



Kanton Zürich  
Gesundheitsdirektion  
**Kantonales Labor Zürich**

# **Development of a novel multiresidue method to detect growth promoters**

**Anton Kaufmann**

**Official food control authority of the canton of Zürich; Switzerland**

**04.06.2019, Oslo**

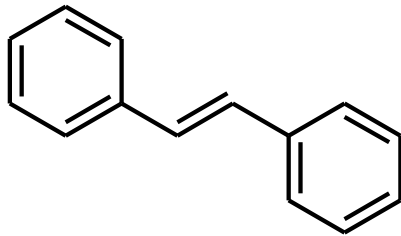
# Content

- I      What are growth promoters?**
- II     The analyte & matrix puzzle**
- III    Extraction und Clean-up**
- IV    Chromatography und detection**
- V     Validation**
- VI    Interpretation**

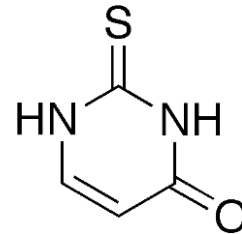
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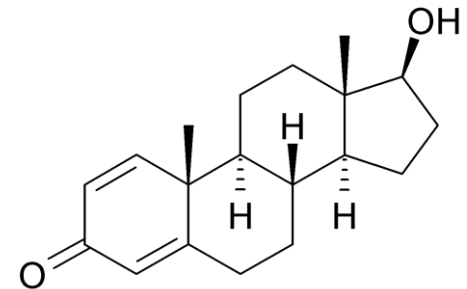
# Compound groups



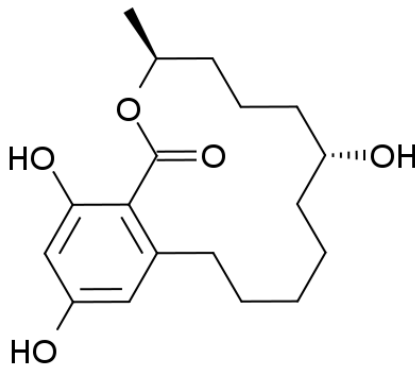
**A1: Stilbens**



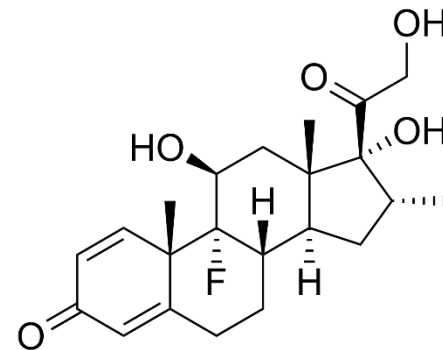
**A2: Thyreostatika**



**A3: Steroide**



**A4: Resorcylic acid lactones**



**B2f: Glucocorticoide etc.**

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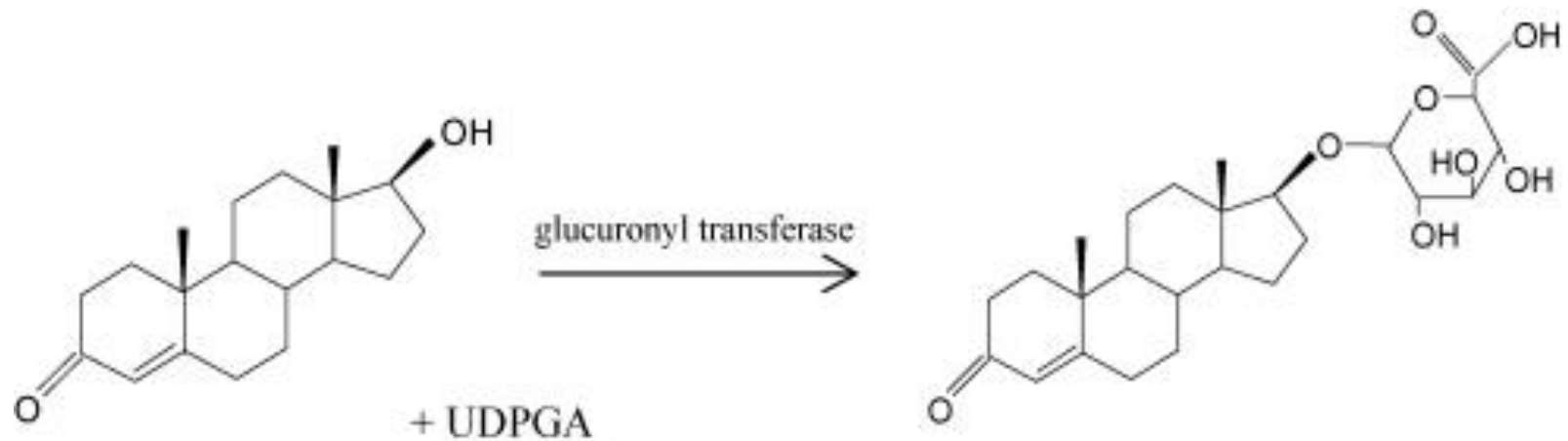
# **Why are currently different methods required to cover all analytes?**

## **Different analyte forms in different matrices**

**Urine: Analyte is glucuronidated**

**Liver: Analyte is present in the unconjugated form**

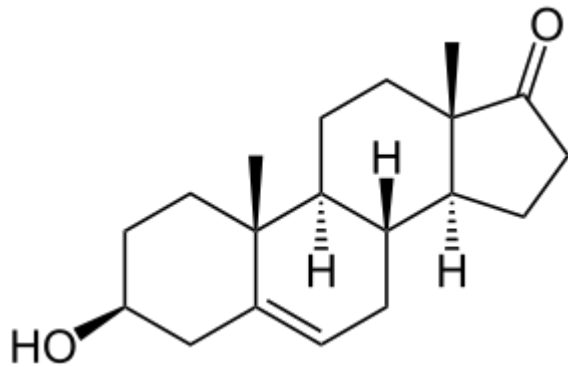
# unbound or glucuronidated ?



**Therefore, prior enzymatic hydrolysis is required**

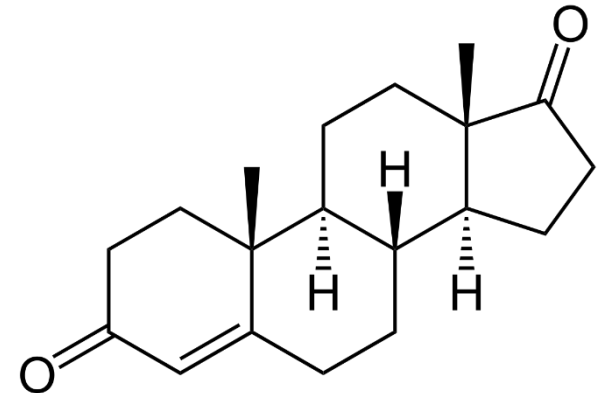
# Why different methods ?

Using enzymes can be a double edged sword



**DHEH**

*Helix pomatia*  
=  
vineyard snail



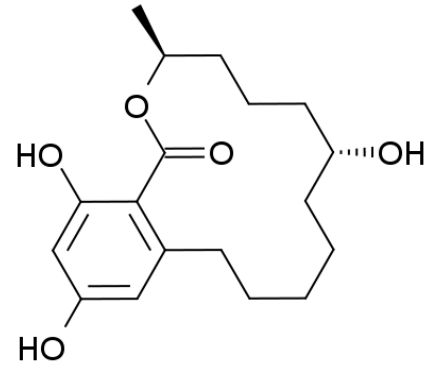
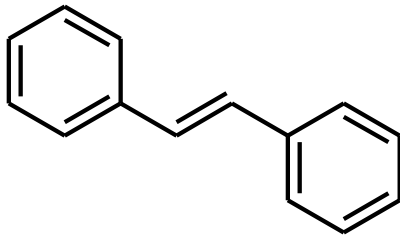
**Androstendione**

That is why utilizing glucuronidase von *Escherichia coli*



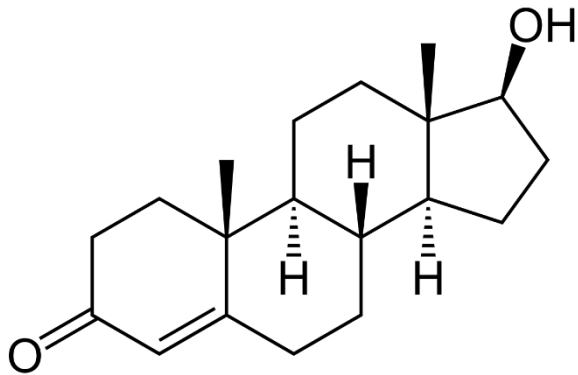
# Why different methods ?

## Extraction and Clean-up



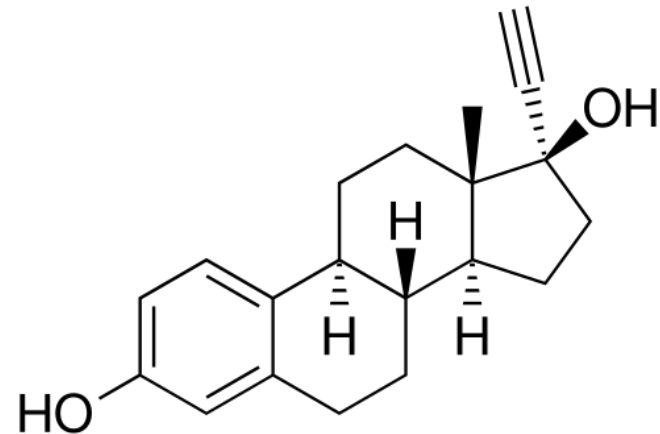
# Why different methods ?

## Ionization



**Testosterone**

**Positive ionization**



**Ethinylestradiol**

**Negative ionization**

# Why different methods ?

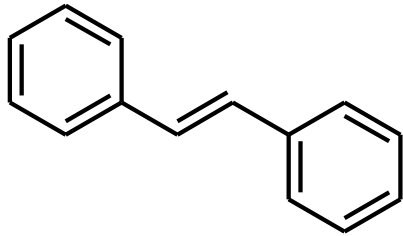
## Sensitivity

- **LC-MS sensitivity is insufficient for Estrogens**
- **Derivatization of estrogens and analysis with GC-MS or LC-MS/MS**

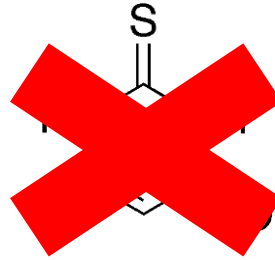
# **Why development of a single multiresidue method?**

- **We were requested to analyze all the Swiss national residue control program growth promoter samples (without receiving any additional manpower or instrumentation)**
- **This would have resulted in additional 1032 samples per year**
- **Reducing this number to «just» 432 samples by developing a new multiresidue method was the motivation to engage into method development**

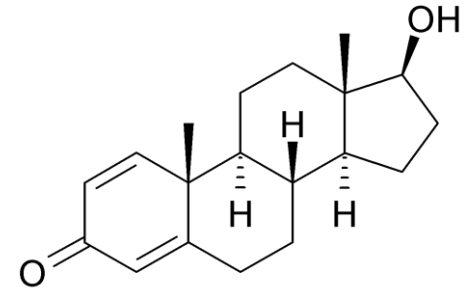
# Covered compound groups



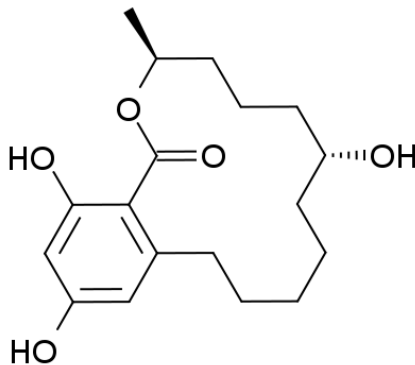
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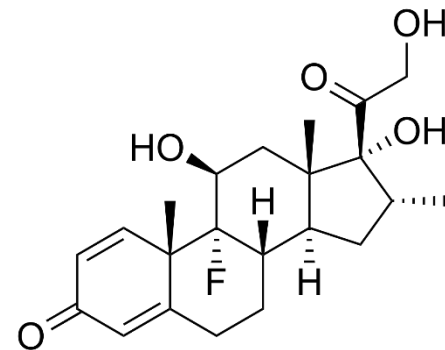
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# **Extraction with ethyl acetate**

- **Good recoveries when spiking samples and immediately extracting them**
  
- **Partially very poor recoveries after enzymatic hydrolysis**
  - **Are some analytes degraded by the used enzyme ?**
  - **Are some analytes irreversibly bound to the matrix ?**

# **Deactivation of native enzymes**

**If an added enzyme can induce analyte degradation (e.g. helix pomatia) then native enzymes present in some matrices (e.g. liver) should be capable in doing the same !**

**- Thermic denaturation of native enzymes**

**Question: Do analytes survive such a heat treatment?**



# Denaturation and recovery



→ **Analysis**



→ **Analysis**



→ **Analysis**

# **The analytes are thermostabil but:**

- **Some (few) analytes spiked in some matrix could not anymore be extracted (virtual zero recovery)**
- **Yet, these problematic analytes could be easily extracted if spiked into denaturated matrix**

**Question: What happens  
(enzymatical degradation or adsorption) ?**

# Test-system:

## Extraction of dienstrol-diacetate dissolved in an aqueous solution of albumin



**Recovery = 0 %**



**Recovery > 90 %**



**Recovery > 90 %**

# **Citation from a scientific paper**

**F. N. Andrews «Hormonal relationships and Applications» (1959)**

**«Dienstrol diacetate is» has been «used in commercially poultry feeds»**

**«The results suggests that dienstrol diacetate is not stored the edible tissues .. «**

**Our experiments showed that it is most likely too well stored !!**

# **Analogy: QuEChERS**

**QuEChERS: Quick, Easy, Cheap, Effective, Rugged, Safe**

**QuEChERS is currently THE pesticide clean-up methodology.  
It is spreading now to other analytical fields**

- **Add mixed standard solution to the sample, directly add acetonitrile, homogenize, add salt, wait for phase separation, analyze**

**Does the obtained analyte recovery represents the analyte recovery from an incurred sample ??**

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# Chromatography

- **Chromatography is important, since many analytes show the same elemental composition and frequently the same MS/MS transitions**
  
- **Many matrix compounds are steroids or behave like steroids**

# Mobile phase

- **Acids are only useful additives for positive ionizable analytes**
  
- **Negative ionizable analytes give the best response if eluted with wasser / acetonitrile**

**Unbuffered mobile phases can give instable separations**



# **Ammonium Fluoride**

## **(positive aspects)**

- **NH<sub>4</sub>F enhances the sensitivity of some problematic ESI negative analytes while not affecting the sensitivity of ESI positive analytes**
- **NH<sub>4</sub>F was reported to be used in some medical diagnostic applications where selected steroids were detected**
- **No reported work exists for the use of NH<sub>4</sub>F in the field of vet drugs**

**(people do not read papers outside of their narrow field of work)**

# **Ammonium Fluoride**

## **(negative aspects)**

- **1 mMol/l NH<sub>4</sub>F is insufficiently buffering the mobile phase. So why not adding some formic acid ?**
- **Clogging of ESI capillary. Three autosampler pressure sensors were ruined in sequence !!!**
- **NH<sub>4</sub>F und acid produces HF !**

# **Ammonium Fluoride (controlled)**

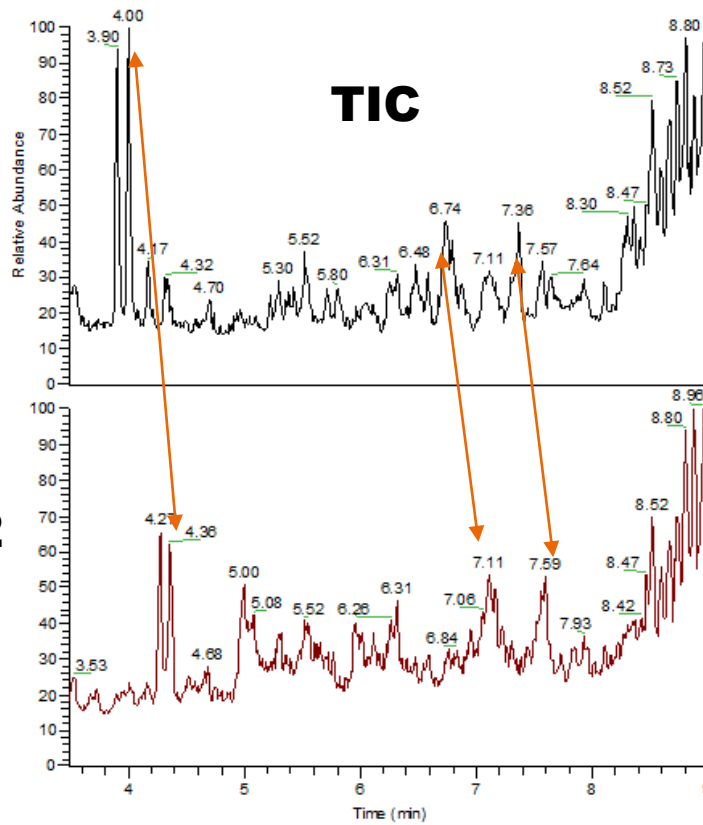
- **0.2 mMol/l NH<sub>4</sub>F without acid for the new methode**
- **Separation column deteriorates now after 1000 instead of 2000 samples**
- **Separation columns once in contact with NH<sub>4</sub>F should not be used for other separations**
- **LC seems to tolerate low levels of neutral NH<sub>4</sub>F**

# Poor reproducibility

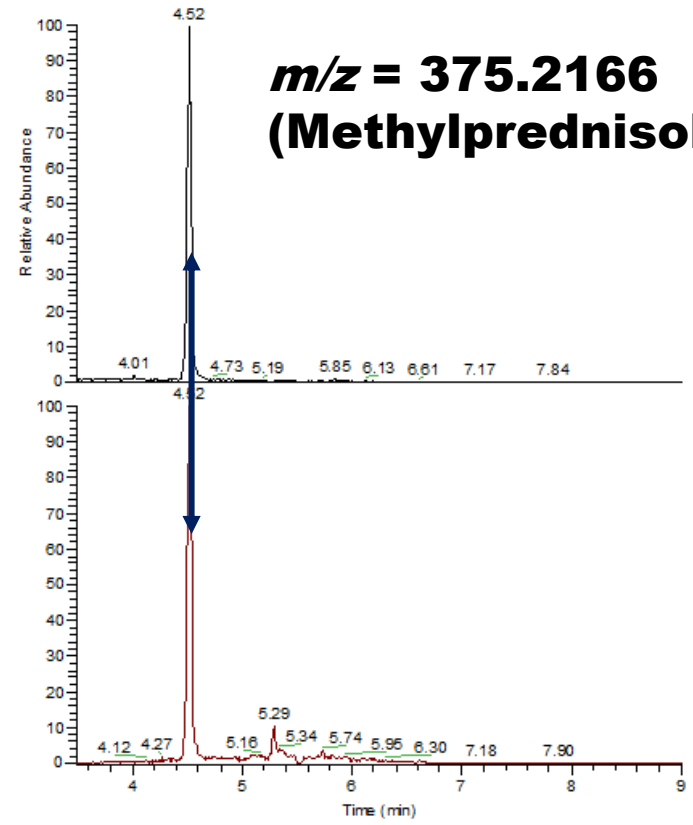
- **Steroide show rather stable retention times (non-polar, undissociated compounds)**
- **But measured peak areas showed poor reproducibility**
- **Signal suppression varied from sample to sample**

# Stability of retention times

**Sample 1**



**Sample 2**



# **The matrix overrules the mobile phase**

- **Using an unbuffered mobile phase, the sample solution pH defines the elution pH**
- **Dissociating matrix compounds move as chromatographic bands and change the local mobile phase pH in a highly dynamic way**
- **Depending on their concentration, they affect their own retention time and that of other dissociable compounds**
- **Irreproducible signal suppression effects result when these compounds partially co-elute with analytes**

# **«Removal of ions» from the matrix extracts**

- **HRMS helped us to quickly identify the responsible compounds as fatty acids**
- **Doing the EtAc extraction und acid conditions shifts the fatty acid distribution equilibrium towards the EtAc phase**
- **Therefore a weak alkaline pH should retain the fatty acids in the aqueous phase.**

# Removal of fatty acids from sample extract

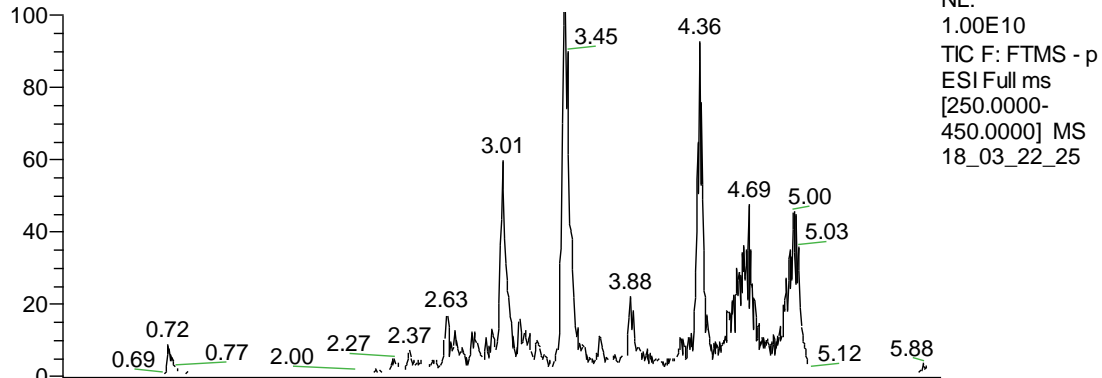
D:\TraceFinderData\...Data\18\_03\_22\_25

03/22/18 21:51:15

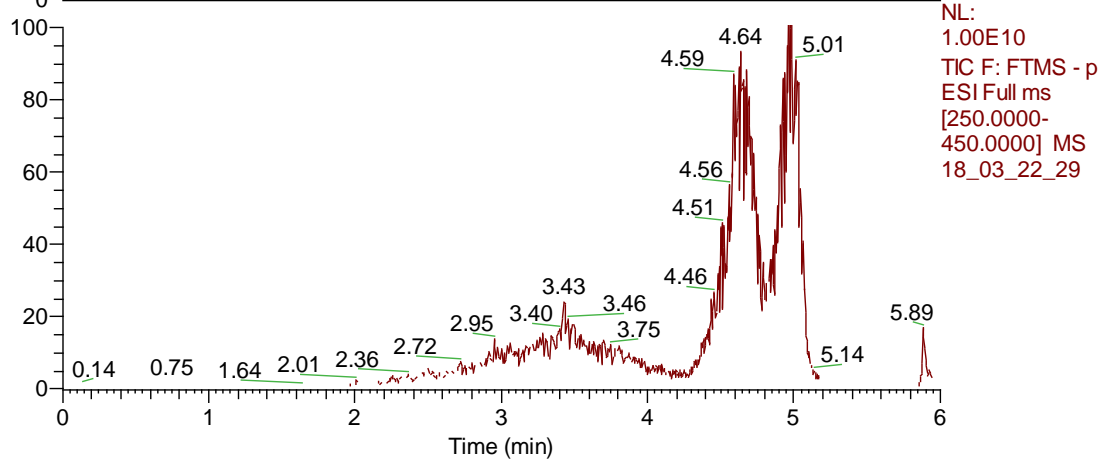
Blut EtAc pH 5.2 Spike

RT: 0.00 - 6.01

**pH = 5.2**



**pH = 9.0**

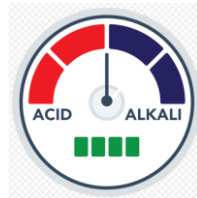




# Prinzipiele der final method (Enzymatic step)



**+ Buffer**



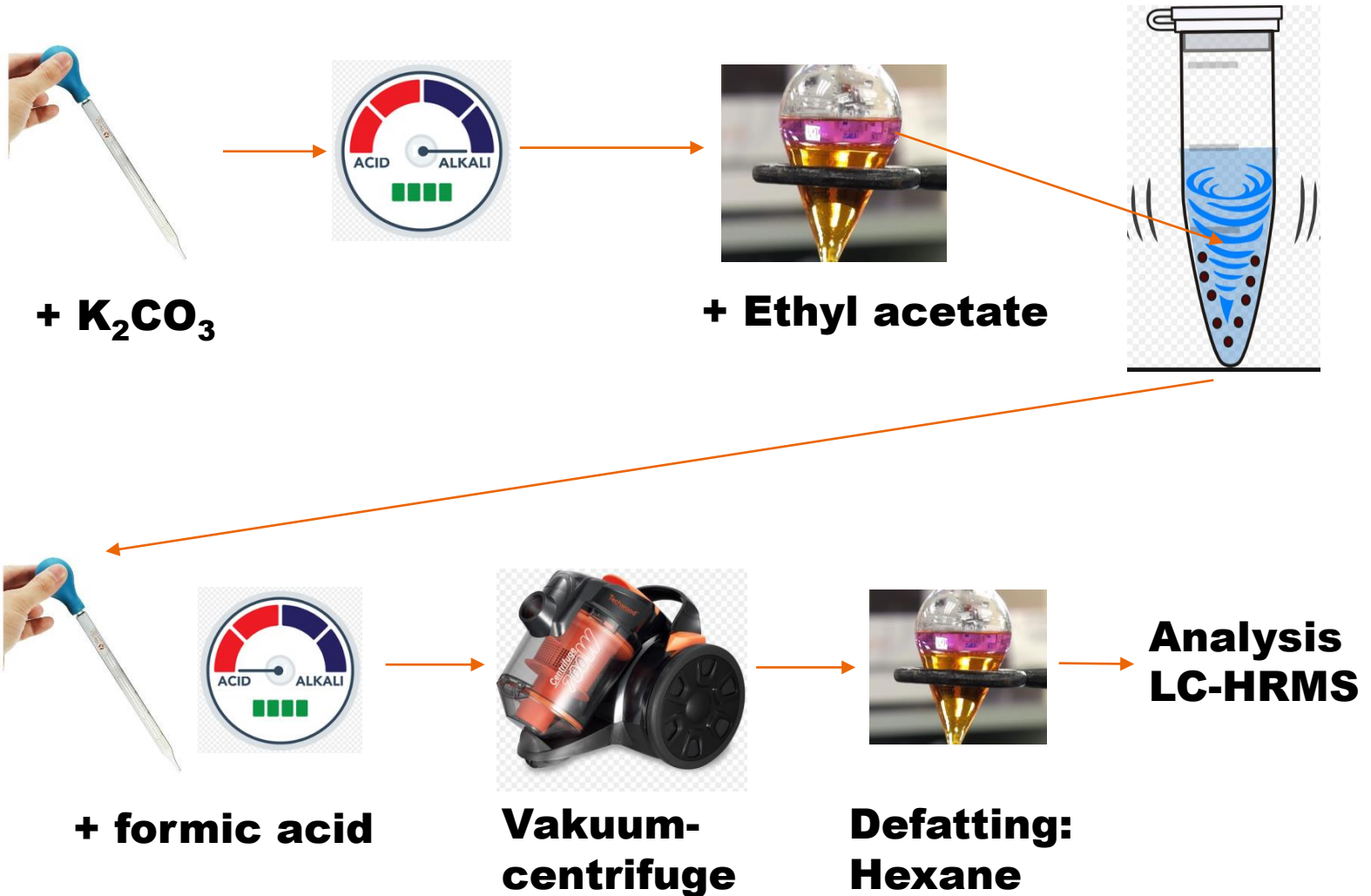
**homogenization**



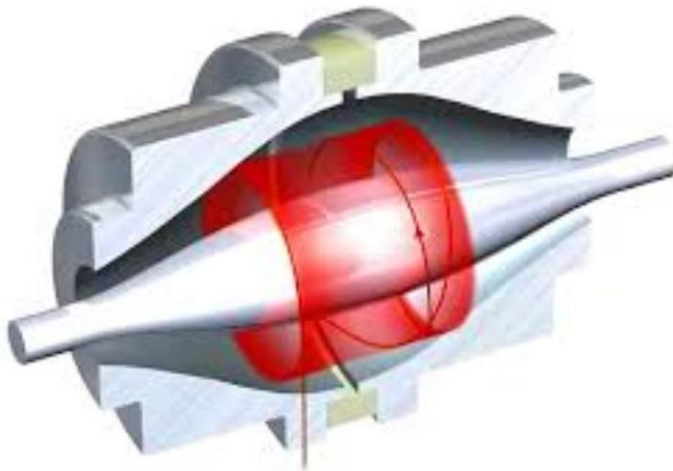
**+ Glucuronidase**

**Incubation (over night)**

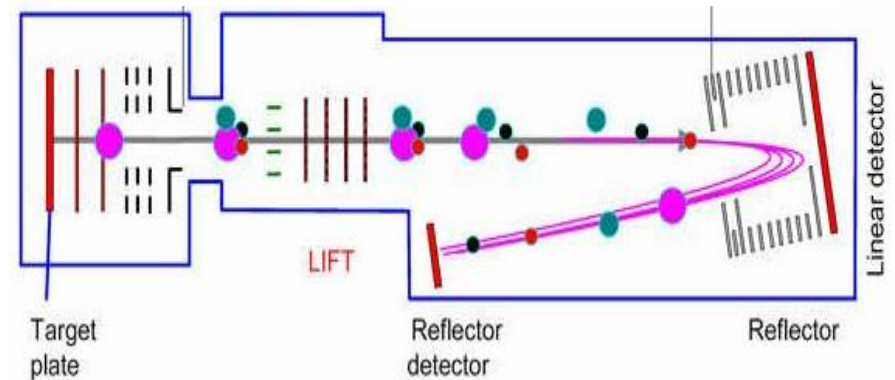
# Prinzipiele der finalen Methode



# HRMS Measurment



**Q- Orbitrap**



**Q- TOF**

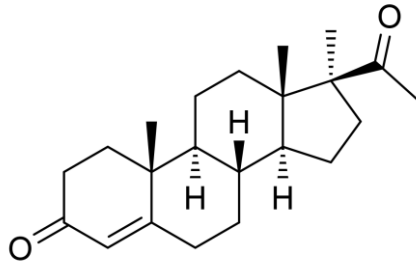
**Full Scan combined with Q-HRMS  
(Quantification & Confirmation)**

# HRMS Detection

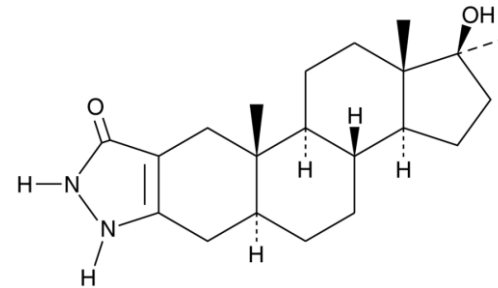
- **HRMS Full Scan alternates with targeted MS/MS**
- **Two injection (ESI plus und ESI minus)  
with identical separation column and mobile phase**

# MS/MS is not always the best (low abundance and non characteristic fragments)

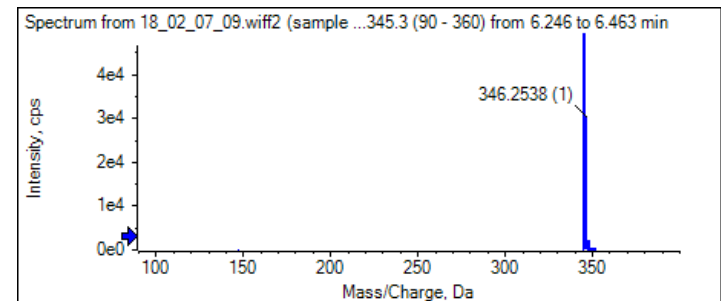
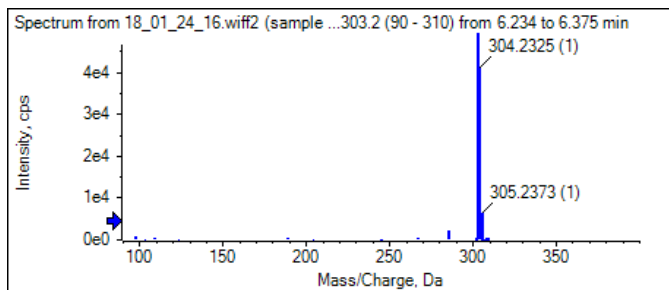
## Methylprogesterone



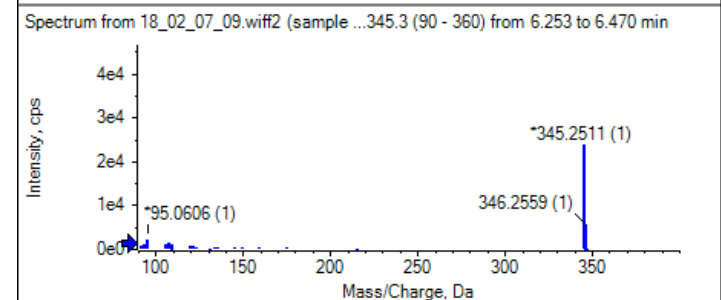
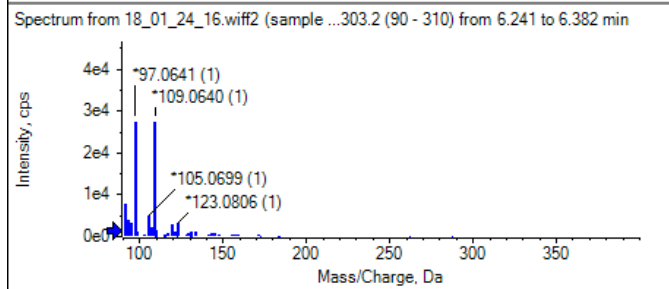
## Hydroxy Stanozolole



10 eV

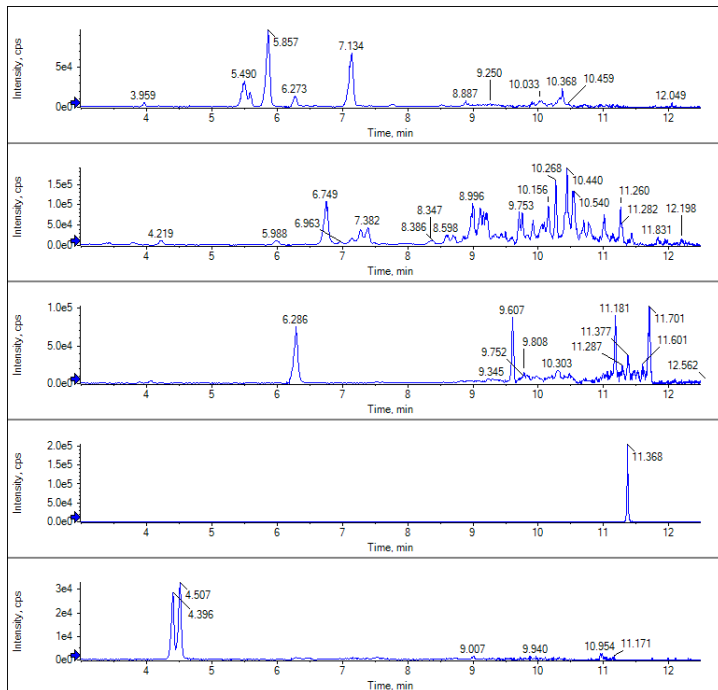


50 eV

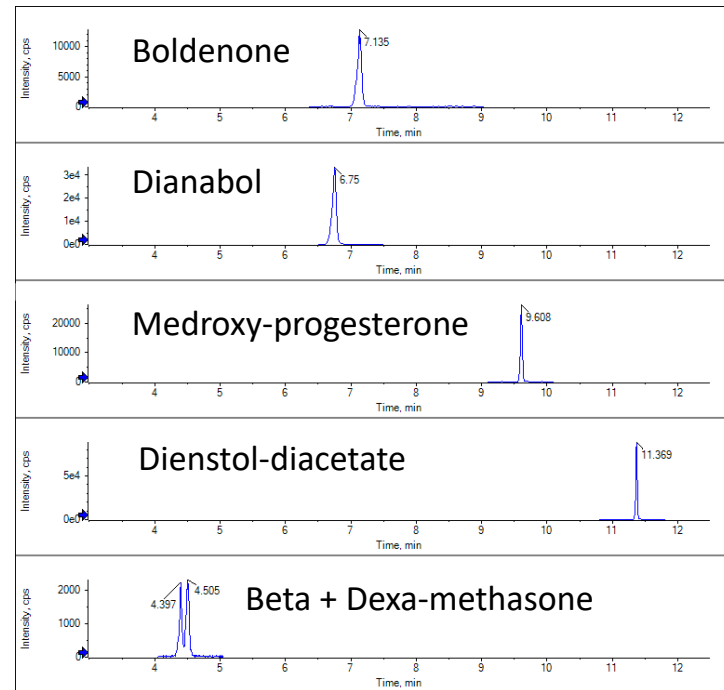


# Yet MS/MS shows superior selectivity

Full Scan



Targeted MS/HRMS



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# Validation

- **Matrix: Urine, muscle, serum, whole blood, liver**
- **Instruments: Q-Exactive und X-500 Q-TOF**
- **Strategy: Select the «most blind» among 24 blind samples**
- **Methodology: According EU Commission decision ( $CC\alpha$ ,  $CC\beta$ )**



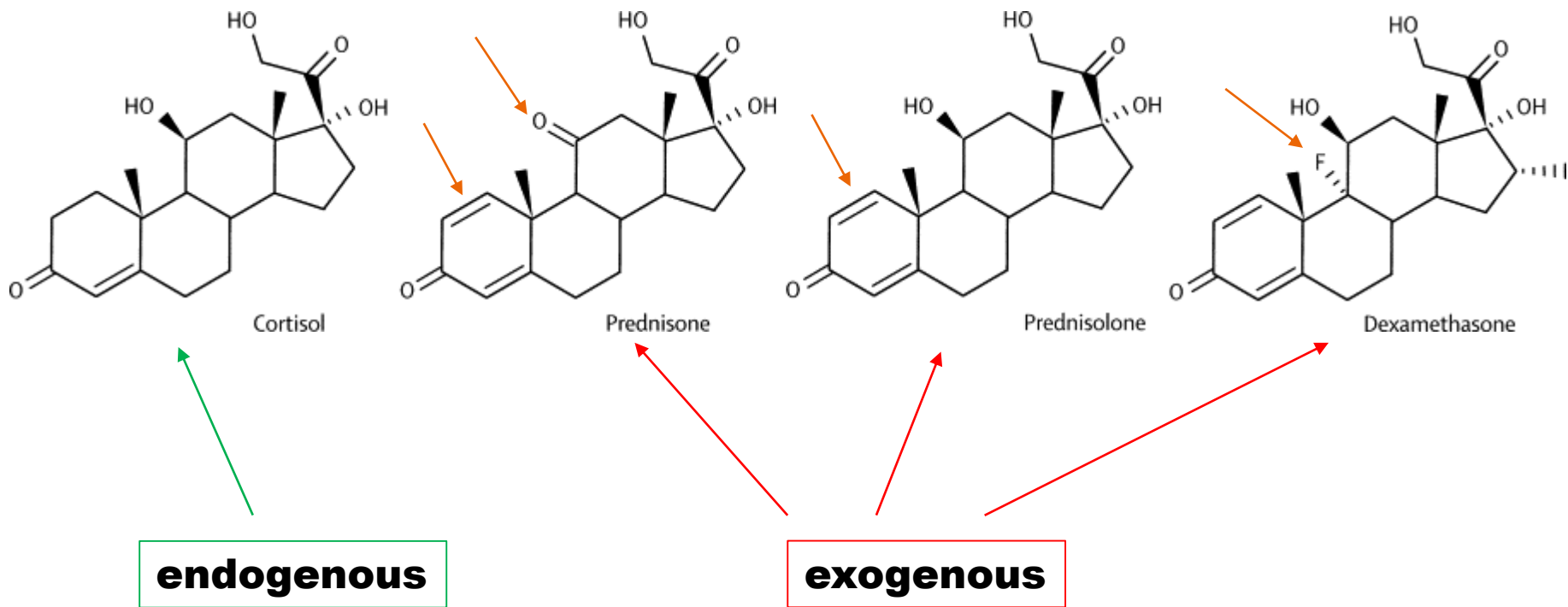
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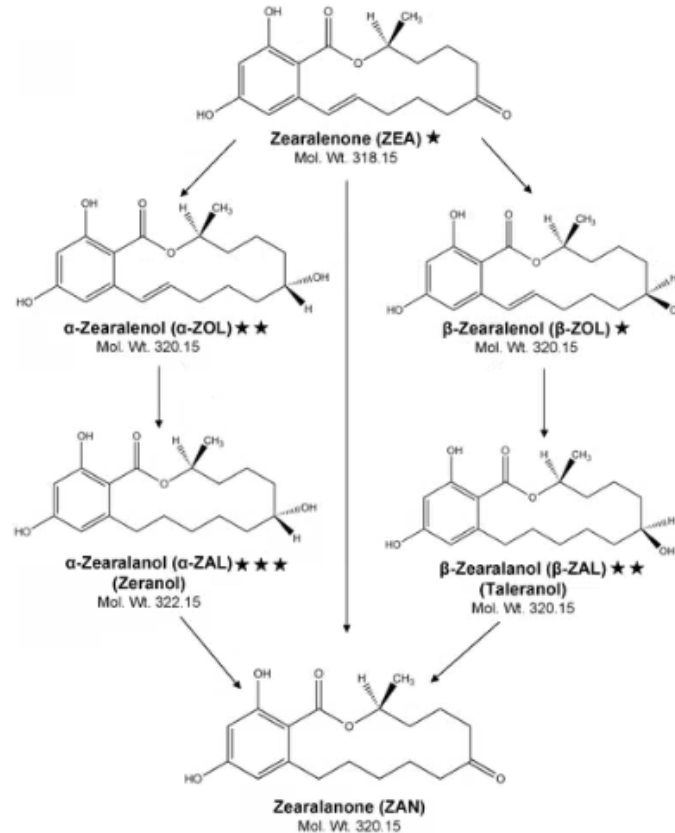
# Interpretation of measured values

- **Exogenic steroids**
- **Pseudo exogenic steroids (as a result of injured animals)**
- **Formation during the sampling procedure  
(e.g. urine sample contaminated with feces)**
- **Endogenic steroids**

# Siamese twins !



# The mycotoxine zearalenone can be converted into the “growth promotor zeranol”



# **Interpretation of measured values**

- **Depending on age or sex, feed etc. different decision levels**
  
- **Isotope ratio MS may be an «expensive» option**

# Take home messages

- **Extracting a spiked or incurred sample is not the same**
- **Look at trees (analyte) but don't forget the forest (matrix)**
- **LC-MS mobile phases tend to be insufficiently buffered (retention time stability of some compounds)**
- **Look what is going on outside your narrow community (use of ammonium fluoride in medicine diagnostic environment)**
- **Accurate measurements may be easier than the correct interpretation of results.**



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