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ALTERNATIVE BIOLOGICAL FLUID FOR METABOLOMIC APPROACHES TO SCREEN STEROID ABUSE IN BEEF CATTLE

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Introduction

In the European Union administration of all hormonal growth promoting substances in livestock production is prohibited. Nevertheless the use of "cocktails" or natural steroids as well as of new compounds with unknown structure is very difficult to prove with the traditional analytical techniques. Metabolomics has emerged as the latest of the so-called –omics disciplines and shows great potential to determine hundreds to thousands of metabolites at once over a wide range of concentrations. Recently, this technique has been successfully applied in bovine urine for the identification of biomarkers after experimental treatments with different growth promoting substances in farm. Taken into account its capacity to accumulate steroids [1], the aim of this work was to assess the potentiality of bile as matrix for metabolomic studies in order to control steroid abuses in cattle.

Experimental

Sixteen male beef cattle (10 - 14 months - Charolaise) were implanted for 70 days with REVALOR®-XS (Merck Animal Health) containing pellets of trenbolone acetate and estradiol. At the same time sixteen male beef cattle were kept as a control group. The sample preparation is reported in the scheme. After SPE each reconstituted bile sample was injected three times using the condition listed below. Ions with identical elution profiles and related m/z values (representing different adducts or isotopes of the same compound) were extracted as molecular features (MFs) characterized by retention time (RT), intensity in apex of chromatographic peak and accurate mass. Alignment of retention times and m/z values was carried out across the sample set using a tolerance window of 0.25 min and 15 ppm, respectively. Stepwise reduction of MFs number was performed based on frequency of occurrence, sample variability, abundance of the respective MFs in classes (groups) and results of *t*-test.

LC-HRMS conditions

LC SYSTEM: Agilent 1290 (Agilent Technologies)			
Column	Synergi 4µ Hydro-RP80A (150 x 2.0 mm, Phenomenex)	**Injection volume**	3 µL
Mobile phases	Formic acid 0.1 % in H_2O / Formic acid 0.1 % in CH_3CN	**Flow**	0.2 mL/min

MASS SPECTROMETER: Agilent 6550 accurate-mass Q-TOF via Jet Stream Technology (Agilent Technologies)	
Ionization	ESI negative
Acquisition mode	Full scan (100-1100 *m/z*)
Data analysis and processing	MassHunter Workstation - Mass Profile Professional

Sample preparation

Weight 120 mg of lyophilized bile

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Add Testosterone-d3 (IS)

↓

Add acetate buffer (pH = 5.0)

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SPE purification

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Reconstitute in 1.5 mL methanol/water 50/50, v/v (CAF-d5 as IS). Dilute 1:25

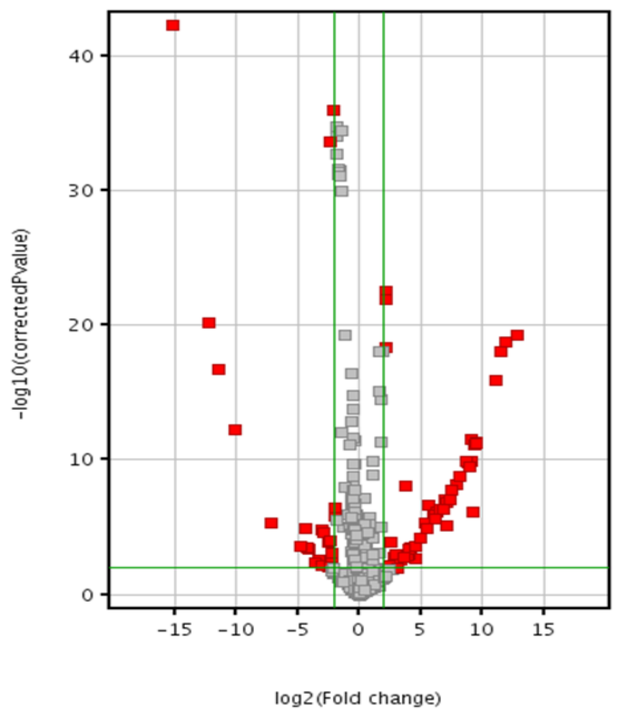

Figure 1 – "Volcano plot" of the 667 features (m/z, RT) obtained after comparison of bile metabolic fingerprint from untreated and treated animals.

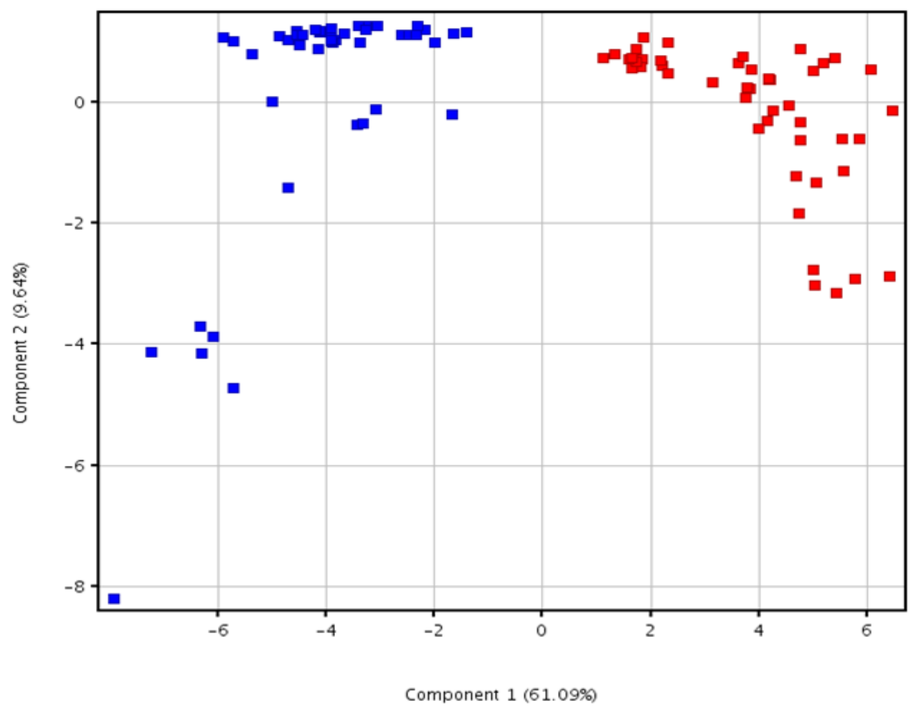

Figure 2 – PCA plot of bile samples from control (■) and treated animals (■) after LC-TOF-MS measurements and *t*-test (p value < 0.01) with Benjamini-Hochberg correction (one of the treated bile samples has been removed due to problems in its preparation).

Results

Figure 1 represents the "volcano plot" obtained after comparison of metabolic fingerprints generated from bile samples from control and treated group. From this analysis a total of 667 MFs were detected which are represented by squared, characterised by a *m/z* and a retention time (RT). Each ion is represented according to its *p*-value (y-axis) and its abundance ratio (x-axis). On this graph ions located in the left side correspond to those which are in average more abundant for control animals and similarly, ions located on the right side are on average more abundant in treated animals. MFs exhibiting *p*-values below 0.01 were considered as MFs for which the differences between the two groups were statistically significant (red squares). In Figure 2 the Principal Components Analysis (PCA) shows a very good discrimination between the control and the treated bile samples. The first two components demonstrated 70.7% variance.

Conclusions

In the best of our knowledge this biological fluid has not been previously tested for metabolomic experiments in veterinary doping. The responsible MFs for segregation between control and treated animals should be identified. Although complete identification cannot be performed solely on the basis of MS data, the accurate mass are very useful for initial identification purposes. In addition further experiments to acquire also the accurate MS/MS spectra are in progress.

References

[1] R. Galarini, S. Moretti, D. Giusepponi, G. Saluti, G. Biancotto, R. Angeletti. *Proceedings of 16th International Symposium on Recent Advances in Food Analysis* (RAFA), November 5–8, Prague, Czech Republic, p.461 (2013).

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