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A new reliable sample preparation for high throughput focused steroid profiling by gas chromatography–mass spectrometry

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ABSTRACT

The use of steroid hormones as growth promoters in cattle has been banned within the European Union since 1988 but can still be fraudulently employed in animal breeding farms for anabolic purposes. If an efficient monitoring of synthetic compounds (screening and confirmation) is ensured today by many laboratories, pointing out suspicious samples from a natural steroids abuse remains a tricky challenge due to the difficulty to set relevant threshold levels for these endogenous compounds. The development of focused profiling or untargeted metabolomic approaches is then emerging in this context, with the objective to reveal potential biomarkers signing an exogenous administration of such natural steroids. This study aimed to assess sample preparation procedures based on microextraction and adapt them to high throughput urinary profiling or metabolomic analyses based on gas chromatography–mass spectrometry measurement. Two techniques have been tested and optimised, namely solid phase microextraction (SPME) and microextraction by packed sorbent (MEPS), using five model steroid metabolites (16α-hydroxyandrosterone, 2α-hydroxytestosterone, 11-keto,5β-androstanedione, 6α-hydroxyestradiol and 7β-hydroxypregnenolone). The considered performance criteria included not only the absolute response of the targeted compounds but also the robustness of the materials, and the global aspect of the diagnostic ion chromatograms obtained. After only five successive urinary extractions, a clear degradation of the SPME fiber was observed which led to discard this method as a relevant technique for profiling, whereas no degradation was observed on MEPS sorbent. Repeatability and recovery yields were calculated from urine samples fortified at 500 $\mu g\,L^{-1}$ and extracted by MEPS. They were found respectively below 11% and above 60% for all model compounds. Detection limits were in the 5–15 $\mu g\,L^{-1}$ range depending on the compounds, and a good linearity was observed on the 10–75 $\mu g\,L^{-1}$ range ($R^2 > 0.99$). This methodology was applied on urine samples collected from control *versus* androstenedione-treated bovines, revealing a significant concentration increase for several well-known metabolites such as etiocholanolone, 5α-androstane-3β,17α-diol, 5β-androstane-3α,17α-diol and 5-androstene-3β,17α-diol. Finally, these results allowed to confirm the suitability of the developed strategy and give to this new MEPS application a promising interest in the field of GC–MS based steroid profiling and metabolomic.

1. Introduction

The use of steroid hormones as growth promoters in cattle has been banned in the EU since 1988 [1,2]. In spite of this regulation, steroids can be fraudulently used in animal breeding for their anabolic properties [3,4], compelling the member States to implement efficient monitoring strategies. The screening diagnostic for natural hormones such as testosterone, estradiol and progesterone clearly remains a difficult challenge, while such natural steroids abuse can nevertheless be confirmed for instance by gas chromatography–combustion-isotope ratio mass spectrometry (GC–C-IRMS) using isotopic deviation measurements ($\delta^{13}C$) in urine [5,6] or by GC–MS/MS through the identification of administrated ester forms in hair [7–9]. Even if these confirmation methods have been well proven, they remain relatively time-consuming and expensive and are therefore not adapted to screening purposes. One possible approach consists in establishing some physiological reference thresholds in terms of "basal" endogenous concentration levels. However, this option is facing a major difficulty because of the high variability of the endogenous metabolites in urine demonstrated by Arts et al. [10] and more recently by Nielen et al. [11]. In this general context, emerging approaches for steroids

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misuse screening are based on focused profiling [12–14] or more global untargeted metabolomic [15,16], both expected to reveal some biomarkers allowing to sign the administration of natural steroids. One can estimate that gas chromatography–mass spectrometric-related techniques remain the method of choice for steroids profiling, considering either the relative low polarity of this class of chemicals which is not well adapted to atmospheric pressure ionisation techniques, and/or the presence of numerous isomer forms which cannot easily be separated with LC [12–14]. Paradoxically, analytical developments in GC–MS based metabolomic appear incomparably less common than in LC–MS based metabolomic. In particular, the question of sample preparation has to be re-investigated for these new approaches due to novel constraints compared to conventional targeted methods. Thus, high throughput capabilities, well-balanced selectivity, good repeatability and compatibility with GC injection, appear as crucial parameters to consider. Solid phase (SPE) or liquid–liquid (LLE) extraction are very common options but relatively time-, solvent- and matrix-consuming. Alternatively, the generation of microtechniques such as solid phase microextraction (SPME) or microextraction by packed sorbent (MEPS) may appear as a valuable way forward combining GC-compatible extracts and automation of protocols while minimising the quantity of solvent used.

SPME was developed in 1990 and was improved during the 1990s by Pawliszyn [17,18]. It consists in a solventless technique based on the more or less specific adsorption of the analyte on a fiber coated with a polymeric material such as polyacrylate (PA) or polydimethylsiloxane (PDMS). SPME is frequently used for the analysis of volatile compounds [19–21] in the headspace mode. For steroids, which are not considered as volatile, PA fiber is commonly used and the extraction is performed in direct immersion of the fiber into the matrix [22–26]. Then, an "on fiber" TMS-derivatisation with BSTFA is carried out to achieve the compatibility with GC separation (increasing volatility in decreasing stationary phase interactions) [22–25]. Compared to other classical extraction procedures like SPE or LLE, SPME finally presents the advantage of being a miniaturised, solventless, sensitive and reusable technique. Moreover, SPME can be fully automatic with direct desorption into the GC inlet.

MEPS is a more recent technique, developed in 2003 by Abdel-Rehim [27,28]. It consists in a miniaturisation of SPE in keeping with the same principle. Indeed, a small amount (1–4 mg) of sorbent is packed inside a cartridge directly placed in a syringe (100–250 μL) between the barrel and the needle. Different types of sorbents are commercially available such as reversed (C18, C8, C2), normal (silica) or ion exchange (SCX) stationary phases. The sorbent can be used several times with an adapted washing and reconditioning to avoid carry-over and to keep the adsorption power of the phase. Because of miniaturisation, extraction time, sample size and solvent volumes are considerably reduced and the elution extract is directly compatible with an on-line injection in LC, GC or CE (capillary electrophoresis). Several MEPS applications in complex matrices were already reported [29] in various areas such as anticancer drugs in plasma by LC–MS/MS [30], antimicrobial substances in urine by CE–MS [31], anaesthetic in blood and urine by LC–MS/MS or GC–MS [29,32], cocaine residues in urine by DART-ToF [33] or brominated flame retardants (BFR) in water by GC-ToF [34]. However and to our knowledge, this technique has never been yet evaluated for measuring steroid hormone-related compounds in urine.

The aim of the present study was to assess these two microextraction techniques (SPME and MEPS), in the scope of performing rapid, repeatable, and high throughput steroids profiling by GC–MS. Special emphasis was made on potential hydroxylated steroid metabolites as such biotransformation products are expected to be affected by exogenous administration of a parent drug [35]. Thus, the present development and optimisation of SPME and MEPS were carried out on five model hydroxylated steroid compounds. The proposed procedures were finally applied to real urine samples collected from control *versus* androstenedione-treated animals, in order to confirm the relevance and robustness of these strategies.

2. Experimental

2.1. Chemicals, reagents, and materials

All reference steroids including 16α-hydroxyandrosterone (16OHAAN), 2α-hydroxytestosterone (2OHT), 11-keto,5β-androstanedione (11KAAD), 6α-hydroxyestradiol ($6OHE_2$), 7β-hydroxypregnenolone (7OHPGN), methyltestosterone (MT) and epitestosterone-d_3 (aT-d_3) were purchased from Steraloids (Newport, RI, USA). Each steroid stock solution was achieved at 1 mg mL^{-1} by diluting steroids powder in an appropriated volume of ethanol. The working standards were prepared by diluting stock solutions in ethanol. All the solutions were stored at −20 °C. Derivatisation reagents N-methyl-N-trimethylsilyl-trifluoroacetamide (MSTFA), N,O-bis(trimethylsilyl) trifluoroacetamide (BSTFA) and trimethyliodosilane (TMIS) were provided by Fluka chemical corporation (Ronkonkoma, NY, USA), while dithiotreitol (DTE) was obtained from Sigma–Aldrich (St. Louis, MO, USA). β-Glucuronidase from *Escherichia coli* was from Roche Diagnostics GmbH (Mannheim, Germany). Ethanol, methanol, ethyl acetate and hexane were of analytical grade and purchased from Carlo-Erba Reagents (Rodano, Italy). Sodium chloride (NaCl) was from Merck (Darmstadt, Germany). Deionised water was obtained with Nanopure system from Barnstead (Dubuque, IA, USA). SPME polyacrylate fiber and 0.75 mm i.d. glass liner were obtained from SUPELCO (Bellefonte, PA, USA). MEPS syringe and C18 MEPS BIN (Barrel Inserts and Needle) were provided by SGE Analytical Science (Ringwood, Australia).

2.2. Animal experiment

One young bull and one heifer (both 13 months old) were treated (oral route) by 250 mg of androstenedione (Vetranal, Sigma–Aldrich). Urine control samples were collected 4 days, 3 days and just before treatment (−4,−3,0). Treated samples were collected 4 h, 20 h, 30 h, 48 h, 3 days and 4 days after injection for the heifer (0+, 1m, 1a, 2, 3, 4) and 20 h, 48 h, 3 days and 4 days after injection for the bull (1–4). The bull also received diuretic (Dimason furosemide, Intervet) by intravenous injection around 10 min before each collection. All urine samples were frozen at −20 °C directly after sampling.

2.3. Sample pre-treatment

Urine samples were unfrozen at room temperature and submitted to an enzymatic deconjugation step using β-glucuronidase from *E. coli* at 37 °C overnight, as described by Buisson et al. [5]. Samples were then centrifuged at 1200 × *g* (5 °C) for at least 10 min. For MEPS, the extraction was performed directly on the resulting supernatant. For SPME, a filtration on 10 kDa filters was performed and the obtained filtrates were freeze-dried. For steroid profiles comparison, performed only in the case of MEPS extraction, a normalisation of the dried material was used as a way to face with the dilution factor issue typically encountered with urine. After centrifugation, the supernatants were then freeze-dried and all samples were reconstituted in water at 80 mg mL^{-1}.

2.4. Extraction

2.4.1. Solid phase microextraction (SPME)

50 μL of each standard solution (10 ng μL^{-1}) was introduced into a vial, and evaporated to dryness under N_2 stream. The dry standard or urines (pre-treated as previously described) were redissolved in 2 mL water containing 300 mg mL^{-1} NaCl. Then the polyacrylate (PA) fiber was immersed into the vial and the extraction was performed under magnetic stirring during 60 min at 70 °C. After extraction, the fiber was rinsed for 2 min into water to remove salt from the fiber. Trimethylsilylation was then performed at 70 °C in headspace mode in a sealed vial containing 20 μL of BSTFA during 60 min.

2.4.2. Microextraction by pack sorbent (MEPS)

Both for the optimisation and the evaluation of this technique, a control urine sample was fortified with all selected model compounds (5 hydroxylated steroids) at 500 μg L^{-1}. The C_{18} MEPS sorbent was first conditioned with 100 μL MeOH and 100 μL H_2O. Then, three samplings of 100 μL were carried out before washing the phase with 100 μL H_2O and 80 μL hexane. The "steroid" fraction was eluted with 2× 90 μL of a MeOH/ethyl acetate mixture (30:70, v/v). Finally the sorbent was rinsed successively with 2× 90 μL of the elution mixture and 2× 100 μL water to avoid any carry-over phenomenon. The extracts were evaporated under N_2, and trimethylsilylation with 20 μL of MSTFA/DTE/TMIS (1000:5:5, v/m/v) mixture was performed at 60 °C during 50 min before injection.

2.5. GC–MS measurement

An Agilent 6890 series gas chromatograph coupled with an Agilent 5973N simple quadripole mass analyser (Agilent Scientific, USA) was used. Chromatographic separation was achieved with a DB-5MS column (30 m × 0.25 mm i.d., d_f: 0.25 μm) (Agilent). Helium was used as carrier gas at a constant flow of 1 mL min^{-1}. Injections were performed using 0.75 mm i.d. glass liner for SPME (10 min desorption) or 4 mm i.d. glass liner containing glass wool for liquid samples extracts (2 μL injected), operating in the splitless mode (5 or 1.5 min for SPME and MEPS, respectively). Inlet temperature was fixed at 280 °C. Two different oven ramps have been used: one for the method development and a second one for the steroid profiling application which included a specific gradient in order to optimise the steroids separation. For method development, the oven was configured as follow: 5 min at 120 °C (5 min), 25 °C min^{-1} to 200 °C (0 min), 3 °C min^{-1} to 300 °C (10 min). For the steroids profiling application, the temperature gradient was: 120 °C (1.5 min), 20 °C min^{-1} to 200 °C (0 min), 5 °C min^{-1} to 220 °C (0 min), 1 °C min^{-1} to 240 °C (1 min), 5 °C min^{-1} to 300 °C (10 min). GC–MS transfer line and source were, respectively, heated at 320 and 230 °C. The electron voltage was set at 70 eV. Mass acquisition was performed in full scan mode in the range m/z 50–650. Extracted ion chromatograms were used to characterise the response of targeted compounds, internal standard (aT-d_3) and external standard (MT). BSTFA derivatives of 16OHAAN, 2OHT, 11KAAD, 6OHE_2, and 7OHPGN led to the diagnostic ions m/z 306, 433, 302 (non-derivatised), 414 and 386, respectively. MSTFA/TMIS/DTE derivatives of the same compounds led to the diagnostic ions m/z 507, 520, 503, 414 and 533, respectively. For MT and aT-d_3 diagnostic ions m/z 446 and 435 were respectively monitored.

2.6. Evaluation criteria

The evaluation of the tested SPME and MEPS techniques was based on several criteria, among which the repeatability of the extraction, including the material stability (fiber or sorbent) after several consecutive extractions, was particularly considered. The estimated recoveries of selected steroids in urine sample were also a main parameter. Another important endpoint was the general visual aspect of resulting chromatograms, including the signal offset, the number of saturated peaks, the shift on retention times and number of peaks attributed to the column or the SPME fiber (bleeding). Recovery was calculated using this formula: R = (abundance of the analyte in the extract/abundance of the analyte with a direct injection after derivatisation at the same concentration) × 100. Linearity was studied by calculating the coefficient of determination (R^2) of the calibration curve on the 10–75 ng μL^{-1} range. Detection limits were estimated on a signal to noise ratio equal to 3.

2.7. Data processing and analysis

The comprehensive comparison of our sample profiles originated from different sub-populations (i.e. urine samples from control *versus* treated animals) imposed the deconvolution and processing of the raw data, which is not achievable manually but requires the use of appropriate software solutions [36]. The HP-Chemstation Data Analysis software was first used for visualizing chromatograms and associated mass spectra, and also for converting original data into a more exchangeable file format (.cdf) compatible with the open source software XCMS. This one is performing peak alignment, detection and integration across all the analysed samples. As a result, a table reporting the abundances of each detected chromatographic peak (characterised by a couple m/z ratio and retention time) was generated. This data table was then analysed using multivariate statistical techniques such as principal component analysis (PCA) with dedicated software (SIMCA-P+ Umetrics, Sweden).

3. Results and discussion

3.1. Solid phase microextraction (SPME)

According to the literature [22–25], extraction was performed by an immersion of the polyacrylate fiber in urine, followed by an on-fiber derivatisation with BSTFA in headspace mode. Several parameters known to have direct influence on the extraction process were optimised such as ion strength, extraction and derivatisation duration and temperatures.

3.1.1. Optimisation of the ion strength

Different NaCl concentrations from 100 to 300 mg mL^{-1} were tested to evaluate the ion strength effect (Fig. 1a). For the five considered model compounds, the measured signal abundance increased with salt concentration, according to a classical "salting-out effect" already described for other compounds [22,24,25]. Indeed, a saturation of the aqueous phase implies a loss of solubility for all the analytes in solution, which involve a better fiber adsorption. Considering these results, a NaCl concentration of 300 mg mL^{-1} was retained for the next experiments. Nevertheless, the main drawback of such a high salt concentration is the saturation of adsorbent correlated with a salt desorption in the GC inlet, leading to an incompatibly with the expected volatility properties for GC analysis. Therefore, a fiber washing step in ultrapure water (H_2O UP) was used (10 mL H_2O UP, 70 °C, stirring, 2 min) just after the extraction to discard the main part of adsorbed salt.

3.1.2. Optimisation of the extraction duration

The extraction duration was assessed between 5 and 90 min (Fig. 1b). As it was expected, the adsorption efficiency on the fiber was found to be considerably influenced by this parameter, with around 2-fold increase for 60 min compared to 30 min. The best

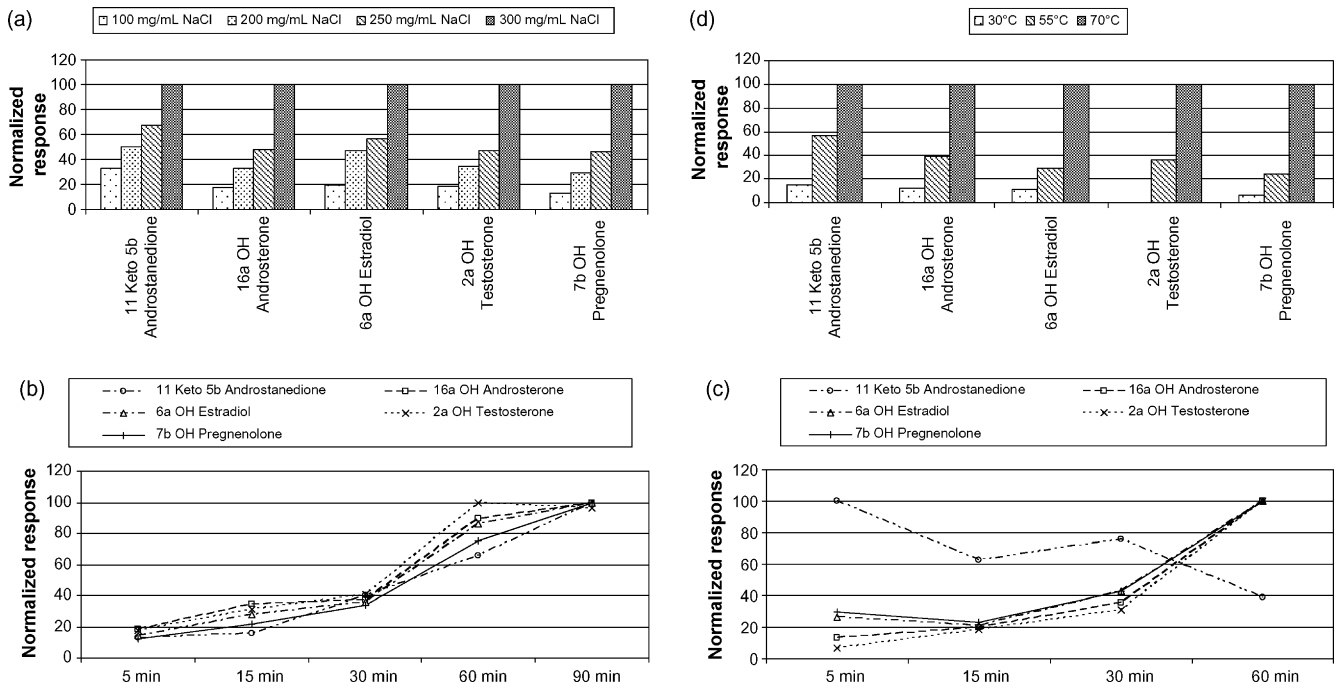

Fig. 1. Optimisation of the SPME extraction/derivatisation: (a) Ion strength effect, (b) extraction duration effect; (c) derivatisation duration effect; (d) temperature effect. All the abundances were expressed in normalised value based on the maximal abundance of each compounds.

response was reached at 60 min for 2OHT and at 90 min for the other targeted compounds. As the response between 90 and 60 min not decreased more than 35%, 60 min was finally selected as extraction duration to earn time.

3.1.3. Optimisation of the derivatisation duration

The derivatisation duration was also investigated. Thus, after extraction and fiber washing, PA fiber was exposed in headspace into a vial containing 20 μL of BSTFA at 70 °C for 5, 15, 30 or 60 min (Fig. 1c). For all these experiments, the TMS-derivatives of 16OHAAN, 2OHT, $6OHE_2$ and 7OHPGN, as well as the native 11-keto-5β-androstanedione (11KAAD) were monitored. For all derivatised molecules, the observed signal abundances were found to increase with the reaction duration, indicating a higher derivatisation yield with increasing time. Conversely, the special case of 11KAAD, which decreases from 100% at 5 min to 38% at 60 min, can be explained by a degradation of compound or by the apparition of secondary derivative forms. The first possibility is suggested to

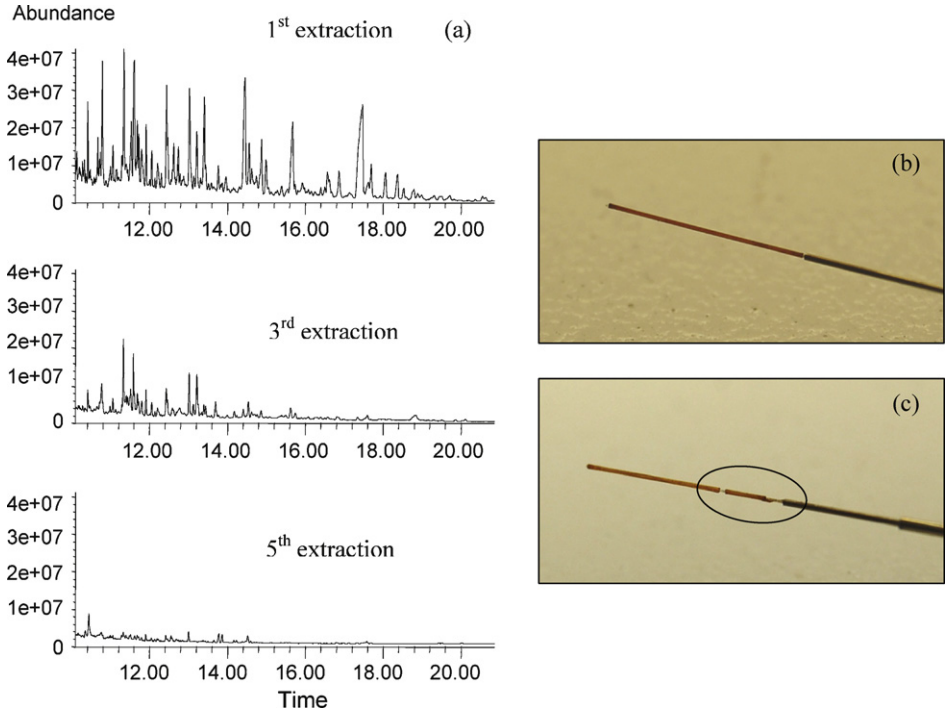

Fig. 2. (a) TIC obtained from the first, the third and the fifth consecutive extractions with the same new SPME fiber. (b) Picture of the SPME fiber before the 1st extraction and (c) after the 5th extraction.

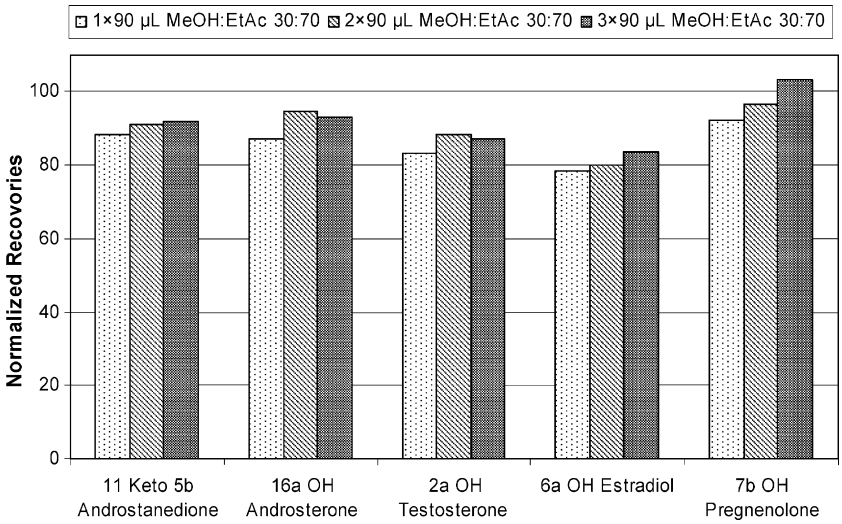

Fig. 3. Normalised response of each steroid associated with recovery yield of each steroid after different volumes of the elution mixture (MeOH:ethylacetate 30:70 v/v).

be the good one because no TMS-derivatives were observed on the TIC. As it exists certainly more steroids with hydroxylated functions than steroids with only ketone ones as urinary bovine metabolites, 60 min was finally retained for the derivatisation duration.

3.1.4. Optimisation of the extraction and derivatisation temperatures

For a practical point of view, temperatures of extraction and derivatisation must be identical if a future automation is envisaged. Thus, three temperatures were tested (Fig. 1d). The influence of the extraction temperature was more specifically evaluated on 11KAAD, which is not concerned by the derivatisation reaction. Temperature was confirmed to be a key factor influencing the extraction, with a 10-fold increase between 30 and 70 °C. According to these results, the extraction and derivatisation temperatures were both set at 70 °C.

3.1.5. Application to urine samples

Before investigating quantitative data and evaluating the usefulness of this technique for profiles or global fingerprints comparison, the repeatability of this approach was assessed. Five SPME preparations were performed on the same urine sample. The obtained chromatographic traces are shown in Fig. 2a. A significant loss of raw signal was demonstrated after 3 and 5 extraction–injection cycles, despite a sample pre-treatment including a centrifugation and a 10 kDa filtration. In order to determine if a physical alteration of the fiber occurred, the microscopic structure of the fiber was examined (Fig. 2b). The fiber degradation was then confirmed. The complexity of the urine from bovine is suspected to cause this physical damage. Indeed, the optimisation of the method was led on a standard mixture diluted in ultrapure water and the fiber was immersed in this solution before being exposed to headspace derivatisation into a vial containing BSTFA. For all these experiments only one fiber was used without any damage to the coating, which allows the assessment of extractions on urine samples. In parallel, several extractions were performed in 5-fold diluted urine. No fiber damage was clearly observed but total ion currents were very poor and not informative enough to perform an exhaustive steroidomic profiling. In conclusion, these results seem to indicate that the urinary matrix played a great role in the coating deterioration. Some authors [37–40] also reported potential pitfalls with the direct immersion mode in complex matrices such as urine or blood and recommended the use of headspace mode whenever possible.

SPME approach was finally given up in the frame of the present work due to the degradation phenomenon, which prevented all comparison between samples, and appeared incompatible with high throughput analysis.

3.2. Microextraction by pack sorbent (MEPS)

The used C_{18}-MEPS protocol was adapted from a C_{18}-SPE procedure described by Buisson et al. [5]. From this basis, urine sampling and elution volumes have been specifically optimised to fit with the present work objectives.

3.2.1. Elution volume

After one sampling of 100 µL of the five reference model steroids (500 µg L^{-1} in water), different volumes of elution mixture (methanol/ethyl acetate, 30:70, v/v) have been tested (1×, 2× and 3× 90 µL) and the recoveries were calculated and reported in Fig. 3. For each target compound, an insignificant signal increase (from 2% to 7%) was observed between 1 or 2 elution volumes of 90 µL while the recoveries remaining at the same level for 2 or 3 elution volumes (±5% variability). Despite this non-significant increase between 1 and 2 elution volumes, 2× 90 µL was finally retained to ensure that other more hydrophobic metabolites are correctly eluted. In this condition, global recoveries were estimated to be upper 79% for all compounds, which was considered satisfactory.

3.2.2. Sampling volume

Two parameters must be taken in account in order to optimise the sampling volume: the aspect of the chromatogram (saturation of the signal and global intensity of the baseline) and the signal to noise ratio of each targeted compound (Fig. 4). The global signal measured after only one sampling of 100 µL did not appear informative enough considering the number of peaks detected. After 5-fold sampling, the raw chromatogram presented a significant number of saturated peaks, which could lead to an inaccurate measurement (error on signal intensity and retention times). Moreover, the background level was found significantly higher for 5 sampling compared to 3, so that the signal to noise ratio obtained for 16OHAAN, 2OHT began to decrease. A good compromise appeared to be 3× 100 µL despite the real increase of the $6OHE_2$, 7OHPGN, 11KAAD signal to noise ratio between 3 and 5 samplings. As profiles were performed in scan mode, "reliable" chromatograms, i.e. narrow peak, good peak shape and low baseline, were preferred as the maximisation of the abundance values.

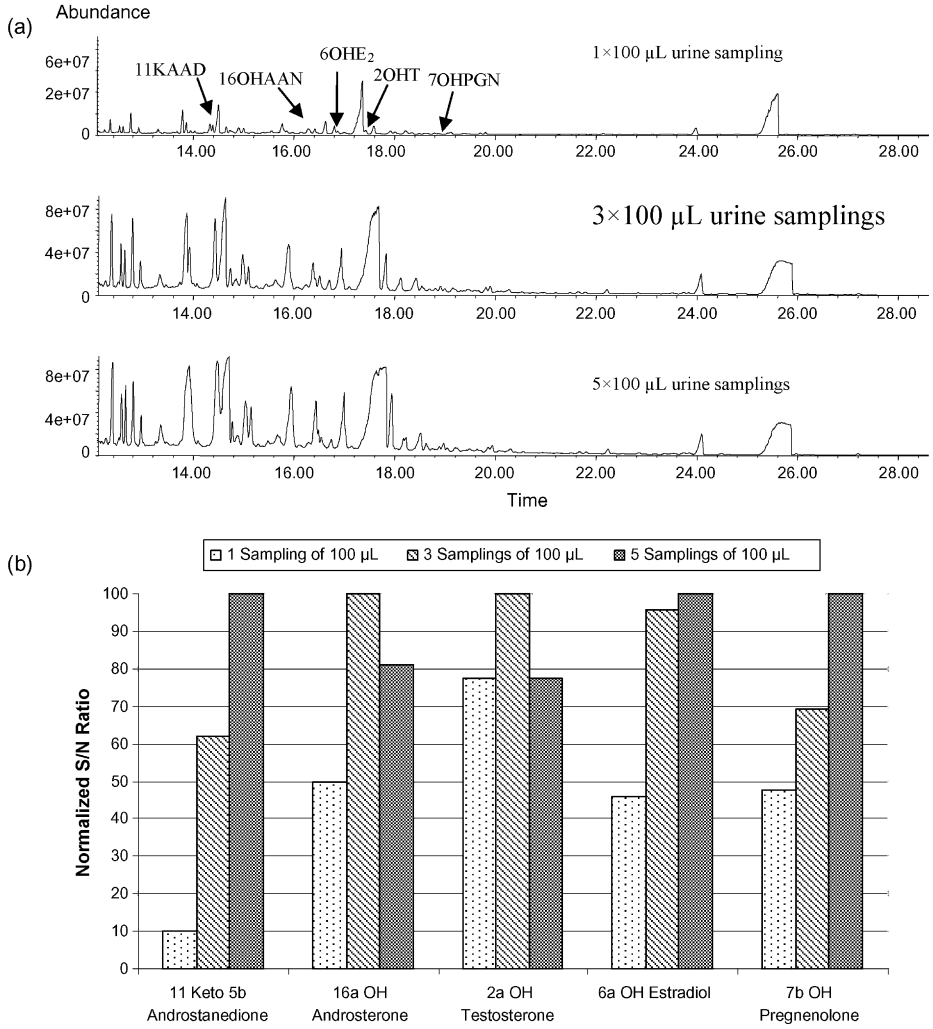

Fig. 4. Optimisation of sampling volumes on spiked urine at 500 ng mL^{-1}. (a) TIC profile and (b) normalised S/N ratio of targeted compounds.

3.2.3. Method performances

Only one bin was used for all the previously described experiments, which correspond to around 25 extractions on water and/or urine samples. Consequently, the C_{18} sorbent appeared to be quite robust for a relative long-term utilisation. Some quantitative data obtained on spiked urine samples are presented in Table 1. The repeatability of the method was evaluated on 10 extractions of the same urine. Except for 2OHT for which a disturbing coelution was observed, repeatability (relative standard deviation of the signal monitored) was found to be lower or around 10%, that was estimated to be very satisfying for a steroidomic profiling exercise. Comparing recoveries in spiked water (Fig. 3) and spiked urine (Table 1), 11KAAD and 2OHT exhibited a lower recovery in urine, certainly due to matrix effect. All the other recoveries were quite identical in water or urine, and were higher than 80%, which was also estimated to be satisfying. The linearity was checked between 10 and 75 ng mL^{-1} except for 11KAAD (20–75 ng mL^{-1}). The coefficient of determination was always above 0.99, indicating that the MEPS sorbent (4 mg of C_{18} stationary phase) was not saturated and allowed to envisage quantification of some metabolites present in urine in this concentration range. Last but not least, the estimated detection limit reached with these extraction conditions ($\leq$15 µg L^{-1}) appeared compatible with the level of steroids found in urine during 4–5 days after treatment with anabolic agents [41,42]. Finally, all these performances have demonstrated the robustness and the efficiency of this MEPS technique, which appears to be a good choice to perform and compare steroids profiles.

3.3. Application

In order to prove the benefits of MEPS, 16 urine samples collected from control and androstenedione-treated animals were

Table 1
Performance of the MEPS//GC–MS method: recoveries, repeatability, detection limit and determination coefficient of target compounds in spiked urine. For recoveries and repeatability, urine was spiked at 500 µg L^{-1}. For 11KAAD determination coefficient was calculated for concentrations between 20 and 75 ng mL^{-1}.

Compounds	Recoveries (%)	Repeatability RSD (%) (n = 10)	LOD (ng mL^{-1})	Determination coefficient (R^2) (10–75 ng mL^{-1})
11-Keto-5β-androstanedione	60	7	15	0.991
16α-OH androsterone	98	5	5	0.993
2α-OH testosterone	68	11	5	0.994
6α-OH estradiol	81	4	5	0.991
7β-OH pregnenolone	93	3	5	0.998

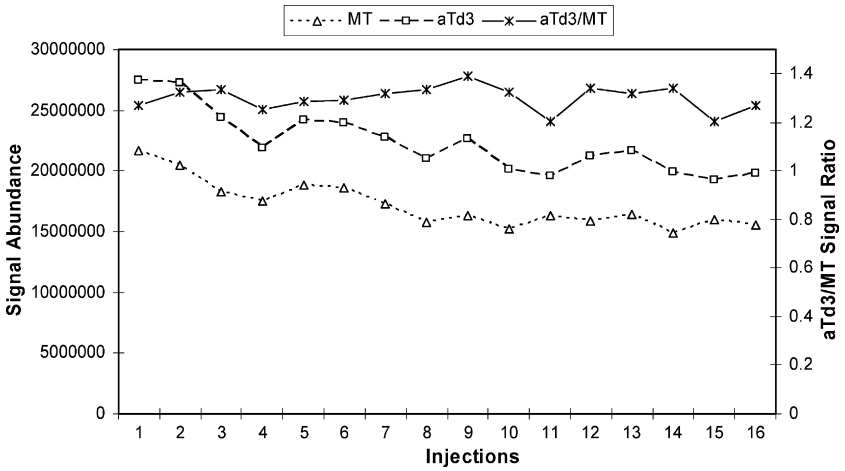

Fig. 5. Abundance of external (MT) and internal standard (EpiT-d_3) signal (left scale) during a sequence of 16 urine samples and their associated signal ratio (EpiT-d_3/MT) (right scale).

extracted according to the optimised MEPS protocol, derivatised and injected in scan mode in GC–MS.

3.3.1. Quality control

The quality of the sample preparation and the monitoring of any potential issue during the entire process including sample preparation and also GC–MS measurement were checked using aT-d_3 as internal standard and MT as external standard, the two compounds being added respectively before the sample preparation and before the derivatisation step. The abundances monitored for these internal and external standards during all the sequence are reported in Fig. 5. The signal of MT and aT-d_3 decreased in the same way. The loss of 25% of signal after 16 urine sample injections illustrated a small deviation certainly due to the fouling of the instrument. However, the constant ratio (RSD < 4%) MT/aTd_3 confirms that the MEPS extraction was repeatable and allows a total normalisation of each sample profile by the internal or/and external standard. Thus and despite the fact that MT is not a perfect external standard for all compounds in urine, all the peak areas extracted using the XCMS software were divided by the area of MT peak before statistical analysis.

3.3.2. Statistical analysis

The visual examination of typical total ion chromatograms obtained for a control and a treated animal did not reveal any significant difference between the two samples (not shown), so that such potential existing differences cannot be extracted without appropriate bioinformatics tools. Thus, after processing with the XCMS software, all ions (couple *m*/*z*; rt) detected in the samples were analysed by multivariate statistics. Even if the number of observations (urine sample) was limited in the present case, the analysis by PCA of the whole analytical information extracted from each fingerprint is proposed to illustrate the relevance of such profiles. A first principal component analysis (PCA) was performed on the basis of all the entire data set, i.e. including all urine samples originated from male and female animals (Fig. 6). PCA score-plot

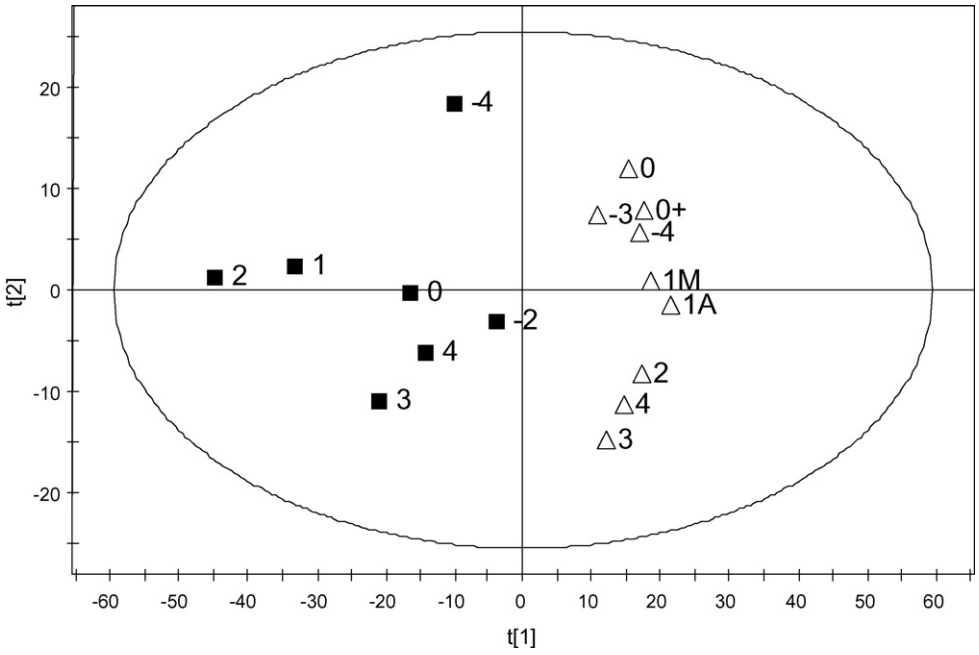

Fig. 6. Principal component analysis (PCA) score plot of all samples (square = males, triangle = females). Number represents the day of urine collection (−4, −3, −2 day and just before injection (0) or 4 h (0+), 20 h (1m), 30 h (1a), 2, 3 and 4 days after).

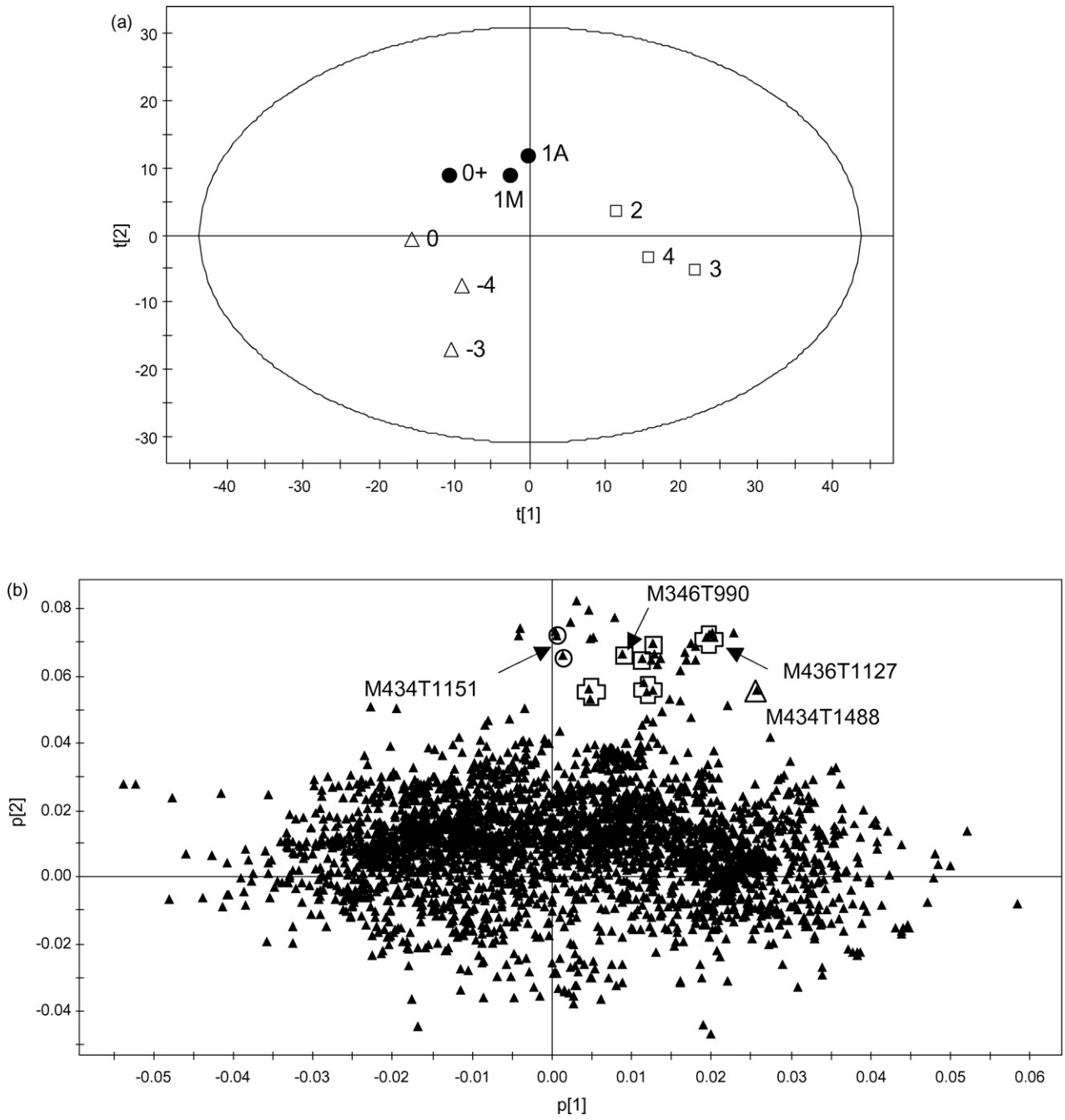

Fig. 7. (a) PCA score plot of female samples [cross = control urines, circles = urine collected between 6 and 30 h after injection, square = urine collected at least 48 h after injection. Number represents the day of urine collection (−4, −3 day and just before injection (0) or 4 h (0+), 20 h (1m), 30 h (1a), 2, 3 and 4 days after)]. (b) The associated loading plot (each variable is characterised by a *m*/*z* and a retention time value "M*x*T*y*", variable bordered by circle are associated to compounds at rt = 1151 s, by square to rt = 990 s, by triangle to rt = 1488 s and by cross to rt = 1127 s).

shows clearly two separated groups on the first component, which reported 54% of the total information: the triangles representing the female group and the squares the male ones. On the second component (reporting 10% of the global information), a slight distinction between control and treated animals was revealed but only in the female group. Because male and female samples presented unsurprisingly very different urinary profiles, and because more samples were collected from the heifer, a second PCA was further performed on the basis of only female samples (Fig. 7a). The score-plot shows three different groups: one with all the control samples on the left, one with samples corresponding to urines collected maximum 30 h after treatment on the top and the last one on the right with urines collected 48 h and more after treatment. The associated loading plot (Fig. 7b) highlights the variables (*m*/*z*, rt) mainly responsible for this separation of these three groups. On the top or on the right area of this graph, ions are up-regulated in treated animals while on the left they are down-regulated or absent for the same animals. Moreover, ions on the top have their maximum abundance between 6 and 30 h after the treatment. Four ions in this area were hypothesised to be TMS-steroid derivatives *m*/*z* (Fig. 7b). After confronting these experimental spectra with an in-house library, these 4 compounds were unambiguously identified. They correspond to 5α-androstane-3β,17α-diol (M436T1227), 5β-androstane-

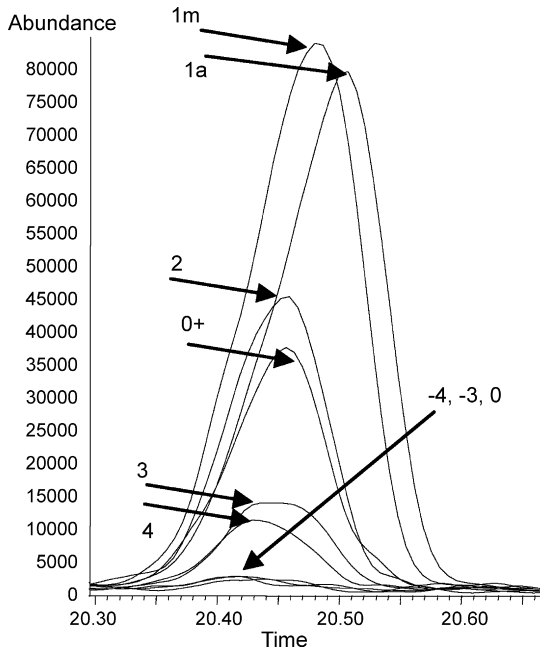

Fig. 8. Chromatograms of ions M436T1227 (5α-androstane-3β,17α-diol). Number represents the day of urine collection (−4, −3 day and just before injection (0) or 4 h (0+), 20 h (1m), 30 h (1a), 2, 3 and 4 days after).

3α,17α-diol (M346T990), etiocholanolone (M434T1151) and 5-androstene-3β,17α-diol (M434T1488). The identities of these compounds were confirmed by new analyses of the corresponding reference substances. These steroids were already described [6] as metabolites excreted after administration of testosterone. Ions M436T1227 corresponding to 5α-androstane-3β,17α-diol were extracted from the TIC (Fig. 8). This confirms what statistical analysis highlighted, namely this compound is highly excreted after treatment than before. These results finally allowed confirming the efficiency and suitability of our analytical approach to acquire and analyse steroidomic profiles that remains promising for further investigations.

4. Conclusion

The objective of this study was to evaluate the capabilities of two microextraction techniques, namely SPME and MEPS, in the scope of performing steroid profiling and metabolomic from bovine urine samples using GC–MS-based measurement. SPME was not found to be adapted to our need due to a very short fiber lifetime observed when immersion in urine was performed. Conversely, MEPS was found to be a robust and efficient approach to analyse urinary steroid fractions in a short time. Indeed, one extraction is achieved in 3 min and can be fully automated, opening the way to high throughput analyses. Only two MEPS cartridges and few solvents were used for the entire study, which made this extraction technique fast, reliable, cheap and finally a technique of choice for such profiling exercises. In this way, a successful discrimination between control and androstenedione-treated bovines was achieved on the basis of their urinary profiles. 5α-Androstane-3β,17α-diol, 5β-androstane-3α,17α-diol, etiocholanolone and 5-androstene-3β,17α-diol were found to be involved in the separation between the two groups. Therefore we have demonstrated that MEPS can be used to perform urine fingerprints by GC–MS to identify potential biomarkers of natural hormones administration. Moreover, other profiles will additionally be obtained by comprehensive chromatography (GC × GC), which should improve the peak capacity and the quantity of analytical information detected.

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