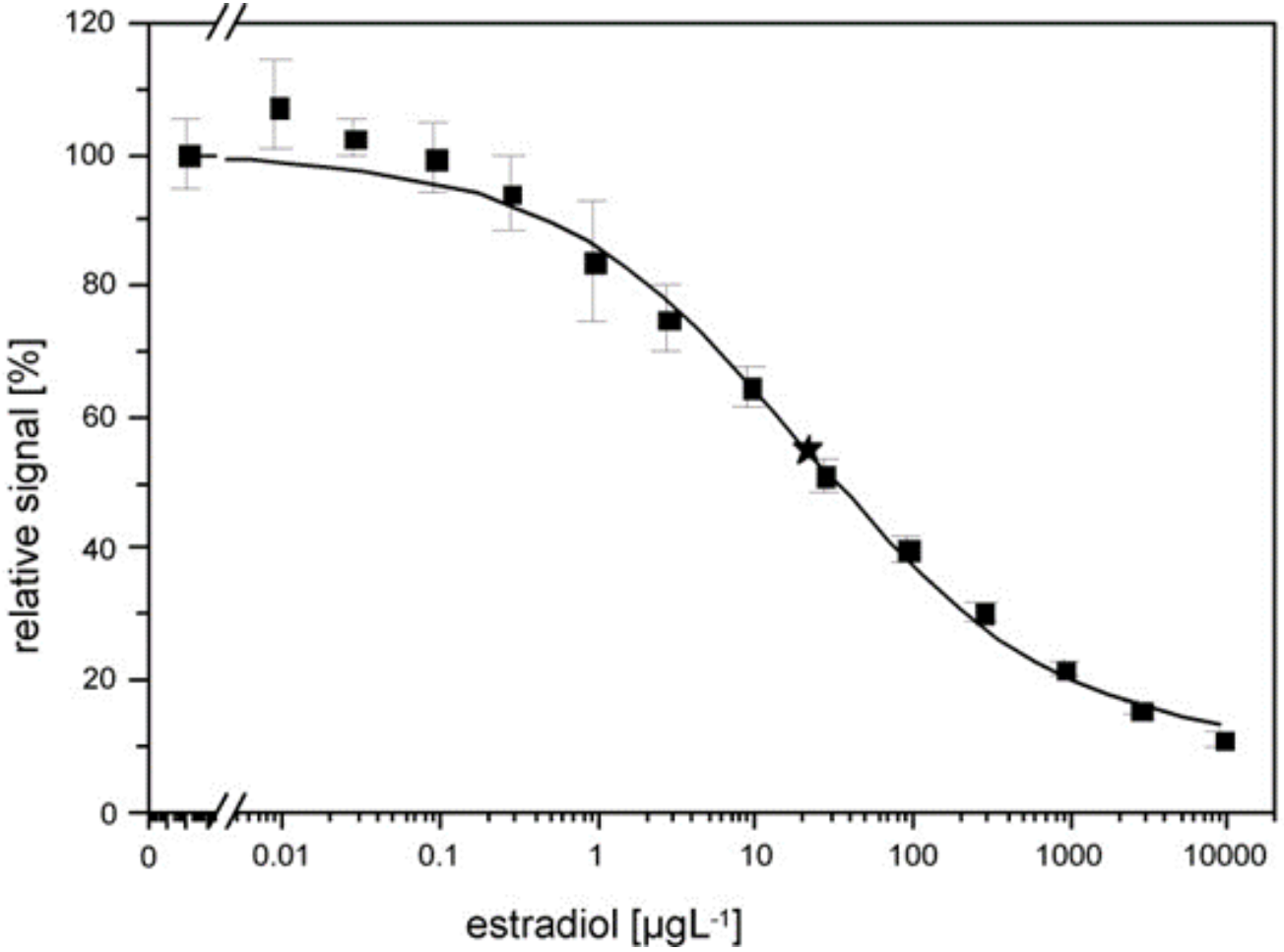
ELISA – Principle

Enzyme Linked ImmunoSorbent Assay



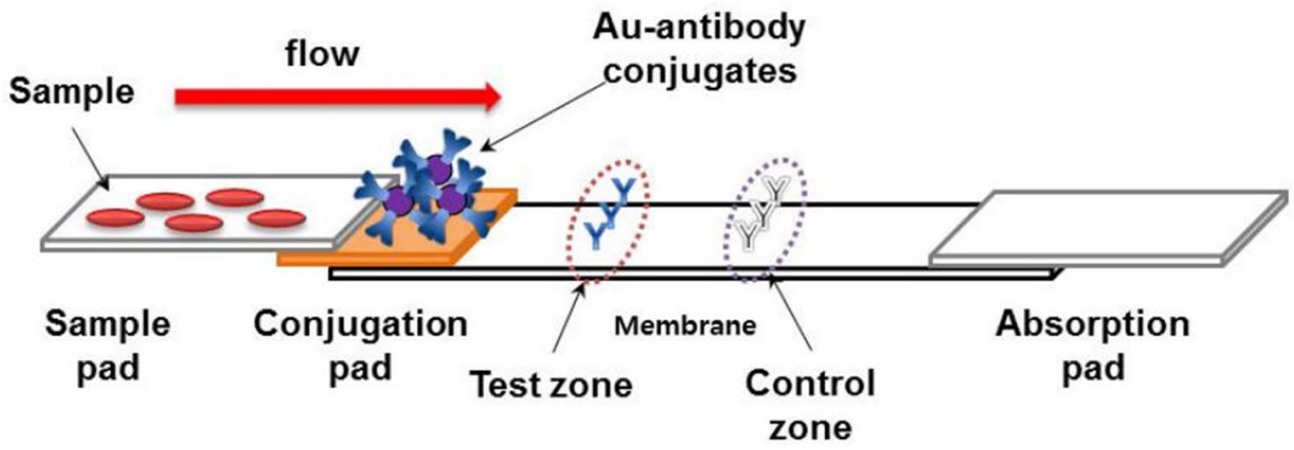
ELISA – Standard Curve

Semi logarithmic plot of results from a competitive assay



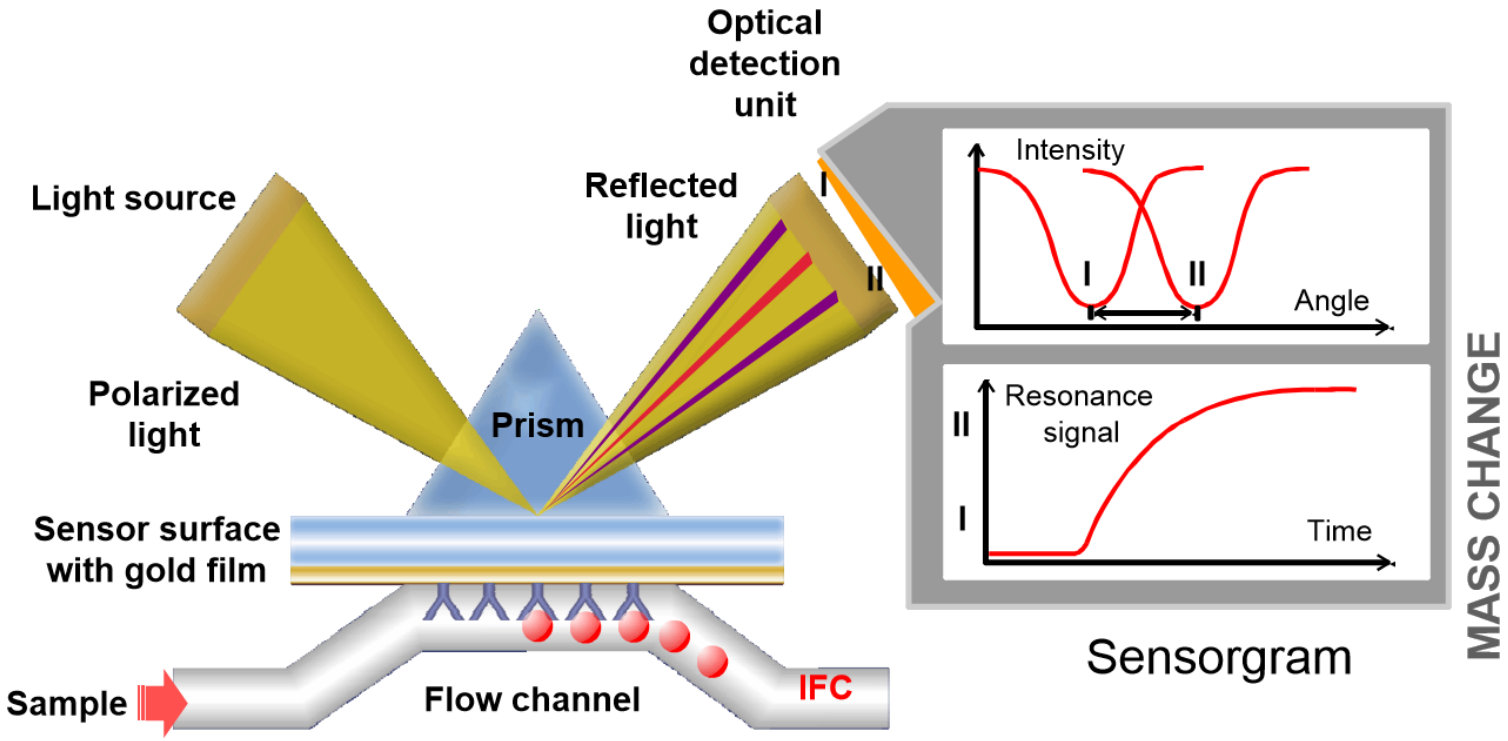
LFD – Principle (1)

Lateral Flow Device



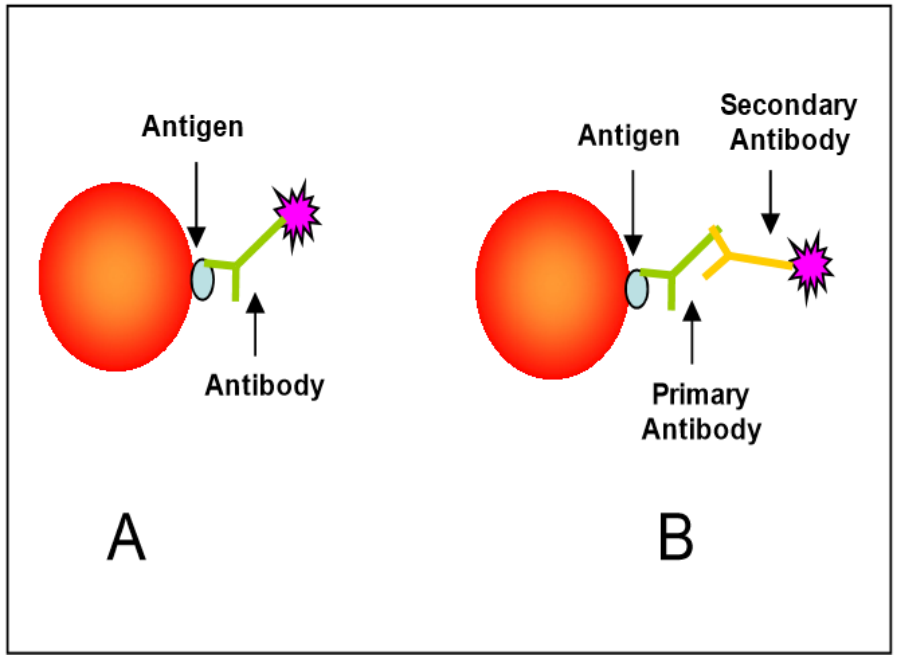
Surface Plasmon Resonance (SPR) Biosensor

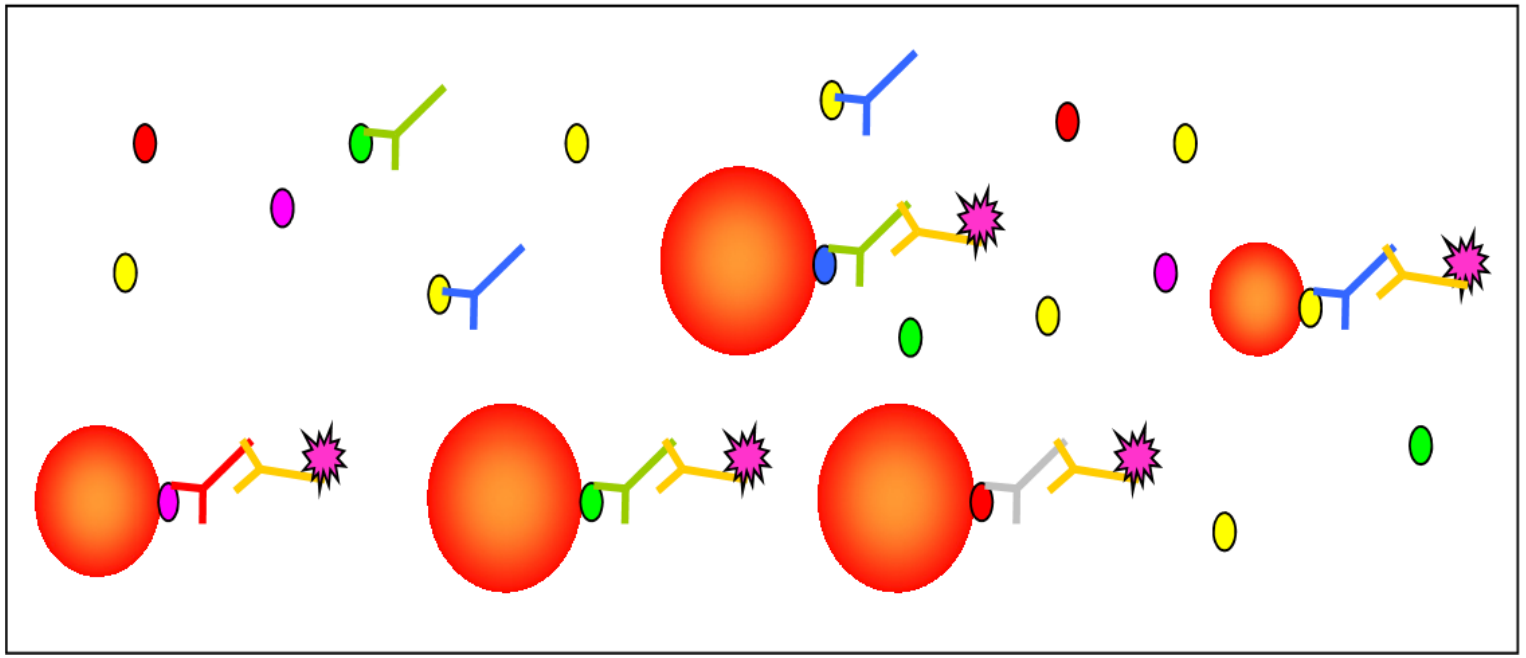
Principle of the Surface Plasmon Resonance (SPR) biosensor. Binding of biomolecules to the surface increases the refractive index, which induces a shift in the SPR angle. The shift is directly proportional to the mass increase.



Multipexing immunoassay with microsphere

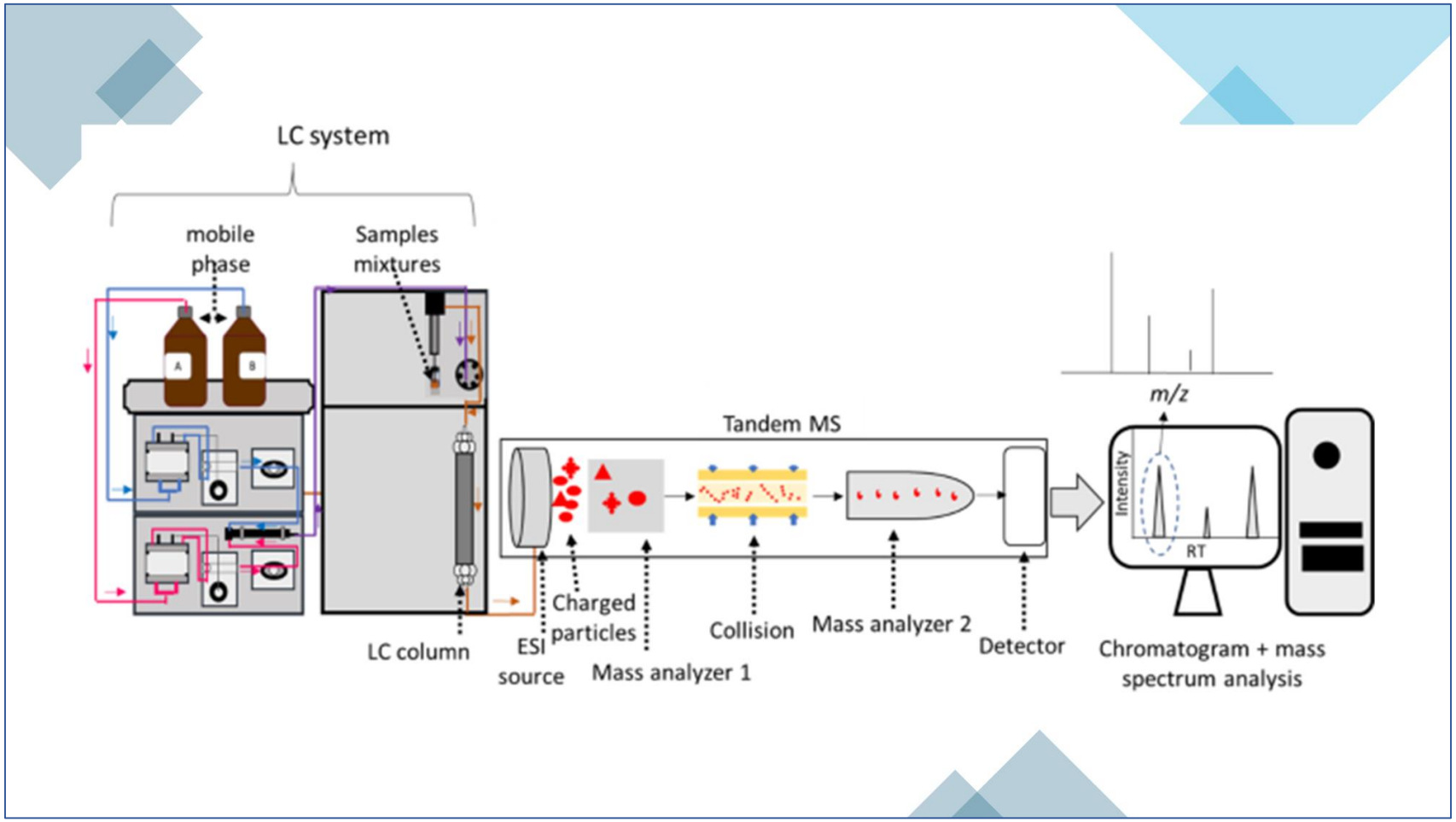
General principle of the direct and indirect immunoassays in which the antigen to be assayed is bound to a microsphere (A and B); Principle of multiplexing in an assay using 3 microspheres of different sizes and four different antibodies.





LC-MS/MS – Principle

Liquid Chromatography coupled to tandem Mass Spectrometry



Basic Explanation of IRMS (1)

- Ratio isotopes $^{13}C/^{12}C$ used to know the origin of steroids excreted in urine
- Endogenous hormones and their metabolites in animals is mainly determined by feed
- Exogenous administred steroids contain less ^{13}C

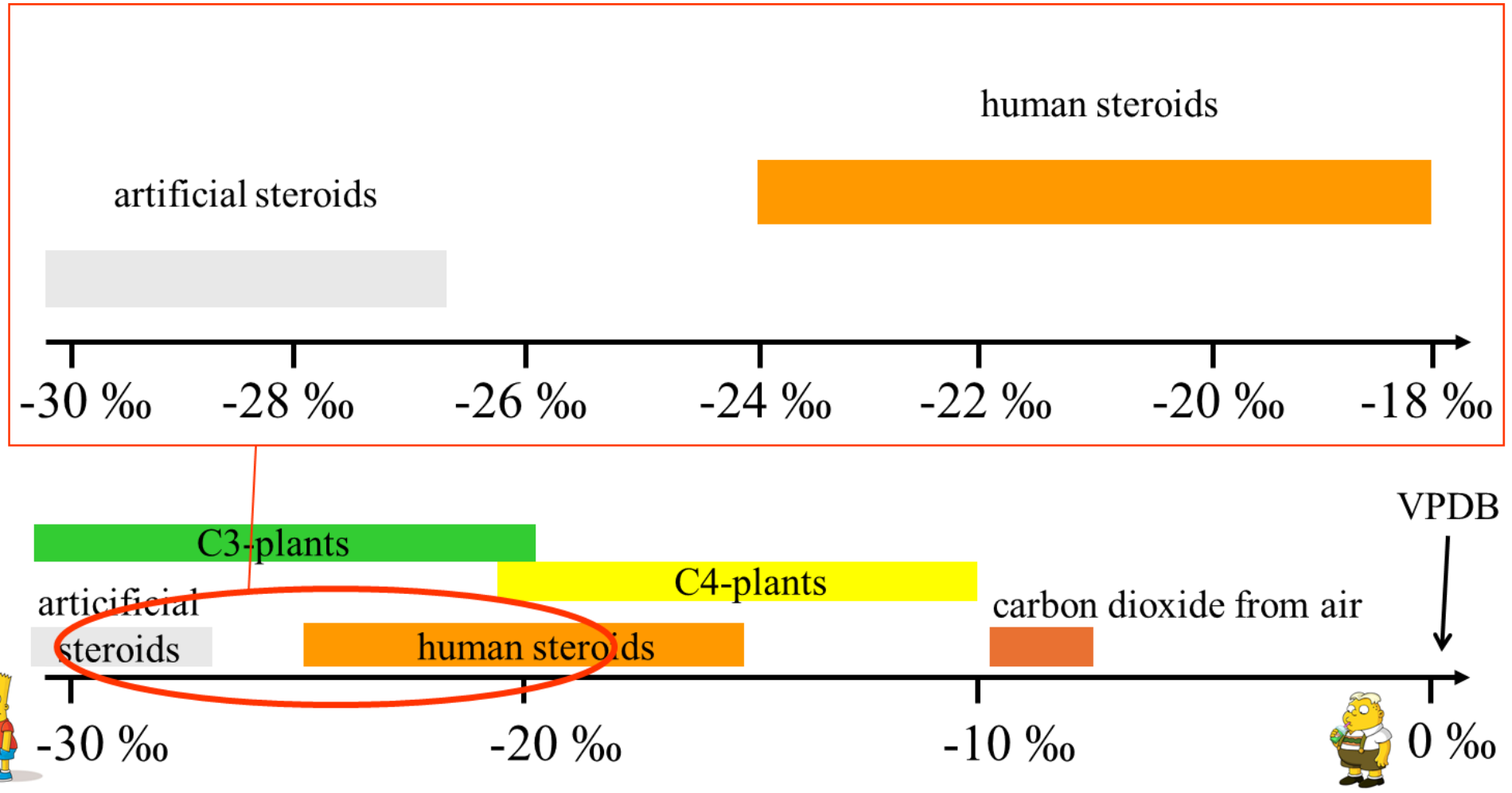
Carbon isotope ratio: $R = {}^{13}C/{}^{12}C$

δ values of carbon: $\delta^{13}C_{VPDB}\ (‰) = (R_{sample}/R_{VPDB} - 1) \times 10^3$

$\Delta\ \delta^{13}C_{VPDB}\ (‰) = \delta^{13}C_{ERC} - \delta^{13}C_{M}$

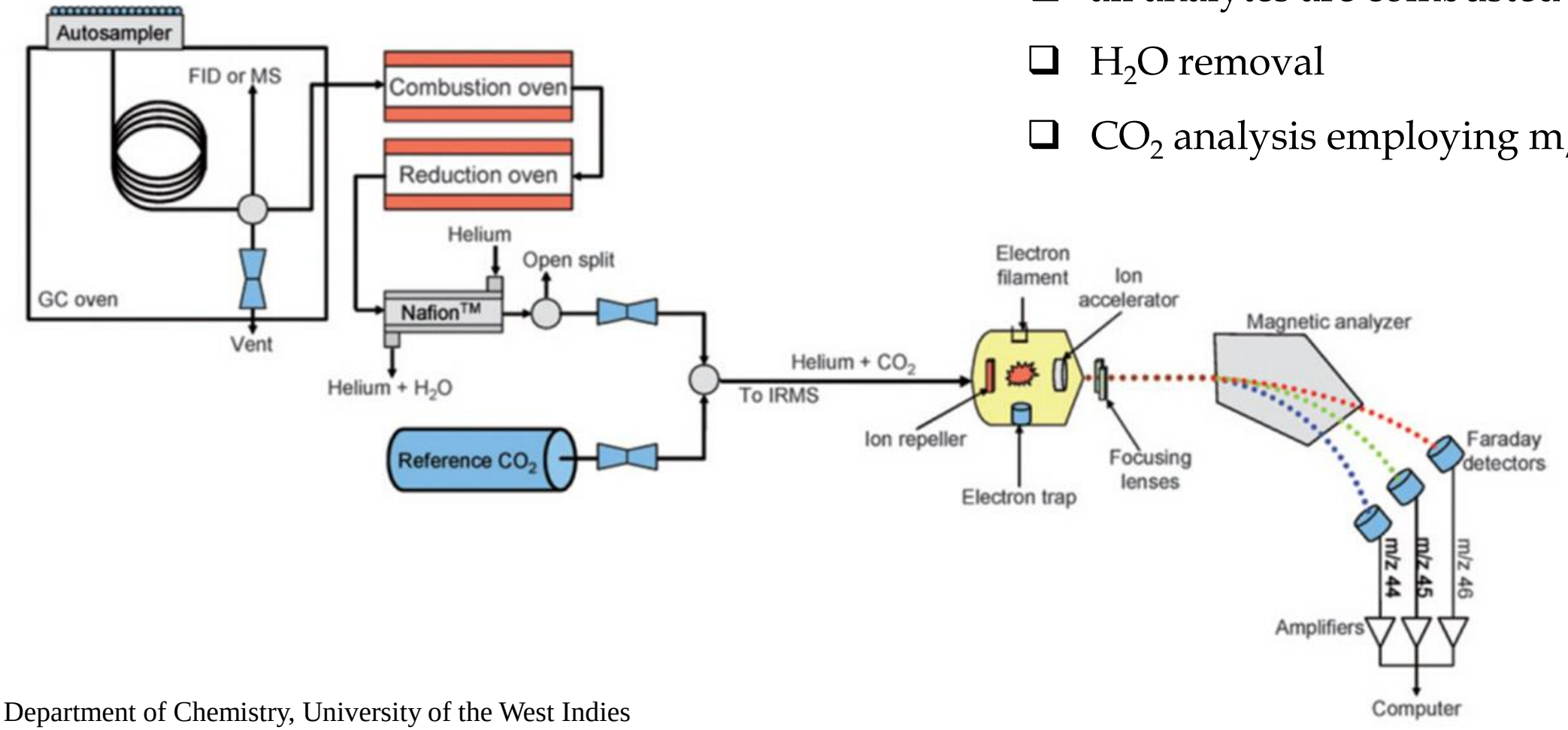
ERC: endogenous reference compound
M= metabolite

Isotope Ratio Mass Spectrometry (2)



Carbon Isotope Ratio (CIR) Measurements (3)

- all analytes are combusted to CO_2 and H_2O
- H_2O removal
- CO_2 analysis employing m/z 44, 45 and 46



Carbon Isotope Ratio (CIR) Measurements (4)

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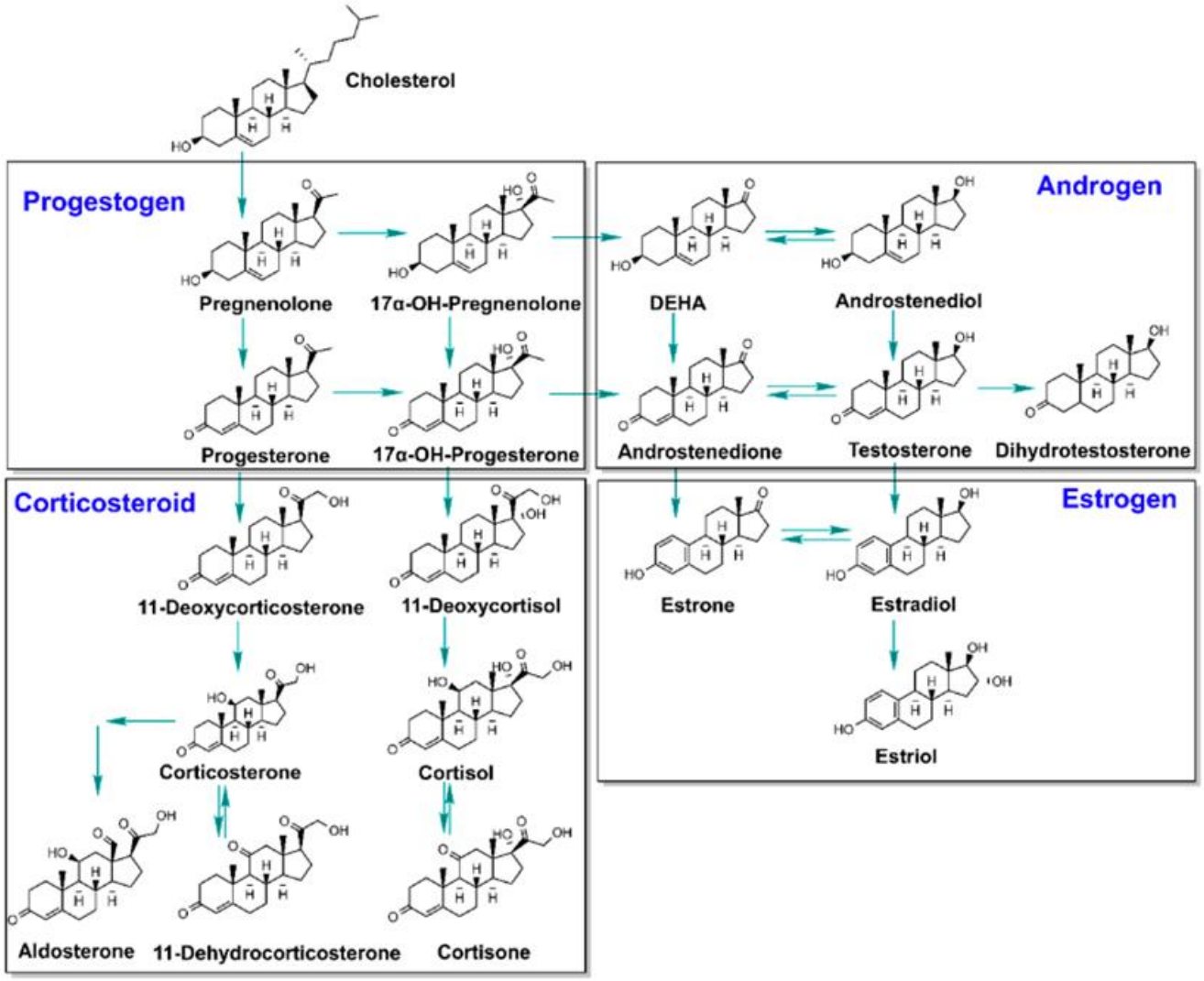
Criteria of Validation of Methods

Classification of analytical methods by the performance characteristics that have to be determined

		Detection limit CCß	Decision limit CCα	Trueness / recovery	Precision	Selectivity / specificity	Applicability/ ruggedness/ stability
Qualitative methods	S	+	–	–	–	+	+
	C	+	+	–	–	+	+
Quantitative methods	S	+	–	–	+	+	+
	C	+	+	+	+	+	+

S = screening methods; C = confirmatory methods; + = determination is mandatory.

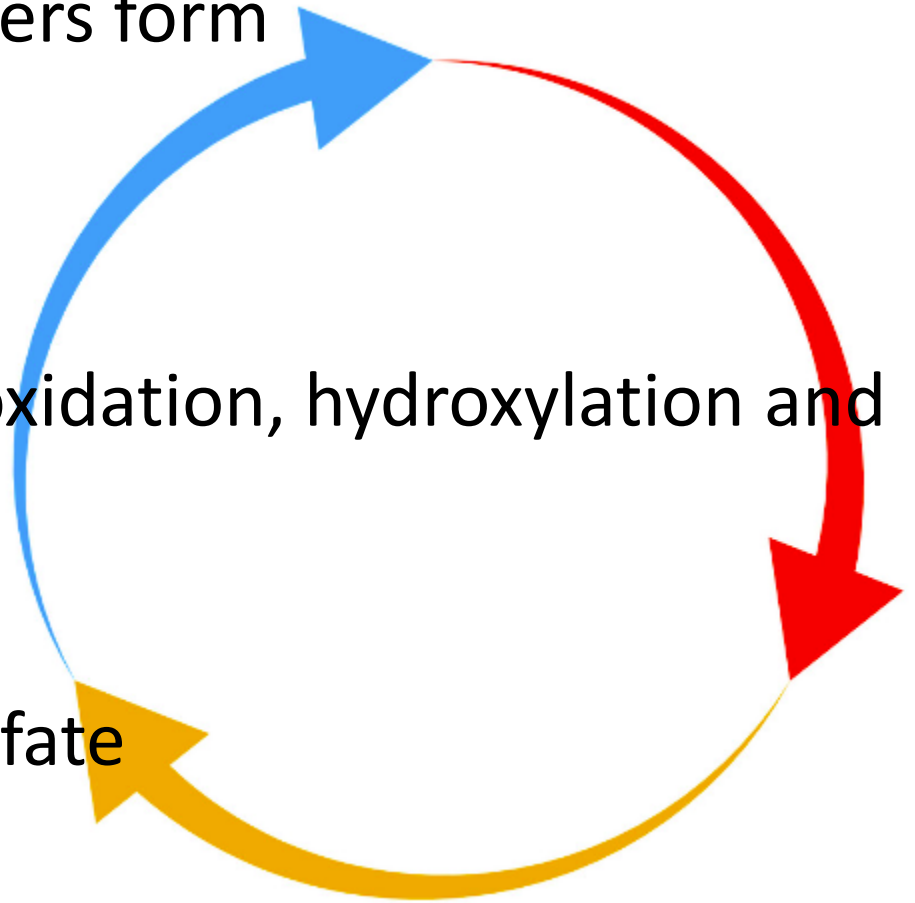
Molecular Structure and Metabolic Pathways of Steroids



Li et al., 2022

Metabolism

- Compounds are highly metabolized → monitoring parent and metabolites
- Most of steroids are administrated on β and esters form
- First step is hydrolysis of ester on injection site
- Epimerization of β to α form
- Phase 1 of metabolism introduce polar group: oxidation, hydroxylation and reduction to increase elimination
  - In liver with CYP450
- Phase 2: conjugation with glucuronic and/or sulfate
  - In kidney



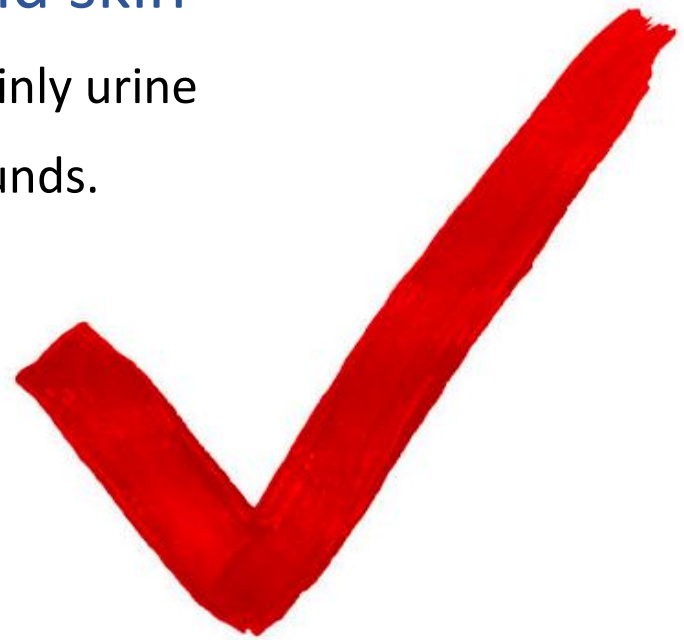
Maximum Residue Limits (MRL) of Several Steroids

In bovine tissues (µg/kg) as defined by JECFA

Compound	Species	Matrices	MRL
Testosterone	Bovines	Muscle, Liver, Kidney, Fat	Not specified
Estradiol	Bovines	Muscle, Liver, Kidney, Fat	Not specified
Trenbolone Acetate	Bovines	Muscle	2 µg/kg (β-trenbolone)
		Liver	10 µg/kg (α-trenbolone)
Progesterone	Bovines	Muscle, Liver, Kidney, Fat	Not specified
Melengestrol acetate	Bovines	Muscle	1 µg/kg
		Liver	10 µg/kg
		Kidney	2 µg/kg
		Fat	18 µg/kg
Zeranol	Bovines	Muscle	2 µg/kg
		Liver	10 µg/kg

Natural Growth Promoting Substances in Biological Samples

Hormonal active compounds can be present in biological samples like edible tissues, serum or plasma, fat, hair and skin



- ❑Antithyroid agents: The presence of especially thiouracil in biological samples, mainly urine
- ❑ Steroids: Steroids clearly form the largest and most complicated group of compounds.
  - Natural hormones : 17β-estradiol, 17β-testosterone and Progesterone
  - Nortestosterone
  - Boldenone
  - 1-Testosterone
- ❑Zeranol: The production of the Fusarium toxin zearalenone in animal feed
- ❑Protein and peptide hormones, Growth hormones, bST and recombinant bST in milk and plasma
- ❑IGF-1 and related growth factors
- ❑Corticosteroids. Natural compounds (cortisol, cortisone). The presence of prednisolone.

Physiological Levels of Natural Hormones

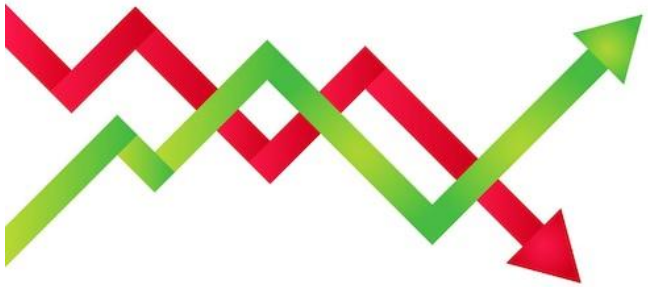
Variation according to sex, age and season and some pathological conditions

❑Testosterone

- Male bovines of 4 to 7 months → 1 to 4 ppb – Adult bulls → 10 ppb
- Pulsatile secretion: nyctemeral variation
- Lower plasma levels in treated bulls due to negative feedback from pituitary gland
- Admistration on esters form: Benzoate, Propionate, Enanthate, …

❑17 ß Oestradiol

- Adult cow during estrus phase → O.O1 to 0.04 ppb
- Admistration on esters form: Benzoate, Cypionate, …



❑Progesterone

- Hormone of pregnancy level between 0 and 10 ppb
- Administrated in association with estrogens and androgens to calm the sexual aspect

Examples: Steroid Chemical Structures

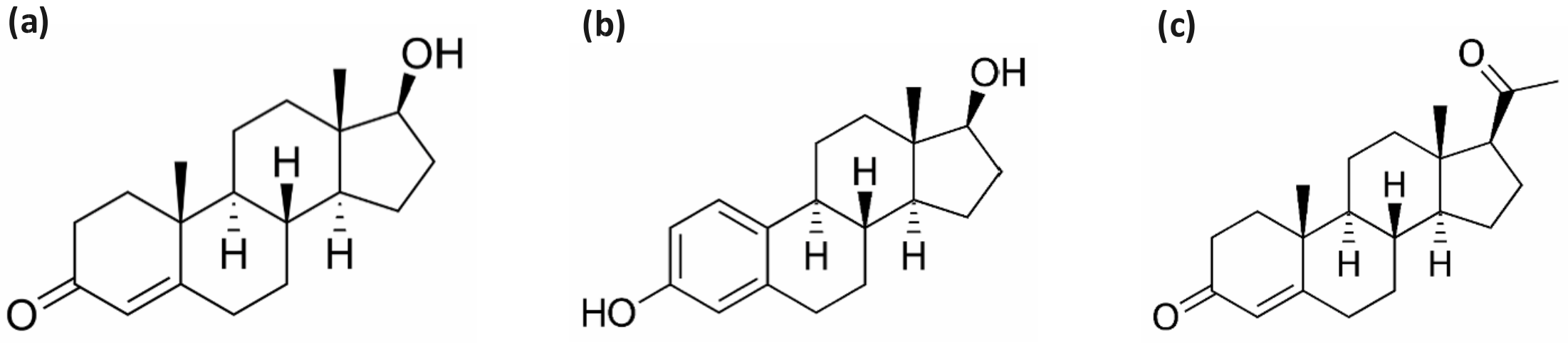
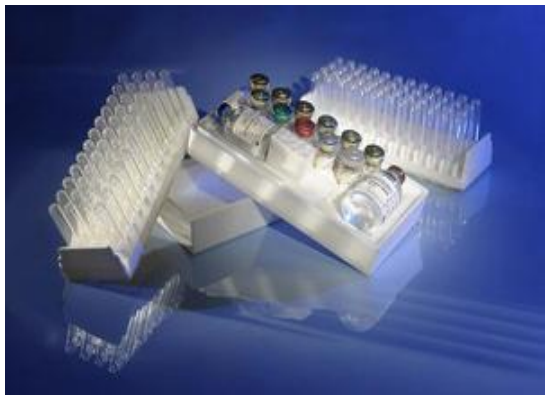


Fig. 1. Chemical structures of (a) testosterone, (b) estradiol and (c) progesterone.

Analytical Methods for Natural Hormones

- Screening
  - Histology – prostate for male veal calves
  - Immunoassay – RIA and Elisa from plasma (ppb and ppt level)

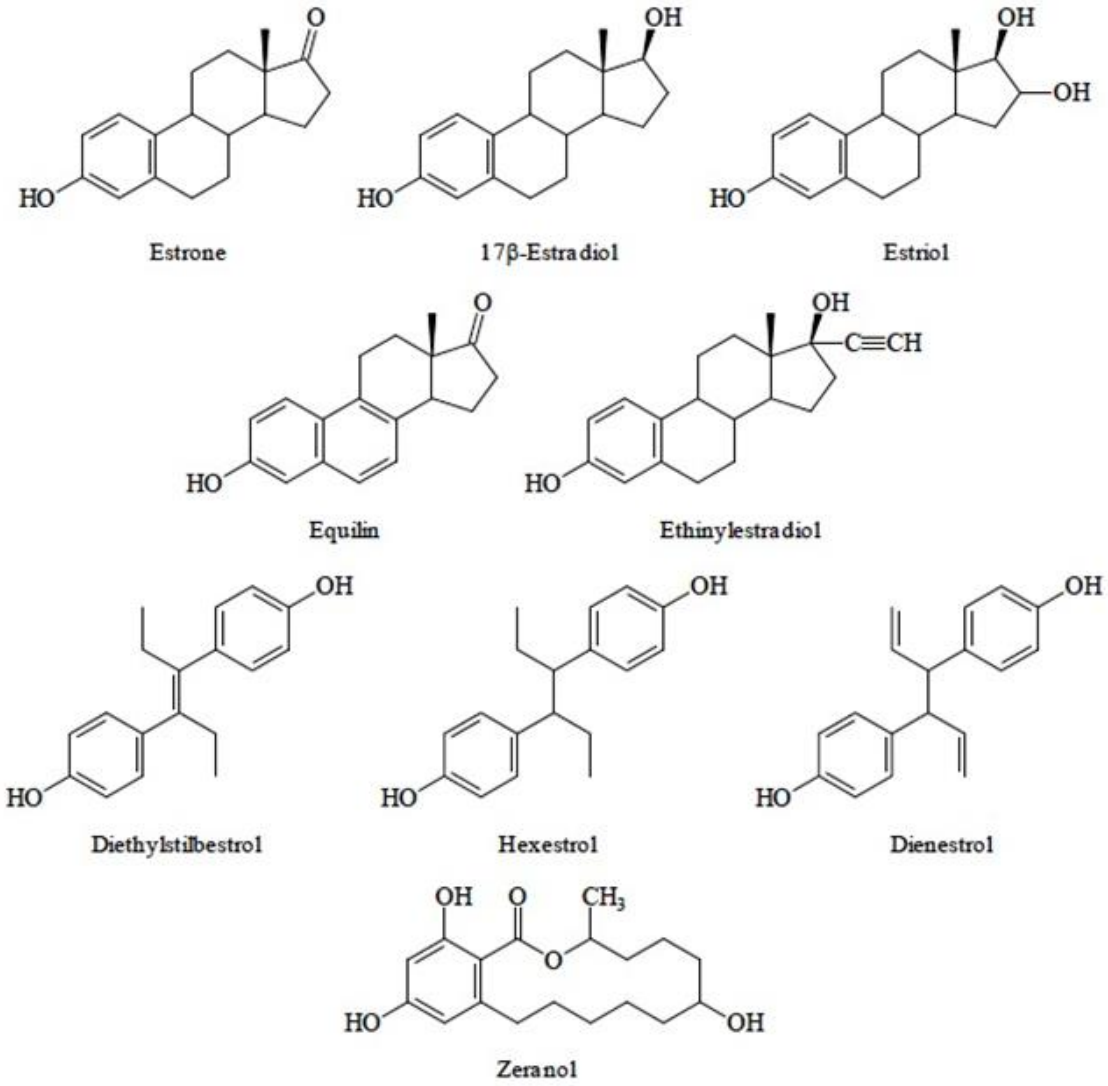


- Confirmatory
  - Steroid esters

    From injection sites collected at slaughterhouse

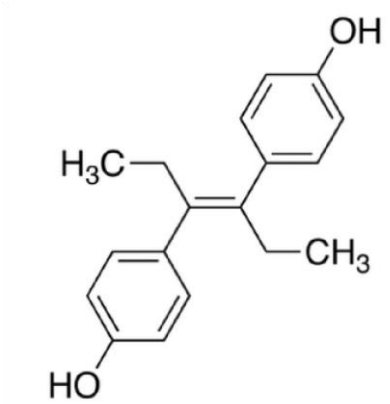
    From hair sample – old usage, risk if environmental contamination
  - Isotope Ratio Mass Spectrometry methods (IRMS)
  - Difference of endogenous and synthetic forms

Chemical Structures: Main Estrogens

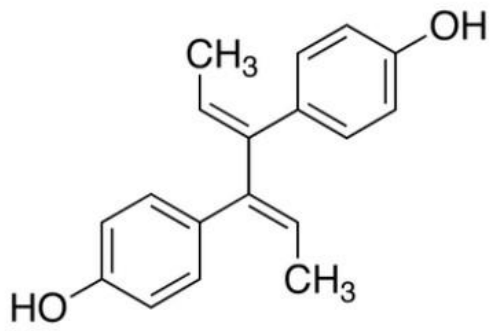


Synthetic Hormones - STILBENES

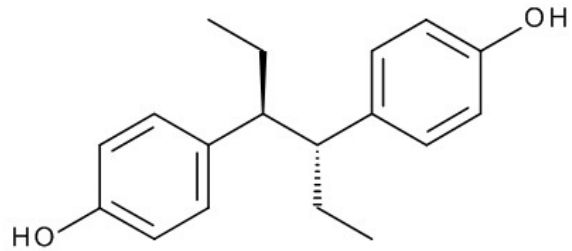
- Synthetic substance with estrogenic effect used in in the 80's
- Banned in all countries → carcinogenic effect
- Excreted in urine on conjugated form
- Detection and quantification by Immuno Assay and Mass Spectrometry



Diethylstilbestrol



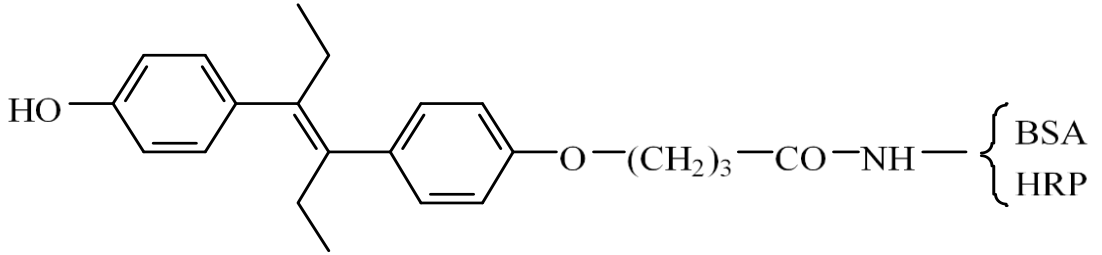
Dienoestrol



Hexoestrol

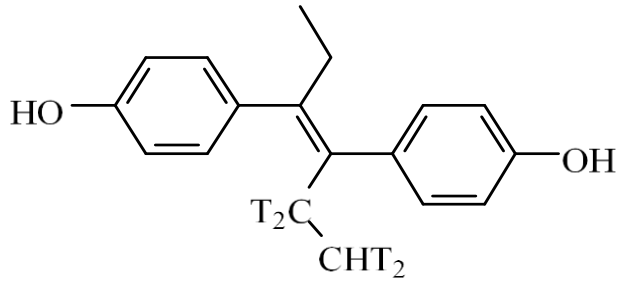
Synthetic Hormones - STILBENES

Chemical structures of the immunogen, the radioactive tracer, and the enzyme conjugate in the DES assay.



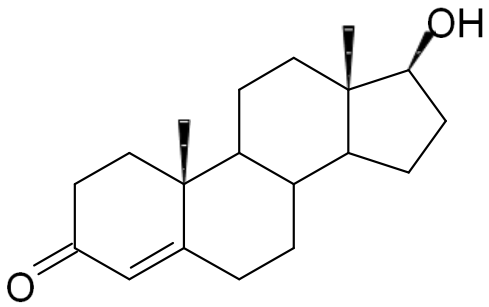
mono-4-O-(carboxypropyl)-DES-BSA : anticorps

mono-4-O-(carboxypropyl)-DES-HRP : conjugué enzymatique

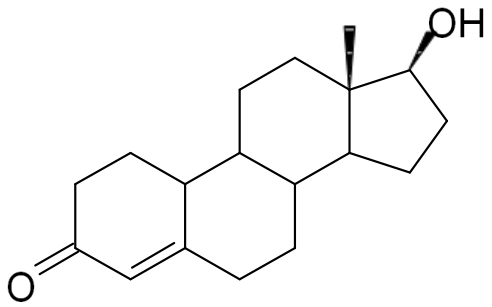


[monoethyl-3H] Diethylsilbestrol : traceur radioactif

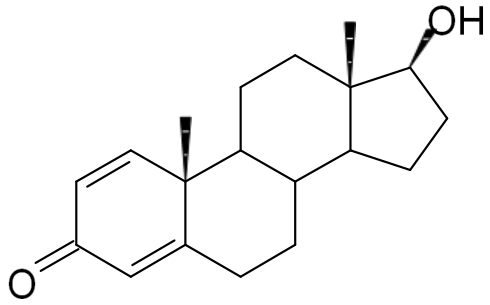
Chemical Structures: Testosterone & Main Synthetic Androgens



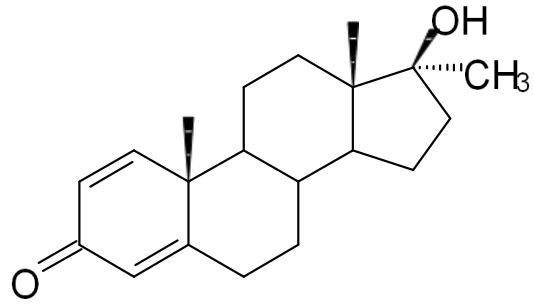
Testostérone



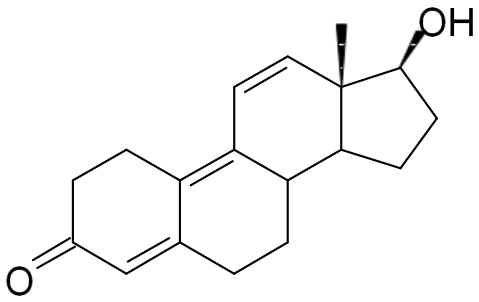
Nortestostérone



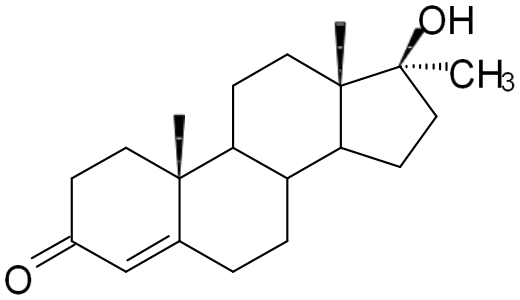
Boldénone



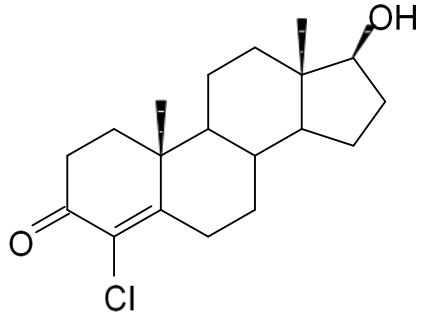
Méthylboldénone



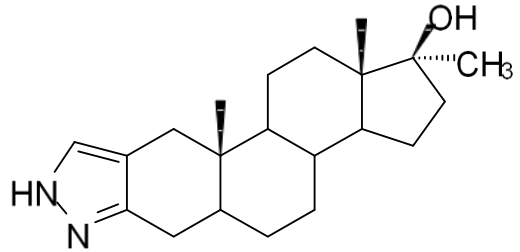
Trenbolone



Méthyltestostérone

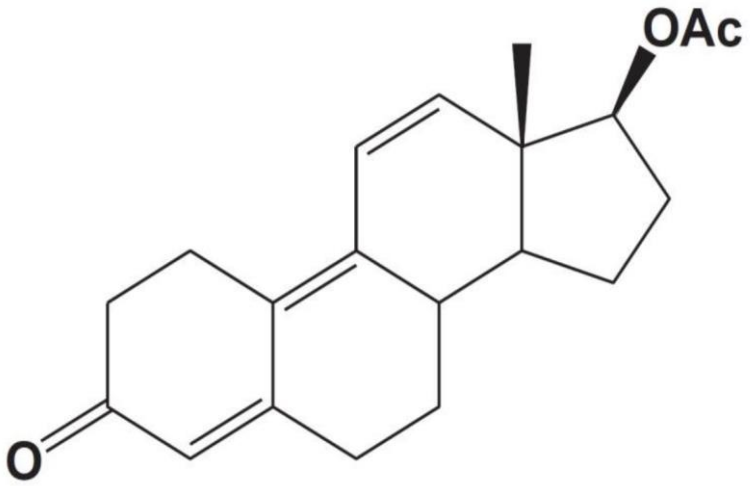


Chlortestostérone



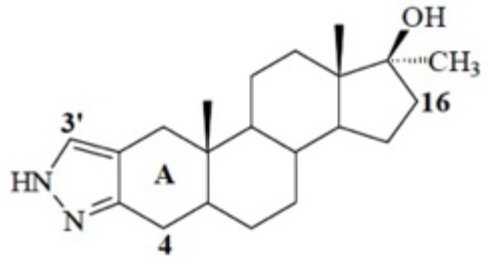
Stanozolol

Synthetic Hormones – ANDROGEN - TRENBOLONE



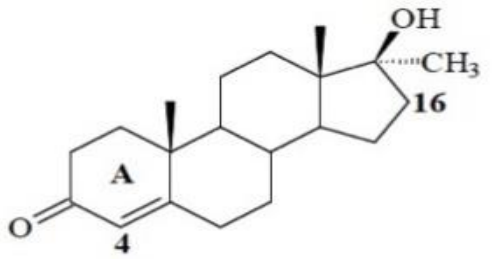
- Powerful anabolic developed by Roussel Uclaf
- Authorized in several countries (USA, ….)
- Androgenic action
- Used in implant form (base of the ear) in combination with oestradiol
- Metabolized in α form, mainly in urine - form, mainly in faeces
- Detection by IA (CR antibody with the metabolite) and LC/MS-MS

Synthetic Hormones – ANDROGEN – STANAZOLOL (1)

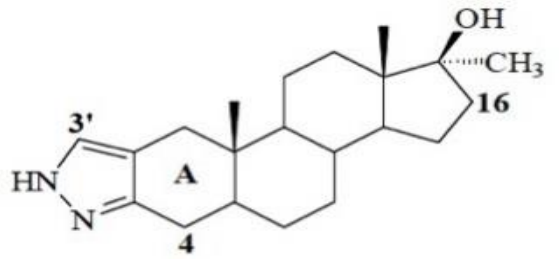


Synthetic Hormones – ANDROGEN – STANAZOLOL (3)

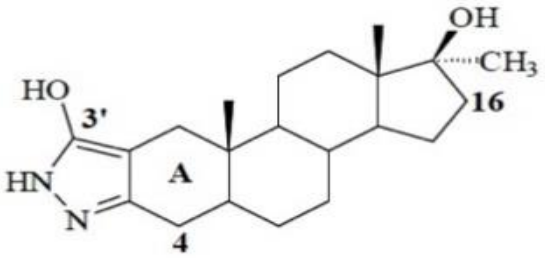
Chemical structures of methyltestosterone, stanozolol, and the metabolites of stanozolol



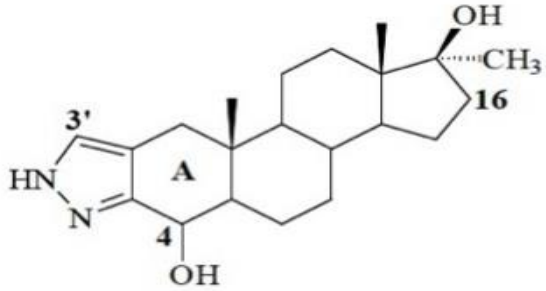
Méthyltestostérone



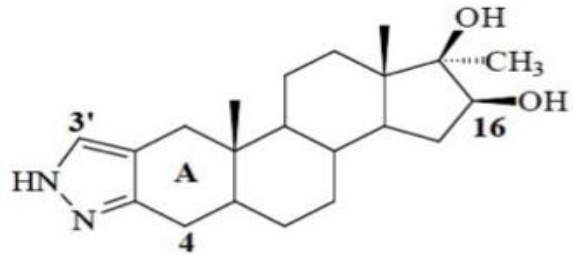
Stanozolol



3'-hydroxystanozolol



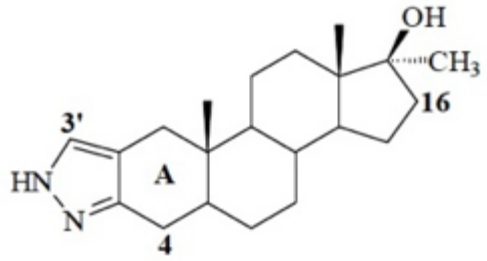
4β-hydroxystanozolol



16β-hydroxystanozolol

Synthetic Hormones – ANDROGEN – STANAZOLOL (4)

- Stanozolol is a synthetic anabolic steroid that has been widely used in sports for performance. Ben Johnson, 1988 in Olympics at Montreal.
- Also used in animal production
- In human stanozolol is metabolized to 3'-hydroxystanozolol and 4 β -hydroxystanozolol, whereas in bovines, 16 β hydroxystanozolol is the main metabolite (see previous slide)
- Elisa for detection in bovine urine



Antibody: stanozolol-17-carboxymethyloxime hapten coupled to KLH.

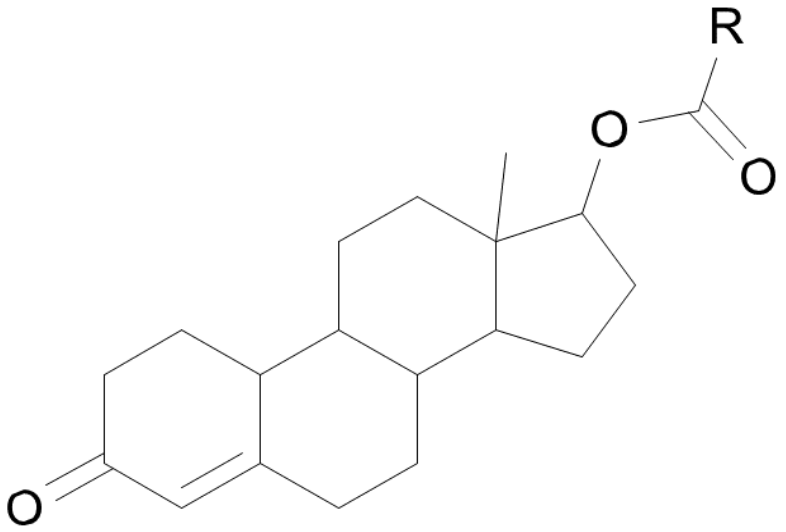
Conjugate: stanozolol-17-carboxymethyloxime hapten coupled to HRP

Cross reactivity: 100% cross-reactivity between stanozolol and 16 β-hydroxystanozolol, 0.01% and 0.3% with 3'-hydroxystanozolol and 4 β -hydroxystanozolol.

- Detection by GC/MS and LC/MS-MS

Synthetic Hormones – Androgen – NORTESTOSTERONE

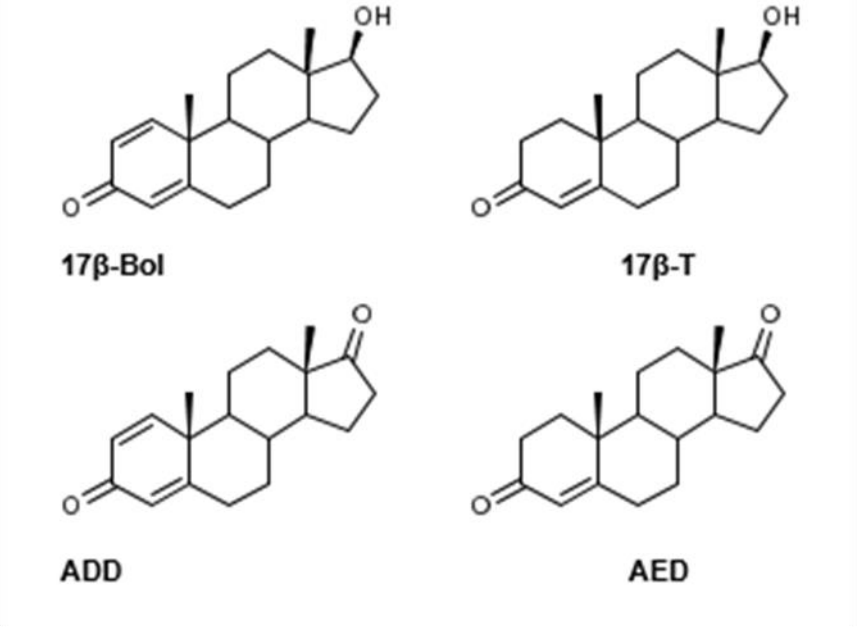
- Injected on ester form R: decanoate, propionate, laurate, …
- Metabolize on α form in urine
- Detection by ELISA - Multi LC-MS/MS
- 17β -NT is endogenous in boars and stallion
- 17α -NT is endogenous in pregnant cows and neonatal calves



17 β-9-Nortestosterone or Nandrolone

Synthetic Hormones – Androgen – BOLDENONE (1)

- ❑Differs from testo by only 1 double bound on A ring
- ❑ADD and AED (keto) are precursor
- ❑Use on ester form
- ❑Detection by multi LC-MS/MS in urine
- ❑Several metabolites (9) in urine of treated animals



17β boldenone (**17β-Bol**),17βtestosterone (**17β-T**), androsta1,4-diene-3,17-dione (**ADD**), androst-4-ene 3,17-dione (**AED**)

Synthetic Hormones – Androgen – BOLDENONE (2)

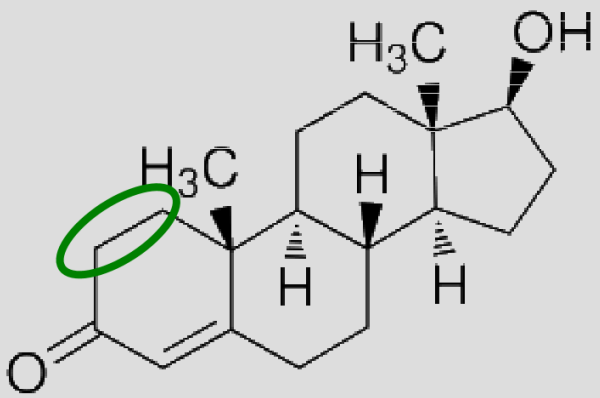
Metabolites of boldenone are present in urine in different untreated and treated animal species

	Untreated		Treated	
	Male	Female	Male	Female
Pig	17β-Bol	–	n.k.	n.k.
Cattle	17α-Bol	–	17α-Bol, 17β-Bol and metabolites	17α-Bol, 17β-Bol and metabolites
Horse	17β-Bol	n.k.	n.k.	n.k.

–, No metabolites present; n.k., Not known.

Synthetic Hormones – Androgen – BOLDENONE (3)

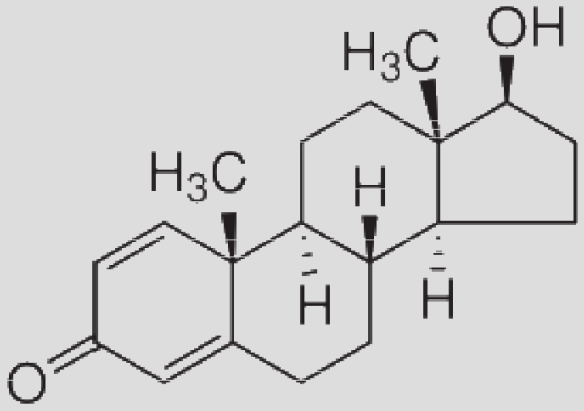
Testosterone



3-oxo-4-ene

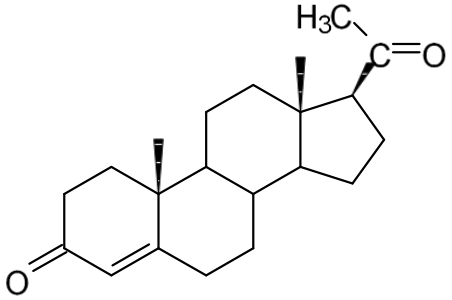
Δ^1 SDH

Boldenone

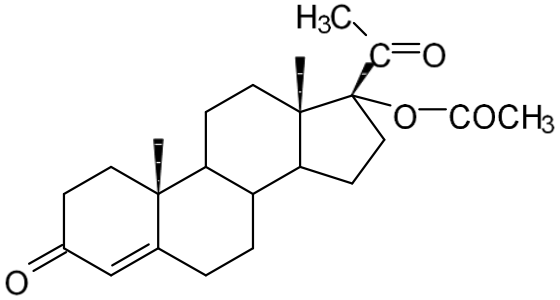


- This enzymatic process introduces a double bond between the C1 and C2 atoms of the steroid's A-ring
- Micro-organism from faeces: transformation of 17 β T to 17 β Bol
- In testis of male horses and pigs production of 17 β Bol through aromatization of oestrogens

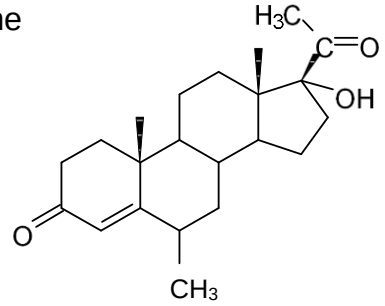
Chemical Structures: Progesterone & Main Synthetic Progestagens



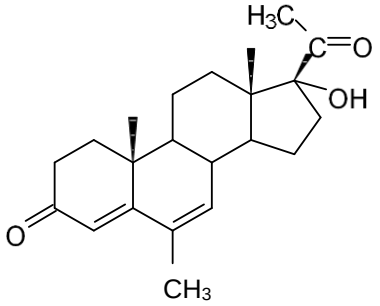
Progestérone



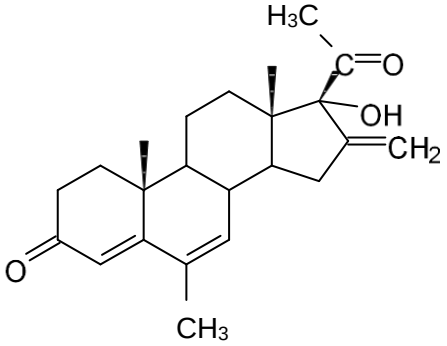
Acétoxyprogestérone



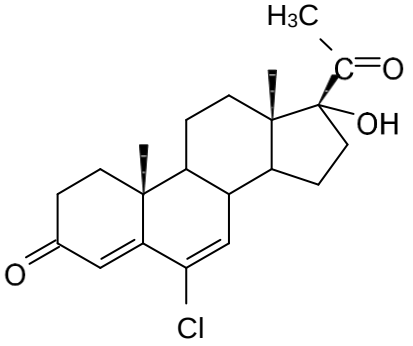
Médroxyprogestérone



Mégestrol

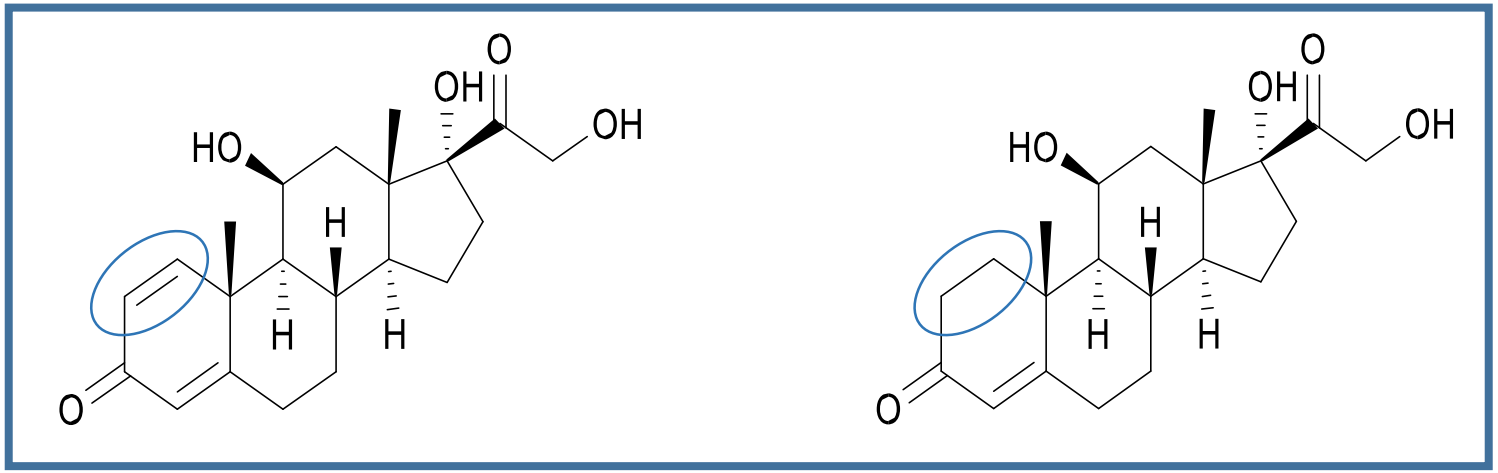


Mélengestrol



Chlormadinone

Synthetic Hormones – Glucocorticosteroid – PREDNIOSOLONE (1)

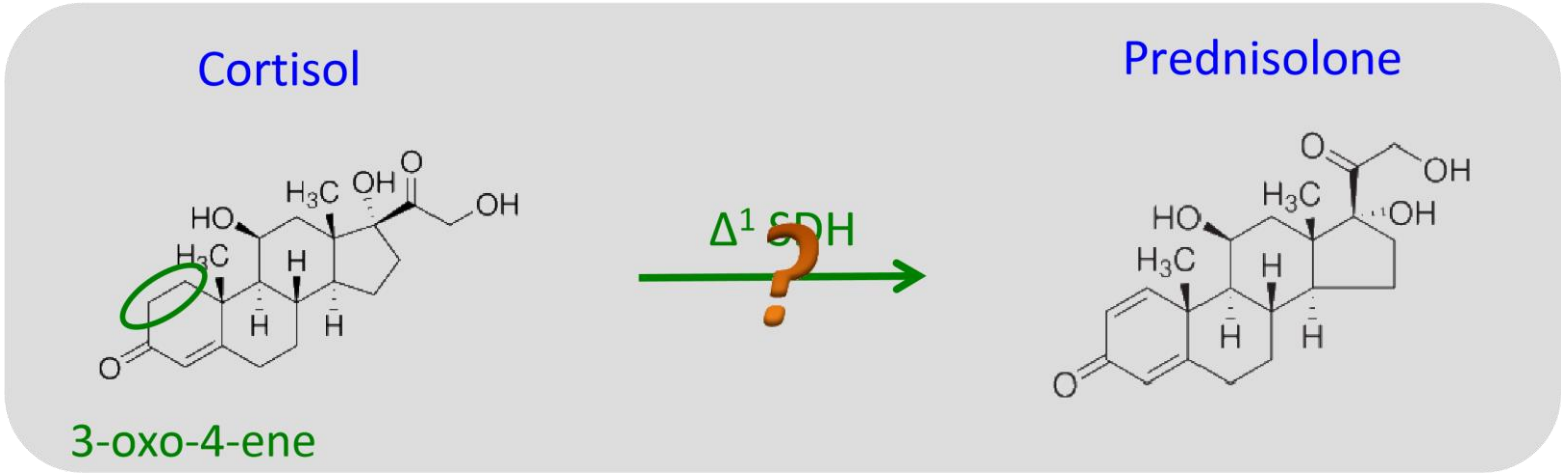


Molecular structures of prednisolone and cortisol.

- ❑Corticosteroid used for inflammatory and auto-immune problems
- ❑Cortisol is endogenous and secreted by the adrenal gland
- ❑Hormone of the stress
- ❑Detection by LC-MS/MS

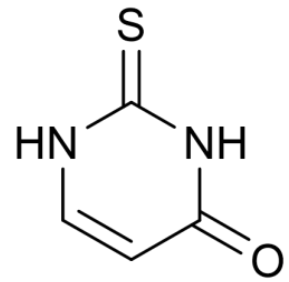
Synthetic Hormones – Glucocorticosteroid – PREDNISOLONE (2)

- Presence of prednisolone in bovine, porcine and equine urine samples
- After stress (transport, injection of ACTH,..) following 1 stress, cortisol levels increase and formation of prednisolone from cortisol
- Threshold level
  - 5 µg/l prednisolone for porcine urine
    - < 5 µg/l: compliant sample
    - > 5 µg/l: "suspect" sample
  - ratio prednisolone/cortisol in liver



Thyrostats - Thiouracil

- Thyrostats (Thiuoracil, Tapazol, methylthiouracil…..) are orally active drugs increase the weight - mainly due to increased water absorption and retention
- Inhibiting thyroid hormone production (gland at the base of the neck)
- Thyrostats have been classified as “possibly carcinogenic to humans”
- Banned in Europe since 1981
- +/- 5 grams per day are administered to reach weight gain and this results in concentrations in urine of over 100 µg L−1.

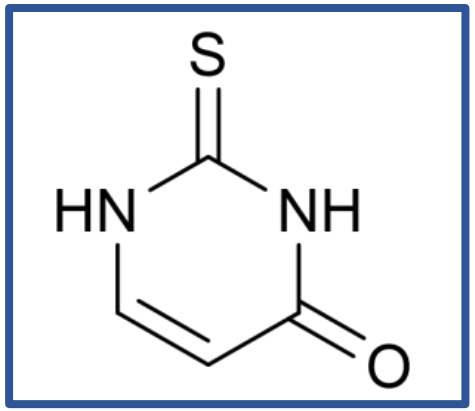


Thiouracil: chemical structure

- Screening: weight of thyroid gland
- HPTLC
- LC-MS/MS
- LC-IRMS ($^{13}C/^{12}C$)

Origin of Thiouracil

- Cruciferous and brassicaceous vegetables contain substances called goitrogens which impair iodine uptake by the thyroid (hypothyroidism).



- Concentrations in urine did not exceed 10 µg L−1 in cattle and 30 µg L−1 in pig urine. Threshold level 10 ppb for cattle and 30 ppb for pigs

- 6-methyl-thiouracil was identified as indicator of thiouracil abuse, whereas 4-thiouracil was indicative for endogenous formation.

Flowchart Decision on Thiouracil Finding in Porcine & Bovine Animals

10 µg/L

2-TU Threshold

Threshold

< Threshold

4-TU Present

Yes

Conform

No

Non-conform

30 µg/L

2-TU Threshold

Threshold

< Threshold

4-TU Present

Yes

Conform

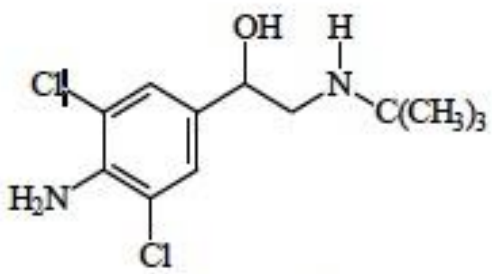
No

Non-conform

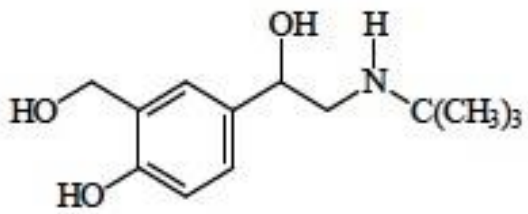
β-Agonists

- Not hormones - Chemical structure similar to catecholamines like adrenaline
- Drug used for respiratory disorders and induction of parturition in cows
- Fat distributing agent – Decrease quantity of fat and increase the amount of muscle
- Active *per* os -eliminated in urine and faeces without being metabolized
- Ractopamine and Zilpaterol are authorized in some country for pig production
- Poisoning due to clenbuterol misdosing after consummation of liver
- Symptoms of twitching, tachycardia, palpitation, …
- Detection by Immunoassay and LC-MS/MS

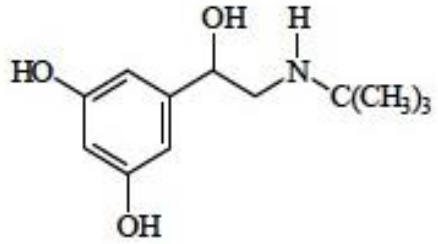
Chemical Structures of Several β-Agonists



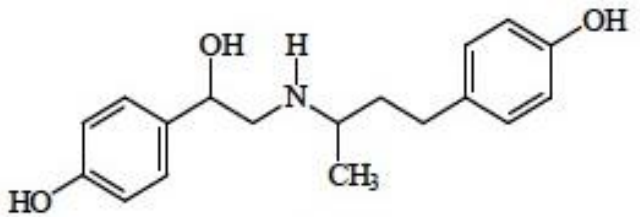
Clenbuterol



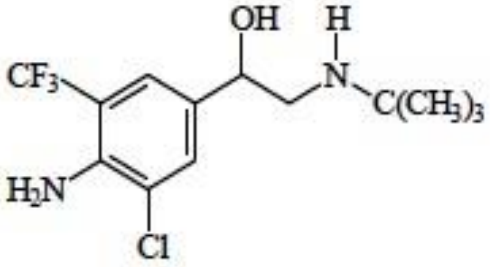
Salbutamol



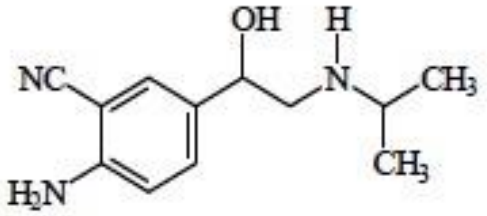
Terbutaline



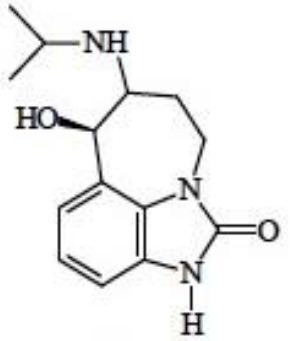
Ractopamine



Mabutérol



Cimatérol



Zilpatérol

β-Agonists: Generic Immunoassay

Main characteristics of RIAs and ELISAs for detecting β –agonists

Tests	A	B	C	D	E	F
Concentration at 50 % of B/B0 (ng/ml)	0.40	0.13	1.05	1.13	0.43	0.32
Cross-reactions (%)						
Clenbuterol	100	100	100	100	60	115
Salbutamol	8	100	18	82	100	100
Mabuterol	100	60	143	40	44	65
Cimbuterol	100	100	96	99	18	-
Terbutaline	9	30	8	34	20	31
Clenpenterol	-	62	41	31	50	-
Mapenterol	-	30	61	8	32	-
Cimaterol	3	15	9	8	9	13

A. RIA : antibody anti-clenbuterol – tracer ^{3}H clenbuterol.

B. RIA : antibody anti-salbutamol – tracer ^{3}H clenbuterol.

C. ELISA : antibody anti-clenbuterol – conjugate clenbuterol-HRP.

D. ELISA : antibody anti-salbutamol – conjugate clenbuterol-HRP.

E. ELISA : antibody anti-clenbuterol and anti-salbutamol – conjugate clenbuterol-HRP.

F. ELISA : antibody anti-salbutamol – conjugate salbutamol-HRP.

Conclusion

The fight against hormones remains an ongoing challenge.

The following points should be borne in mind:

- ❑ Analysis of toxicological data (JECFA, ...)
- ❑ Risk analysis by regulatory agencies
- ❑ Implementation of controls
  - Control plan: Control location, matrix of interest, number of samples, ...
  - High-performance analytical laboratory
- ❑ Proportionate penalties in the event of a positive result


thank you
danke
謝謝
рахмат
ngiyabonga
teşekkür ederim
Баярлалаа
спасибо
faafetai lava
mersi
kia ora
barka
welalin
tack
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dank je
misaotra
matondo
paldies
grazzi
mahalo
tapadh leat
gracias
хвала
asante
manana
obrigada
tenki
vinaka
спасибі
blagodaram
kiitos
dankie
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nanni
nandri
bedankt
mauruuru
hvala
gràçie
bayarlalaa
köszönöm
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tanemirt
grazie
terima kasih
rahmet
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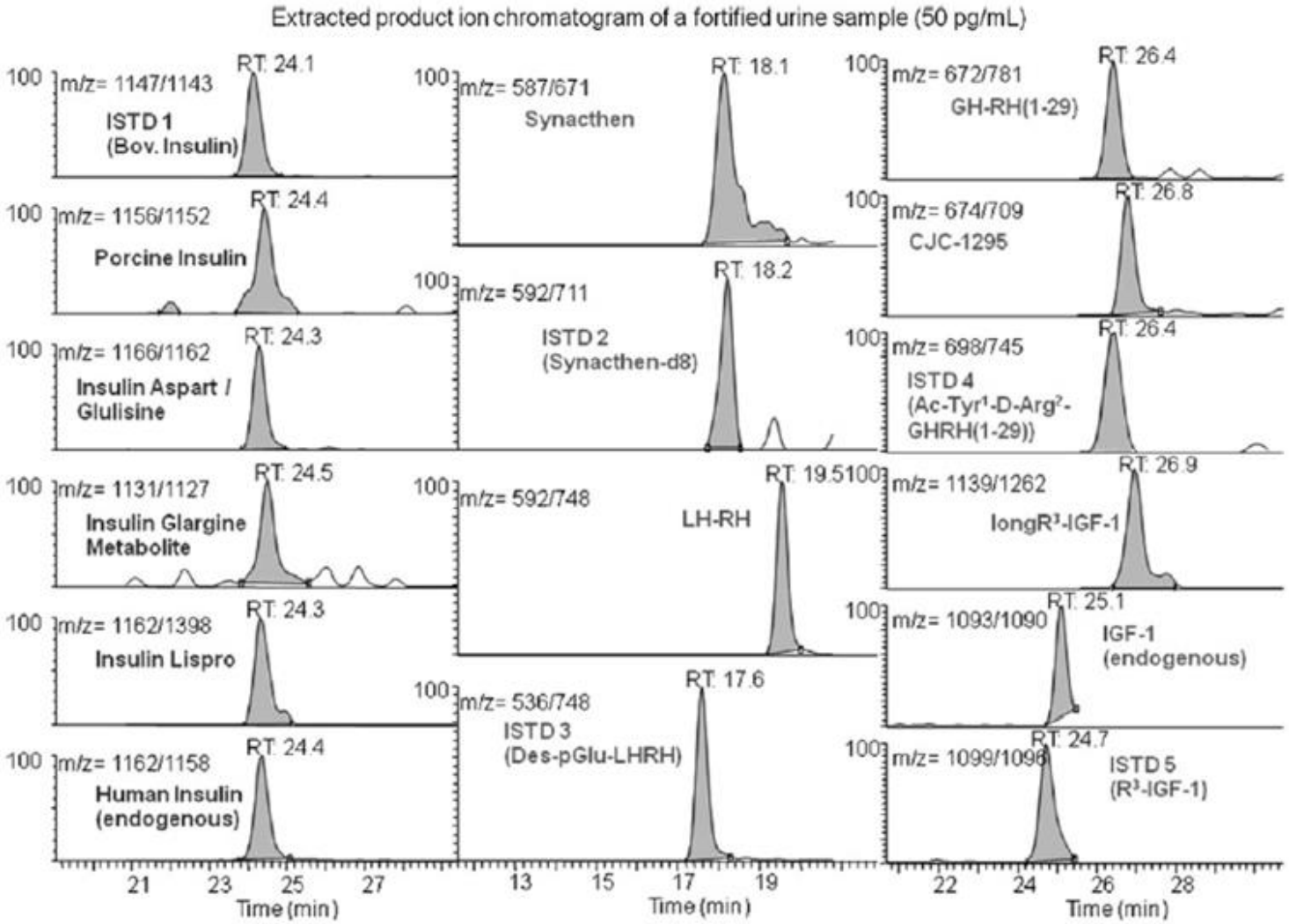
kam sah hamnida
diolch
dhanyavadagalu
shukriya
mercé
мерси
شكرًا لك
তোমাকে ধন্যবাদ
감사합니다
xièxie
ευχαριστώ
merci

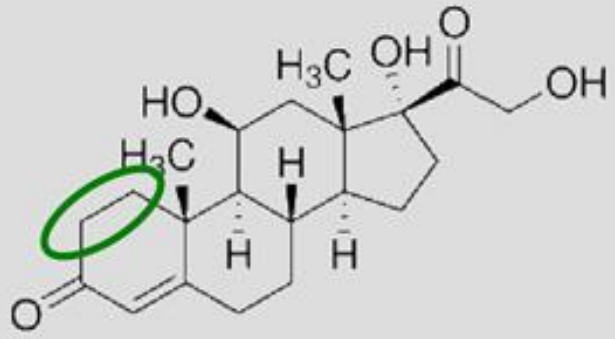
Chromatograms of Fortified Urine Sample with Peptides



Extracted product ion chromatograms (LTQ) of a urine sample fortified with 50 pg/mL of different insulins, Synacthen, LH-RH, GH-RH(1–29), CJC-1295 and LongR3- IGF-1. All target peptides were simultaneously extracted from 5 mL of urine with the described procedure.

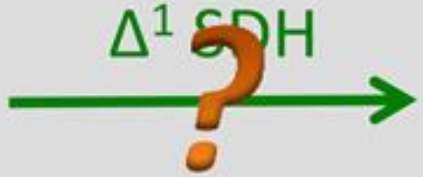
Biotransformation of Cortisol to Prednisolone

Cortisol

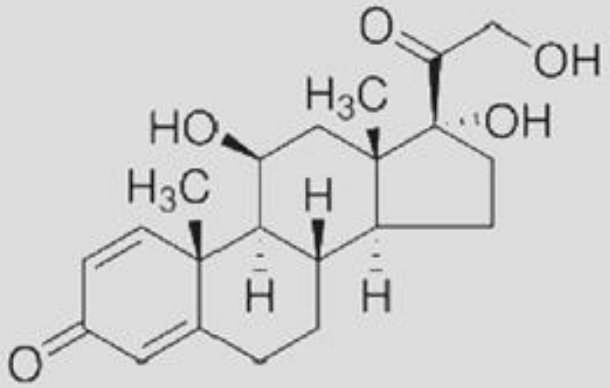


3-oxo-4-ene

Δ1 5DH



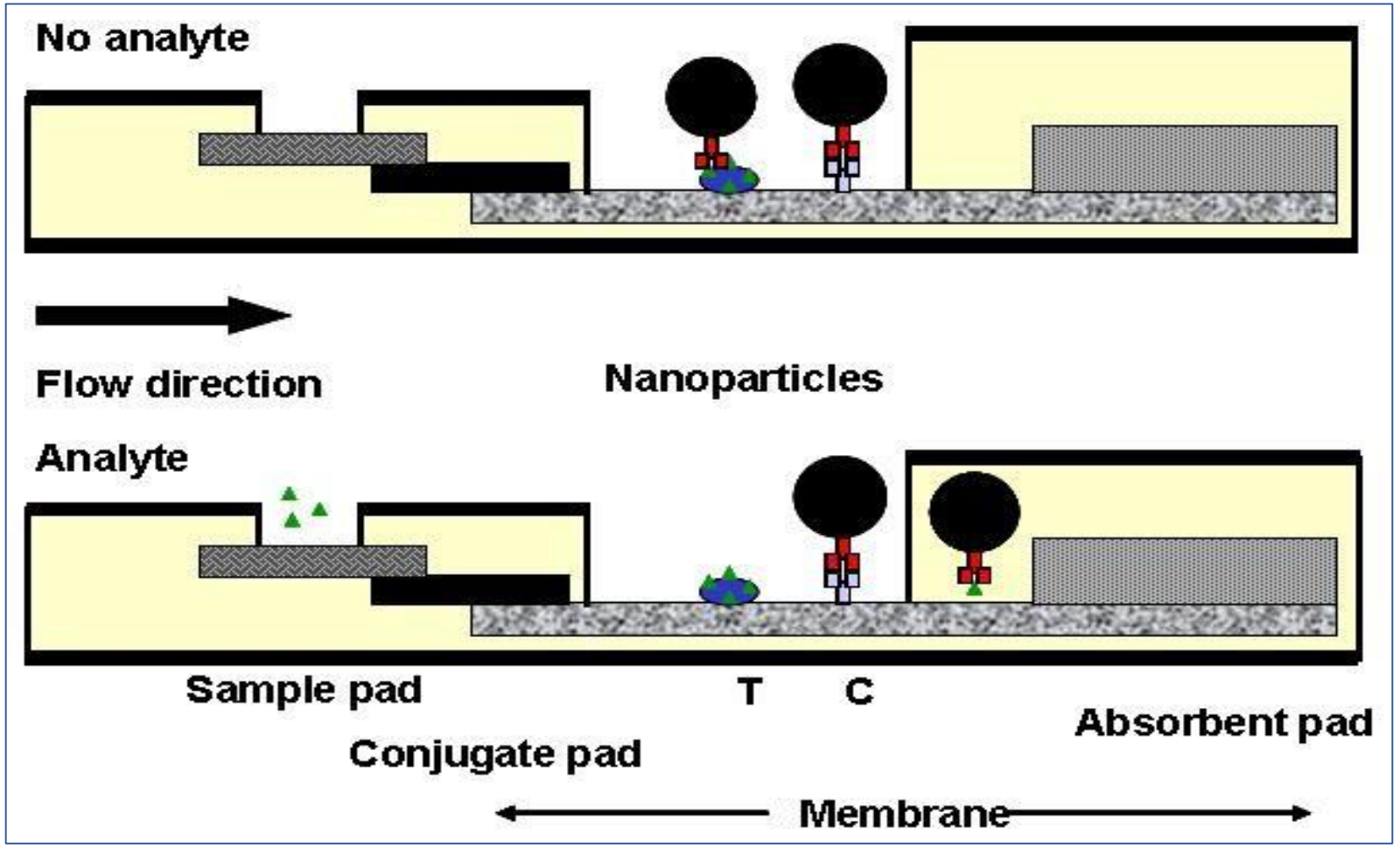
Prednisolone



Biotransformation of Testosterone to Boldenone

LFD – Principle (2)

Lateral Flow Device



Role of the Steroid

- Steroid hormones play critical roles in the regulation of water and salt homeostasis, metabolism, stress response, the initiation and maintenance of sexual differentiation and reproduction, and can be classified into 4 major groups according to their structure and function:

  a) oestrogenic: reproductive hormones in females,

  b) androgenic: male reproductive hormones,

  c) progestagens: gestation hormones,

  d) corticosteroids: stress hormones.

- Steroid hormones are excreted mainly via the urine in the form of glucuronides, sulphates, diglucuronides, disulphates and sulphoglucuronides

Steroidgenesis

The steroid pathway showing relations between individual steroids