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Comprehensive two-dimensional gas chromatography improves separation and identification of anabolic agents in doping control

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ABSTRACT

The application of comprehensive two-dimensional gas chromatography coupled to time-of-flight mass spectrometry (GC × GC-TOFMS) for the analysis of six anabolic agents (AAs) in doping control is investigated in this work. A non-polar–polar column configuration with 0.2 μm film thickness (d_f) second dimension (^{2}D) column was employed, offering much better spread of the components on ^{2}D when compared to the alternative 0.1 μm d_f ^{2}D column. The proposed method was tested on the "key" AA that the World Anti-Doping Agency (WADA) had listed at the low ng mL^{-1} levels (clenbuterol, 19-norandrosterone, epimethendiol, 17α-methyl-5α-androstane-3α,17β-diol, 17α-methyl-5β-androstane-3α,17β-diol and 3′-OH-stanozolol). The compounds were spiked in a blank urine extract obtained by solid-phase extraction, hydrolysis and liquid–liquid extraction; prior to analysis they were converted to the corresponding trimethylsilyl (TMS) derivatives. The limit of detection (LOD) was below or equal to the minimum required performance limit (MRPL) of 2 ng mL^{-1} defined by WADA, and the correlation coefficient was in the range from 0.995 to 0.999. The method allows choosing an ion from the full mass spectra which shows the least interference from the matrix and/or the best sensitivity; this can only be done if full scan mass spectral data are available. The advantage of GC × GC over classical one-dimensional GC (1D GC), in terms of separation efficiency and sensitivity, is demonstrated on a positive urine control sample at a concentration of 5 ng mL^{-1}. The obtained similarity to the in-house created TOFMS spectra library at this level of concentration was in the range from 822 to 932 (on the scale from 0 to 999). Since full mass spectral information are recorded, the method allows the retro-search of non-target compounds or new "designer steroids", which cannot be detected with established GC–MS methods that use selected ion monitoring (SIM) mode.

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1. Introduction

The very low concentrations of anabolic agents (AAs), and the complex matrices in which they are found (urine, sera, and other biological materials), require a powerful technique for separation and unambiguous identification. At present gas chromatography coupled to a quadrupole mass analyzer (GC-qMS) is a technique of choice which exhibits high specificity and sensitivity, especially when selected ion monitoring (SIM) mode is applied. The separation is based on using a traditional narrow bore capillary column, and detection necessarily relies on monitoring pre-defined diagnostic ions in pre-defined time windows. The extraction process is generally standardized, comprising of solid-phase extraction (SPE), hydrolysis of conjugates (metabolites) with β-glucuronidase and liquid–liquid extraction (L/LE) by using diethylether or *tert*-butylmethylether (TBME). Prior to GC–MS analysis, the AA are derivatized to their corresponding trimethylsilyl (TMS) derivatives since they usually contain one or more hydroxyls and/or keto groups and generally show poor chromatographic behavior if not derivatized [1]. The most characteristic and preferably the most abundant ion is used for quantification, and 2–3 other ions (qualifiers) are used for confirmation purposes. Most of the current methods in doping analysis, if not all of them, are still "transparent" to the potentially new "designer" AA at the lowest level of detection, since they rely on pre-defined ions to be monitored. Full scan mass spectral techniques coupled to classical one-dimensional GC (1D GC) with classical injection are not sensitive enough to detect low level concentrations down to 1 ng mL^{-1}, as defined by the World Anti-Doping Agency (WADA) [2].

In the last few decades many attempts have been made to improve the selectivity and the sensitivity of the GC–MS approach. Improvements in the oven temperature program [3,4] in order to obtain better signal-to-noise ratio (S/N), the use of ion-trap tandem mass spectrometry [5,6], high-resolution mass spectrometry

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[7–10], hybrid mass spectrometry (high-resolution–time-of-flight) [11], and combustion/isotope ratio mass spectrometry [12], are amongst the most common. An attempt was made by Mazzarino et al. [13] to shorten the runtime while retaining the resolution and limit of detection by applying fast GC with a 5 m narrow bore column (0.1 mm I.D.; 0.1 μm film thickness). However, most methods are based on improvements of the selectivity, although usually by making the detector increasingly "blind" to the matrix. Such an example is the shift from full scan mode to SIM mode in low resolution MS, then to high resolution MS (in SIM mode), and finally to MS/MS or MS^n. Several new approaches are proposed for increasing the sensitivity and selectivity of the methods in doping control by using an extra step in purification of the samples. In this manner immunoaffinity chromatography (IAC) [14] and high performance liquid chromatography (HPLC) fractionation [15] are employed. None of these approaches is based on chromatographic separation improvements since the limit of the separation efficiency of the 1D GC step has largely been reached.

Recently a new approach for separation improvement has been proposed, by using comprehensive two-dimensional gas chromatography (GC × GC) [16–19] in doping analysis. The use of GC × GC is perhaps one of the most significant innovations in terms of separation efficiency improvement since the introduction of narrow bore capillary columns. GC × GC also reportedly exhibits improved sensitivity, alongside separation power, compared to traditional 1D GC [20,21]. The separation is performed on two sequential columns: the first one (^{1}D) approximates a conventional 1D GC separation, while the second one (^{2}D) must act as a fast-eluting high-efficiency column, usually 0.5–2 m in length, with 0.1 mm I.D. and 0.1 μm film thickness (d_f). The separation mechanisms of the stationary phases should be as different as possible in order to maximize their orthogonality, giving rise the significant increase in separation power. The interface between the two columns (modulator) has to trap/accumulate in the predefined period (3–8 s) all the components which elute from the ^{1}D column and then release them rapidly as narrow adjacent fractions into the ^{2}D column. The efficient trapping and fast releasing of the components is usually achieved by cryo-focusing, producing ultra-narrow peaks at the end of the ^{2}D column. Co-eluting components on ^{1}D, where the separation is based on the first mechanism, are separated on the orthogonal ^{2}D column, where the separation is based on the second mechanism. GC × GC has been successfully applied in petrochemical [22,23], food [22,24], environmental [25], forensic [26–28], and essential oils [29,30] analysis of complex matrices, showing for the first time some new features such as structured chromatograms. Apart from improved peak capacity and structured chromatograms, GC × GC exhibits an increase in the signal height when compared to classical GC [17,19].

Silva et al. [18] reported analysis of key WADA AA in urine using GC × GC-TOFMS at the lowest purported concentration, demonstrating that this technique is highly sensitive and specific in screening of these AA. Alternatively, it can be employed as a full spectra confirmatory method when coupled to TOFMS as a detector. The separation on a second dimension column was based on a 1 m OV-1701 column (0.1 mm × 0.1 μm), however, the AA were spread in a rather narrow band of approximately 1 s out of the 6 s modulation period, along with matrix compounds also within this region. This tends to reduce the extent of matrix separation. No data are provided on linearity and TOFMS spectra similarity to a commercial or in-house MS library.

The present report describes further improvements in doping analysis, specifically the increased peak capacity (separation power) through better spread of AA and matrix components over the 2D space, and sensitivity and identification power of the TOFMS as a detector coupled to GC × GC. Linearity ranges and limit of detections for the tested substances are established, and advantages of GC × GC over 1D GC in term of sensitivity, separation and identification power are demonstrated through several examples. The bias of TOFMS detector against the higher masses, observed in the results of Silva et al. [18] but not reported, has been stressed in respect of the 19-norandrosterone case.

The performance of the present proposed method was confirmed using spiked urine extracts obtained after SPE, hydrolysis with β-glucuronidase and L/LE with TBME. The proposed method is "non-transparent", since it permits full mass spectral information to be retained both for targets or non-targets. Finally the method was quantified for a urine positive control sample (UPC) spiked with the "key" WADA AA at a concentration of 5 ng mL^{-1}, which was prepared at a WADA accredited anti-doping laboratory.

2. Experimental

2.1. Reagents and chemicals

Clenbuterol (**1**), 19-norandrosterone (**2**), epimethendiol (**3**), 17α-methyl-5α-androstane-3α,17β-diol (methyltestosterone-M1 metabolite, **4**), 17α-methyl-5β-androstane-3α,17β-diol (methyltestosterone-M2 metabolite, **5**), 3′-OH-stanozolol (**6**), and methyltestosterone (IS, 99.3% purity) were purchased from National Measurement Institute (NMI, Pymble, Australia). Their structures are given in Fig. 1. *N*-methyl-*N*-(trimethylsilyl)-trifluoroacetamide (MSTFA) and *tert*-butylmethylether (TBME) were purchased from Sigma–Aldrich, ammonium iodide (NH_4I), potassium carbonate (K_2CO_3) and anhydrous sodium sulfate from BDH Chemicals (Kilsyth, Australia), methanol (HPLC grade), ethanethiol and phosphorouspentoxide (P_2O_5) from Merck (Darmstadt, Germany). β-Glucuronidase (from *Escherichia coli*, K12) was supplied from Roche (Mannheim, Germany). All chemicals and reagents were of analytical grade or higher. Water used in the experiments was of Milli-Q® (Millipore) grade.

2.2. Sample preparation

Stock solutions of AA were prepared by dissolving a known amount of each in HPLC grade methanol to a concentration of 0.2 mg mL^{-1}. Working solutions and standard mixtures were prepared by progressive dilution of the stock solutions. A stock solution of internal standard (methyltestosterone) was prepared at a concentration of 1 mg mL^{-1} in methanol, and the working solution at a concentration of 5 μg mL^{-1}. The derivatization mixture (MSTFA–NH_4I–ethanethiol) was prepared in a ratio 1000:2:6 (v/w/v). Solutions were stored at 4 °C when not in use.

Urine samples spiked with AA were prepared in the concentration range from 0.5 to 20 ng mL^{-1} by adding an appropriate volume of the standard mixture and 25 μL of the internal standard (IS) solution to the blank urine extracts; they were prepared according to the widely accepted sample preparation procedure [12,31] for AA. Briefly, 2.5 mL urine was applied to a 500 mg C-18 SPE column (Bond Elut, Varian), previously conditioned with 3 mL methanol and 3 mL water. AA were eluted with 3 mL methanol and the solvent was evaporated under a stream of nitrogen. 1 mL of phosphate buffer (pH 7.0) and 50 μL β-glucuronidase were added to the residue and the mixture was incubated in a water bath at 55 °C for 1 h. After cooling, 0.75 mL potassium carbonate solution (5%) was added, shaken for 5 min and AA were extracted with two portions of 2.5 mL TBME. The two extracts were combined, dried under a stream of nitrogen, and spiked with AA and IS at appropriate concentration, dried again under nitrogen and kept in a desiccator over P_2O_5 for at least 20 min. Prior to

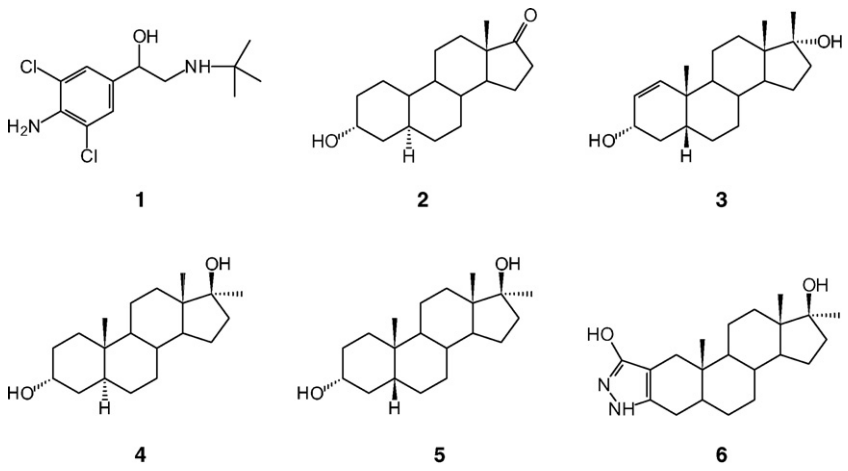

Fig. 1. Structures of the WADA "key" AA: (**1**) clenbuterol, (**2**) 19-norandrosterone, (**3**) epimethendiol, (**4**) M1 metabolite, (**5**) M2 metabolite, and (**6**) 3′-OH-stanozolol.

analysis, the residue was derivatized by dissolving in 50 μL of derivatization mixture (MSTFA–NH_4I–ethanethiol) with heating at 80 °C for 30 min. Standard mixtures of derivatized AA were prepared by drying aliquots of diluted solutions, and derivatized as described.

A urine positive control sample (UPC) spiked with 5 AA (**1**, **2**, **3**, **5** and **6**) at a concentration of 5 ng mL^{-1} was prepared at the National Doping Control Centre (NDCC), Mahidol University in Bangkok, Thailand (WADA accredited laboratory). The sample was used for checking the performance of the present method.

2.3. Instrumentation

2.3.1. GC × GC-FID

The GC equipped with a flame ionization detector (GC × GC-FID) used in the study was an Agilent 6890 system (Palo Alto, CA, USA) with a longitudinal modulation cryogenic system (LMCS; Chromatography Concepts Pty Ltd., VIC, Australia) [32]. The column configuration used with this system was 30 m BPX5 (0.25 mm I.D.; 0.25 μm film thickness (d_f)) as a first dimension (^{1}D) column coupled to a 1 m BPX50 (0.1 mm I.D.; 0.1 μm d_f) as a second dimension (^{2}D) column, both columns from SGE Scientific (Ringwood, Australia). The oven temperature program was from 140 °C (hold for 1 min) to 200 °C at 40 °C min^{-1}, then to 240 °C at 4 °C min^{-1}, then to 330 °C at 15 °C min^{-1} (hold for 5 min). The injector and detector temperatures were 280 and 320 °C, respectively, and the sampling frequency was 100 Hz. Hydrogen was used as a carrier gas at a flow rate of 1.1 mL min^{-1} and 1 μL of the sample was injected in split mode at a split ratio of 10:1. The modulation period was 5 s, and temperature of the modulator system (T_M) was varied from 0 to 200 °C, during the optimization study. CO_2 was used as a coolant in the LMCS and nitrogen as a flush gas at a pressure of 15 psi. Agilent ChemStation software was used for data acquisition and processing.

2.3.2. GC × GC-TOFMS

A LECO time-of-flight mass spectrometer (TOFMS) model Pegasus III (LECO Corp., St. Joseph, MI, USA) connected to an Agilent 6890 GC was used in GC × GC-TOFMS experiments. The TOFMS detector was operated at 1600 V and applied electron ionization voltage was 70 eV. Data collection rate was 100 Hz over the mass range from 45 to 700 amu. The temperatures of the transfer line and ion source were 280 and 230 °C, respectively, and data acquisition and processing were performed by ChromaTOF software (LECO Corp., St. Joseph, MI, USA). A separate GC × GC-TOFMS-based in-house library for improved identification was generated using standard solutions at a concentration of 1 μg mL^{-1} for all AA, in the same manner as in our previous work [17]. The National Institute of Standards and Technology algorithm (NIST MS Search 2.0 Program) was used for mass spectra searching.

Two complementary column configurations were used for AA separation and identification in this experiment: polar/non-polar (P/NP) and non-polar/polar (NP/P).

2.3.2.1. Polar/non-polar column configuration (P/NP). P/NP consisted of 30 m BPX50 (0.25 mm I.D.; 0.25 μm d_f) as ^{1}D and 1 m BPX5 (0.1 mm I.D.; 0.1 μm d_f) as ^{2}D column. Oven temperature program was from 80 °C (1 min) to 180 °C at 40 °C min^{-1}, then to 240 °C at 4 °C min^{-1} and finally to 330 °C at 15 °C min^{-1} (hold for 5 min). Temperature of the injector was 280 °C and 1 μL of sample was injected in splitless mode (1 min purge time) at a carrier gas (helium) flow rate of 1.5 mL min^{-1}.

2.3.2.2. Non-polar/polar column configuration (NP/P). Two different ^{2}D columns were applied in NP/P, differing only in d_f. The first one (NP/P1) was the same as listed for GC × GC-FID analysis above and the second one (NP/P2) was of the same length and I.D., but with a 0.2 μm d_f BPX50 phase. Two chromatographic methods for each NP/P set were applied, differing only in the oven temperature program and carrier gas flow rate. The oven program for the first (NP/$P1_S$; S: short runtime) was from 120 °C (hold for 1 min) to 200 °C at 20 °C min^{-1}, then to 230 °C at 3 °C min^{-1}, and finally to 320 °C (hold for 5 min) at 8 °C min^{-1}, at a flow rate of 1.5 mL min^{-1}, while the oven program for the second one (NP/$P1_L$; L: long runtime) was from 120 °C (hold for 1 min) to 320 °C (hold for 5 min) at 4 °C min^{-1}, at a flow rate of 1.3 mL min^{-1}. Accordingly, a 0.2 μm d_f ^{2}D column film thickness was used in NP/$P2_S$ and NP/$P2_L$ chromatographic methods. 1 μL sample was injected in splitless mode (2.5 min purge open time).

2.4. Experiments in 1D GC mode

All experiments using 1D GC were carried out under the same conditions as in GC × GC, except the modulator was off and the acquisition rate of TOFMS detector was 20 Hz. Thus in this case the 1D system will comprise a long column directly coupled to a short column and whilst this can be termed a multi-chromatography system according to Hinshaw and Ettre [33] and discussed elsewhere [34], the second very short column is anticipated to lead to negligible variation in peak properties such as width.

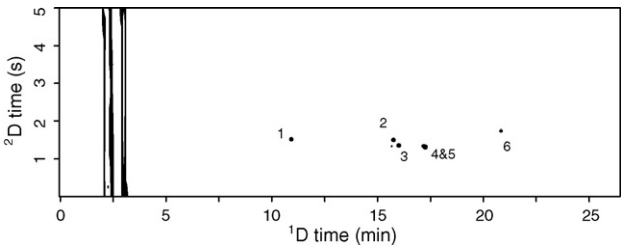

Fig. 2. GC × GC-FID contour plot of the TMS derivatized WADA "key" AA on BPX5/BPX50 column configuration: (1) clenbuterol, (2) 19-norandrosterone, (3) epimethendiol, (4) M1 metabolite, (5) M2 metabolite, and (6) 3′OH-stanozolol.

3. Results and discussion

3.1. GC × GC-FID separation of anabolic agents

It was found in our previous work [17] that the peak shape of steroids on the ^{2}D column depends on the modulator temperature (T_M). The GC × GC separation of the standard mixture of AA was initially accomplished by GC × GC-FID, where T_M was optimized for the best separation and narrowest peak width. An acceptable separation was achieved in 21 min, except for **4** and **5** which co-elute (Fig. 2), at 5 s modulation period but the best modulation temperature was found to be dependent on the retention time of the AA, and presumably their boiling points or vapour pressure. The peak width at half height ($w_{1/2}$) of each anabolic agent was constant over a T_M range from 0 °C until a characteristic (steroid-dependant) temperature, at which stage the peak width of the anabolic agent starts to increase. For instance, the peak of clenbuterol (first eluted compound) started to broaden at 60 °C T_M (Fig. 3), but the peak of 3′OH-stanozolol maintained its $w_{1/2}$ of ~45 ms up to 160 °C. Onset of peak broadening for the rest of the AA was observed between these two temperature limits. In contrast to our previous work [17], here we have not observed an increase of the peak width towards lower temperatures, down to 0 °C (except for **6** where the minimum achieved T_M at its elution temperature was 60 °C). This is probably due to the different nature of these AA, and the use of a different derivatization reagent capable of derivatizing not just the hydroxyls but also keto groups. In order to maximize the separation efficiency (narrowest peaks) and to minimize the CO_2 consumption, we decided to perform all further experiments holding T_M at 80 °C. The 2D plot in Fig. 2 shows the separation of the 6 AA in a standard mixture at a concentration of 10 μg mL^{-1} (split ratio 1:10), at 80 °C T_M.

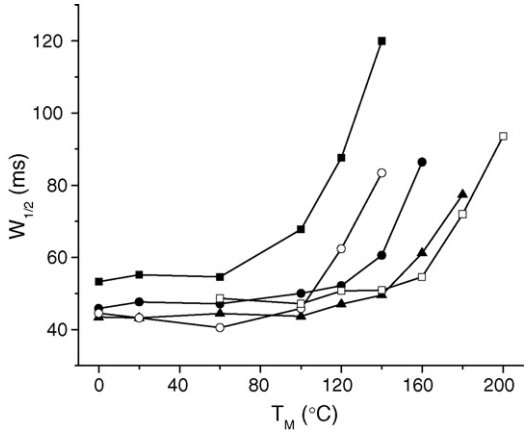

Fig. 3. The influence of modulator temperature (T_M) on peak width ($w_{1/2}$) for the WADA "key" AA. Clenbuterol (■); 19-norandrosterone (●); epimethendiol (▲); M2 metabolite (○) and 3′OH-stanozolol (□).

The AA were detected at a concentration down to 0.2 μg mL^{-1} in a standard mixture (splitless mode), which theoretically corresponds to 4 ng mL^{-1} spiked in urine (at a concentration factor of 50), but the lack of identification power of the FID detector limits its applicability in complex mixtures, since the analyte signals may be poorly recognized in the presence of much higher signals from the matrix.

3.2. GC × GC-TOFMS separation and identification of anabolic agents

Since BPX50/BPX5 (P/NP) has been successfully applied in steroid separation [17] this column configuration was first employed. Despite the good peak shapes (data not shown) of **1**–**5**, with an average $w_{1/2}$ of 90 ms, it was not possible to elute 3′OH-stanozolol at a concentration lower than 0.5 μg mL^{-1}, corresponding to a concentration of 10 ng mL^{-1} in urine at a concentration factor of 50. The injector temperature was varied from 260 to 320 °C, initial oven temperature from 80 to 180 °C, and the flow rate from 0.8 mL min^{-1} to 1.5 mL min^{-1}, and **6** could not be eluted at concentrations below 0.5 μg mL^{-1}. This is probably because of the length (30 m) and the polarity of the ^{1}D column (BPX50) and its activity towards polar components. 3′OH-stanozolol is known as the most problematic steroid in anti-doping control when GC is applied [4,35,36]. Because of this, experiments were continued on the non-polar/polar (NP/P) column configuration, BPX5/BPX50.

3.2.1. Short runtime vs. long runtime

Since the focus of this work was the separation and identification of the key WADA AA in a urine matrix, and not on the extraction efficiency, they were spiked directly into the blank urine extracts. A 2D plot of a blank urine extract spiked with the AA at a concentration of 2 ng mL^{-1} and IS at a concentration of 50 ng mL^{-1}, by using the method NP/P1$_S$, is shown in Fig. 4A. As a result of the poor spread of the components on the ^{2}D column, unsatisfactory separation from the matrix and identification of the key AA are obtained. For instance, **2**–**5** were detected with a similarity below 600, and **1** and **6** were not detected at all. In order to improve the separation efficiency, especially the spread of the components on ^{2}D, a slower oven temperature program was applied (NP/P1$_L$). The obtained separation and identification of AA were better, but at an overall runtime of 50 min (Fig. 4B).

3.2.2. 0.2 μm d_f ^{2}D column vs. 0.1 μm d_f ^{2}D column

In order to further improve component spread on ^{2}D, we applied a ^{2}D column with 0.2 μm d_f, instead of the original 0.1 μm d_f column, keeping other conditions the same as in NP/P1$_L$. The 2D plot of the blank urine extract spiked with the AA at 2 ng mL^{-1} is shown in Fig. 4C, where the improvement of the peak separation is apparent. A peak table with ^{1}D retention times (^1t_R), ^{2}D retention times (^2t_R), $w_{1/2}$, S/N for selected quantification ions and the similarity to the in-house created TOFMS library for the WADA key AA at 0.1 μg mL^{-1} in standard solution was created (Table 1) under these conditions (NP/P2$_L$). The concentration of 0.1 μg mL^{-1} in the standard solution theoretically corresponds to a concentration of 2 ng mL^{-1} of AA when spiked in an original urine sample, at the concentration factor of 50 (2.5 mL urine to 50 μL final volume of the urine extract). The improvement of the separation on the 0.2 μm d_f ^{2}D column over 0.1 μm d_f is clearly seen from the example of 19-norandrosterone, given in Fig. 5. The increased retention of **2** and the co-eluting endogenous component provided by the thicker film produces a better separation, followed by a better deconvolution of their MS spectra.

Table 1
Peak data table of the WADA key anabolic agents including retention times on each dimension and peak widths at half height. TOFMS similarity against the in-house TOFMS library, selected quantification ions and corresponding signal-to-noise ratios were reported for a 0.1 μg mL^{-1} standard solution. Linearity data are for a concentration range of 2.0–20.0 ng mL^{-1}.

Anabolic agent	^1t_R (s)	^2t_R (s)	$w_{1/2}$ (ms)	TOFMS similarity	Quant. ion	S/N	Correlation coefficient	Equation[a]
Clenbuterol[b]	1670	2.48	108	963	335 (86)	51 (542)	0.996	$y = 0.0138x + 0.0102$
19-Norandrosterone	2125	2.58	103	986	405	51	0.996	$y = 0.0058x + 0.0098$
Epimethendiol[b]	2165	2.64	110	970	358 (143)	18 (398)	0.999	$y = 0.0086x + 0.0076$
M1 metabolite (**4**)	2340	2.44	117	984	255	36		
M2 metabolite (**5**)	2345	2.45	119	982	255	30	0.995	$y = 0.0143x + 0.0109$
IS (Methyltestosterone)	2500	2.91	115	981	301	1808		
3′OH-Stanozolol[c]	2920	3.08	128	840	143	34	0.996	$y = 0.0043x + 0.0002$

[a] y = AAarea/ISarea; x = AA concentration in ng mL^{-1}.
[b] For clenbuterol and epimethendiol, two ions of each are shown with their respective S/N. The former ions are more unique, but are of lower abundance.
[c] Equation obtained for 254 m/z.

3.2.3. Linearity and limit of detection (LOD)

The linearity was recorded by obtaining data over the concentration range from 0.5 to 20 ng mL^{-1}, and the limit of detection (LOD) was established under these conditions (NP/P2$_L$). The correlation coefficients were in the range from 0.995 (for 3′OH-stanozolol) to 0.999 (for epimethendiol), over the concentration range from 2 to 20 ng mL^{-1} for all AA. LOD was defined as the lowest concentration which gave S/N above 10 for the quantification ion (see Table 1) and the minimum acceptable match (MAM) criterion [17] to the in-house library of 700 or higher. This is a rigorous criterion when defining LOD for steroids in anti-doping control, and only possible by using GC × GC-TOFMS at the lowest limit required for reporting the WADA check solution. The obtained LOD was from 1 ng mL^{-1} (for **1**, **2**, **4** and **5**) to 2 ng mL^{-1} (for **3** and **6**).

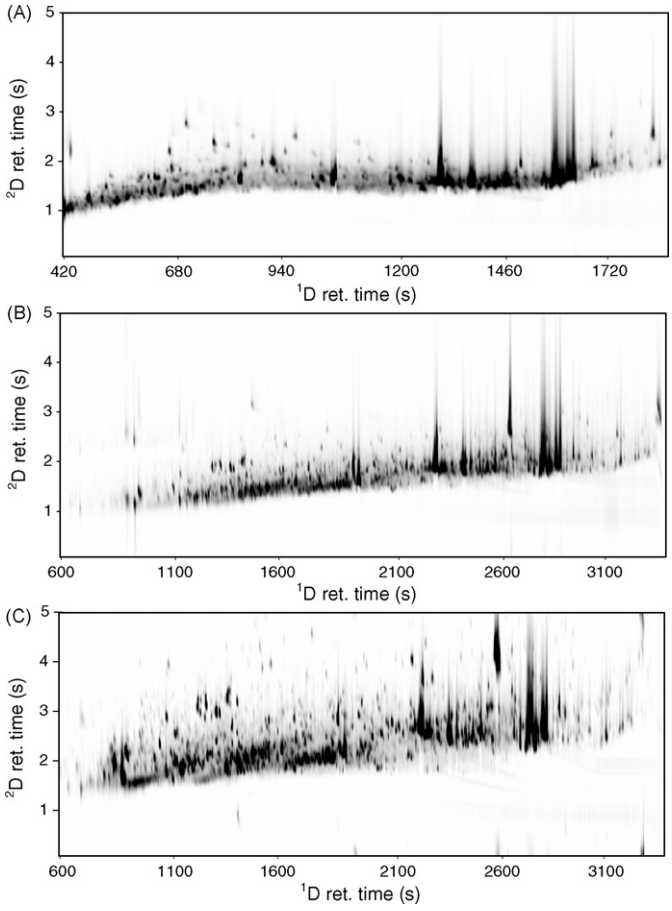

Fig. 4. 2D plots of spiked urine extracts with the WADA key AA at a concentration of 2 ng mL^{-1}, analyzed under NP/P1$_S$ (A), NP/P1$_L$ (B) and NP/P2$_L$ (C) chromatographic conditions.

3.3. GC × GC-TOFMS vs. GC-TOFMS

The high acquisition rate of TOFMS as a detector in GC allows full scan mass spectral information to be acquired not only for the target components, but also for non-targets and the matrix. The similarity matching of each compound's MS spectrum against the MS libraries, even at the LOD, is another benefit of TOFMS. In addition, it allows choosing the best ion for quantification from the full scan mass spectrum, whether it is the most abundant and/or the ion that has least interferences from the matrix. The sensitivity, separation and identification efficiency of the present method (NP/P2$_L$) were checked on a UPC sample (Fig. 6) and a spiked urine extract with WADA key AA at a concentration of 20 ng mL^{-1} (data not shown), in GC × GC and 1D GC mode. In the latter case the modulator was turned off and the acquisition rate was reduced to 20 Hz (in order to closely match the data acquisition conditions in

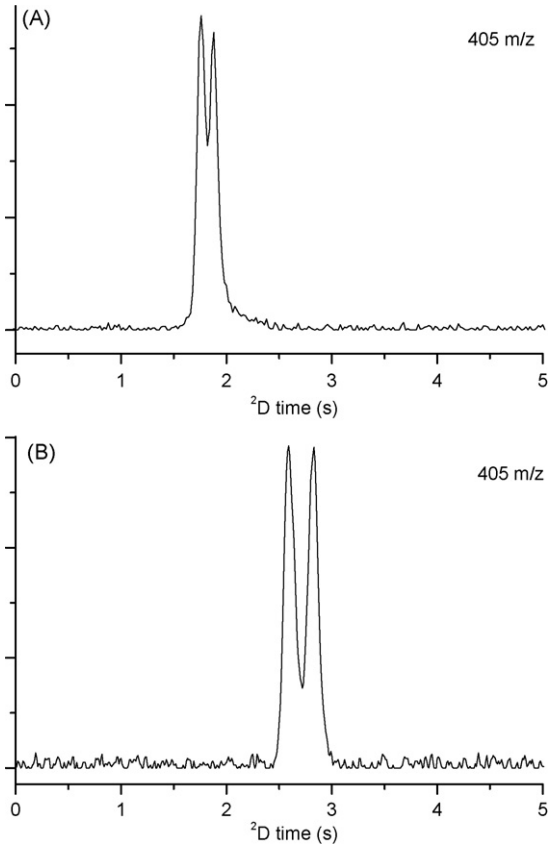

Fig. 5. Extracted ion chromatogram at 405 m/z for 19-norandrosterone, showing the achieved separation from the co-eluting endogenous component on 0.1 μm (A) and 0.2 μm (B) d_f ^{2}D column. (**2**) is the first eluting peak.

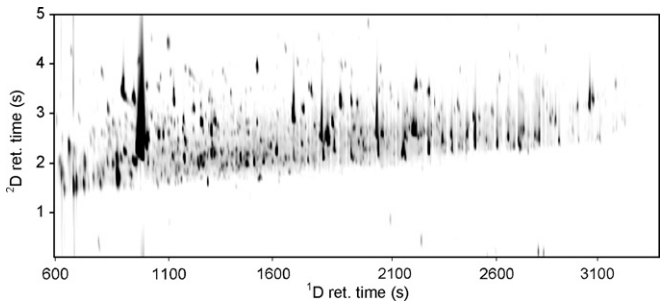

Fig. 6. 2D plot of the UPC sample, analyzed under NP/P2$_L$ conditions.

Table 2
Comparison of similarities to the in-house created TOFMS library between 1D GC-TOFMS and GC × GC-TOFMS obtained spectra for the WADA key anabolic agents spiked in urine at two levels of concentration.

	5 ng mL^{-1} (UPC sample)		20 ng mL^{-1}	
	1D GC	GC × GC	1D GC	GC × GC
Clenbuterol	–	875	–	897
19-Norandrosterone	520	928	854	828
Epimethendiol	–	822	591	936
M1[a] metabolite	–	–	761	938
M2 metabolite	–	932	817	957
3′OH-stanozolol	–	870	–	894

[a] M1 metabolite was not spiked in the UPC sample, since this epimer is not monitored at the MRPL of 2 ng mL^{-1} [1].

classical 1D GC–MS), while the rest of the conditions were kept the same. The results of a comparison (see Table 2) showed that not one anabolic agent in the UPC sample was detected in 1D GC, except **2** with a very low similarity (match 520 on a scale from 0 to 999). Furthermore, at the highest concentration level tested in urine (20 ng mL^{-1}), only **2** and **5** were properly detected in 1D GC (with matches of 854 and 817). **3** was poorly identified (match 591) and **4** was recognized with a higher similarity (match 761) but at a low S/N (14). Clenbuterol and 3′OH-stanozolol were not detected at all under the defined criteria. In comparison, all the AA in both samples were identified in GC × GC mode with an average match

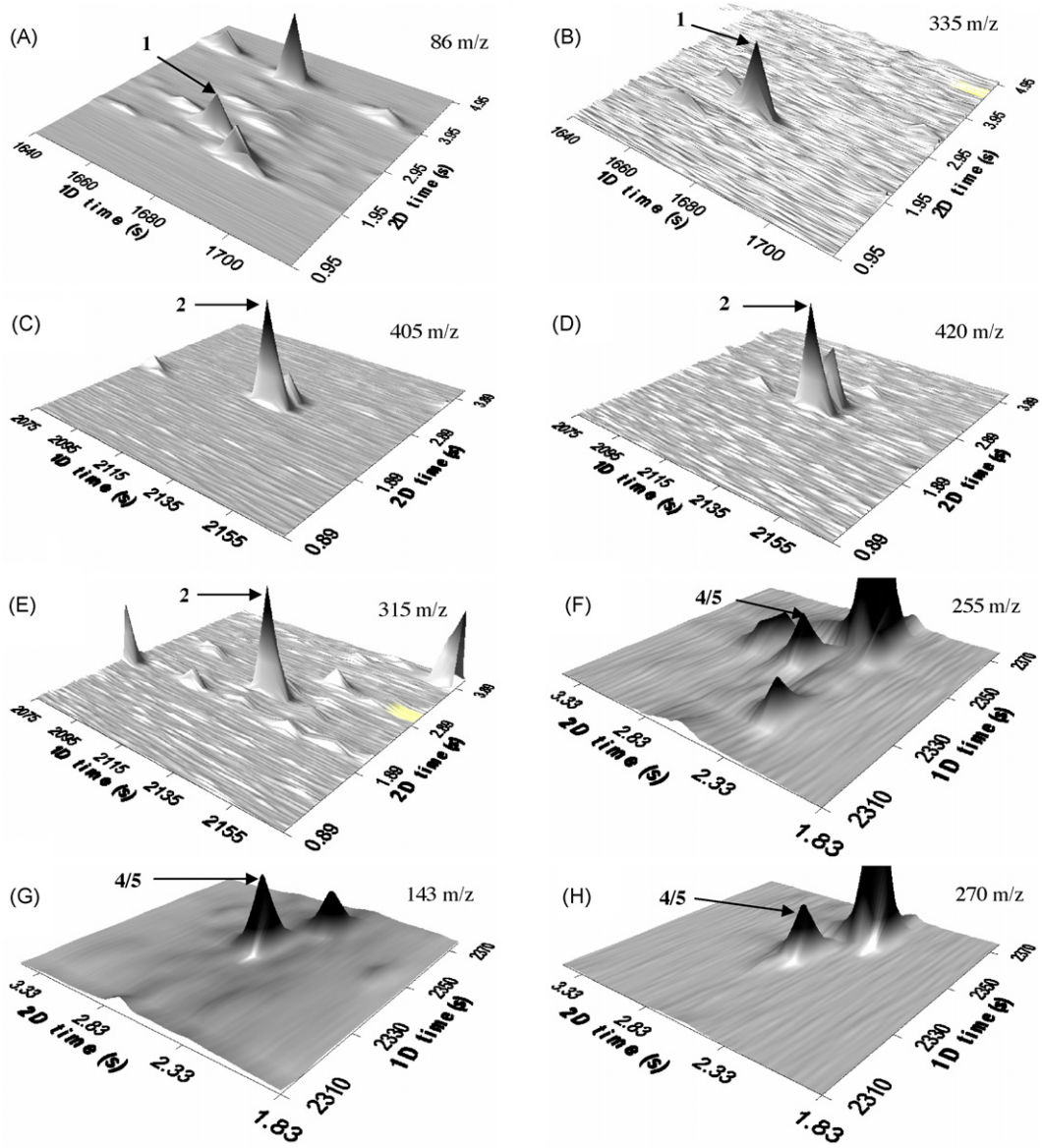

Fig. 7. Extracted 3D plots for: clenbuterol at 86 *m*/*z* (A) and 335 *m*/*z* (B); 19-norandrosterone at 405 *m*/*z* (C), 420 *m*/*z* (D) and 315 *m*/*z* (E); M2 metabolite at 255 *m*/*z* (F), 143 *m*/*z* (G) and 270 *m*/*z* (H), obtained from the UPC sample.

quality of 890 (UPC sample) and 910 (20 ng mL^{-1}). The results of the comparison are given in Table 2. As can be seen from the 2D plot of the UPC sample in Fig. 6, the extract prepared at NDCC is significantly cleaner than the extracts prepared at the ACROSS laboratory, presumably because of the experience of NDCC and optimized conditions applied in sample preparation. The extraction conditions applied at the ACROSS laboratory were not optimized since the focus of the research was on the separation efficiency. The separation and improved dispersion of the components on the ^{2}D column by using the 0.2 μm d_f phase is even more apparent on this sample.

Here a number of advantages of GC × GC over the classical 1D GC were observed in the comparative study of the UPC sample:

1. In the extracted ion chromatogram (data not shown) and 3D plot at 86 *m/z* for clenbuterol (Fig. 7A), an endogenous component co-elutes with **1** on ^{1}D, making quantification difficult in 1D GC if this ion is used. For the 335 *m/z* extracted ion chromatogram (Fig. 7B) there is no interference (co-elution), but this ion is much less abundant compared to 86 *m/z* under these conditions, thus this drastically reduces the sensitivity. As a comparison, S/N of 239 is obtained at the concentration of 0.5 ng mL^{-1} in urine if 86 *m/z* is selected, with no background interference for GC × GC, and S/N of 32 if 335 *m/z* is selected. However, the match quality was not satisfactory to confirm the presence of clenbuterol at this level of concentration, using the applied criteria.
2. In the extracted ion chromatogram and 3D plot at 405 *m/z*, 420 *m/z* and 315 *m/z* for **2** (Fig. 7C–E), an endogenous component co-elutes with **2**, with some common mass fragments (405 *m/z*, 420 *m/z*) as in **2**. The extracted ion area ratio is in favor of the endogenous component at lower concentrations (see Fig. 5, at 2 ng mL^{-1}), and cannot be ignored in 1D GC. It is obvious from Fig. 7E that 315 *m/z* will give the least interference in 1D GC if chosen for quantification, with some loss of sensitivity compared to 405 *m/z*. However, GC × GC offers in this case the choice of any ion for quantification, since full mass spectra are obtained even at the lowest detectable concentrations. The same sensitivity as for 405 *m/z* can be obtained if 225 *m/z* were chosen, with no interfering component (data not shown).
3. In the extracted ion chromatogram (data not shown) and 3D plot at 255 *m/z* for M2 metabolite (Fig. 7F) an endogenous component co-elutes on ^{1}D, making the quantification in 1D GC difficult if this ion is selected. GC × GC overcomes this problem since M2 and the interfering component are separated on the ^{2}D column. However, in the 435 *m/z*, and especially in the 345 *m/z* extracted ion chromatograms (data not shown), there is no interference, but these two ions are less abundant under these conditions when compared to 255 *m/z*, thus reducing the sensitivity by approximately 2-fold. On the other hand, the extracted ion chromatogram and 3D plot at 143 *m/z* (Fig. 7G) lacks interference and can be used as a quantification ion for sensitive determination since 143 *m/z* is the base ion in the mass spectrum of M2. From Table 2, the similarity of the mass spectrum of M2, at this level of concentration, to the in-house TOFMS library is 932. A similar sensitivity as for 255 *m/z* was obtained by selecting 270 *m/z* ion (Fig. 7H), with no co-elution on the ^{1}D column. All these ions (255, 143, 345, 435 and 270 *m/z*) are common for both M1 and M2 metabolites, but at different relative abundances, which helps to differentiate them even if they are separated by only one modulation period (5 s) on ^{1}D column. Deconvolution of their mass spectra is possible if their retentions differ on the ^{2}D column. Fig. 8 shows the two metabolites present in a standard solution. The ion abundance for 255 *m/z* is higher for M1, and that of 270 *m/z* is higher for M2. Since the ratio of ions 255 and 270 *m/z* are not constant in the two peak pulses in Fig. 8, this shows that M1 and M2 are partially resolved on the ^{1}D column. The retention of each compound on the first column can be predicted by a recently proposed metric based on modulated peak distribution [37]. The peaks almost completely overlap on the ^{2}D column.
4. Cryo-focusing and releasing of components on the ^{2}D column permits an elegant integration of the peak area of **6**, even though it showed a high degree of tailing on ^{1}D. The expanded extracted ion chromatogram at 254 *m/z* in Fig. 9 shows nine modulation slices of **6** (due to the tailing on ^{1}D) at a concentration of 0.5 μg mL^{-1} in a standard solution, automatically integrated and summed up by ChromaTOF software. At a concentration of 5 ng mL^{-1} in urine (UPC samples) four slices are detected, integrated and summed up. The identification and integration of tailing peaks can be difficult in 1D GC.

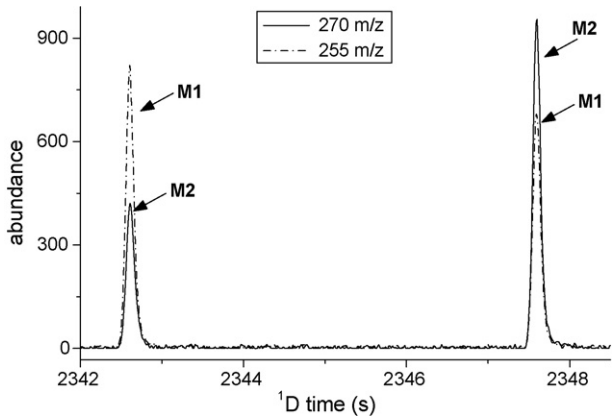

Fig. 8. Mass spectral deconvoluted modulated peaks for ions 255 and 270 *m/z*, corresponding to M1 and M2 metabolites, in a standard solution, showing that the peaks strongly overlap on both the first and second columns.

Among the full scan mass spectral information of the target components, GC × GC-TOFMS also offers the complete spectral information of the matrix (e.g. endogenous sterols and non-target components), allowing post-run search and even quantification if reference materials are available, without the need for re-analysis of the samples. The UPC sample was post-run processed against the in-house created library of 19 endogenous sterols, which were investigated in our previous work [17]. 10 of them were detected and identified with an average similarity of 922. The corrected T/E ratio (testosterone/epitestosterone) for the UPC sample was 0.85, which is within the expected window for normal male

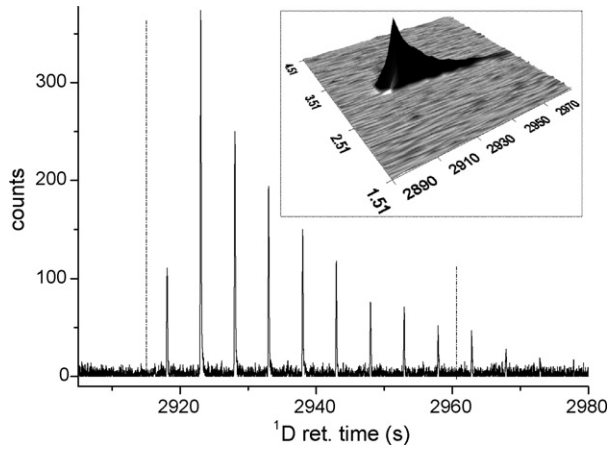

Fig. 9. Extracted ion chromatogram at 254 *m/z* of 3′OH-stanozolol at 10 ng mL^{-1} in standard solution showing the ^1t_R range in which 9 modulations were detected and integrated automatically by ChromaTOF software. The extracted 3D plot in the inset shows the integrated surface.

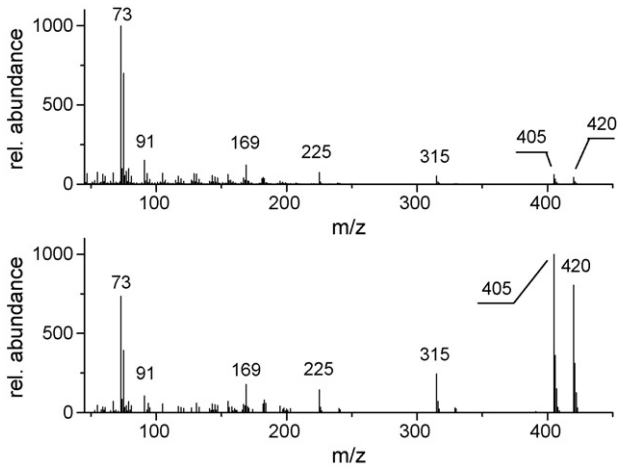

Fig. 10. The comparison of TOFMS spectrum of 19-norandrosterone recorded at 1 μg mL^{-1} in standard solution (A) to its entry in NIST05 quadrupole MS-based library (B).

athlete's urine [1,38,39]. Several tens of other endogenous compounds, generally low molecular mass, were identified against the NIST05 library with a match quality much higher than 900. Higher molecular mass compounds exhibiting higher molecular mass fragments in the mass spectra showed lower similarity to the commercial (quadrupole-based) MS libraries, since bigger difference is observed in the ion intensities to the TOFMS obtained spectra. However, if a more comprehensive TOFMS-based library was created for identified endogenous compounds, the similarity would be expected to be much higher.

The main drawback of using TOFMS as a detector for GC × GC in sterol analysis is some loss of sensitivity when higher mass fragments are chosen for quantification. For instance, 405 *m*/*z* is the base ion in the MS spectrum of **2** in NIST05 (quadrupole-based) library, and 73 *m*/*z* is 73% abundant compared to the base ion. But in the TOFMS spectrum of **2** (recorded at 1 μg mL^{-1} in standard solution), the base ion is 73 *m*/*z* and the ion 405 *m*/*z* is 2.8% abundant only (Fig. 10). This is presumably the main reason for the low similarity of TOFMS spectra against the quadrupole-based MS library, and why best library matching is with a laboratory generated TOFMS library, as observed in our previous work [17]. We point out that the TOFMS was properly tuned according the manufacturer's recommendation.

The prior work reported by Silva et al. [18] employed scanning from 80 to 750 *m*/*z* and so excluded the strong response to the 73 *m*/*z* ion. This ion dominates the mass spectrum of the target anabolic compounds as seen in Fig. 10, and in its absence the reduced intensity of the high-mass ions will appear as apparently more intense. The total ion chromatogram of a urine extract will therefore appear much more complex if the 73 *m*/*z* ion is included in the scan range. The lack of specificity of *m*/*z* 73 ion and its general presence in TMS derivatized samples makes it of low value for diagnostic purposes, and can be considered as contributing to chemical noise in the GC–MS data. The modulation process and peak compression resulting from GC × GC operation leads to somewhat improved sensitivity compared with the analogous 1D GC method.

4. Conclusions

The applicability of GC × GC coupled to TOFMS has been demonstrated in this study. The main advantages of GC × GC over 1D GC, i.e. the increased peak capacity, enhanced sensitivity and improved identification power, were proven through the analysis of the key WADA AA spiked in urine extracts.

In contrast to the traditional GC–MS method in SIM mode, the proposed method offers some unique features. The ^{2}D retention times, the full mass spectra of target and non-target components, as well as the retained full mass spectral information of the matrix, are the benefits of the proposed method. Additionally, the MAM criterion, introduced in our previous work [17], was successfully applied alongside the established WADA rigorous criteria for identification and quantification of AA in urine. Furthermore, the method provides a retro-search (post-run) if any new "designer" drug is present in the sample, but not detected at the time of analysis due to the lack of known spectral information. In this case the search is performed only on the acquired data, with no need of re-analysis. However, the post-run detection of 'designer steroids' would only work if the 'designer steroids' could be extracted by the method. Moreover, GC × GC revealed its real power through some examples of component co-elutions on ^{1}D column in extracted ion mode. The extreme case, where the co-eluted components share the same *m*/*z* masses originating from the same structure fragments, cannot be solved even by using high resolution or tandem mass spectrometry. Separation of co-eluted components in this case is the only solution. We have shown in our experiment a co-elution of 19-norandrosterone with an endogenous component, sharing some of the most characteristic *m*/*z* fragments in their spectra.

A new dimension in doping control analysis has been implemented, this time on the separation side. Instead of increasing the method performance by making the detector more "blind" (like in SIM mode, HRMS, tandem MS) this new method has increased its capability by adding a new "separation" dimension, while retaining the full mass spectral information. We believe that the coupling of the best separation power (GC × GC) to the best detection power (MS/MS) will be probably just a matter of time.

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