LINEAR QUADRUPOLE ION TRAP FOURIER TRANSFORM (LTQ-ORBITRAP) TO FIGHT AGAINST THE ILLEGAL USE OF GROWTH PROMOTERS IN CATTLE
0. INTRODUCTION AND OUTLINE

0.1 Access to accurate masses on competent MS

1-INTRODUCTION : HIGH RESOLUTION, MASS ACCURACY, AND MASS ANALYZERS

2-GROWTH PROMOTERS IN THE EU

3-TARGET APPROACHES

△ Structure elucidation (ecdysteroid metabolites)

△ Protein residues (GH)

4-UNTARGET APPROACHES: BIOLOGICAL SAMPLE FINGERPRINTING

△ Principle

△ Applications

5-CONCLUSION AND PERSPECTIVES
INTRODUCTION
HIGH RESOLUTION MS ADVANTAGES
1. ADVANTAGES OF HIGH RESOLUTION MS

1.0 Resolution acquisition and consequence onto the MS information

Mixture of proteins - R~100.000 @ m/z 400

18.2 mmu
1. ADVANTAGES OF HIGH RESOLUTION MS

1.1 Expression of resolving power pending on mass analyzers

\[ R \rightarrow \frac{m}{\Delta m} \]

But \( \Delta m \) is differently calculated pending on the mass analyzer

- Double sector
- 10% valley
- Equivalent to 5% of peak height

- FTMS, TOF
- FWHM definition
- Equivalent to 50% of peak height

Resolution: 10,000 (10% valley) = 20,000 (FWHM)
1. ADVANTAGES OF HIGH RESOLUTION MS

1.2 Relation in-between mass accuracy and elemental composition

<table>
<thead>
<tr>
<th>Data Bank</th>
<th>Country</th>
<th>Downloadable</th>
<th>N Compound</th>
</tr>
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<tr>
<td>PubChem Compound</td>
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<td>KEGG Compound</td>
<td>Japan</td>
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<td>HMDB</td>
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<td>Yes</td>
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<tr>
<td>BOMET</td>
<td>Japan</td>
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Example of results obtained for 3 target compounds

<table>
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<tr>
<th></th>
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<tbody>
<tr>
<td></td>
<td>M = 209.009</td>
<td>M = 276.096</td>
<td>M = 358.084</td>
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Candidates

<table>
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<tr>
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<th>52731</th>
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<tr>
<td>2 digit</td>
<td>327</td>
<td>3744</td>
<td>6742</td>
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<tr>
<td>3 digit</td>
<td>54</td>
<td>121</td>
<td>486</td>
</tr>
</tbody>
</table>

Error in ppm

ppm

mmu/nominal mass*10^6
1. ADVANTAGES OF HIGH RESOLUTION MS

1.3 First type of application: chemical structure elucidation

**C_{19}H_{25}O_{5}^{32}S**

\[ \text{R} = 69901 \]
\[ z = 1 \]

\[ C_{19}H_{25}O_{5}^{32}S \]

\[ \text{R} = 67804 \]
\[ z = 1 \]

\[ C_{18}^{13}C_{1}H_{25}O_{5}^{32}S \]

**C_{18}^{13}C_{1}H_{25}O_{5}^{32}S**

\[ \text{R} = 69500 \]
\[ z = ? \]

\[ C_{18}^{13}C_{1}H_{25}O_{5}^{32}S \]

**H = 1.0078**

**C = 12.0000**

**C = 13.0034**

**O = 15.9949**

**S = 31.9721**

**S = 32.9715**
1. ADVANTAGES OF HIGH RESOLUTION MS

1.3 Second type of application: mass clean-up

MS/MS – Fish extract

HRMS – Fish extract

PFOA

412.9 > 368.9

PFOS

498.9 > 80

PFUnDA

562.9 > 518.9

PFTeDA

712.9 > 668.9

412.96532; ±10 ppm

562.95578; ±10 ppm

712.94616; ±10 ppm

498.92912; ±10 ppm

562.95578; ±10 ppm

712.94616; ±10 ppm
1. ADVANTAGES OF HIGH RESOLUTION MS

1.4 Mass clean-up: always possible?

KENDRICK graphic and van KREVELEN diagram

\[
\text{KENDRICK Mass} = \text{Experimental Mass} \times \frac{14,0000}{\text{mass(C}_2\text{H}_2)}
\]

Mass defect \( \text{KENDRICK} = \text{Nominal Mass} - \text{Mass KENDRICK} \)

Plot of IUPAC mass defect vs nominal mass

- HRMS \( \rightarrow \) PROBABLY LESS EFFICIENT
- HRMS \( \rightarrow \) USEFUL
1. ADVANTAGES OF HIGH RESOLUTION MS

1.4 Mass clean-up: depending on mass defect

- **ΔM**
- **CH**
- **CHO**
- **M(u)**

**Δ MASS DEFECT VS NOMINAL MASS**

**MOST MATRICES INTERFERENCES**

**ANALYTES WITH MASS DEFECT**

Bruno LE BIZEC, AOAC INTERNATIONAL, 23rd November 2009
1. ADVANTAGES OF HIGH RESOLUTION MS

1.4 Mass clean-up: depending on mass defect

C$_{25}$H$_{44}$O$_2$ClSi$_2$

R=2.000

M$^+$• (37Cl)

468.2473

4-CHLOROTESTOSTERONE

C$_{25}$H$_{44}$O$_2$ClSi$_2$
1. ADVANTAGES OF HIGH RESOLUTION MS

1.4 Mass clean-up: depending on mass defect

4-CHLOROTESTOSTERONE

**C_{25}H_{44}O_{2}ClSi_{2}**

R=5.000

M+● (37Cl)

468.2473
1. ADVANTAGES OF HIGH RESOLUTION MS

1.4 Mass clean-up: depending on mass defect

C$_{25}$H$_{44}$O$_2$ClSi$_2$

R=10.000

M$^+$• (37Cl)

468.2473

4-CHLOROTESTOSTERONE
1. ADVANTAGES OF HIGH RESOLUTION MS

1.4 Mass clean-up: depending on mass defect

\[ R = 20.000 \]

\[ M^+ \bullet (37\text{Cl}) \]

\[ 468.2473 \]

C\text{\textsubscript{25}}H\text{\textsubscript{44}}O\text{\textsubscript{2}}Cl\text{\textsubscript{Si}}\text{\textsubscript{2}}

S/N

R

7000

C\text{\textsubscript{25}}H\text{\textsubscript{44}}O\text{\textsubscript{2}}Cl\text{\textsubscript{Si}}\text{\textsubscript{2}}
1. ADVANTAGES OF HIGH RESOLUTION MS

1.4 Mass clean-up: depending on mass defect

\[ C_{25}H_{44}O_2Si_2 \]

\[ M^+ \]

17\(\beta\)-TESTOSTERONE

\[ 432.2880 \]

R = 2.000
1. ADVANTAGES OF HIGH RESOLUTION MS

1.4 Mass clean-up: depending on mass defect

C_{25}H_{44}O_{2}Si_{2}  

R=5.000  

M^+  

432.2880  

17\beta-TESTOSTERONE
1. ADVANTAGES OF HIGH RESOLUTION MS

1.4 Mass clean-up: depending on mass defect

17β-TESTOSTERONE

C_{25}H_{44}O_{2}Si_{2}

R=10.000

M^{+}•

432.2880
1. ADVANTAGES OF HIGH RESOLUTION MS

1.4 Mass clean-up: depending on mass defect

\[ R = 20.000 \]

\[ C_{25}H_{44}O_{2}Si_2 \]

\[ M^+ \]

432.2880
1. ADVANTAGES OF HIGH RESOLUTION MS

1.4 Mass clean-up: depending on mass defect

 Plasma: 10 ng.L$^{-1}$

17α-E$_2$  
17β-E$_2$

$R = 5000$

m/z: 343.21

HRMS

343 > 343

MS/MS
1. ADVANTAGES OF HIGH RESOLUTION MS

1.5 Accurate mass measurement in mass spectrometry

**Figure 5.** Results from the mass measurement of the [M + Na]^+ ion at m/z 498 (± 10 ppm view).

*Chem. Rev. 2007, 107, 3621–3653*

**Intercomparison Study on Accurate Mass Measurement of Small Molecules in Mass Spectrometry**

Anthony W. T. Bristow and Kenneth S. Webb
LoC Labtech, Middlesbrough, United Kingdom

*J Am Soc Mass Spectrom 2003, 14, 1086–1098*
1. ADVANTAGES OF HIGH RESOLUTION MS

1.6 Mass analyzers able to operate in HRMS, and to provide mass accuracy
1. ADVANTAGES OF HIGH RESOLUTION MS

1.7 Orbitrap®: resolving power and m/z

![Graph showing resolving power vs. m/z for Orbitrap® technology]

- **ICR**
- **7T**
- **12T**

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1. ADVANTAGES OF HIGH RESOLUTION MS

1.8 Orbitrap®: stability

![Graph showing deviation (ppm) over time for m/z 1422 and m/z 524]

- **Deviation (ppm)**
  - Time (hours)
  - 0% (bias)
  - 3 ppm
  - 4 HOURS

![Diagram illustrating stability with +λ and -λ]

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1. ADVANTAGES OF HIGH RESOLUTION MS

1.9 Orbitrap®: speed scanning, and resolution

**Isotopes** $^{13}$C$_2$ et $^{34}$S

C$_{23}$H$_{38}$N$_7$O$_5^{34}$S 526.2607 u  $^{13}$C$_2$C$_{21}$H$_{38}$N$_7$O$_5$S 526.2716 u

Detection: 750 ms; RP: 45,000

Detection: 375 ms; RP: 22,000

Detection: 1500 ms; RP: 90,000
2

GROWTH PROMOTERS

CONTEXT IN EUROPE
2. GROWTH PROMOTERS

2.1 Context in Europe

France/EUROPE

REST OF THE WORLD
2. GROWTH PROMOTERS

2.2 Still some affairs

In 2008
24,888 batches of animal feed samples were inspected
370,000 inspectors were sent to:
> 36,000 feed producers,
> 118,000 feed shops
> 20,000 breeding houses
> 6,932 tons of tainted feed were confiscated and destroyed
> 256 firms operating illegally were closed

Since the milk scandal
The voice of Ireland's farming industry
THE JOURNAL

Farmers seek probe as casualties fail residue tests
La terre a trempé
La terre a trempé

Veaux aux anabolisants :
four éleveurs présentés à la justice
SANTÉ PUBLIQUE DANS LE SUD-OUEST

4. GROWTH PROMOTERS
3
TARGETED APPROACHES
ECDYSTEROIDS
3. TARGETED APPROACHES

3.1 Ecdysteroids: introduction

Natural occurrence
(insects, crustaceans & vegetals)

20-hydroxyecdysone (20E)

C_{27}H_{44}O_{7} ; M_i = 480,30870

Anabolic properties 20E

Availability on the Web

Burdette W.J 1961 Otaka et al. 1968
Purser and Baker 1994
Kratky et al. 1997
3. TARGETED APPROACHES

3.1 Ecdysteroids: characterization of the administered substance

HRMS, R=30,000 ESI-
3. TARGETED APPROACHES

3.1 Ecdysteroids: metabolite detection

M1

m/z 463.3054 – RT = 7.81 min

M2

m/z 495.2952 – RT = 6.36 min

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3. TARGETED APPROACHES

3.1 Ecdysteroids: metabolite identification

14-deoxy-20-hydroxyecdysone

$\text{C}_{27}\text{H}_{44}\text{O}_6; M_i = 464.31379$

$[\text{M-H}]^-$

$m/z_{\text{the}} = 463.30596$

$m/z = 463.30573$

$m/z_{\text{the}} = 303.19602$

$m/z = 303.196$

$m/z_{\text{the}} = 159.10212$

$m/z = 159.102$

$m/z_{\text{the}} = 445.29540$

$m/z = 445.29547$

$\text{C}_{27}\text{H}_{44}\text{O}_6$
3. TARGETED APPROACHES

3.1 Ecdysteroids: metabolite identification

26, 20-dihydroxyecdysone

\[ \text{C}_{27}\text{H}_{44}\text{O}_8; M_i = 496,30362 \]

\[
\begin{align*}
[M-H]^- & = 495.29510 \\
m/z_{\text{the}} & = 175.09704 \\
m/z_{\text{the}} & = 319.19095 \\
m/z_{\text{the}} & = 477.28523 \\
m/z_{\text{the}} & = 511.28986 \\
\end{align*}
\]

LC-(ESI-)-HRMS (R = 30 000)
3. TARGETED APPROACHES

3.1 Ecdysteroids: metabolite quantification

14-deoxy, 20-hydroxyecdysone

20,26-dihydroxyecdysone

GROWTH HORMONE
3. TARGETED APPROACHES

3.2 Growth hormone: introduction

Growth hormone = somatotropin (bST/rbST)
Used as growth promoter + to increase milk production

Synthesis by the pituitary gland
Effects on different organs or tissues
Production of IGF-1
Increase of milk production
190-191 aa sequence
MW = ± 22 kDa
At least 1 aa difference
3. TARGETED APPROACHES

3.2 Growth hormone: characterization

N-terminal (rbGH) peptide by LC-HRMS (Orbitrap™) – R = 30,000

\[ m/z \ 609.31844 \quad \text{z=3} \]

\[ m/z \ 913.47395 \quad \text{z=2} \]

\[ m/z \ 1825.942 \quad \text{z=1} \]
N-terminal (rbGH) peptide by LC-HRMS (Orbitrap™) – R = 30,000
3. TARGETED APPROACHES

3.2 Growth hormone: other possibilities

- **Cal 1**: 0.2 fmol o.c.
- **Cal 3**: 1.4 fmol o.c.
- **Cal 5**: 6.8 fmol o.c.
3. TARGETED APPROACHES

3.2 Growth hormone: quantification, kinetic of elimination in blood

Bovine, Plasma, 4 mL 913,38>774.27
UNTARGETED APPROACHES
BIOLOGICAL SAMPLE FINGERPRINTING
PRINCIPLE
4. UNTARGETED APPROACHES

4.1 Principle of metabolomic

Definition: Global characterisation of a biological system through the simultaneous measurement of all metabolites… accessible to the analysis.

Principle: Generate and compare metabolic fingerprints from (large) sets of biological samples by an appropriate analytical tool (mainly spectroscopy-based techniques).

Objectives:
- Reveal similarities or dissemblance between sub-groups of samples (descriptive modelling).
- Characterise these similarities or dissemblance (explicative modelling).
- Identify specific metabolites (candidate biomarkers) for further use (diagnostic modelling).

Food product

Biological fluid

METABOLOMIC

Chromatograms

Mass Spectrum
4. UNTARGETED APPROACHES

4.1 Principle of metabolomic

SAMPLES

PREPARATION
(EXTRACTION and
PURIFICATION)

LC-HRMS
FINGERPRINTING

1 SAMPLE = 1 FINGERPRINT

INTENSITY

m/z

TIME

LC-HRMS FINGERPRINTING
4. UNTARGETED APPROACHES

4.1 Principle of metabolomic

For each signal (ion m/z), comparison of peak intensities between the 2 groups
4. UNTARGETED APPROACHES

4.1 Principle (ESI -, 70-800 u)

CONTROL ANIMAL

TREATED ANIMAL
4. UNTARGETED APPROACHES

4.1 Principle (EI, 70-800 u)

Bovine urine – Steroids - SPME extraction - BSTFA derivatization
4. UNTARGETED APPROACHES

4.1 Principle: detection of potential candidate biomarkers

“Volcano Plot”: bi-logarithmic representation of all ions \([m/z, \text{rt}]_i\) constituting the fingerprints of interest, i.e. presenting a difference of intensity sufficiently important and significant between the 2 sub-group of analysed samples.

Permit to visualise potential ions of interest, i.e. presenting a difference of intensity sufficiently important and significant between the 2 sub-group of analysed samples.
4. UNTARGETED APPROACHES

4.1 Principle: after identification of candidate biomarkers… biological interpretation

http://www.genome.ad.jp/kegg/pathway/

Jourdan et al., Bioinformatics 2008 24(1):143-145
APPLICATIONS
4. UNTARGTED APPROACHES

4.2 Application: different statistical tools

Objectives: multivariate statistical techniques to reveal the information contained in the large metabolomic data set: ANOVA, PCA, Clustering, LDA, PLS, OPLS, PLS-DA

Supervised methods
PLS-Discriminant Analysis

Kidney samples: control vs. animals treated with anabolic steroids
Extraction with Hexane (kidney samples).
4. UNTARGETED APPROACHES

4.2 Application: critical validation of ion chromatogram - Dose effect

Calves treated with 1, 2 and 4 implants of RevalorS® (140 mg TbAc + 24 mg E2)
4. UNTARGETED APPROACHES

4.2 Application: OPLS, urine sample collected at Day+2, +3, +4

Control calves (6+6) vs. treated calves (3+3) with 500 µg/day of clenbuterol over 6 days
4. UNTARGETED APPROACHES

4.2 Application: extracted ion chromatograms → m/z 132.0 at RT 2.4 min

- m/z : 300.1 ; RT : 17.5 min
- m/z : 312.1 ; RT : 17.3 min
- m/z : 348.1 ; RT : 14.2 min
- m/z : 347.1 ; RT : 20.1 min
- m/z : 132.0 ; RT : 2.4 min
- m/z : 215.2 ; RT : 19.7 min
4. UNTARGETED APPROACHES

4.2 Application: identification of candidate biomarkers

**Creatine**

\[ \text{C}_4\text{H}_9\text{N}_3\text{O}_2 \; ; \; M_i = 131.06978 \]

\[ [\text{M}+\text{H}]^+ \]

\[ m/z_{\text{the}} = 132.07730 \]

\[ m/z_{\text{obs}} = 132.07816 \]

\[ m/z_{\text{obs}} = 132.07811 \]
The rapidly growing metabolomics community is spread around the globe, making communication a major hurdle.

With this in mind, has been developed a Metabolomics Science website with the aim to provide the metabolomics community a useful platform with general information on analytical tools as well as bioinformatics software to perform experiments.
5. CONCLUSION

5.2 Missing criteria: not included in the 2002/657/EC

VALIDATION CRITERIA

Fréquence de distribution des mesures

α = 1%

σB

2,33σB

[0, CCα]

ANALYTICAL CRITERIA

2002/657

HIGH RESOLUTION

FAST/RESOLUTIVE CHROMATOGRAPHY

BIOMARKERS VALIDATION....
LINEAR QUADRUPOLE ION TRAP FOURRIER TRANSFORM (LTQ-ORBITRAP) TO FIGHT AGAINST THE ILLEGAL USE OF GROWTH PROMOTERS IN CATTLE

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