Profiling of metabolomic changes induced by testosterone esters in pig plasma and urine

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Introduction

The use of hormones as growth promoters for fattening purposes in livestock has been banned in the European Union since 1988 by Council Directive 93/22/EC. However, this banned substances are still reported within the framework of European monitoring residue plans. In order to improve reliability of the detection methods, metabolomics approaches to non-targeted screening for detection of anabolic practices using the naturaly steroid hormones or new synthetic growth promoters have been designed recently [1,2]. In this study, metabolic fingerprinting to discriminate between pigs treated with testosteron esters and control animals has been investigated.

Materials and methods

Animals
Twelve 90 day-old male and female pigs (aproximately 28 kg body weigh) were randomly separated into test (8 animals) and control (4 animals) groups. Animals from the test group were treated with an i.m. administration (0.6 ml) of the hormonal preparation (30 mg/ml 17β-testosterone propionate, 60 mg/ml 17β-testosterone phenylpropionate, 60 mg/ml 17β-testosterone isocapronate, 100 mg/ml 17β-testosterone decanoate; Sustanon 250, N.V. Organon, CZ Reg.56/357/91-C).

Sample collection and preparation
Urine samples were collected from 14 to 90 days after treatment in both groups. Plasma samples were collected from day 1 to day 90. All pigs were weighed every week within the experiment. Samples were filtered on centrifugal devices to remove proteins (cut off at 10 kDa, 10000 rpm, 4◦C, 30 min) and obtained filtrates were mixed with 20 μL internal standard (testosterone-D3 in metanol at the concentration of 1 ngL^{-1}, Sigma-Aldrich, CZ).

Q-Exactive mass spectrometry
Each sample (10 μL) was injected into the chromatographic system using an Accera 1200 Series (Thermo Fisher Scientific) on a Hypersil Gold C18 column (2.1x100 mm, 1.9 μm particle size) for separation. Mobile phase consisted in water containing 0.1 % acetic acid (A) and acetonitrile containing 0.1 % acetic acid (B) in gradient mode. LC-HRMS metabolomic fingerprints were acquired on Q-Exactive mass spectrometer (Thermo Fisher Scientific) in positive ESI+ mode. Full scan mass spectra were acquired from 80 to 800 m/z using a mass resolution of 70.000 FWHM in centroid mode. Raw data were processed by SIEVE software and the open-source XCMS software..

Multivariate statistical analysis
Multivariate statistical analyses PCA and OPLS-DA were carried out using SIEVE, XCMS, Statistica 10.0 and SIMCA 14.0 (demo) statistic software.

Results

- Metabolomic fingerprinting for two plasma samples collected from control (a) and the treated (b) pigs (Fig. 1).
- Multivariate statistical analysis PCA (a) and OPLS-DA (b) for plasma samples (Fig. 2).
- Multivariate statistical analysis PCA (a) and OPLS-DA (b) for urine samples (Fig. 3).
- The anabolic effect of testosterone esters (preparations) in pigs (Fig. 4).

Figure 1.

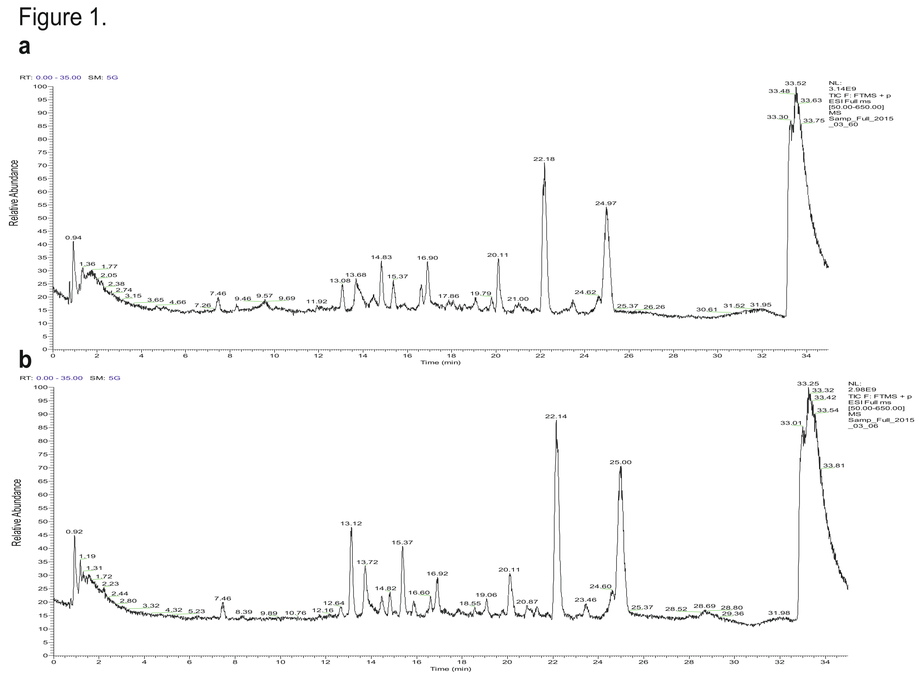

Fig.1 – *Typical examples of UHPLC-(HR)MS total ion chromatograms (TIC) obtained for two plasma samples collected from the control (a) and the treated (b) pigs.*

Figure 2.

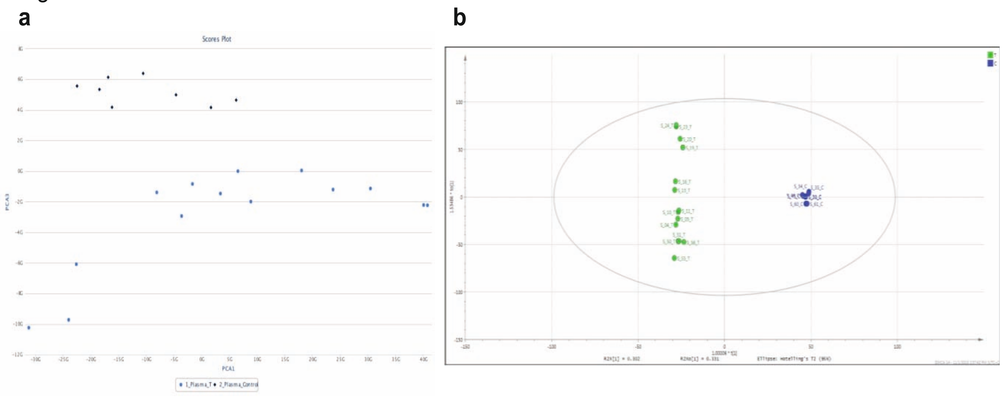

Fig.2 – *The score plot resulting from the PCA (a) and OPLS-DA (b) analysis performed on the generated plasma metabolomic UHPLC-HRMS fingerprints.*

Figure 3.

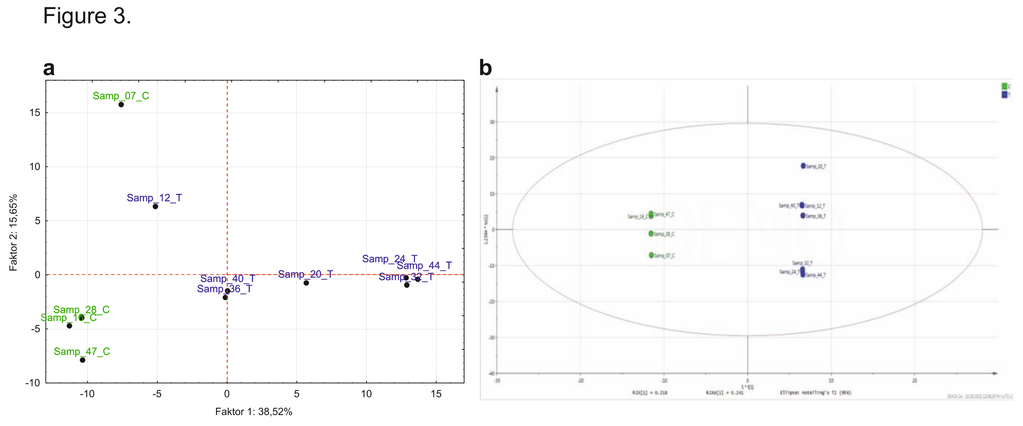

Fig.3 – *The score plot resulting from the PCA (a) and OPLS-DA (b) analysis performed on the generated urine metabolomic UHPLC-HRMS fingerprints.*

Figure 4.

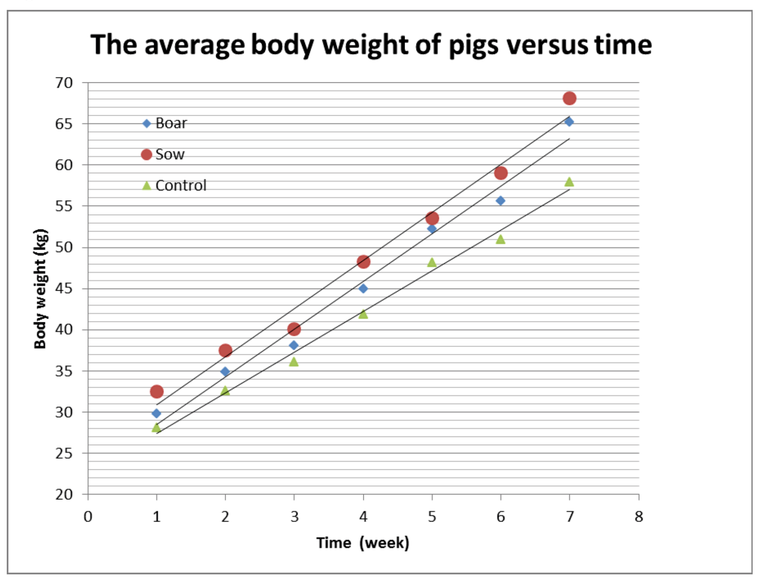

Fig.3 – *The plot of dependency the average body weight of pigs over time fattening demonstrating the anabolic effect of testosterone esters*

Conclusions

- Significant metabolic differences between test and control group in plasma and urine were found on day 28 after application of (esters) testosterone preparation. No significant differences between treated and control group in plasma and urine were found after day 42. Moreover, urine samples were analysed using conventional target analysis by LC-MS/MS. No residues were detected by LC-MS/MS analysis after day 14.
- The study demonstrates that metabolomic analysis may be a useful tool for anabolic practices performance also in pigs.
- A significantly better growth performance ($p < 0.05$) in treated pigs was found when compared with the control animals. Thus, the anabolic effect of testosterone esters in pigs was demonstrated.
- Additional experimental animals will be used to improve statistical significance of the fingerprinting data for potential screening application.

References:

[1] Pinel G. et al.(2010): Targeted and untargeted profiling of biological fluids to screen for anabolic practices in cattle, Trends in Analytical Chemistry, Vol.29, No.11, 1269-1280,
[2] Le Bizec B. et al.(2012): Metabolomics in food analysis: application to the control of forbidden substances, Drug Test. Analysis 4, 59-69.

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