METABOLOMICS ANALYSIS OF LIVER TO REVEAL PROFILES DISRUPTION IN BOVINES UPON STEROID TREATMENT

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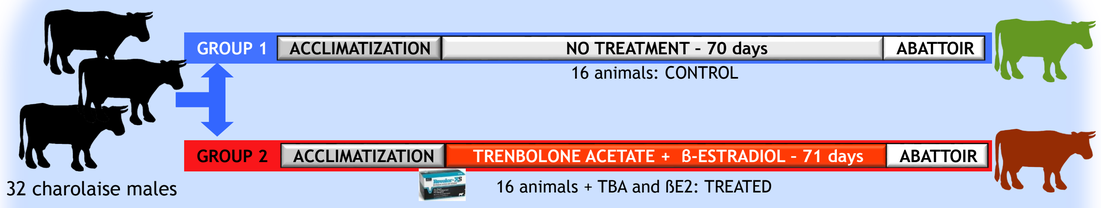
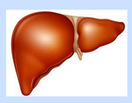
INTRODUCTION

The surveillance of illegal anabolic practices in bovine meat production is necessary to guarantee consumers' health. Screening strategies based on the recognition of indirect biological effects are considered by the community as promising tools to overcome some limitations of classical analytical methods and might therefore concur to ensure safer food for the consumer.
Given that hormonal therapies influence the physiology of an organism, strategies based on the detection of metabolic changes that occur following anabolic practices are promising approaches to identify their misuse [1-3]. The present work is aimed at characterizing the metabolic profile induced in liver by administration of anabolic steroids, and to identify potential disturbances in the hepatic metabolism. A total of 32 liver samples, 16 from untreated bulls and 16 from bulls treated with an ear implant (Revalor-XS®) containing trenbolone acetate (200 mg) and estradiol (40 mg), were analyzed following a LC-HRMS-based metabolomics analysis using RP and HILIC chromatographic separations.

SAMPLE COLLECTION AND PREPARATION



EXTRACTION OF METABOLITES



2 g OF EACH LIVER SAMPLE

FREEZE- DRYING

WEIGHT 50 mg

AQUEOUS EXTRACTION 1.5 mL MeOH/H_2O (50/50)

SUPERNATANT COLLECTION

VACUUM DRY

RESUSPENSION IN PRESENCE OF INTERNAL STANDARDS

QC PREPARATION

MIX AND ALIQUOT EXTRACTS FROM ALL PROCESSED SAMPLES

VACUUM DRY

RESUSPENSION IN PRESENCE OF INTERNAL STANDARDS

DATA ACQUISITION AND PROCESSING

HRMS

Full Scan 70-1000 m/z
70,000 resolution (at 200 m/z)
Positive ESI
Negative ESI

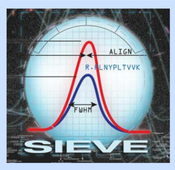
HILIC

Phase A: H_2O + 10 mM ammonium acetate + 10 mM acetic acid
Phase B: ACN
Flow: 0.3 mL min^{-1}
Run time: 30 minutes

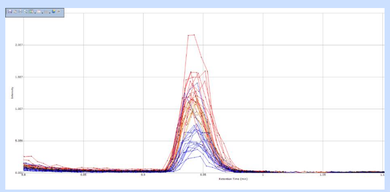
REVERSED PHASE

Phase A: H_2O + 0.1% acetic acid
Phase B: ACN + 0.1% acetic acid
Flow: 0.4 mL min^{-1}
Run time: 30 minutes

PROCESSING

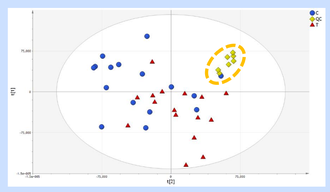


Chromatographic peaks allignment
Frames extraction
Peaks integration
Selection of interesting features
Putative identification based on m/z



STATISTICAL HANDLING

4 DATA MATRICES WERE ANALYSED



Unsupervised PCA was run to verify the acceptability of the analytical session using QC samples

Frames were filtered for:
- Treated/Control ratio (T/C < 0.7 or T/C > 1.5)
- Student T-test p value (< 0.05)
- CV % measured for QC samples in a given working session (< 30%)

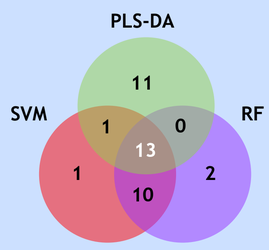
3 MULTIVARIATE ANALYSES WERE PERFORMED FOR SELECTION OF BIOMARKERS

SVM
Support Vector Machine

PLS-DA
(Partial Least Square - Discriminant Analysis)

RF
(Random Forest)

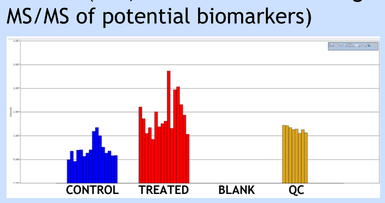
Significant frames common to all statistical tools, among the top ranking 25 features, were selected as potential markers to be identified

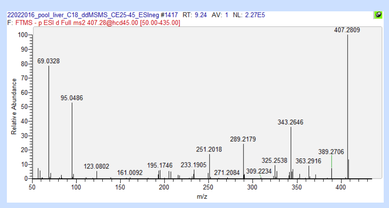


IDENTIFICATION OF BIOMARKERS

MS/MS STRUCTURAL ELUCIDATION

QC samples were analysed using FullScan + DDA (140,000 resolution + targeted MS/MS of potential biomarkers)





RT and MS/MS were compared to those of refence material

DATA COMPARISON

6 out of 8 potential markers of anabolic treatment have been identified by another lab, analysing (LABERCA) the same liver tissues with a different acquisition and processing platform

MEASURED m/z	IDENTIFIED COMPOUND	T/C VALUE	p VALUE
ESI⁻: 146.0446	DL-Glutamic acid	0.66	4.17 E-05
ESI⁻: 151.0249	Xanthine	0.69	1.94 E-03
ESI⁻: 181.0703	Galactitol	0.66	4.62 E-05
ESI⁻: 193.0347	Glucuronic acid	1.59	9.05 E-04
ESI⁻: 218.1026	Panthotenic acid	0.62	1.94 E-03
ESI⁺: 260.1862	Hexanoyl-carnitine	1.62	2.97 E-05
ESI⁻: 407.2809	Cholic acid	0.59	5.11 E-03
ESI⁻: 464.3023	Glycocholic acid	0.46	3.43 E-03

8 potential markers have been identified

CONCLUSIONS

Different multivariate statistical tools were applied to the datasets to select common metabolites for classification of samples and to reveal potential biomarkers on the basis of their significant changes in concentrations after administration of sexual steroids.
The identity of 8 candidate biomarkers was confirmed using reference standard material and fragmentation spectra. Moreover, a subset of biomarkers was also validated by a different laboratory that performed the same analyses using an independent instrumental and elaboration platform, confirming the robustness of the results achieved.
The identified metabolites can be considered as candidate markers of the proposed anabolic treatment with sexual steroids.
Results have been published in: Metabolomics 13, 7, 80 (DOI: 10.1007/s11306-017-1220-0).

References

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2 - Jacob C, Dervilly-Pinel G, Biancotto G, Monteau F, Le Bizec B.. Metabolomics 2015;11(1):184-197.
3 - Kouassi Nzoughet J, Dervilly-Pinel G, Gallart-Ayala H, Biancotto G, Le Bizec B.,. Metabolomics 2015;11(6):1884-1895.

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