

RIVM WORKSHOP
 Bilthoven, The Netherlands (20th October 2006)


LABERCA and the BOLDENONE STORY
 MARKERS AND CURRENT METHODS

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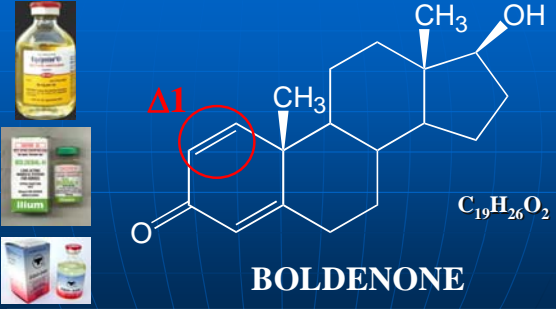
Laboratoire d'Etude des Résidus et Contaminants dans les Aliments
 Ecole Nationale Vétérinaire de Nantes - France - www.laberca.org

OUTLINE



- 1 . Introduction
- 2 . First attempt to point out a metabolite marker
- 3 . Second attempt presumed successful
- 4 . GC-C-IRMS
- 5 . Conclusions

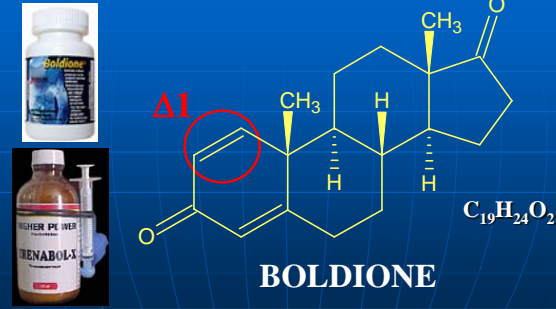
1 . INTRODUCTION
 1.1 – BOLDENONE CHEMICAL STRUCTURE



CC12CCC3C(C1)C(=O)CC=C3C2 $C_{19}H_{26}O_2$

BOLDENONE

1 . INTRODUCTION
 1.2 – BOLDENONE VARIANT



CC12CCC3C(C1)C(=O)CC=C3C2 $C_{19}H_{24}O_2$

BOLDIONE

1 . INTRODUCTION
 1.3 – SINCE THE EU EXPERT GROUP CONCLUSIONS: NEW THINGS?

EU EXPERTS WORKING GROUP on BOLDENONE DRAFT 2.5 (5/5/2003) p. 1/26


EU EXPERT REPORT on BOLDENONE: DRAFT 2.5

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2 . MARKER METABOLITE: THE HOLY GRAAL

2.1 – OBJECTIVES

Find a candidate diagnostic metabolite marker fulfilling the following conditions:

- Always present whatever the treatment
- Sufficiently abundant for the long-term detection of steroid abuse
- Never found in non-treated animals

2 . MARKER METABOLITE: THE HOLY GRAAL

2.2 – ANIMAL EXPERIMENT

- A** • oral treatment with boldenone + ADD
1 day oral treatment with ADD (0,2 mg/ml) + β -BOLD (2 mg/ml), single administration dose = 5 mg/calf/diet
- I** **B** • oral treatment with boldenone esters
3 days oral treatment with Boldenone esters (BOLD acetate, BOLD benzoate, BOLD propionate, 2+2+1) dose =100 mg/calf/diet
- C** • oral treatment with ADD
6 days oral treatment with ADD, dose = 100 mg/calf/diet
- po** • oral treatment with boldenone esters
5 days oral treatment with BOLD esters, dose = 100 mg/calf/diet
- NL** **IM** • intramuscular injection of boldenone undecylenate
250 mg IM BOLD esters
- F** **po** • oral treatment with ADD

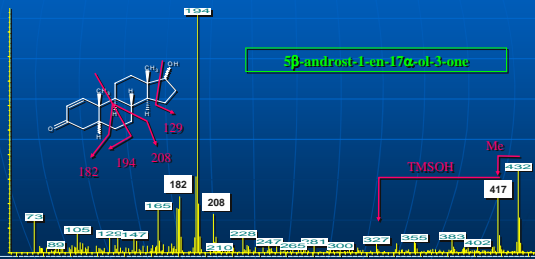
2 . MARKER METABOLITE: THE HOLY GRAAL

2.3 – CHEMICAL STRUCTURE ELUCIDATION

M2

M^+ =432
GC=11.55 min
LC= VI-VII

BOLD+ADD (po)	BOLD ESTERS (po)	ADD (po)	BOLD ESTERS (po)	BOLD ESTERS (IM)
++	+	+++	+++	++



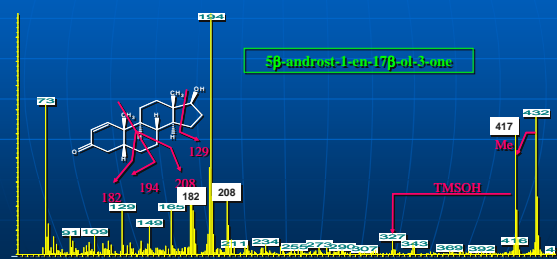
2 . MARKER METABOLITE: THE HOLY GRAAL

2.3 – CHEMICAL STRUCTURE ELUCIDATION

M4

M^+ =432
GC=12.00 min
LC= VII

BOLD+ADD (po)	BOLD ESTERS (po)	ADD (po)	BOLD ESTERS (po)	BOLD ESTERS (IM)
++	+	+	+	++



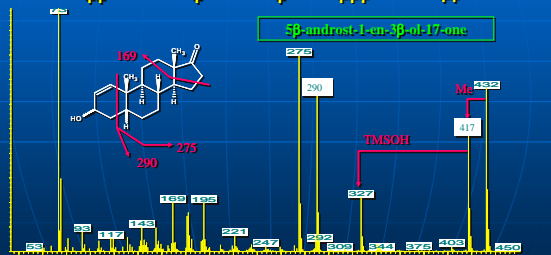
2 . MARKER METABOLITE: THE HOLY GRAAL

2.4 – FIRST ATTEMPT FAILED

M5

M^+ =432
GC=12.39 min
LC=VI-VII

BOLD+ADD (po)	BOLD ESTERS (po)	ADD (po)	BOLD ESTERS (po)	BOLD ESTERS (IM)
++	+	+	+++	++



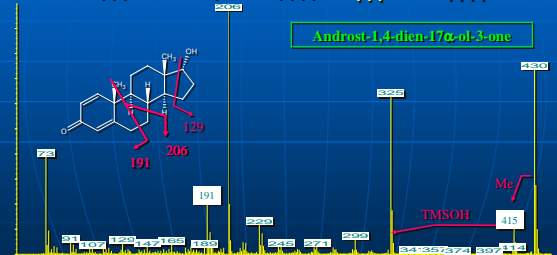
2 . MARKER METABOLITE: THE HOLY GRAAL

2.4 – FIRST ATTEMPT FAILED

M8

M^+ =430
GC=13.02 min
LC= VII

BOLD+ADD (po)	BOLD ESTERS (po)	ADD (po)	BOLD ESTERS (po)	BOLD ESTERS (IM)
+++	+	+++	+++	+++



2 . MARKER METABOLITE: THE HOLY GRAAL

2.4 – FIRST ATTEMPT FAILED

	Metabolite structure	RT (min)	A	B	C	im	po
M1	β -androst-ene-3,17dione	1192 min	ND	ND	++	+	++
M2	β -androst-en-17 α -ol-3-one	1155 min	++	+	+++	++	+++
M3	α -androst-en-17 ζ -ol-3-one	1267 min	ND	ND	+	+	++
M4	β -androst-en-17 β -ol-3-one	1200 min	++	+	+	++	+
M5	β -androst-en-3 β -ol-17-one	1239 min	++	+	+	++	+++
M6	β -androst-en-3 α -ol-17-one	1283 min	ND	ND	ND	++	ND
M7	Androst,4-diene β ,17dione	1321 min	ND	ND	++	ND	++
M8	Androst,4-dien-17 α -ol-3-one	1302 min	+++	+	++++	++++	+++
M9	Androst,4-dien-17 β -ol-3-one	1338 min	+	ND	++	++	ND

2 . MARKER METABOLITE: THE HOLY GRAAL

2.4 – FIRST ATTEMPT FAILED

- DETERMINE BOLD + ADD METABOLITES IN URINE
M1, M2, M3, M4, M5, M6, M7, M8, M9

- PRESENT WHATEVER THE TREATMENT
M2, M4, M5, M8

- SUFFICIENTLY ABUNDANT
M2, M4, M8

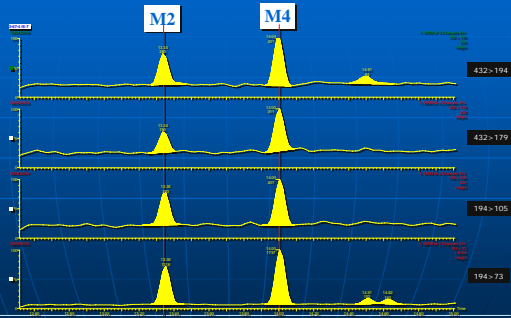
- NOT FOUND IN NON-TREATED ANIMALS

M2, M4

2 . MARKER METABOLITE: THE HOLY GRAAL

2.4 – FIRST ATTEMPT FAILED

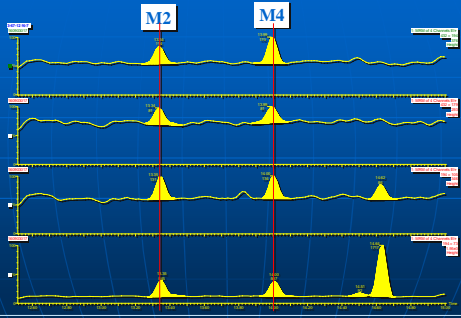
BOLD+ADD (po)



2 . MARKER METABOLITE: THE HOLY GRAAL

2.4 – FIRST ATTEMPT FAILED

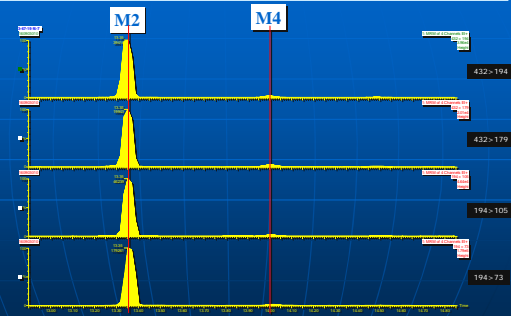
BOLD ESTERS (po)



2 . MARKER METABOLITE: THE HOLY GRAAL

2.4 – FIRST ATTEMPT FAILED

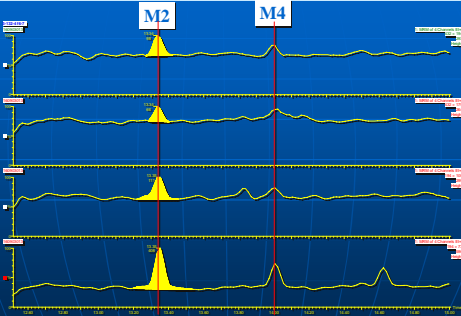
ADD (po)



2 . MARKER METABOLITE: THE HOLY GRAAL

2.4 – FIRST ATTEMPT FAILED

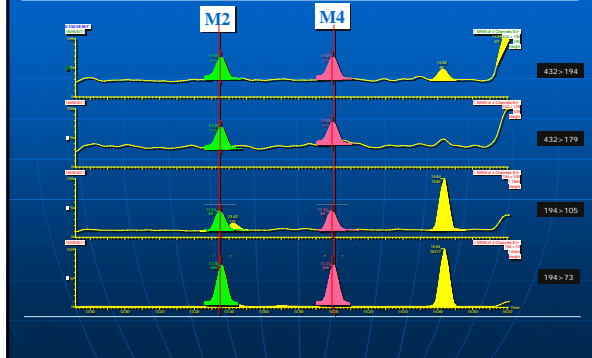
BOLD ESTERS (IM)



2. MARKER METABOLITE: THE HOLY GRAAL

2.4 – FIRST ATTEMPT FAILED

Non treated (17 α BOLD>0)



OUTLINE

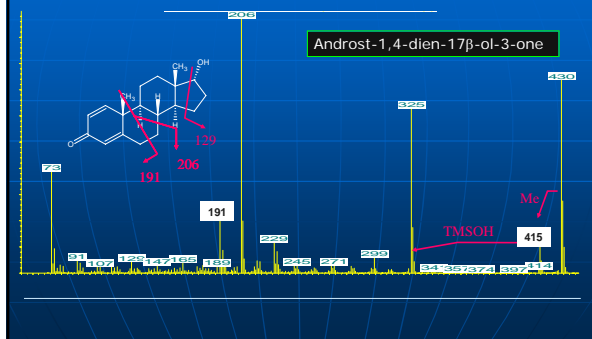


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3. SECOND ATTEMPT PRESUMED SUCCESSFUL

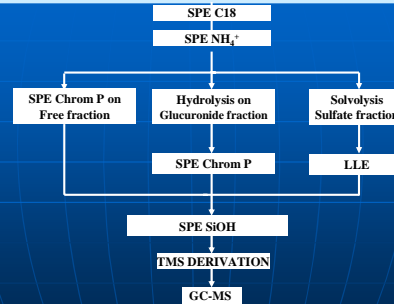
3.1 – 17 α - and 17 β -BOLDENONE

M9 M^+ =430
GC=13.38 min
LC= VII



3. SECOND ATTEMPT PRESUMED SUCCESSFUL

3.2 – ANALYTICAL STRATEGY FOR PHASE II METABOLISM STUDY

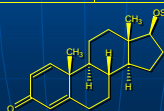


3. SECOND ATTEMPT PRESUMED SUCCESSFUL

3.3 – PHASE II METABOLISM: TENDANCY

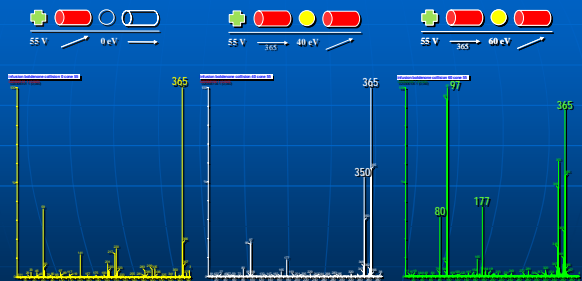
A=ORAL TREATMENT WITH BOLDENONE+ADD

Metabolite structure	Glucuronide	Sulfate	Free
M2 5 β -androst-1-en-17 α -ol-3-one	100	0	0
M4 5 β -androst-1-en-17 β -ol-3-one	100	0	0
M5 5 β -androst-1-en-3 β -ol-17-one	100	0	0
M8 17 α -boldenone	94	1	5
M9 17 β -boldenone	78	22	0
M7 Androsta-1,4-diene-3,17-dione	0	0	100

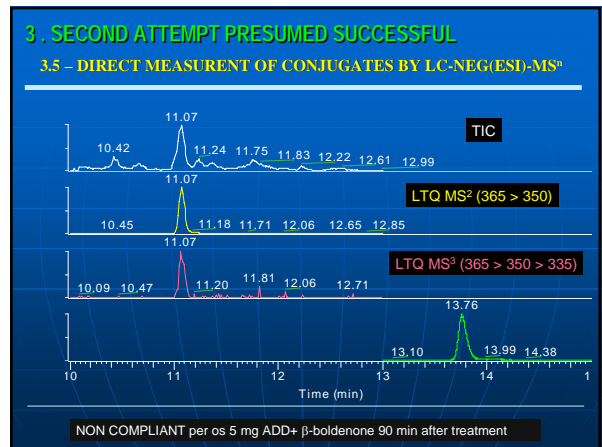
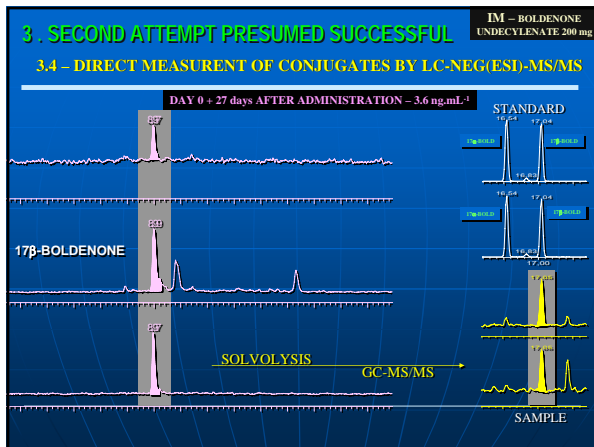
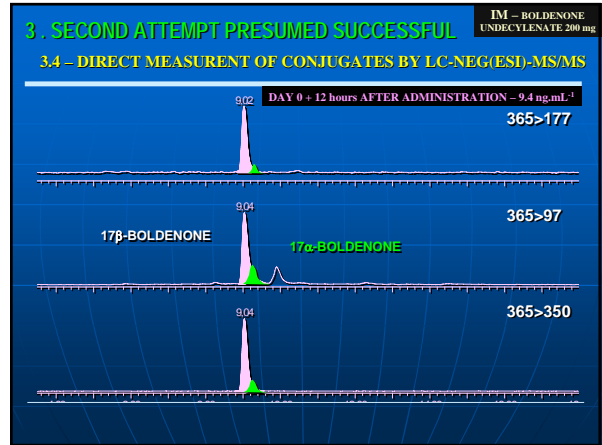
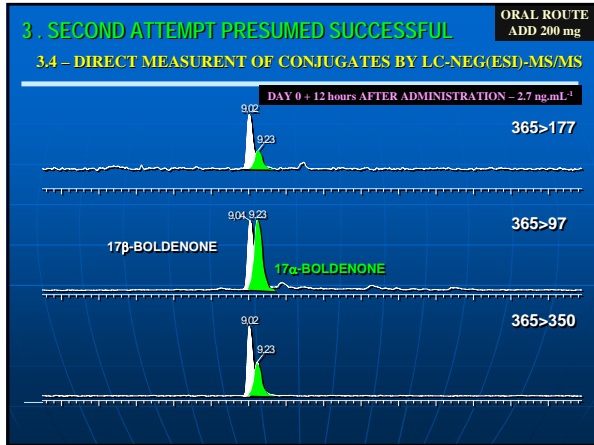
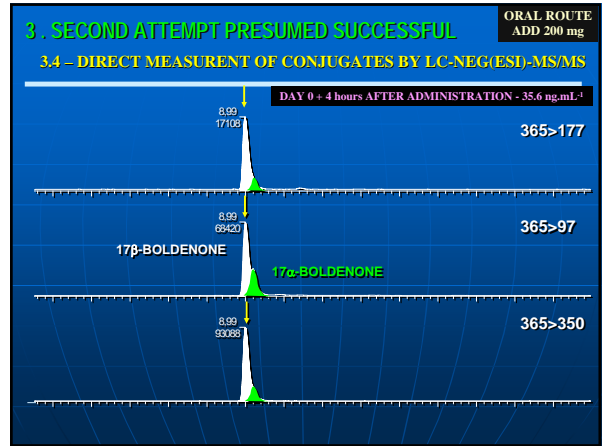
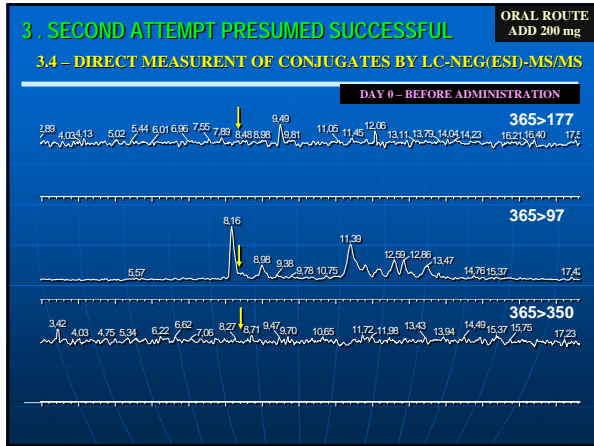


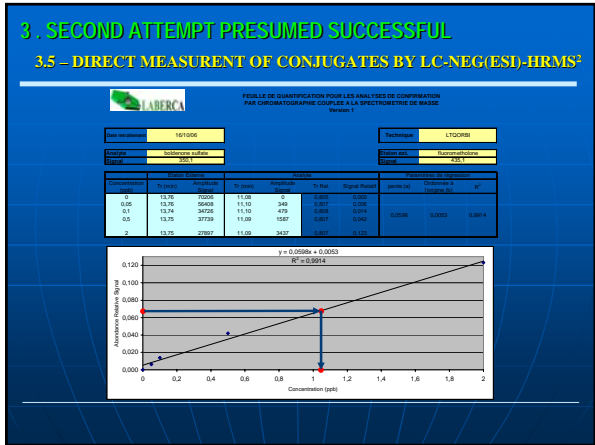
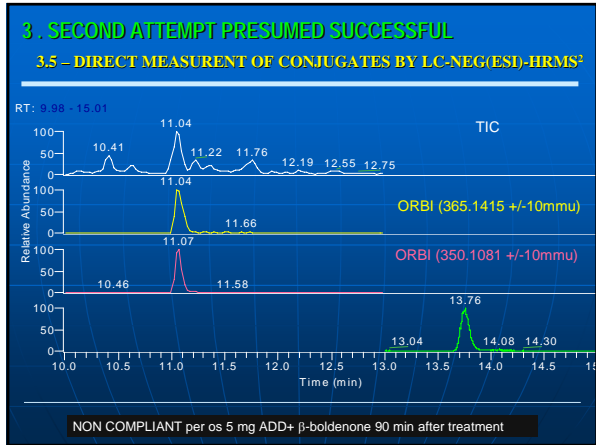
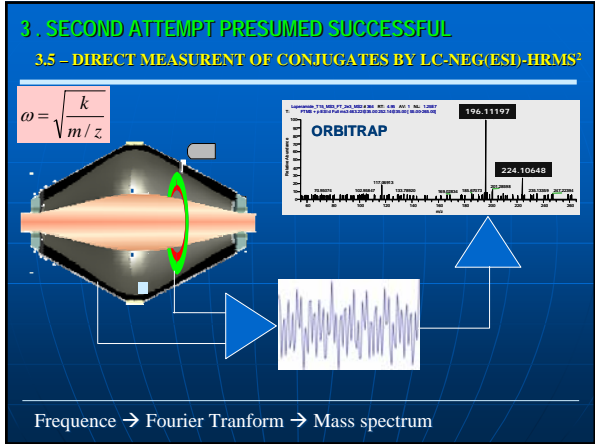
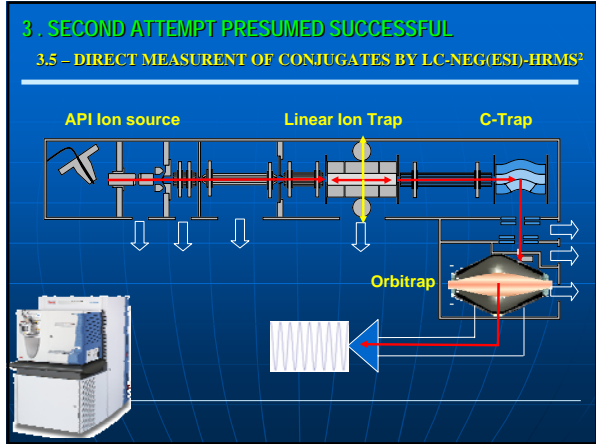
3. SECOND ATTEMPT PRESUMED SUCCESSFUL

3.4 – DIRECT MEASUREMENT OF CONJUGATES BY LC-NEG(ESI)-MS/MS

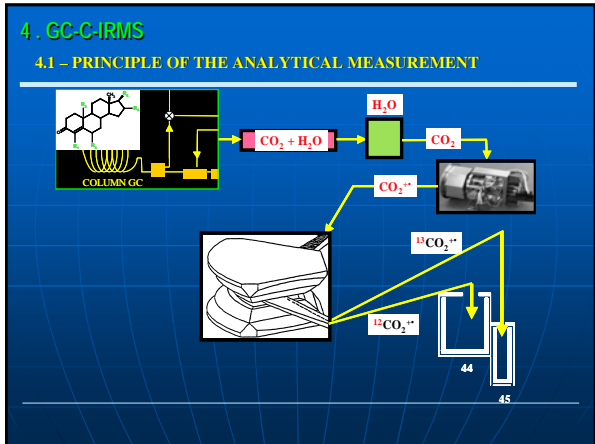


LC-MS/MS, NEG-ESI, SRM



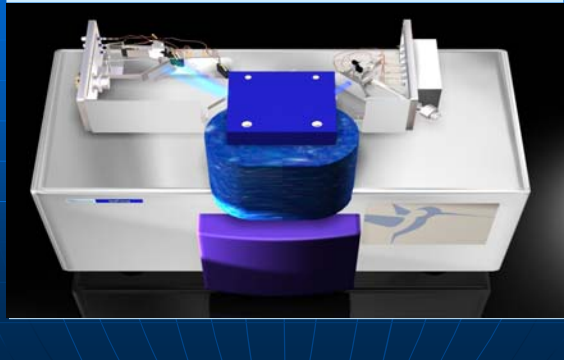


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4. GC-C-IRMS

4.1 – PRINCIPLE OF THE ANALYTICAL MEASUREMENT

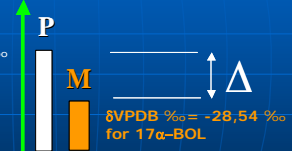


4. GC-C-IRMS

4.2 – GC-C-IRMS APPLIED TO 17 α -BOLDENONE

Study of $^{13}\text{C}/^{12}\text{C}$ isotopic compositions of 17 α -boldenone (after ADD treated bovine)

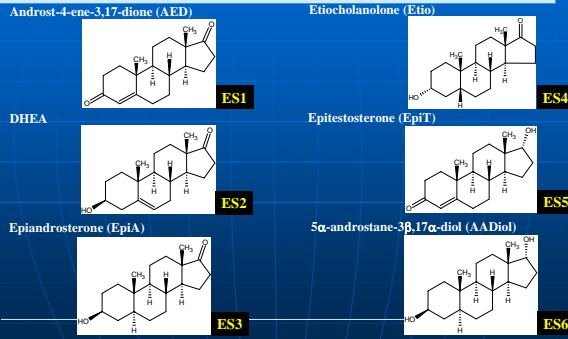
$\delta\text{VPDB } \text{‰} = -23 \text{ ‰}$
for ERC



LOD = 20 ng.mL⁻¹

4. GC-C-IRMS

4.3 – NON DISCUSSED OBSERVATIONS IN OUR FIRST STUDY



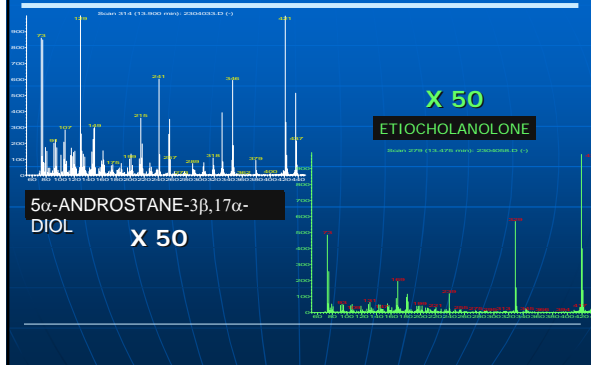
4. GC-C-IRMS

4.3 – NON DISCUSSED OBSERVATIONS IN OUR FIRST STUDY

	A	B	C
	ADD+BOLD	BOLD ESTERS	ADD
ES1	X2	↗ (ND - J ₀)	↗ (ND - J ₀)
ES2	X2	X40	X3
ES3	X4	X30	X10
ES4	Etio	X3	X50
ES5	X3	X20	X2
ES6	AAdiol	X5	X50

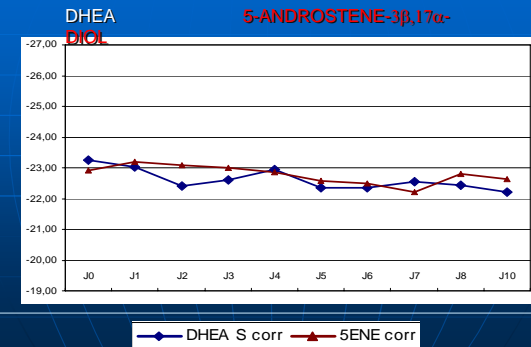
4. GC-C-IRMS

4.4 – GC-C-IRMS MONITORING OF 2 ABUNDANT NATURAL STEROIDS



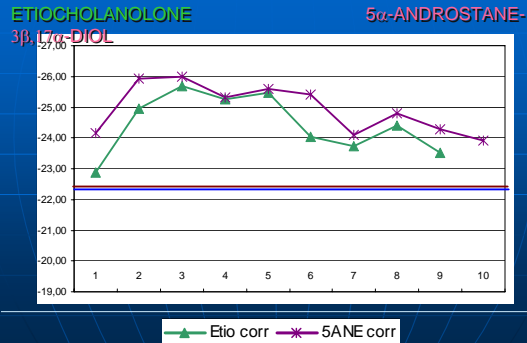
4. GC-C-IRMS

4.4 – GC-C-IRMS: RESULTS REGARDING ERCs



4. GC-C-IRMS

4.4 – GC-C-IRMS: RESULTS REGARDING PRESUMED METABOLITES



4. GC-C-IRMS

4.2 – RESULTS

Classical testosterone ERCs are not affected by boldenone treatment whereas $\delta^{13}\text{C}_{\text{VPDB}}$ -values of etiocholanolone and 5 α -androstane-3 β ,17 α -diol depleted after boldenone administration ($\delta^{13}\text{C}_{\text{VPDB}}$ down to -26%). This experiment demonstrated unambiguously the metabolic link between boldenone and these two testosterone metabolites which can be used to confirm (by GC-C-IRMS) the illegal administration of boldenone (and probably ADD) to cattle.

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5. CONCLUSION

5.1 – SUMMARY

- Summary of observations
 - Identification of 9 phase I metabolites
 - M2 and M4 candidate markers ~~NOT~~ not validated (few male urine samples showed signals for these two metabolites)
 - ~~5 β -androst-1-en-17 α -ol-3-one (M2)~~ ~~5 β -androst-1-en-17 β -ol-3-one (M4)~~
 - Phase II metabolite study led to the identification of a specific sulphate conjugate, **17 β -boldenone-17-SO₃H**
 - Studies of ¹³C/¹²C isotopic compositions of boldenone metabolite. First results on ADD treated bovine showed
 - => $\delta_{\text{VPDB}}^{13\text{C}} = -28,54 \text{ ‰}$ for 17- α BOL (LOD 20 ng.mL⁻¹);
 - => significant depletion of etiocholanolone + 5 α -androstane-3 β ,17 α -diol