

47th Annual Florida Pesticide Residue Workshop
18th-21st July 2010, St Pete Beach, Florida, USA



CONTROLLING HORMONAL RESIDUES IN ANIMAL BIOLOGICAL MATRICES: FROM CONVENTIONAL TARGETED METHODS TO EMERGING GLOBAL 'OMIC APPROACHES

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www.laberca.org – www.sarraf-educ.org

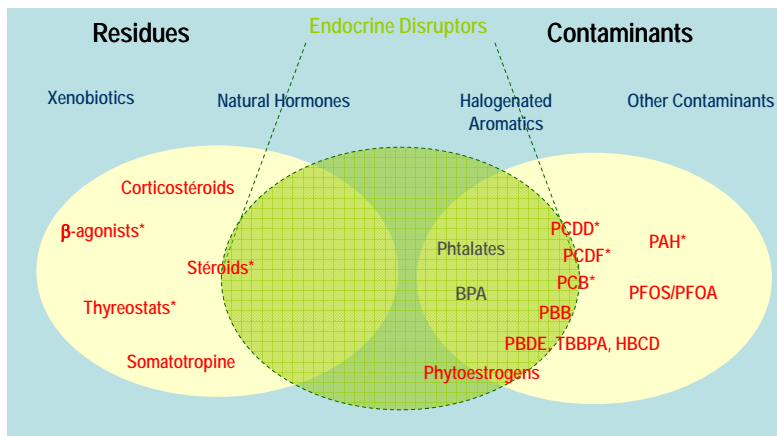
Outline

1. Introduction

2. Targeted approaches

3. Untargeted approaches

4. Conclusion



* National Reference Laboratory (NRL) for the General Directorate for Food (French Ministry of Agriculture)



UPLC-MS/MS (x 1)



LC-HRMSⁿ (x 1)



HPLC (x 2)



GC-MS (x 3)



GC-MS/MS (x 2)



LC-MS/MS (x 2)



GC-HRMS (x 3)



GC-C-IRMS (x 2)



- **GROUP A - Substances having anabolic effect and unauthorized substances**
 - 1 - Stilbens, stilben derivatives, and their salts and esters
 - 2 - Thyrostats
 - 3 - Steroids
 - 4 - Resorcylic acid lactones including zeranol
 - 5 - β -agonists
 - 6 - Compounds included in Annex IV of EC n°2377/90
- **GROUP B - Veterinary drugs and contaminants**
 - 2f - Corticosteroids
- **Analytical Criteria & Validation**

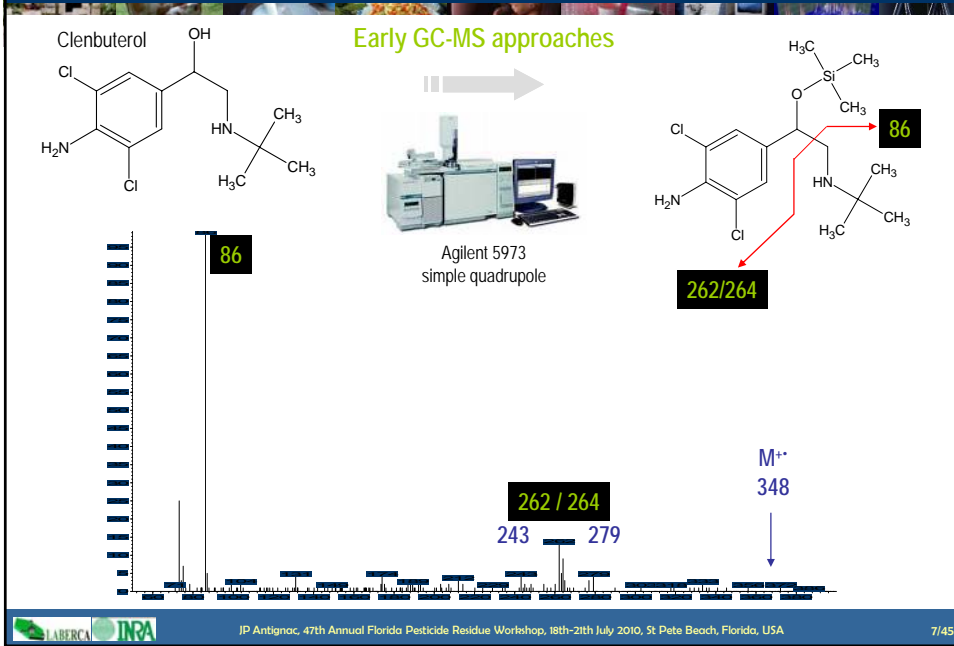
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1. Introduction
2. Targeted approaches
3. Untargeted approaches
4. Conclusion

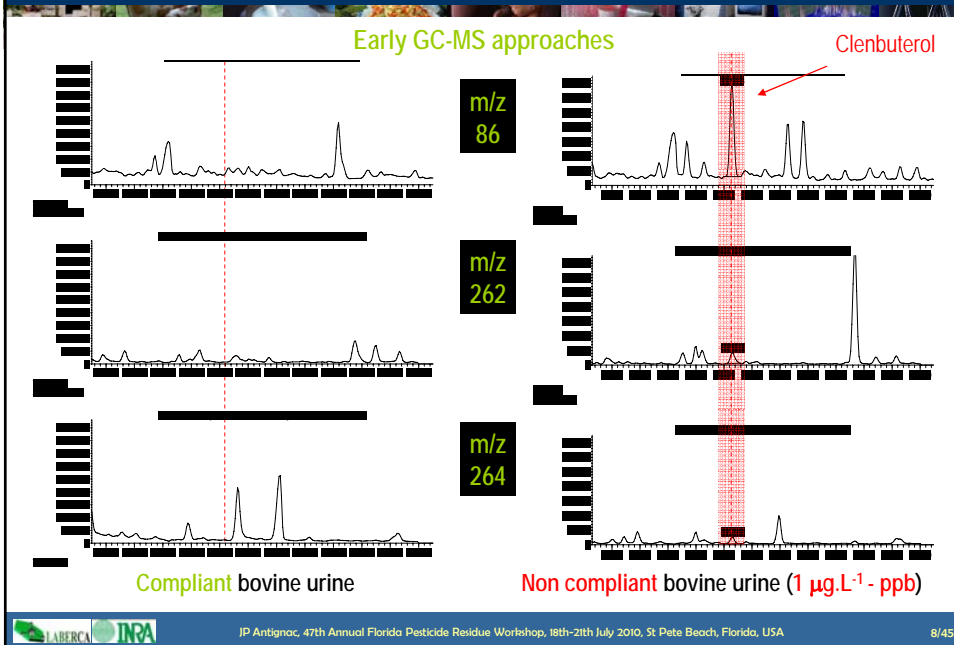
2. Targeted approaches

Beta-agonists



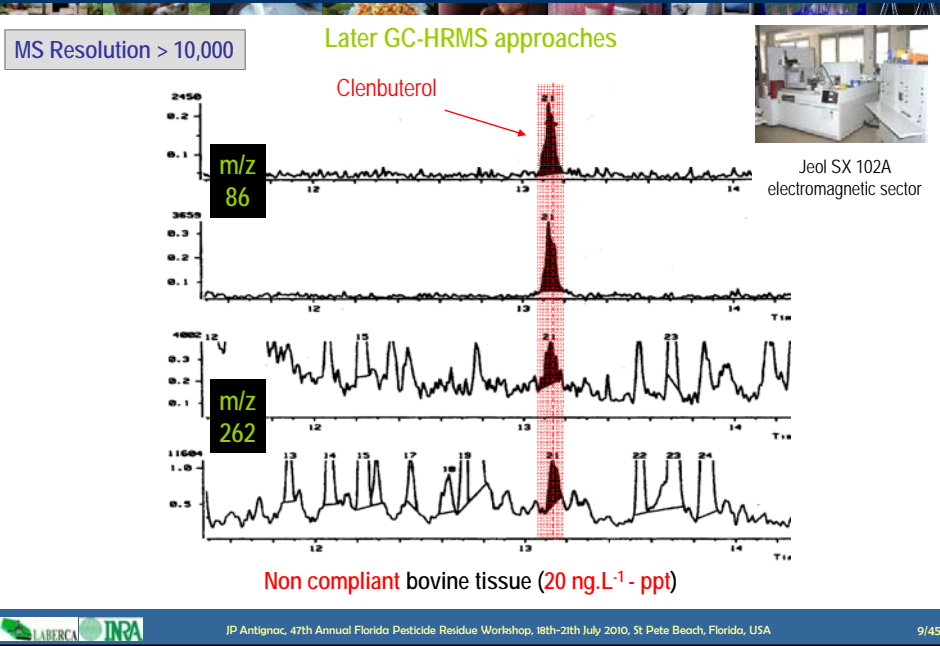
2. Targeted approaches

Beta-agonists



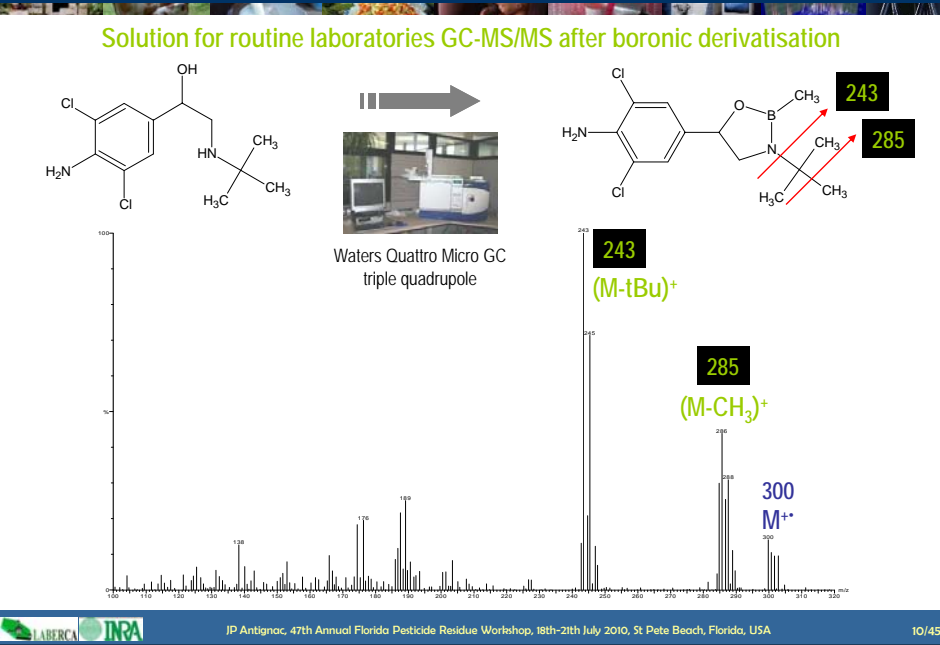
2. Targeted approaches

Beta-agonists



2. Targeted approaches

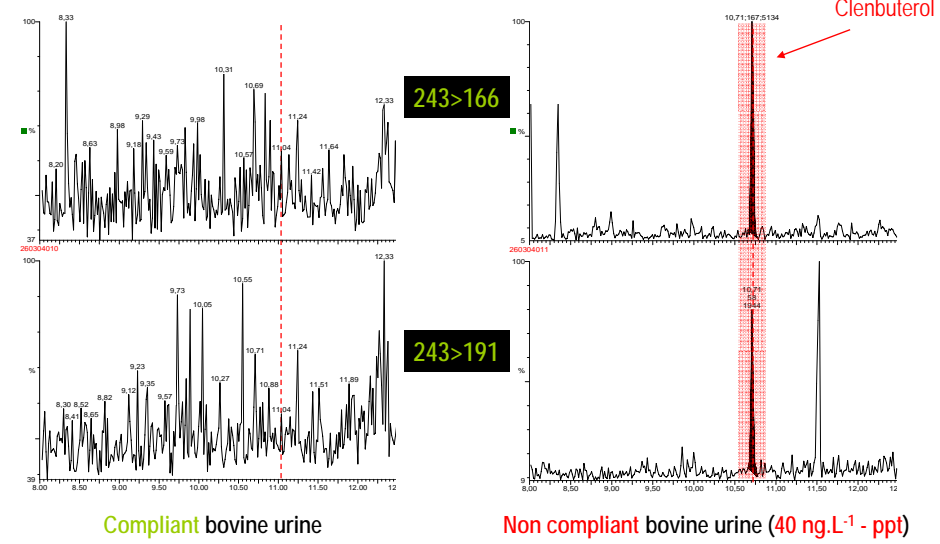
Beta-agonists



2. Targeted approaches

Beta-agonists

Solution for routine laboratories GC-MS/MS after boronic derivatisation



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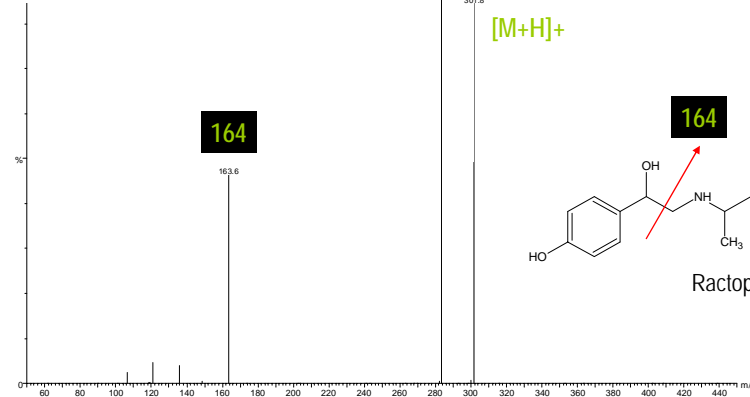
2. Targeted approaches

Beta-agonists

Nowadays LC-MS/MS approach



Waters Xevo TQ / Agilent 6410 triple quadrupole



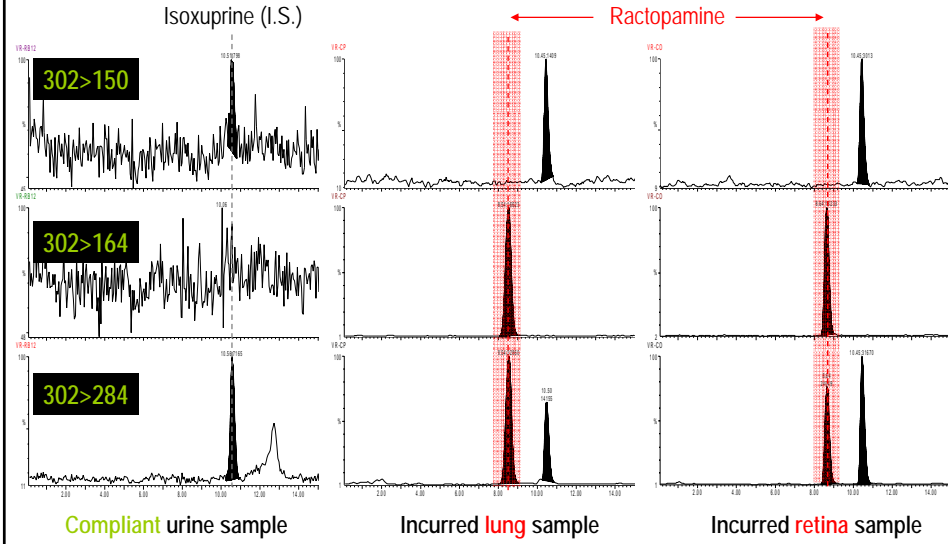
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2. Targeted approaches

Beta-agonists

Nowadays LC-MS/MS approach



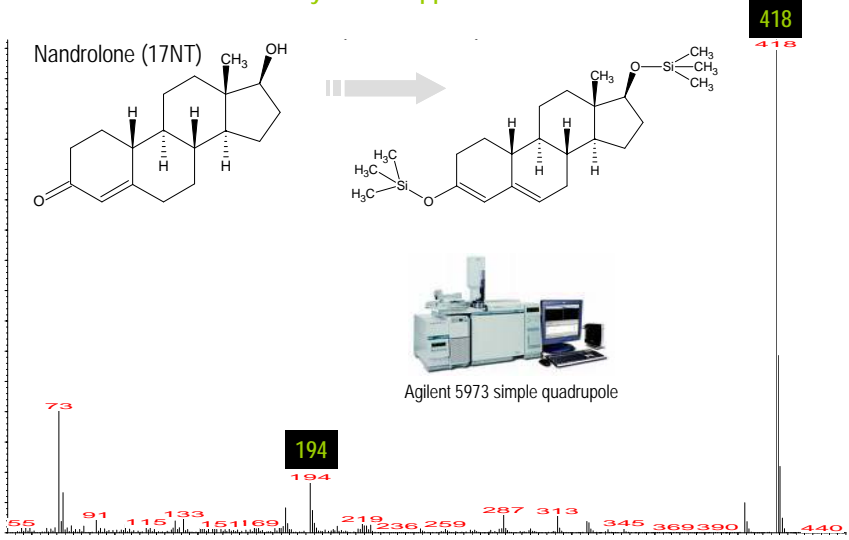
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2. Targeted approaches

Anabolic steroids

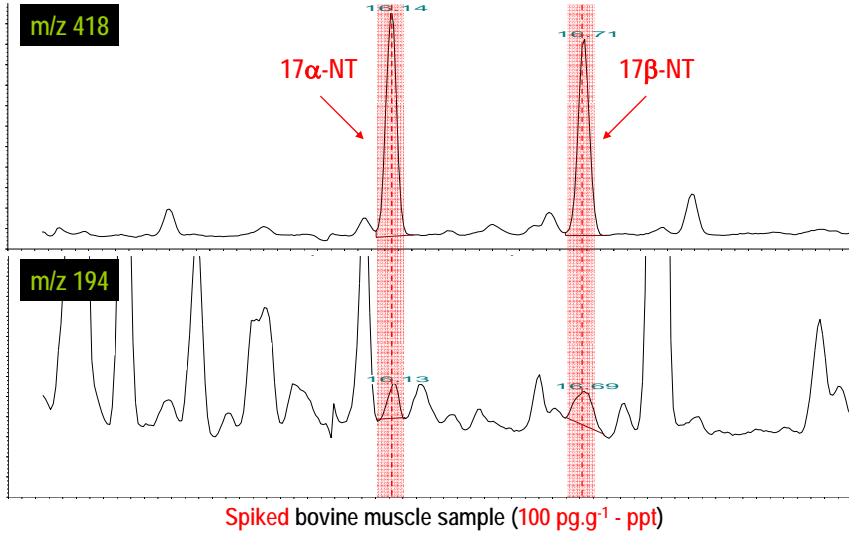
Early GC-MS approaches



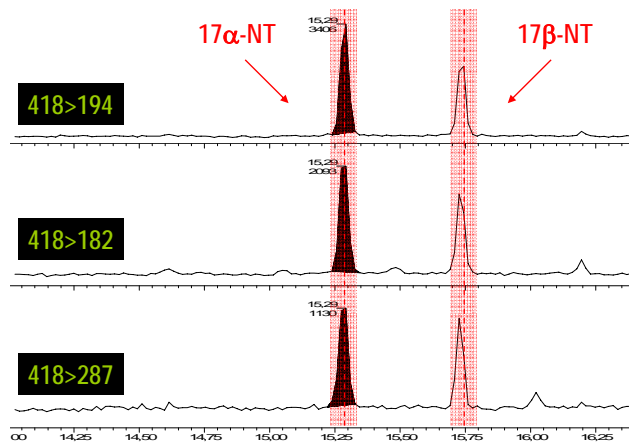
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Early GC-MS approaches



Nowadays GC-MS/MS approaches

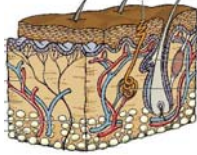


Waters Quattro Micro GC / Agilent 7000 triple quadrupole



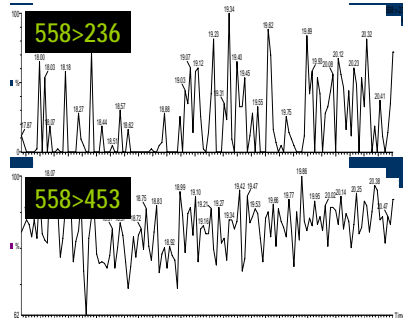
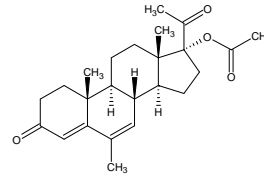
2. Targeted approaches

Anabolic steroids

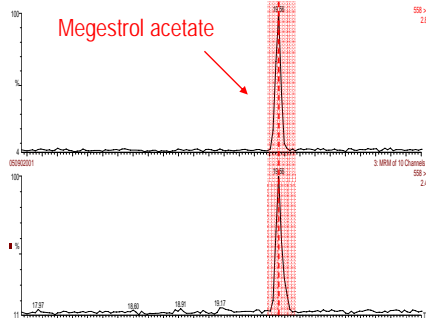


Nowadays GC-MS/MS approaches

Anabolic steroid esters in hair
(Exemple of Megestrol acetate)



Compliant bovine hair sample



Non compliant bovine hair sample

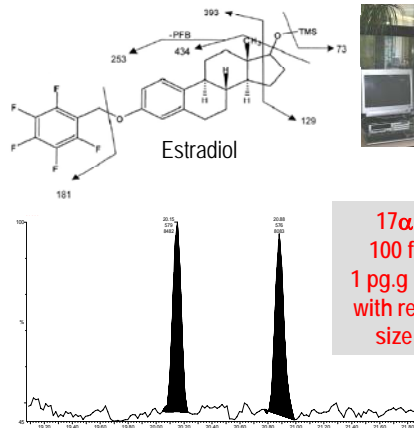
2. Targeted approaches

Anabolic steroids

Most hyphenated targeted GC-MS/MS developments

Still GC-MS/MS (QqQ) measurement... but...

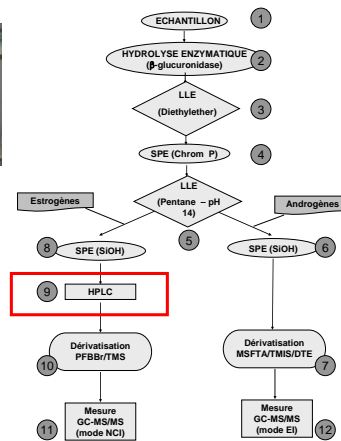
Pentafluorobenzyl bromide derivatisation and negative chemical ionisation



17 α and 17 β -E2
100 fg on-column
1 pg.g (ppt) on matrix
with reduced sample
size (around 1g)

Sample preparation

Until an ultimate purification



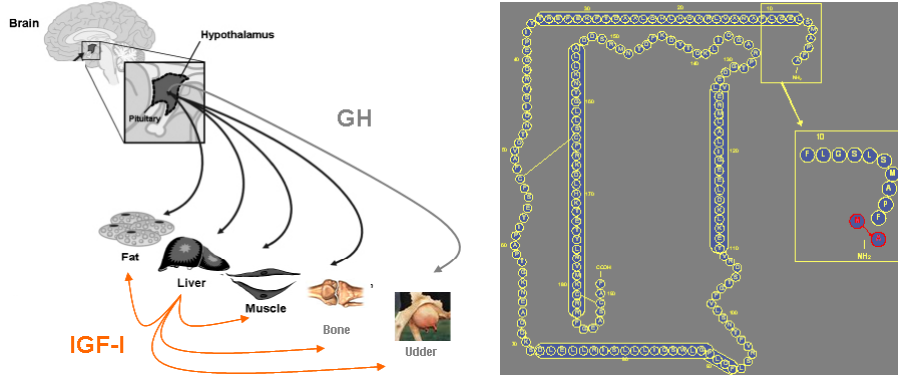
Courant et al., Anal. Chim. Acta 2007:586:105 & J. Agric. Food Chem. 2008:56(9):3176 & J. Clin. Endocr. Metab. 2010:95:82.

2. Targeted approaches

Growth hormone

Detection of the administration of growth hormone (somatotropin, GH) in lactating cow

- **Question:** How to ensure the measurement, and the differentiation from its endogenous form, of this peptidic hormone?

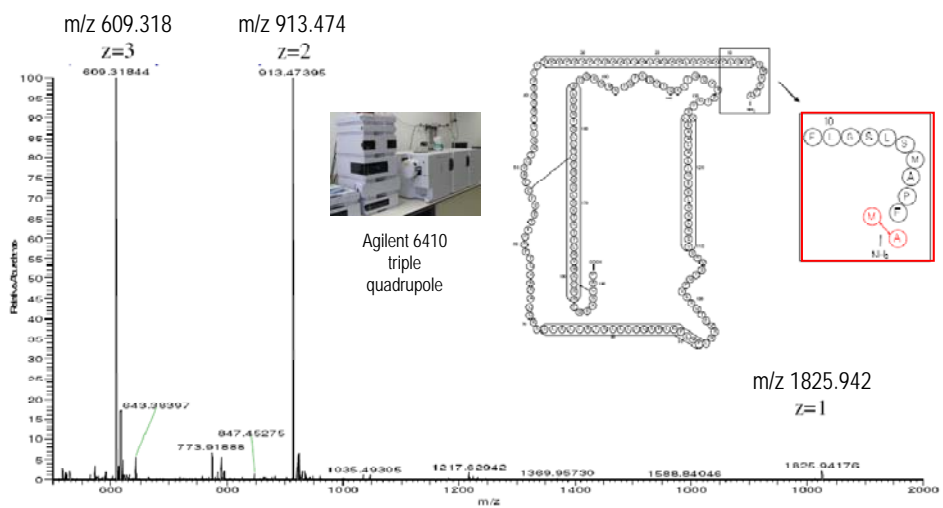


- **Challenges:** extremely low residual concentration levels, elevated mass/molarity ratio, endogenous and exogenous forms differing only by 1 amino acid.

2. Targeted approaches

Growth hormone

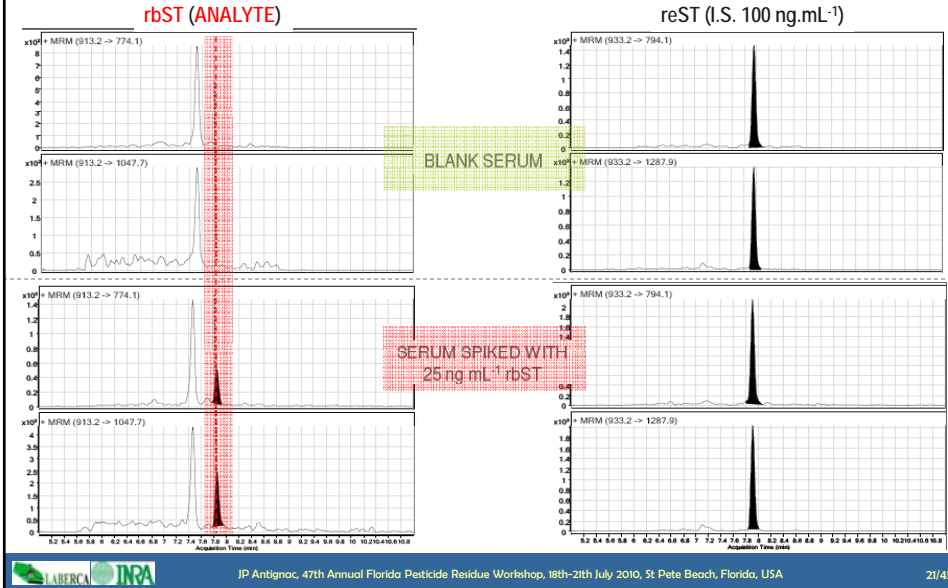
Approach 1: measurement of the N-term peptide by LC-MS/MS



2. Targeted approaches

Growth hormone

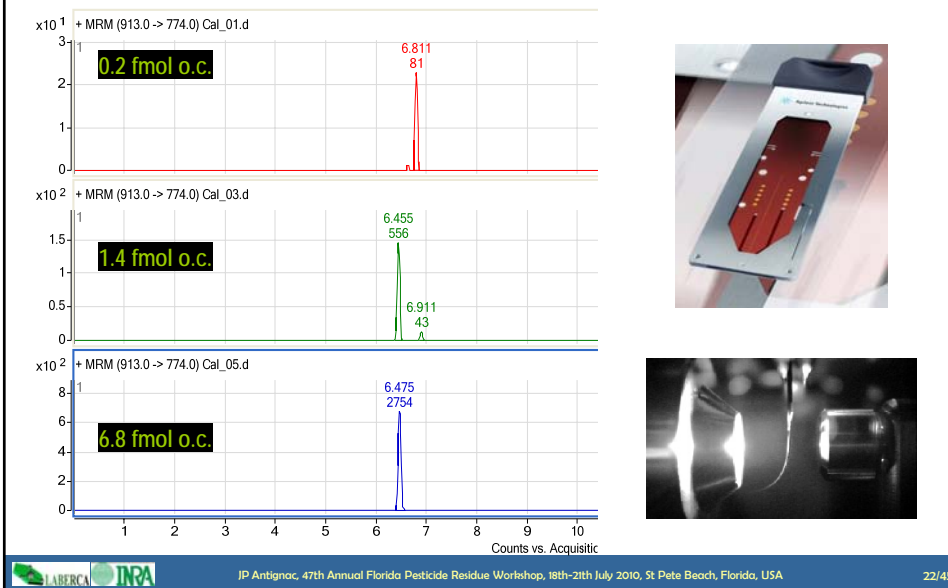
Approach 1: measurement of the N-term peptide by LC-MS/MS



2. Targeted approaches

Growth hormone

Approach 2: measurement of the N-term peptide by LC-CHIP - MS/MS



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3. Untargeted approaches

Metabolomic: an emerging field of exponential development

Year	Number of publications
2000	~10
2001	~20
2002	~30
2003	~50
2004	~100
2005	~200
2006	~400
2007	~700
2008	~1100
2009	~1600
2010	~2300

Results of the "metabolomic or metabonomic" request assessed on <http://www.sciencedirect.com> (13th April 2010).

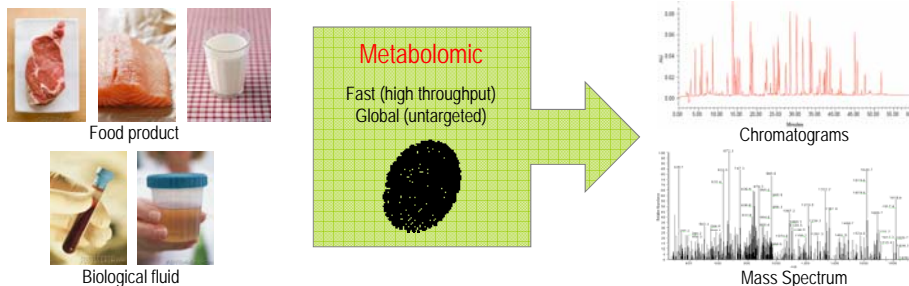
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3. Untargeted approaches

Metabolomic: basic concept and definition

Definition: global characterisation of a biological system through the simultaneous measurement of any metabolite present in this system and accessible to the analysis

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



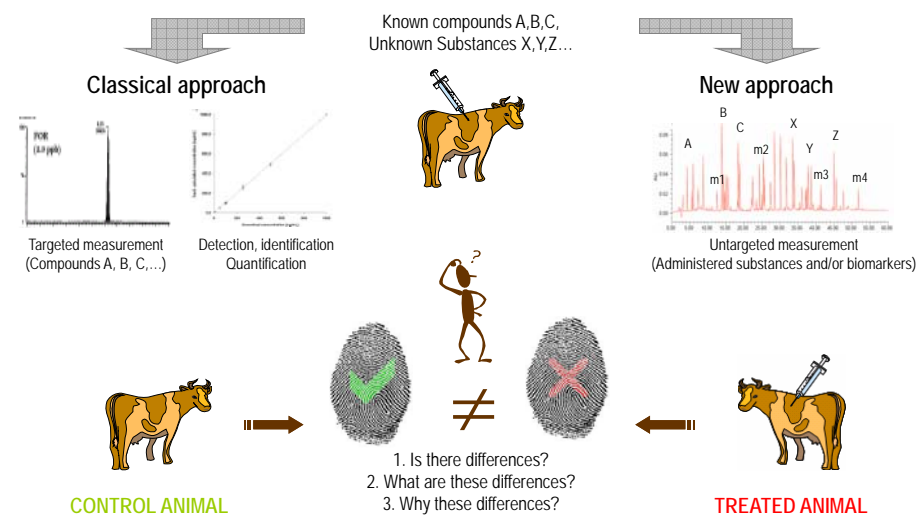
- Objectives:**
- List each detected compound constituting the metabolic profiles : systematic metabolomic
 - Reveal similarities or dissimilarities between sub-groups of samples : differential metabolomic
 - Use the metabolic profiles (or biomarkers) for diagnostic purpose : quantitative metabolomic
 - Investigate metabolic perturbations without identification imperative : systemic metabolomic

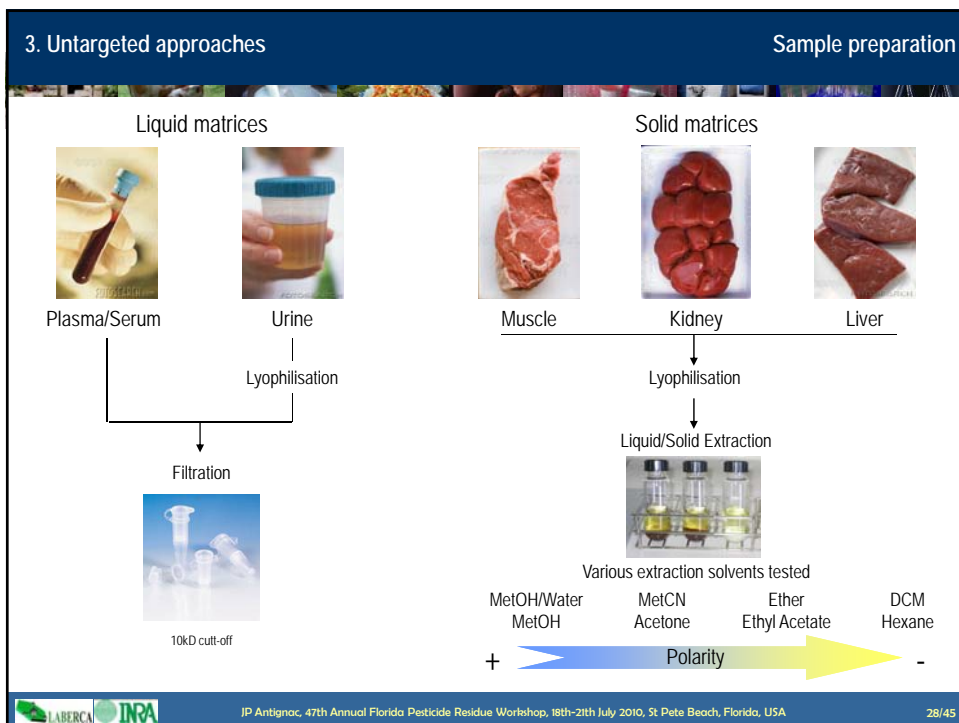
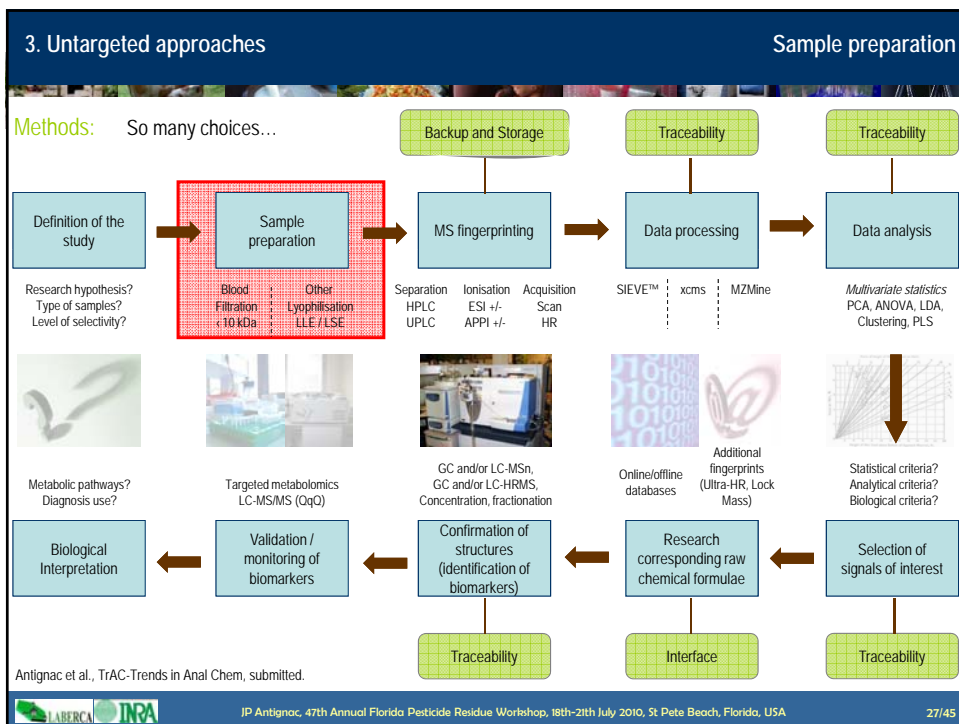


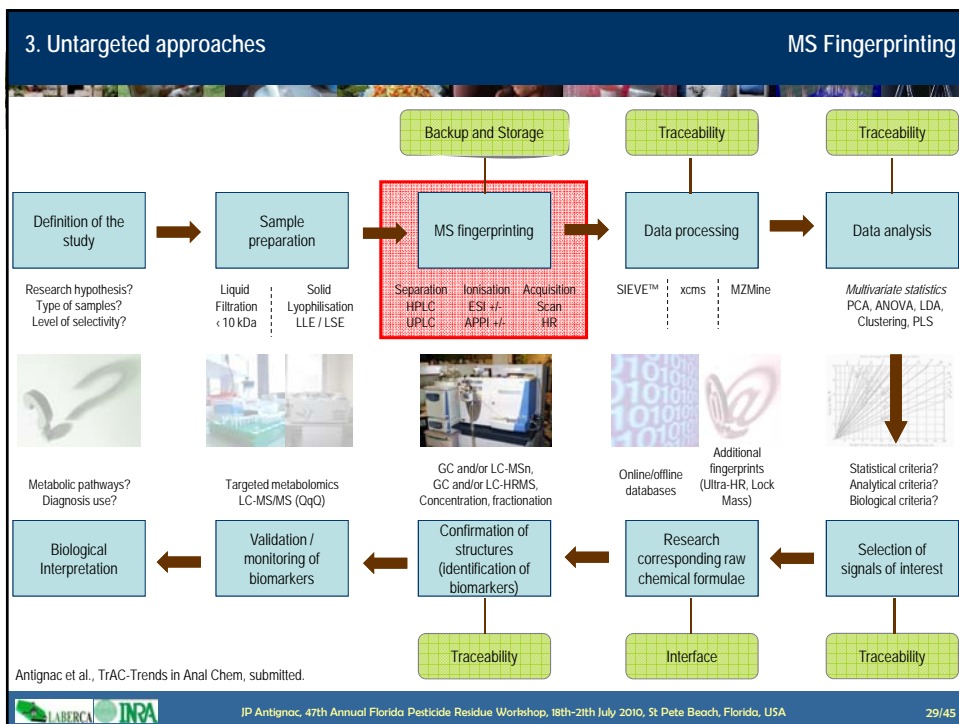
3. Untargeted approaches

MS-based metabolomic at LABERCA: various contexts / projects

Metabolomic as new screening strategy for controlling use of growth promoters in cattle







3. Untargeted approaches

MS Fingerprinting

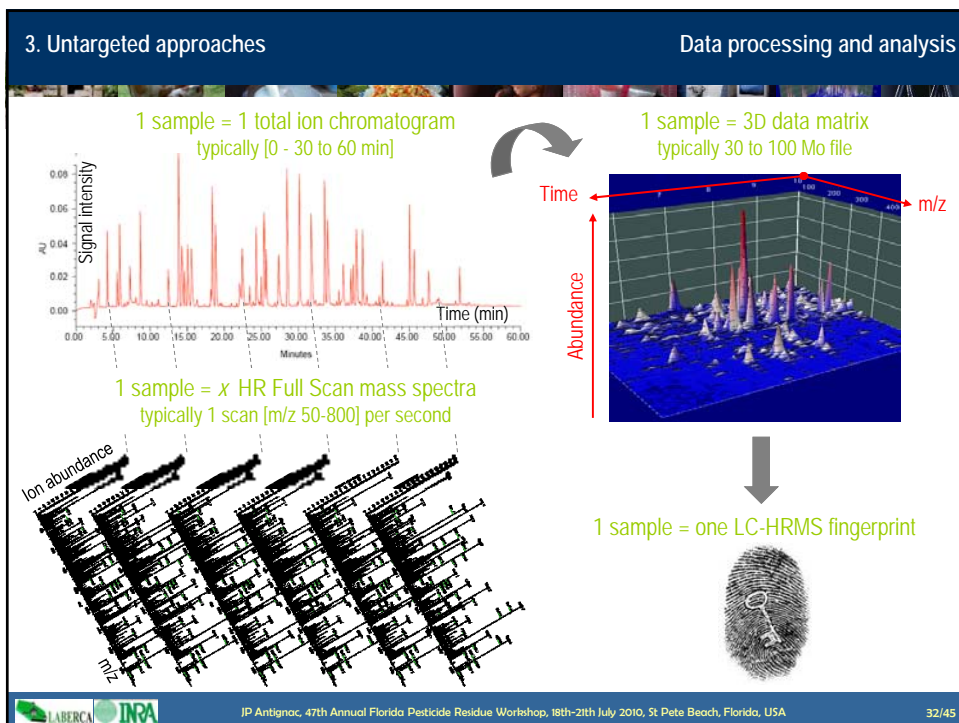
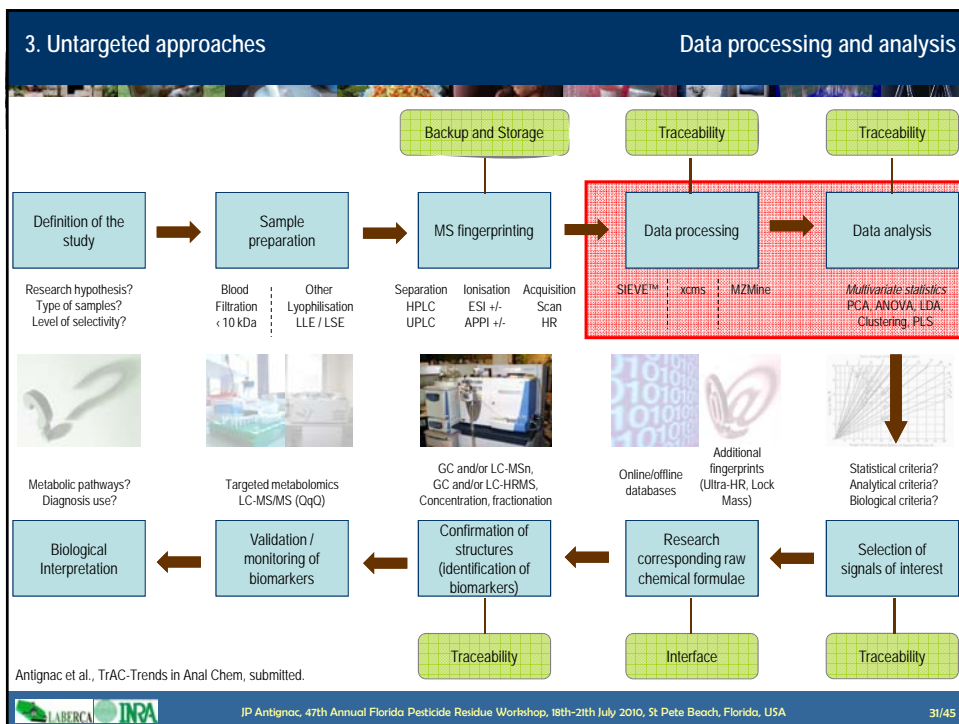
Thermo LTQ-Orbitrap™ instrument... but also... TOF and Q-TOF

- What Chromatographic conditions ?
Choice of a fast-LC reverse phase separation system
- What tuning conditions ?
Choice of a "generic" tuning optimization process
- What measurement repeatability?
Systematically evaluated for each application
- Need for signal normalization?
Various methods tested : total signal, single or multiple IS
- What quality control procedures?
Reference substances and samples, randomization,...

Full Scan mode (m/z 50 to 800... or higher)
High resolution (30,000 to 100,000)

LABERCA INRA

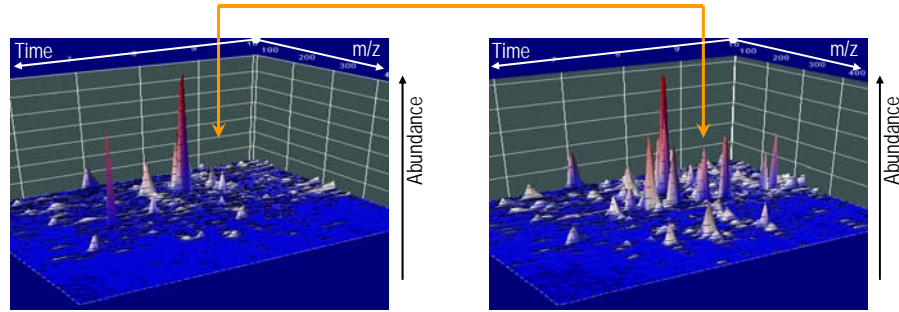
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3. Untargeted approaches

Data processing and analysis

Data processing: peak alignment, peak picking and peak comparing
(Compare the signal abundances observed for similar ions in two or more groups of samples)



Typical « Control » Sample
(ex. from control animal)

Typical « Test » Sample
(ex. from anabolised animal)

Statistical criteria F (ANOVA) or t (student test) associated to a statistical confidence level (p-value)



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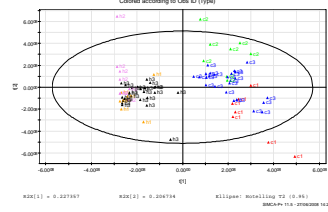
3. Untargeted approaches

Data processing and analysis

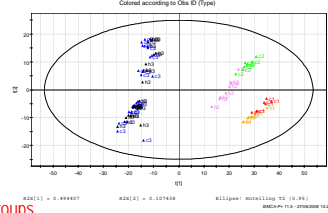
Data analysis: extract the most useful information from the "metabolic soup"
(peak sorting and multivariate statistical analyses)

ID / statistical descriptors	Samples ...	
	Control	Test
1	0.0000	0.0000
2	0.0000	0.0000
3	0.0000	0.0000
4	0.0000	0.0000
5	0.0000	0.0000
6	0.0000	0.0000
7	0.0000	0.0000
8	0.0000	0.0000
9	0.0000	0.0000
10	0.0000	0.0000
11	0.0000	0.0000
12	0.0000	0.0000
13	0.0000	0.0000
14	0.0000	0.0000
15	0.0000	0.0000
16	0.0000	0.0000
17	0.0000	0.0000
18	0.0000	0.0000
19	0.0000	0.0000
20	0.0000	0.0000
21	0.0000	0.0000
22	0.0000	0.0000
23	0.0000	0.0000
24	0.0000	0.0000
25	0.0000	0.0000
26	0.0000	0.0000
27	0.0000	0.0000
28	0.0000	0.0000
29	0.0000	0.0000
30	0.0000	0.0000
31	0.0000	0.0000
32	0.0000	0.0000
33	0.0000	0.0000
34	0.0000	0.0000
35	0.0000	0.0000
36	0.0000	0.0000
37	0.0000	0.0000
38	0.0000	0.0000
39	0.0000	0.0000
40	0.0000	0.0000
41	0.0000	0.0000
42	0.0000	0.0000
43	0.0000	0.0000
44	0.0000	0.0000
45	0.0000	0.0000
46	0.0000	0.0000
47	0.0000	0.0000
48	0.0000	0.0000
49	0.0000	0.0000
50	0.0000	0.0000

Unsupervised analyses (ex. PCA)



Supervised analyses (ex. OPLS)



Ratio/Fold: difference of abundance observed in average between control and test groups
p-value: statistical confidence level associated to this observed differences



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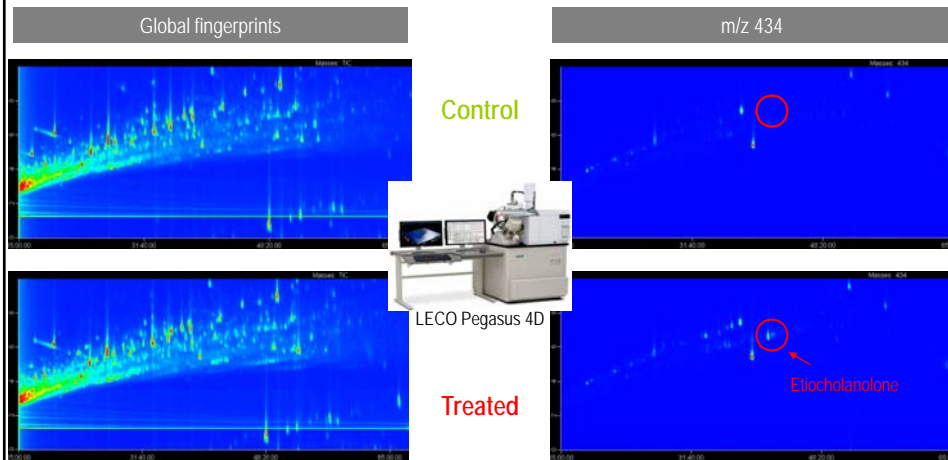
3. Untargeted approaches

STERNAT project: natural steroid hormones

Analytical approach 2 : GCxGC-(EI+)-HRMS

Control versus treated bovines with androstenedione (250 mg, oral route)

Sample preparation focused on total free steroids i.e. : deconjugation, extraction by MEPS C₁₈, TMS derivatisation



Anizan et al., manuscript in preparation.

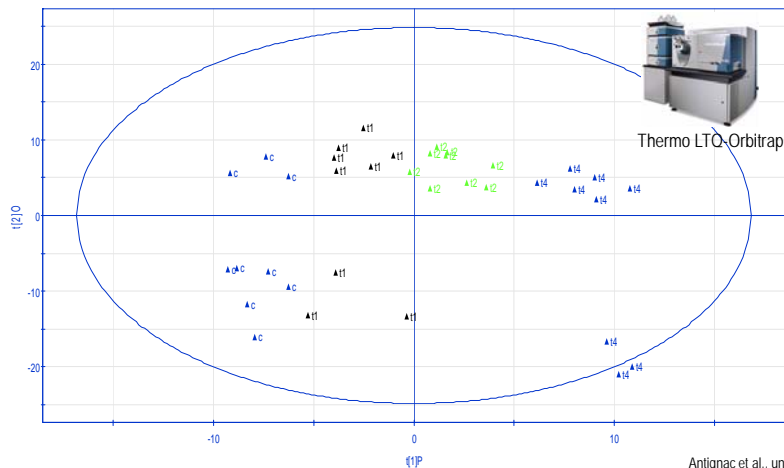
3. Untargeted approaches

STERNAT project: natural steroid hormones

Analytical approach 3 : LC-(ESI+)-HRMS

Control (n=5) versus treated (n=3x5) calves with 1, 2 ou 4 RevalorS® implants (140 mg TbAc + 24 mg E₂)

OPLS performed on metabolic fingerprints generated from kidney samples (D90) after hexane extraction

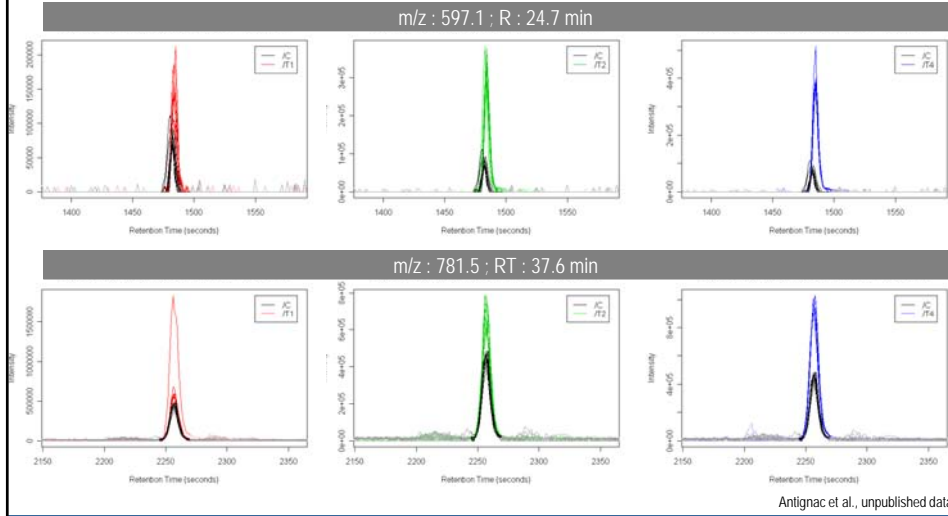


Antignac et al., unpublished data.

3. Untargeted approaches

STERNAT project: natural steroid hormones

Analytical approach 3 : LC-(ESI+)-HRMS
 Control ($n=5$) versus treated ($n=3 \times 5$) calves with 1, 2 ou 4 RevalorS® implants (140 mg TbAc + 24 mg E₂)



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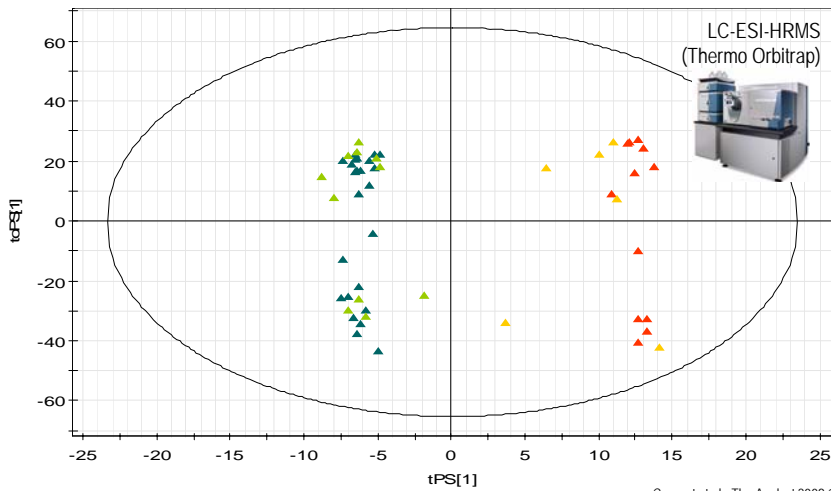
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3. Untargeted approaches

CRIPAGO project: β -Agonists

Control ($n=6+6$) versus treated ($n=3+3$) calves with clenbuterol (*per os*, 500 μ g/day for 6 days)

OPLS performed on metabolic fingerprints generated from urine samples (D2, D3, D4) after lyophilisation/filtration



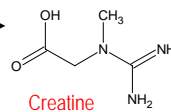
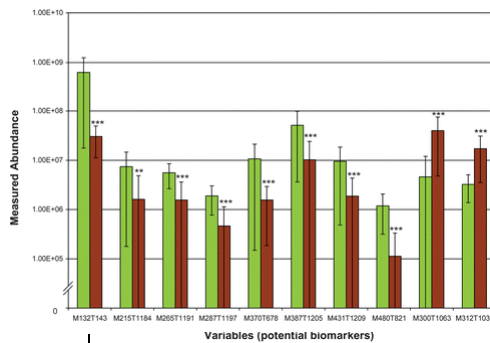
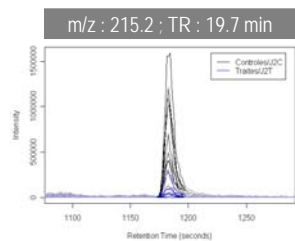
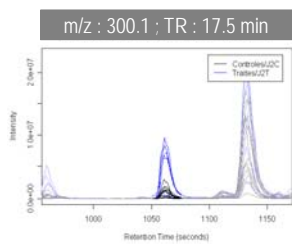
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3. Untargeted approaches

CRIPAGO project: β -Agonists

Control ($n=6+6$) versus treated ($n=3+3$) calves with clenbuterol (*per os*, 500 $\mu\text{g}/\text{day}$ for 6 days)



$[\text{M}+\text{H}]^+$
 $m/z = 132,07730$
 $\text{C}_4\text{H}_9\text{N}_3\text{O}_2$; $M_i = 131,06978$

Courant et al., The Analyst 2009;134:1637-1646.



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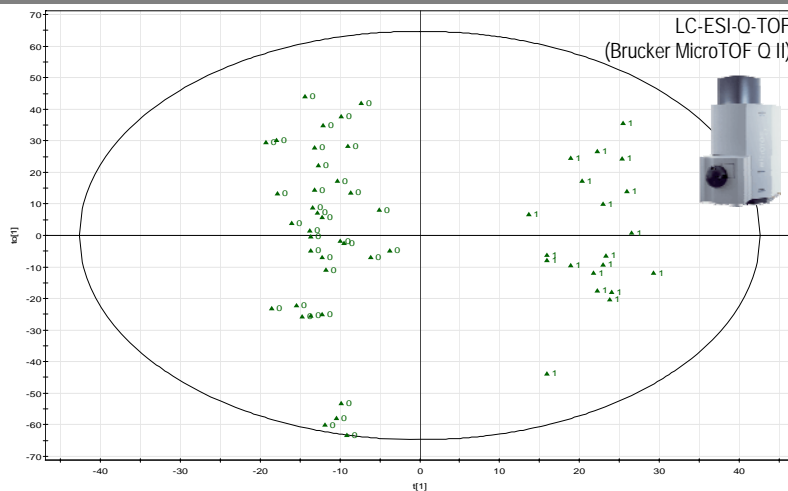
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3. Untargeted approaches

SOMATOPRINT project: Growth hormone

Control ($n=7$) versus treated ($n=6$) horses with reGH (SC injections, 18 $\mu\text{g}/\text{kg}/\text{day}$ for 2 weeks)

OPLS performed on metabolic fingerprints generated from plasma samples (D3 to D25) after filtration



Kieken et al., Anal. Bioanal. Chem. 2009;394:2119-2128 & Metabolomics 2010, in press.



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Horses ($n=2$) before and after treatment with reGH (SC injections, 18 and 25 $\mu\text{g}/\text{kg}/\text{day}$ for 2 weeks)

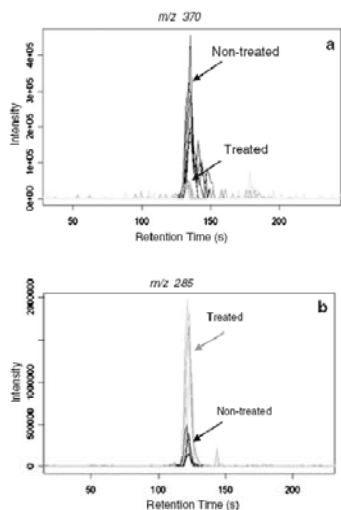


Table 1 List of ions that were the most correlated to the discriminant axis and that were selected as potential biomarkers

ION ID	[M+H] ⁺	Retention Time (min)	Fold
M401T121	400.920	2.02	43.52
M285T123	284.804	2.05	10.08
M301T101	301.011	1.68	17.00
M303T130	303.034	2.17	2.96
M370T136	369.969	2.27	26.20
M136T144	136.048	2.40	14.43
M190T1537	189.546	25.62	1.53
M215T118	214.844	1.97	1.81
M377T1368	377.144	22.80	3.88
M372T1566	372.273	26.10	1.60
M87T169	87.098	2.82	1.67
M436T1540	436.158	25.67	1.74
M30411469	304.161	24.48	1.38
M259T1615	259.096	26.92	1.53
M315T178	315.225	2.97	1.51
M338T1318	338.086	21.97	1.52
M222T135	222.019	2.25	1.53
M211T124	210.872	2.07	4.29
M313T123	312.837	2.05	4.14
M311T123	310.841	2.05	4.76

Kieken et al., Anal. Bioanal. Chem. 2009;394:2119-2128 & Metabolomics 2010, in press.

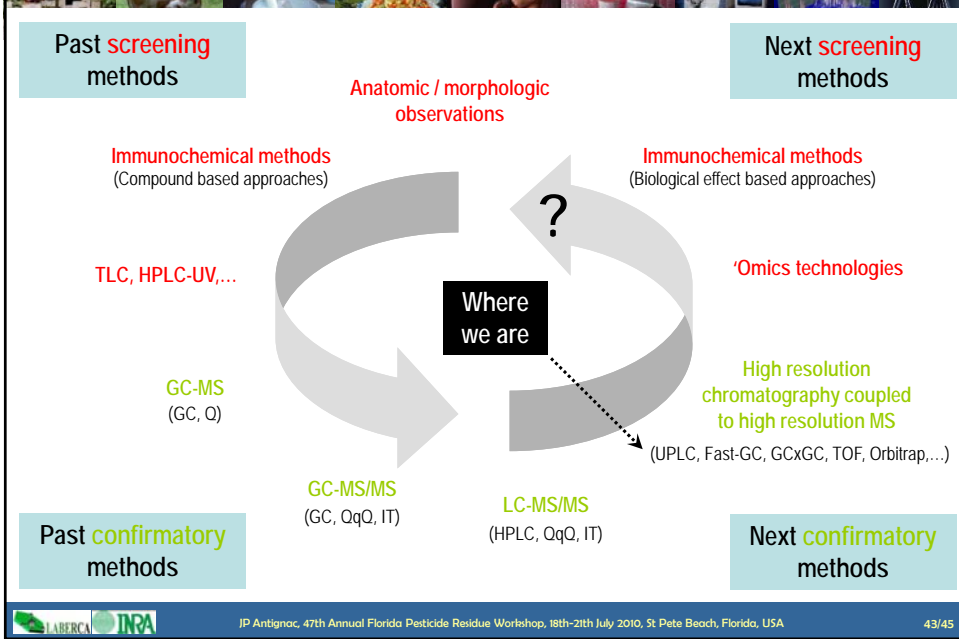


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From Targeted approaches...

Exhaustive list of known compounds



... To Untargeted approaches

Opened and without *a priori* globally detected substances / signals / effect



Acknowledgements

Our "residue" and metabolomic research teams

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Fanny Kleken
Sébastien Anizan
Flavia Hanganu

- **Post-doc student**

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Gwenaëlle Le Gall

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Our financial supports



and you for your attention

Head of the group

Pf. Bruno Le Bizec

