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# Enhancing detectability of anabolic-steroid residues in bovine urine by actively modulated online comprehensive two-dimensional liquid chromatography – high-resolution mass spectrometry

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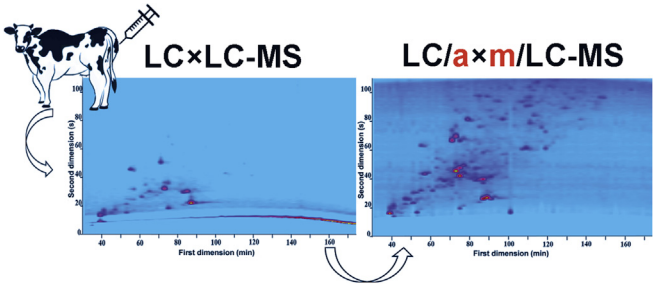
## HIGHLIGHTS

- An online comprehensive two-dimensional liquid chromatography (LC × LC – MS) method for the residues analysis is presented.
- Stationary-phase-assisted active-modulation improves detectability of anabolic steroids.
- Signals are enhanced and signal-to-noise ratios increase by up to a factor of seven.

## GRAPHICAL ABSTRACT

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## ABSTRACT

In this study we describe an approach to enhance the sensitivity of an online comprehensive two-dimensional liquid chromatography (LC × LC) high-resolution mass spectrometry method for the separation and detection of trace levels of anabolic-steroid residues in complex urine matrices.

Compared to one-dimensional liquid chromatography (1D-LC), LC × LC methods offer higher separation power, thanks to the combined effect of two different selectivities and a higher peak capacity. However, when using state-of-the-art LC × LC instrumentation, the price paid for the increase in separation power is a decrease in sensitivity and detectability of trace-level analytes. This can be ascribed to the sample dilution that takes place during each of the two chromatographic steps. The way in which fractions are collected and transferred from the first to the second column is also of paramount importance, especially the volume and the solvent composition of the fractions injected in the second column.

To overcome the detection limitation, we present an active-modulation strategy, based on concentrating the fractions of the first-dimension effluent using a modulation interface that employs trap columns. We obtained a signal enhancement for anabolic-steroid compounds in a bovine-urine sample by a factor of 2.4–7.6 and an increase in the signal-to-noise ratio up to a factor of 7 in comparison with a standard loop-based modulation interface. In addition, thanks to the increased sensitivity of our method,

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a substantially larger number of peaks were detected (76 *vs.* 36). Moreover, we could reduce the solvent consumption by a factor of three (160 mL *vs.* 500 mL per run).



## 1. Introduction

In the livestock industry the use of hormonal active growth promoters can be perceived as an economically profitable choice. Injecting steroid-esters in food-producing animals is a way of getting more tender and leaner muscle meat. However, steroids accumulate in human bodies and their presence has been connected to diseases such as cancer [1], reproductive disorders and infertility [2] and cardiovascular diseases associated with a high blood pressure [3,4]. Consequently, the use of growth-promoting compounds (substances that have an anabolic effect, *i.e.* "hormones") has been strictly prohibited in the European Union (EU) since 1986 [5]. Following EU legislation [6], liquid chromatography - mass spectrometry (LC-MS) has been widely applied for identification and confirmatory analysis of hormones residues.

Many routine methods based on LC-MS and LC-tandem mass spectrometry (LC-MS/MS) have been described in the literature [7–15]. LC-MS methods using high-resolution MS (HRMS) enable the screening of a high number of compounds in one run. LC-MS/MS methods, using multiple-reaction monitoring (MRM), are often used for quantitative purposes. Such methods can be very selective and they provide a good deal of qualitative information, allowing appropriate identification of target compounds.

However, despite the relative universality of MS or $MS^2$ methods, matrix-related signal-suppression effects are of serious concerns in the analysis of complex biological samples. The intensity of signals observed in MS experiments depends on the amount of analyte introduced in the source, but may also depend on the amounts of other simultaneously introduced interferences. High acquisition rates help to reduce the effect of the matrix on component identification in all MS modes, because the greater number of scans across the chromatographic peak enhance the chances of obtaining pure-component spectra. This leads, however, to lower signal-to-noise values, loss of mass accuracy and less-reliable quantification [16]. For instance, van der Heeft et al. reported that LC-MS experiments with mass-resolving power of 7500 FWHM (Full Width at Half Maximum) (for UHPLC-Orbitrap MS) and 10,000 FWHM (for UHPLC-QTOF) did not suffice to unequivocally identify all the steroids present in the extracts of bovine hair due to interfering matrix compounds [14]. Kellmann et al. [17] studied the resolution power required for full-scan MS screening methods for residue analysis, varying the mass resolving power in the range of 10,000 to 100,000 FWHM. Due to the complexity of the matrix, a very high resolving power (≥50,000 FWHM) was found to be required for detection of analytes at low-concentration levels, which is not always feasible.

To minimize the signal-suppression effect and to enhance the overall analytical performance an efficient liquid-phase separation prior to MS detection is essential.

On-line comprehensive two-dimensional liquid chromatography (LC × LC) is a powerful separation tool for the analysis of complex samples in a variety of application fields. Because of the combination of different separation mechanisms, it has a great potential for separating highly similar analytes in complex matrices, decreasing matrix effects and improving analytical performance.

Despite the high number of recent publications on LC × LC separations of complex samples related to food (*e.g.* Refs. [18,19]), there are only a few studies describing applications in food contamination and residue analysis [20,21]. Furthermore, the first dimension ($^1$D) in what are claimed to be LC × LC systems is mostly used as an automated solid-phase extraction (SPE) sample clean-up procedure before the actual 1D-LC separation [22–27].

The majority of the studies in LC × LC have been focusing on the numbers of resolved peaks on the chromatogram and on obtaining a high peak capacity, while the detection sensitivity is of minor interest. However, there are a number of applications where the detection sensitivity is the key objective of the analysis such as is the case for steroid analysis.

The absence of studies involving trace-level residues can be ascribed to the reduced sensitivity of the LC × LC technique compared to 1D-LC, which impairs the analyses of compounds of interest present at very low concentrations.

The major source for this limitation of LC × LC methods is the accumulated dilution of the analytes that takes place during the two chromatographic steps involved in the process, plus any dilution that may occur during collection, storage and transfer of the sample fractions. The relationship between analyte dilution and detection limits has been discussed in several papers [28–31]. It was shown [29,32] that the on-column dilution, split ratio of the effluents, and solvent strength of the $^1$D effluents in the $^2$D system greatly affect the detection performance.

There are several ways to overcome these restrictions to some extent. The standard modulation interface in LC × LC consists of a 2-position 8- or 10-port switching valve, equipped with two identical loops, which serve alternatingly as fraction collector for the $^1$D effluent and injector for the $^2$D separation. The most frequently used strategies to reduce analyte dilution, which do not require complex modification of the interface, are (*i*) to focus the analytes at the top of the second-dimension ($^2$D) column by diluting the $^1$D effluent with a solvent that is a weak $^2$D eluent [33,34] and (*ii*) to use solid adsorbents to re-focus the analytes [35–39].

To our knowledge, LC × LC has found very little application in residue analysis. In the present study we aimed to demonstrate a significant improvement in detection sensitivity for steroid-compounds screening in complex bovine-urine matrices by LC × LC-MS. We developed an "active-modulation" modulation approach, which is based on the collection and concentration of the fractions from the $^1$D separation prior to injection on the $^2$D column. The standard loop-type interface for LC × LC ("passive modulation") is modified by inserting trap columns instead of the loops, with the aim to reduce the volume of the $^1$D effluent fractions, so as to improve the detection sensitivity and to overcome the dilution effect. In this study, we set out to explore whether the smaller volumes allows narrower $^2$D columns to be used, so that splitting the $^2$D effluent prior to the MS detector can be avoided. We also aim to demonstrate that this active-modulation setup allows replacing of the $^1$D effluent by a different $^2$D injection solvent, so that the separation efficiency can be enhanced and incompatibility with the detector can be avoided.

## 2. Experimental

### 2.1. Chemicals and materials

Acetonitrile (ACN), methanol (MeOH) and 1-butanol (all LC-MS grade) were purchased from Biosolve (Valkenswaard, The Netherlands), UHPLC-MS grade water was provided by Avantor Performance Materials (Deventer, The Netherlands). Formic acid (FA, LC-MS grade) was from Sigma-Aldrich (Steinheim, Germany). The standards of stanozolol from Sequoia Research Products (Pangbourne, United Kingdom), α/β-testosterone, α/β-nortestosterone, β-boldenone, α-trenbolone, 1,4-androstadiene-3,17-dione (ADD), methylboldenone, α-methyltestosterone were obtained from Steraloids (Newport, RI, United States), norethandrolone, clostebol, tetrahydrogestrinone (THG), and 16β-OH-stanozolol were obtained from NMI (Sydney, Australia). The chemical structures of the compounds are reported in Table 1. Stock solutions were prepared by dissolving compounds in methanol at 1000 mg $L^{-1}$ and stored at - 20 °C. Dilutions were finally prepared in acetonitrile/water/formic acid (10/90/0.1, *v*/*v*).

### 2.2. Sample preparation

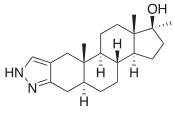
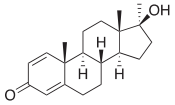
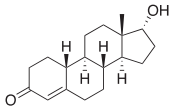
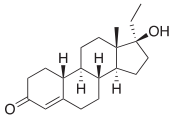
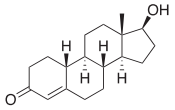
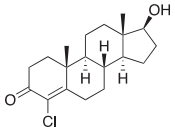
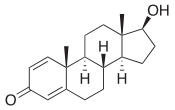
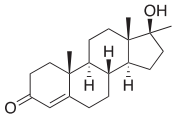
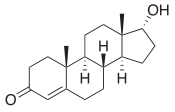
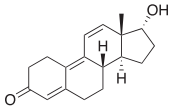
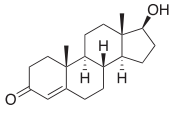
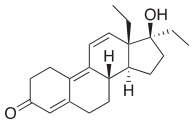
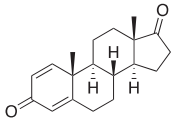
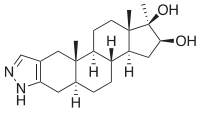
Bovine-urine samples were taken from different farms in the Netherlands. All samples were stored in the dark at - 20 °C until assayed.

The sample-preparation method used in this study was derived from an existing standard operating procedure within the co-authors laboratory (RIKILT). Briefly, after performing enzymatic hydrolysis of a 2 mL aliquot of the urine as described earlier [9], the samples were passed through a $C_{18}$ disposable solid-phase-extraction (SPE) cartridge (6 mL, 500 mg; Bond Elut, Agilent Technologies, Santa Clara, USA), previously conditioned with 5 mL of MeOH followed by 5 mL of water. After sample loading, SPE $C_{18}$ columns were washed with 5 mL of MeOH/water (40/60, *v*/*v*) after which the analytes were eluted with 5 mL of MeOH/water (80/20, *v*/*v*). The eluate was collected and evaporated at 50 °C under a gentle stream of nitrogen until dryness. The residue was dissolved in 100 µL of ACN/water (90/10, *v*/*v*) and transferred into a tapered injection vial.

### 2.3. Instrumentation and chromatographic conditions

All chromatographic experiments in this study were carried out on an Agilent 1290 Infinity 2D-LC system (Agilent Technologies, Waldbronn, Germany), consisting of two Infinity 1290 binary pumps (G4220A), an Infinity 1290 autosampler, two Infinity 1290 thermostatted column compartments (G1316C) and a 2-position 8-port switching valve (G4236A) configured for back-flush injection. All Agilent modules were controlled by a computer with Agilent

**Table 1**
Chemical structures and detection details of anabolic-steroid standards used in this study.

| Compound# | Name | Molecular formula, $[M+H]^+$ | Chemical structure | Compound# | Name | Molecular formula, $[M+H]^+$ | Chemical structure |
|---|---|---|---|---|---|---|---|
| **1.** | **Stanozolol** | $C_{21}H_{32}N_2O$ 329.2587 |  | **8.** | **Methylboldenone** | $C_{20}H_{28}O_2$ 301.2162 |  |
| **2.** | **17α-nortestosterone** | $C_{18}H_{26}O_2$ 275.2006 |  | **9.** | **Norethandrolone** | $C_{20}H_{30}O_2$ 303.2324 |  |
| **3.** | **17β-nortestosterone** | |  | **10.** | **Clostebol** | $C_{19}H_{27}ClO_2$ 323.1772 |  |
| **4.** | **17β-boldenone** | $C_{19}H_{26}O_2$ 287.2006 |  | **11.** | **17α - methyltestosterone** | $C_{20}H_{30}O_2$ 303.2324 |  |
| **5.** | **17α-testosterone** | $C_{19}H_{28}O_2$ 289.2162 |  | **12.** | **17α-trenbolone** | $C_{18}H_{22}O_2$ 271.1693 |  |
| **6.** | **17β -testosterone** | |  | **13.** | **Tetrahydrogestrinone (THG)** | $C_{21}H_{28}O_2$ 313.2089 |  |
| **7.** | **1,4-androstadiene-3,17-dione (ADD)** | $C_{19}H_{24}O_2$ 285.1849 |  | **14.** | **16β-OH-Stanozolol** | $C_{21}H_{32}N_2O$ 345.2463 |  |

OpenLAB CDS Chemstation C.01.04 software (Agilent Technologies, Santa Clara, CA, USA).

The detector was a hybrid quadrupole – time-of-flight (MicrOTOF, Bruker Daltonics, Bremen, Germany) mass spectrometer, equipped with an electrospray interface (ESI, Bruker Daltonics) with the following parameter settings: capillary voltage, 3500 V; nebulizer gas pressure, 3 bar; drying-gas flow rate, 8 L $min^{-1}$; temperature, 200 °C. Measurements were carried out in the positive-ionization mode with a scan range from *m/z* 50 to 1000 and a scan frequency of 6 Hz. The Q-TOF system was externally calibrated on a daily basis with sodium formate clusters. MS measurements were controlled and the data were acquired using a second computer with DataAnalysis v.4.1 software (Bruker Daltonics, Bremen, Germany).

For selectivity evaluation and optimization the following columns were examined: Ascentis Express $C_8$ (150 × 2.1 mm i.d., packed with 2.7-μm core-shell particles, 90 Å pore size), Zorbax Eclipse Plus Phenyl – Hexyl Rapid Resolution HT (50 × 4.6 mm i.d., 1.8-μm fully porous particles, 95 Å pore size) and Zorbax SB-CN Rapid Resolution HD (50 × 4.6 mm i.d., 1.8-μm particles fully porous, 80 Å pore size) all supplied by Agilent Technologies (Wilmington, DE, USA); Kinetex PFP column (150 × 2.1 mm i.d., 1.7-μm core-shell particles, 100 Å pore size; Phenomenex, CA, USA). Unless specified otherwise in the text, all these columns were tested in one-dimensional runs using water/ACN and water/MeOH gradients from 40% to 100% of organic modifiers and a constant concentration of 0.1% (*v/v*) FA. The gradient times were adapted to obtain an effective separation window.

*LC×LC-MS analysis.* All $^1$D separations were performed on an Ascentis Express $C_8$ column (150 × 2.1 mm i.d., 2.7-μm particles; Supelco, Bellefonte, CA, USA) with 0.1% (*v/v*) FA in $H_2O$ (A) and methanol (B) containing 0.1% (*v/v*) FA as the mobile-phase components. Gradient elution was performed from 65% B to 95% B in 175 min, followed by a fast return to the initial composition of 65% B and equilibrated for 33 min at a flow rate 10 μL $min^{-1}$. The separation was carried out at ambient temperature. The injection volume was 1 μL of the standard solution or 40 μL of urine extract. For LC × LC experiments two identical 20-μL loops were installed in the switching valve to collect the fractions coming from the $^1$D. For the $^2$D experiments a cyano column (Zorbax SB-CN, 30 × 4.6 mm i.d., 1.8-μm particles, 80 Å pore size; Agilent Technologies) was used. The separation was carried out at 30 °C with mobile phase $H_2O$ (A) and ACN (B) both containing 0.1% (*v/v*) FA and 1% (*v/v*) 1-butanol. The latter additive served to reduce the column equilibration time [40]. The gradient was programmed from 5% B to 32% B in 0.06 min, kept at 32% B until 1.79 min, returned to the initial conditions of 5% B and equilibrated for 0.2 min. The $^2$D flow rate was set to 2.5 mL $min^{-1}$. A T-piece was used to split the $^2$D effluent, such that only one third of the total flow was send to the MS. The modulation time was 2 min.

For the *Actively Modulated LC×LC-MS* experiments the same hardware and $^1$D conditions as described above were used. An additional Shimadzu LC-20AD pump was used for the dilution of the $^1$D effluent with water. The two streams were connected using a stainless-steel T-piece installed before the modulation interface. The dilution flow was 0.1 mL $min^{-1}$, *i.e.* 10 times as large as the flow of $^1$D effluent. The two cyano trap columns (Zorbax SB-CN, 5 × 2.1 mm i.d., 1.8-μm particles, 80 Å pore size; Agilent Technologies) were placed in the positions of the two loops in the switching valve and the valve was configured for back-flush operation. The setup is discussed in Section 3.3 below. The $^2$D separation was performed on a Zorbax SB-CN cyano column (50 × 2.1 mm i.d., 1.8-μm fully porous particles, 80 Å pore size; Agilent Technologies) at 40 °C and a flow rate of 0.8 mL $min^{-1}$, with $H_2O$ (A) and ACN (B), both containing 0.1% (*v/v*) FA and 1% (*v/v*) 1-butanol, as mobile-phase components. The gradient elution was from 20% B to 30% B in 0.01 min, from 30% B to 33% B in 1 min, then to 45% B in 0.6 min, returned to the initial conditions of 30% B and then equilibrated for 0.4 min. The modulation time was 2 min.

Peak-detection evaluation was performed by subtracting background spectra followed, by using the "Sophisticated Numerical Annotation Procedure" (SNAP) peak-finder algorithm with the signal-to-noise threshold of 3 and a quality factor of 0.5. LC × LC-MS data visualization was performed using GC Image R 2.5 software (GCimage, Lincoln, NE, USA).

### 2.4. Calculations

The peak capacity ($n_c$) in gradient-elution LC was calculated using the following equation [41].

$$n_c = 1 + \left( \frac{t_G}{1.7 \cdot \left( w_{1/2} \right)} \right) \quad (1)$$

where $t_G$ is the gradient time and $w_{1/2}$ is the average peak width at half height (FWHM).

The dilution of the complete two-dimensional chromatographic process can be estimated from Ref. [65]:

$$^{2D}DF = {}^1DF \times {}^2DF = \frac{2\pi \times {}^1\sigma \times {}^2\sigma \times {}^2F}{V_i \times {}^2t_w} \quad (2)$$

where $^1\sigma$ and $^2\sigma$ are the standard deviations of the first and second dimension peaks in time units, $^2$F is the 2nd dimension flow rate, $V_i$ is the injection volume and $^2t_w$ is the modulation time.

## 3. Results and discussions

### 3.1. Stationary-phase selection and orthogonality evaluation

To achieve the highest possible resolving power, LC × LC requires coupling of methods with separation mechanisms that are as different ("orthogonal") as possible. To alter the selectivity of a method, one can choose a different retention mechanism (column chemistry) and/or manipulate the composition of the eluent (for example, the organic component [42] [43], or the pH [44]).

As anabolic steroids are small neutral molecules with similar structures (Table 1), ion-exchange chromatography (IEC) and size-exclusion chromatography (SEC) are not suitable for their separation. *n*-Alkyl-chain reversed phases, such as $C_8$ and $C_{18}$, are most-commonly used for steroids analysis [7–10]. A high degree of orthogonality can be provided by coupling reversed-phase LC (RPLC) with normal-phase LC (NPLC). Several NPLC-based separation of steroids and their derivatives were reported on diol [45] [46], amino [47] or bare-silica columns [48], using apolar mobile phases (*e.g.* combinations of *n*-hexane and dichloromethane). However, because of eluent incompatibility and immiscibility issues, combining such a separation with a reversed-phase system (aqueous eluents) is difficult. Ideally, this requires a sophisticated modulation interface, in which the $^1$D effluent is either evaporated [49–51] or exchanged [52]. Several polar stationary phases were tested (*e.g.* Inertsil Diol (GL Sciences), Hypersil GOLD Amino (Thermo Fisher Scientific) and Luna silica (Phenomenex)), utilizing solvents miscible with RPLC eluents (*i.e.* acetonitrile or methanol). However, our results showed a lack of retention or poor separation of hydrophobic steroids compounds (results not shown).

To the best of our knowledge the only paper showing the successful combination of non-polar and polar types of materials for steroid analysis is that of Liu et al., where a $C_{18}$-based separation

with water/acetonitrile mobile phases was coupled to a method using a polyamine stationary phase and acetonitrile-rich solvents [53]. This method was, however, designed to analyse steroid glycosides, which are characterized by a higher hydrophilicity, due to the presence of additional hydroxyl structural groups on the sugar moieties. Hydrophilic-interaction liquid chromatography (HILIC) employs mobile phases containing high percentages of acetonitrile and low amounts of water and it is more compatible with RPLC than NPLC [54], [55]. However, HILIC is specifically suitable for highly polar analytes and HILIC × RPLC (or RPLC × HILIC) but is unlikely to provide sufficient separation for such hydrophobic compounds as anabolic steroids. Furthermore, in case free steroids are analysed, the polar constituents of the samples are typically removed during the sample-preparation step. Thus, we focused our research on RPLC × RPLC combinations. Beside a good robustness and repeatability and well understood separation mechanisms, the use of different RPLC separations in the two dimensions may provide adequate selectivity with few solvent-compatibility issues. In addition, by choosing an appropriate combination of columns and eluents, one may re-focus the analytes in the $^1$D effluent fractions at the top of the second-dimension column, thus decreasing band broadening and improving sensitivity.

In exploring possible selectivities complementary to hydrophobic separation on alkyl stationary phases, we tested RP separations targeting aromaticity and steric interactions. We compared phenyl − hexyl stationary phases (which are thought to provide different aromatic selectivity based on π-π interactions [56], [57]), fluoro-substituted phases (pentafluorophenyl, PFP, characterized by π-basicity and strong steric interactions, especially for halogenated compounds) and cyano-propyl phases (CN, known for π- π interactions, as well as dipole-dipole interactions [58]). The π-electron density and π-π interaction between steroids and stationary phases is also influenced by the type of solvent used and, thus, significant differences in selectivity are observed when changing the organic solvent from acetonitrile to methanol [43,56,59]. This has been demonstrated by RPLC × RPLC separations with good orthogonality using different solvents in the two dimensions [42].

A typical strategy to study orthogonality based on exploratory runs with a mixture of representative standard compounds. However, such a mixture does not represent the true complexity of the sample matrix and, therefore, does not provide accurate information pertaining to the sample. It also requires a large number of available reference compounds. However, many relevant steroid derivatives cannot be purchased. Therefore, in our study we opted to use the target sample (bovine urine) to study the orthogonality of different one-dimensional separations. This may provide sufficient information to understand each solute's retention behaviour in each dimension. We evaluated the complementarity of different RPLC selectivities using the asterisk approach developed by Camenzuli et al. [60], which is based on experimentally measured retention times of the tracked compounds under different gradient-elution conditions on various columns. A set of equations are used to characterize the use of the separation space and to provide a measure of the orthogonality in the form of the $A_0$ value. In the ideal case of completely orthogonal mechanisms an $A_0$ value of 100% will be obtained. It was shown [60] that the number of components presented in the sample does not greatly affect the $A_0$ value. However, in order to obtain low standard deviation values (below 0.10) the number of components used to compute $A_0$ should exceed 25.

The RP columns used in this study are listed in the Experimental section. All columns were tested for the separation of spiked urine samples (operation conditions are reported in the Experimental section). Retention times of steroid standards and unknown compounds, tracked in the chromatograms based on matching individual *m/z* values, were collected and used for performing the calculations in Microsoft Excel. The orthogonality of the separation was estimated based on the 36 major tracked, confidently matched peaks that were found in the samples, including 14 steroids standards (see Table 1) spiked into the urine.

The highest value of 74% (shown in Fig. 1) was obtained for the combination of a $C_8$ column with methanol as organic modifier and a CN column using acetonitrile. This combination was subsequently selected for analysing the bovine urine samples with LC × LC-MS. Our results suggest that the π-π-interactions of the polar analytes with the cyano and phenyl columns are supplemented by dipole-dipole interactions on the cyano column in RPLC of steroids [56]. A similar $C_8$ × CN column combination was successfully applied for the separation of phenolic acids in herb extracts [61] and for the analysis of Traditional Chinese medicines [62,63]. A summary of the other column/mobile-phase combinations is provided in Table S1 in Supporting information.

### 3.2. LC × LC separation of anabolic steroids in bovine urine samples

Optimization of LC × LC methods may be directed towards different analytical objectives. In our case the aim was to perform residue-monitoring analysis for untargeted screening, focusing in particular on the separation of isomers and closely eluted 'unknowns'. The analysis of these compounds is particularly relevant, because of the frequent introduction in the market of new derivatives.

With these aims in mind, we did not optimize our setup to obtain the highest possible peak capacity (one of the major descriptors of the separation power in LC × LC), but instead targeted the chemical selectivity. During the method development, the peak-capacity values and peak production rate (peak capacity per unit time) were made secondary to the better resolution of isomers.

- Optimization of $^2$D separation method

In LC × LC the total analysis time is dependent on the time of the $^2$D separation, as this defines the sampling time and, therewith, the conditions for the $^1$D separation. Consequently, to achieve a reasonably fast LC × LC separation the $^2$D analysis should be as fast as possible. Hence, one of the most critical aspect of our method optimization was the optimization of the $^2$D separation.

During the method development, we investigated the influence

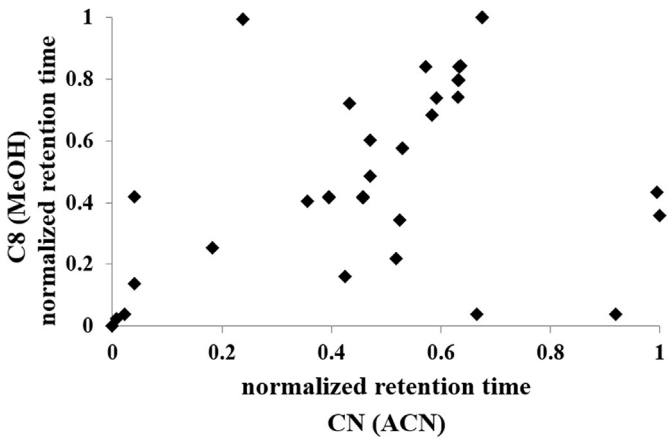



**Fig. 1.** Normalized-retention-time plot for the $C_8$ × CN column combination selected for LC × LC-MS analysis of the bovine urine samples. The prediction is generated by combining two 1D-LC runs. The obtained orthogonality value ($A_0$) is 74%.

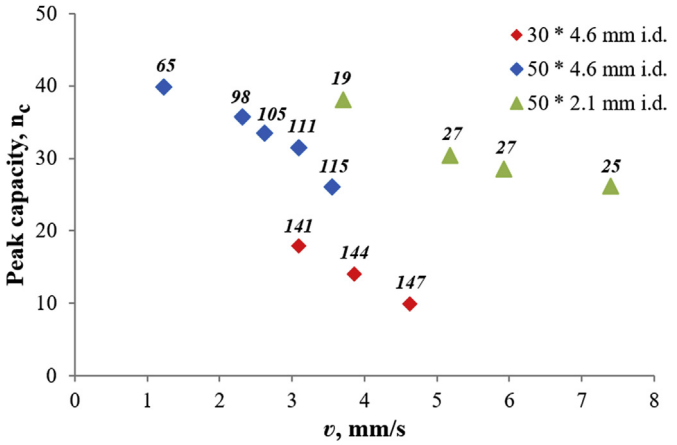



**Fig. 2.** Effect of the linear flow velocity on the peak capacity and on the dilution factor (values indicated above for each point) of the $^2$D separation for three CN columns of different dimensions considered in this study. Chromatographic conditions: mobile phase (A) $H_2O$ and (B) acetonitrile, both containing 0.1% (*v*/*v*) FA; gradient elution from 30% to 40% B (with $t_g/t_0 \approx 10$); $V_{inj} = 1$ µL of standard steroid mixture (Table 1). Dilution factors were calculated according to Eq. (2).

of linear flow velocity on the peak capacity. Short and wide-bore $^2$D columns are typically used to ensure fast separation with a reasonable column back-pressure and a negligible effect of $^2$D injection band broadening. Fig. 2 shows a comparison of the peak-capacity values obtained for cyano columns of different lengths and internal diameters (50 × 4.6 mm i.d., 30 × 4.6 mm i.d. and 50 × 2.1 mm i.d., all packed with 1.8-µm particles). We obtained the highest peak capacity when using the 50 × 4.6 mm i.d. column. However due to flow-rate restrictions (at the maximum back pressure of 60 MPa) we could not achieve an efficient separation in less than 2.5 min (not taking into account the time for column equilibration), even at the highest possible linear flow velocity ($u = 3.8$ mm s$^{-1}$, flow rate of 2.5 mL min$^{-1}$) (See Supporting material, Fig. S1). In order to overcome this limitation we tested a shorter column (30 × 4.6 mm i.d.), while keeping the gradient time constant. This shortened the analysis time, but induced losses in peak capacity and increased dilution (dilution factors specified for each point in the figure).

One of the challenges of our optimization was to preserve good resolution ($R_s > 1$) of the isomers of the steroid standards present in the sample (Fig. 3), while maintaining a short cycle time. To achieve this, we reduced the injection band broadening by programming a step increase from a low percentage of organic modifier to the elution composition of our first analyte (steep increase from 5% B to 32% in 0.06 min). This allowed us to focus the sample at the head of the column and to achieve satisfactory resolution of the ten test analytes within a cycle time of 2 min.

- Optimization of $^1$D separation and LC × LC method

When progressing from the commonly used on-line SPE-LC-MS methods to a comprehensive two-dimensional LC separation, the first-dimension separation must be optimized. Fig. 4 shows the LC-MS extracted-ion chromatograms for the set of steroid standards using a linear methanol-water scouting gradient on a $C_8$. When compared to the analysis of the same sample reported in Fig. 3, great differences in the elution order of the steroids can be observed (roughly 1 through 10 becomes 7, 4, 3, 8, 2, 6, 5, 9, 10, 1). The asterisk approach suggests that this combination of the two columns may provide a good selectivity (great orthogonality) for steroid standards (Table S1).

The optimization of the $^1$D efficiency is summarized in Fig. 5a and Fig. 5b. As expected, an approximate square-root relationship was observed between the peak capacity and the gradient time, with the highest peak capacities observed at the highest $t_g/t_0$ ratio. Fig. 5b shows the relationship between peak capacity and linear flow velocity. It is interesting to note that, despite the use of core-shell technology, this methanol-based separation method seems to work best at intermediate linear flow velocities.

When the modulation time is increased, peak capacity is lost due to undersampling of the $^1$D effluent. However, this may be compensated by the longer time available for the $^2$D separation, which allows an increase in $^2n_c$. It has been demonstrated in several studies [64,65] that the highest peak capacity per unit time will be obtained if $^1$D peaks are sampled between two to three times to the $^2$D. This implies that in the present case, with an optimized $^2$D cycle time (equal to the modulation time) of 2 min, the $^1$D peaks should be roughly 4–6 min wide. However, the peak width for gradient-elution LC cannot be varied at will.

The peak width depends largely on three factors: *i.e.* (*i*) the square root of the plate count of the column ($\sqrt{}^1N$), (*ii*) the column hold-up time ($t_0$) and (*iii*) the retention factor at the moment of elution ($k_e$). Snyder et al. have introduced a simple (Linear Solvent Strength, LSS) model [66,67], which relates $k_e$ to the effective gradient slope $b = S\ B\ t_0$, where $S$ is the slope of the log $k$ *vs.* $\varphi$

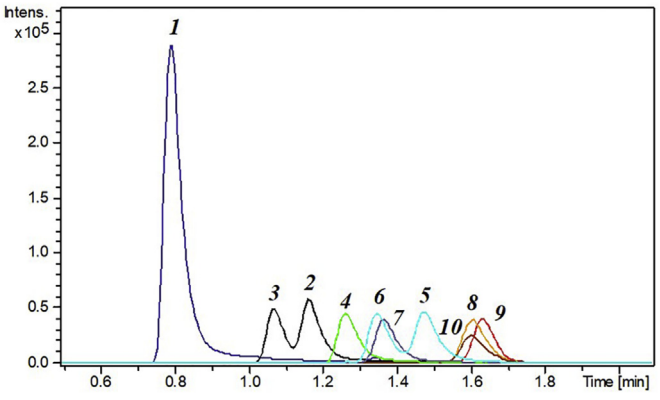



**Fig. 3.** Reconstructed chromatograms of steroid standards (overlaid extracted-ion chromatograms with 1 mDa mass extraction window) separated on a 30 × 4.6 mm i.d. CN column, packed with 1.8-µm particles, under the optimized $^2$D separation conditions. Detailed conditions are reported in Experimental. For analyte abbreviations and identification see Table 1.

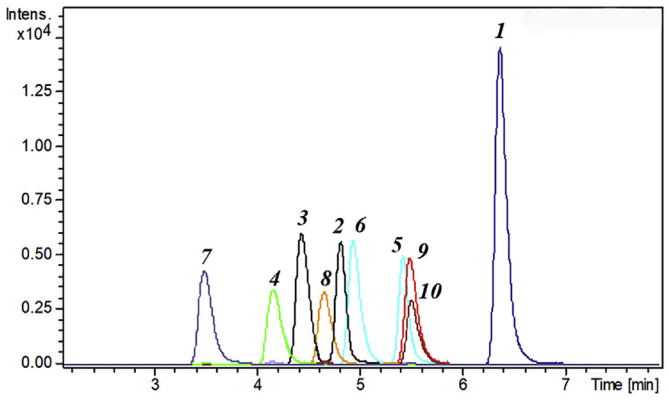



**Fig. 4.** Reconstructed ion chromatogram of the mixture of steroid standards separated on the $^1$D on a 150 × 2.1 mm i.d. $C_8$ column, packed with 2.7-µm particles. Conditions: flow rate 0.2 mL min$^{-1}$, mobile-phase components A: $H_2O$ and B: methanol both containing 0.1% (*v*/*v*) FA. Linear gradient from 65% to 95% B in 8.75 min. For analyte identification see Table 1.

relationship, $B$ is the slope of the gradient (variation of $\varphi$ with time) and $t_0$ is the column hold-up time. The optimum value for $b$ is in the range of 0.2–0.4. This implies that for a given analyte, phase system (given $S$), column and flow rate, the peak width cannot be varied by more than a factor of two to stay within the optimum range. Because small-molecule analytes, such as steroids (molecular weight, MW, 250–350 Da), typically show much smaller $S$ values than large-molecule analytes, such as peptides or proteins [68], relatively short gradients are typically used (*e.g.* $t_g/t_0 = 10\Delta\varphi$, where $t_g$ and $\Delta\varphi = \varphi_{final} - \varphi_{initial}$ are the duration and the span of the linear segment of the gradient, respectively). As a result, a first-dimension peak width of 4–6 min can only be achieved if $t_0$ is very large, i.e. (very) long columns and/or (very) low flow rates.

Therefore, the conditions that we could realistically apply for the $^1$D separation of steroids were sub-optimal in terms of the flow rate used (10 μL min$^{-1}$; linear flow velocity 0.07 mm s$^{-1}$). A $^1$D gradient with a slope of 0.17 %B per minute was used, resulting in an average peak width of 4.5 min and an analysis time of 175 min. Thus the, first-dimension run was optimally sampled with a modulation time of 2 min.

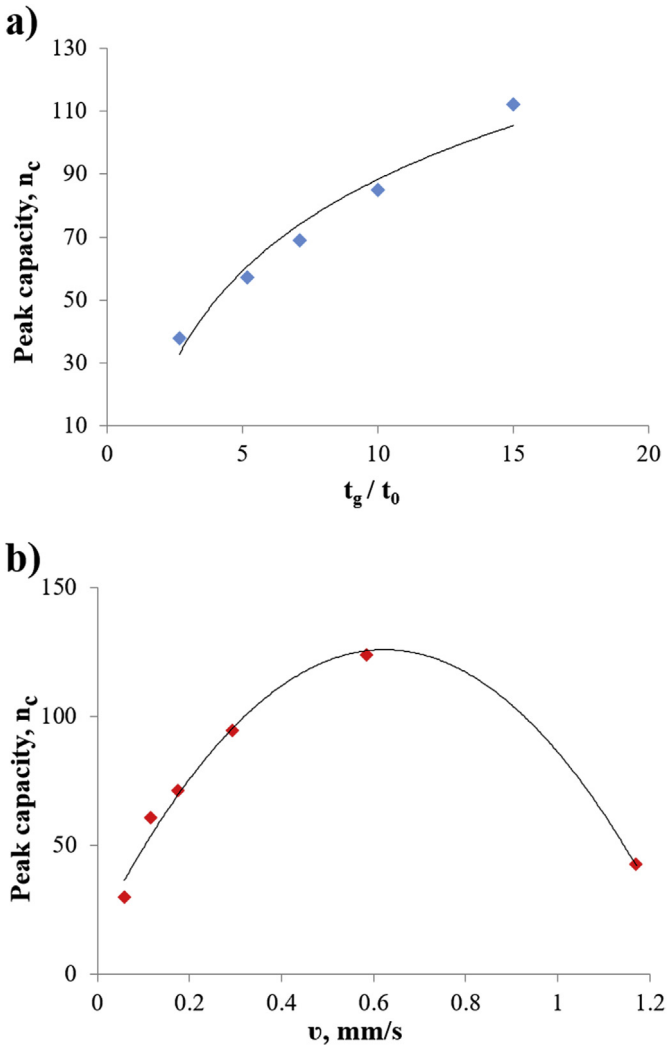



**Fig. 5.** Effect of the gradient time (**a**) and the linear flow velocity (**b**) on the peak capacity of the $^1$D separation on a 150 × 2.1 mm i.d. $C_8$ column, packed with 2.7-μm particles. Conditions: mobile phase (A) $H_2O$ and (B) methanol, both containing 0.1% (*v*/*v*) FA; $V_{inj}$ = 1 μL, ESI (+) detection. (a) Linear gradient from 65 to 95% B at flow rate 60 μL min$^{-1}$; (b) constant $t_g/t_0$ of 5.

The optimized LC × LC method was first applied to the separation of the mixture of steroid standards (Fig. 6a) and, subsequently, to the analysis of the bovine urine sample (Fig. 6b). As can be seen from Fig. 6a we obtained a good spread separation of the standards with the proposed method in the 2D separation space, with a sufficiently high sampling speed to maintain the resolution of the isomers (2, 3 and 5, 6) obtained in the $^1$D separation dimension. When we applied this method for the LC × LC analysis of the urine sample, we observed only a relative small number of features, despite the relative high amount of sample injected (40 μL, concentrated from 2 mL of urine). In part this is due to the fact that a total-ion chromatogram is shown for the urine sample (untargeted-screening mode), whereas overlaid extracted-ion chromatograms, with an inherently higher signal-to-noise ratio are shown for the standard mixture (targeted-screening mode). Another main reason why so few peaks are observed in Fig. 6b is dilution of the analytes during the two chromatographic steps involved in the process and during transfer from the injector to the $^1$D column, at the modulation stage (transfer from $^1$D to $^2$D), and from the $^2$D column to the detector (column and extra-column band-broadening) [29]. The small number of detected peaks discerned in Fig. 6b clearly limits the application of this method for the analysis of low-concentration (trace-level) compounds in a complex mixture, such as urine.

The column diameter greatly affects the dilution and the sensitivity [69–71], but the exact gain is difficult to predict, due to the effect of flow rate on MS response. In practice, scaling down the column i.d. from 4.6 mm to 1 mm has been shown to lead to an increase in sensitivity by a factor of eight [69], while a decrease from 4.6 mm to 0.32 mm resulted in an increase in signal height by

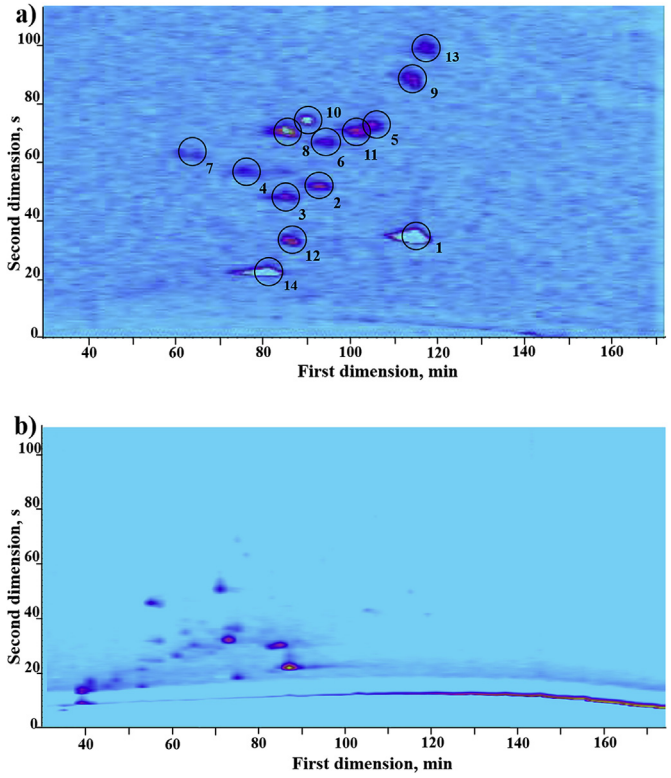



**Fig. 6.** LC × LC-MS separation, performed under the optimized conditions and detected in positive-ionization (ESI-MS) mode. **a**: mixture of steroid standards (overlaid extracted-ion chromatograms with 1 mDa mass extraction window); **b**: bovine-urine sample (total-ion chromatogram). For separation conditions see Experimental. For analyte abbreviations and identification see Table 1.

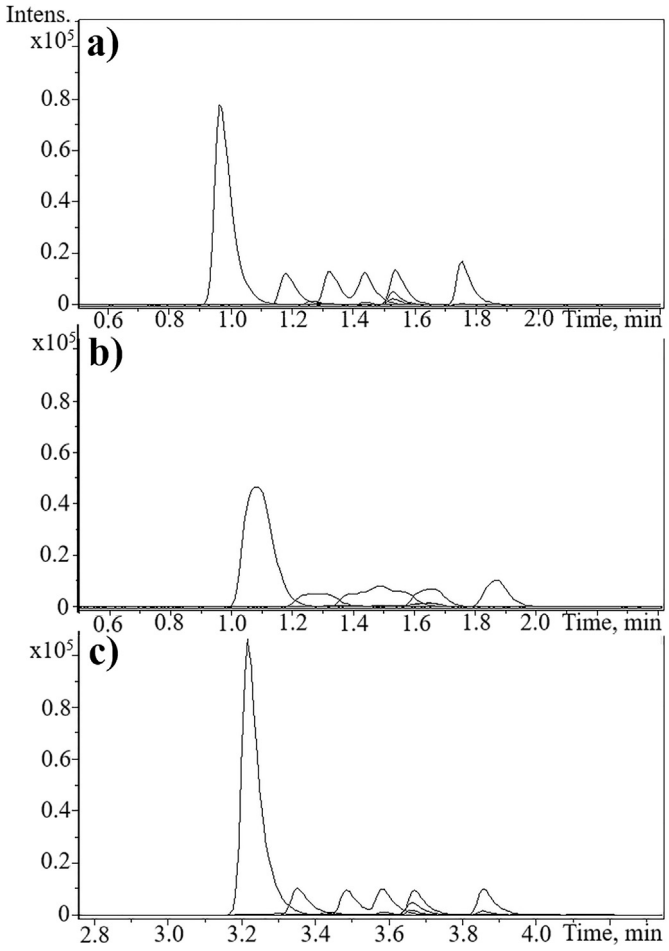



**Fig. 7.** Effect of the injection volume of the steroid standards on the optimized $^{2}$D separation performance using a 50 × 2.1 mm i.d. Zorbax-CN column. For separation conditions see Experimental. **a**: 1 μL sample injection ("ideal" scenario); **b**: large-volume injection (20 μL), mimicking the injection from the sample loop with $^{1}$D effluent (containing ≈ 80% Methanol, a "real" case); **c**: the same 20-μL injection (containing ≈ 80% Methanol) using the modulation approach.

a factor of 200 [70].

Fig. 2 shows a significant reduction of the dilution factor for the 2.1-mm i.d. column in comparison with conventional-bore columns (4.6 mm i.d.). An additional advantage from the decrease of the column i.d. is the lower operating flow rate, which eliminates the need for splitting of the effluent flow prior to the detector, thus further improving the sensitivity.

As we described above (LC × LC optimization section) the use of narrow $^{2}$D columns is attractive in combination with UHPLC technology. Finally, the 50 × 2.1 mm i.d. columns yielded the best compromise between peak capacity, dilution and $^{2}$D cycle time. Thanks to sub-2-μm particle technology we could operate this column at high linear velocities, with limited losses in peak capacity, while at the same time reducing the dilution and solvent consumption. However, we cannot reduce the column diameter without considering injection band broadening. For example, a transfer volume of 20 μL corresponds to ≈20% of the column volume of a 50 mm × 2.1 mm i.d. $^{2}$D column and it would significantly affect the performance of the column ($^{2}n_c$). The elution strength of the sample solvent or of the fraction transferred to the $^{2}$D column also significantly affects the peak heights and peak widths in the resulting separation [29]. To estimate the impact of injection band broadening in practice, we designed experiments to simulate the effect of the transfer volumes under our optimized LC × LC conditions, recomposing our samples in a solvent mimicking the elution composition of the steroids from $^{1}$D (ϕ ≈ 80% methanol). Because of the high concentration of methanol, the sample solvent becomes a strong eluent for the $^{2}$D, which results in serious peak distortion. The effect of the injection volume is clearly demonstrated in Fig. 7, where we show chromatograms obtained by injecting the same mass of standards, diluted in 1 μL and 20 μL solvent (case a and b in Fig. 7, respectively). The 20-μL injection resulted in a significant decrease of the signal intensity (peak height) and, overall, separation performance.

The way to overcome the injection limitations involves replacing the loops in a conventional loop-type LC × LC interface with two small trap columns and using this interface in combination with a dilution flow after the $^{1}$D separation. This active-modulation approach has been denoted a × m [35]. The setup is illustrated in Fig. 8. The $^{1}$D effluent was diluted with an aqueous make-up flow using a T-piece that reduced the elution strength of the $^{1}$D mobile phase and enhanced the trapping. The components captured on the traps were then eluted by the $^{2}$D gradient towards the $^{2}$D separation column in backflush mode.

### 3.3. *Actively modulated HPLC × UPLC analysis of the urine samples*

If the sample components are retained by the trap columns, this approach allows the use of a high dilution flow, reaching volumetric ratios that depend on the binding capacity of the trap column and on the retention factors of the analytes at the resulting solvent composition. Given the hydrophobicity of steroid compounds (logP about 3.0–3.5), their retention factors on the CN-column at a composition of about 5% methanol/95% water were above 20. We opted for a dilution flow rate that was about ten times higher than the $^{1}$D-effluent flow rate. As a result the eventual $^{2}$D injection volume depended only on the porosity and the volume of

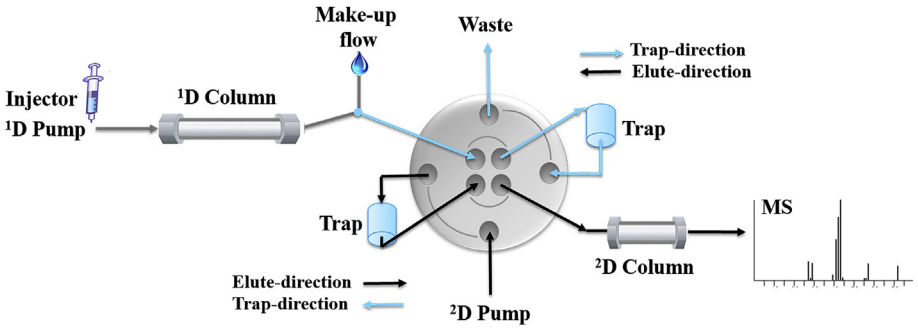



**Fig. 8.** Schematic illustration of the *actively modulated HPLC×UPLC (a×m)* set-up, used in our study.

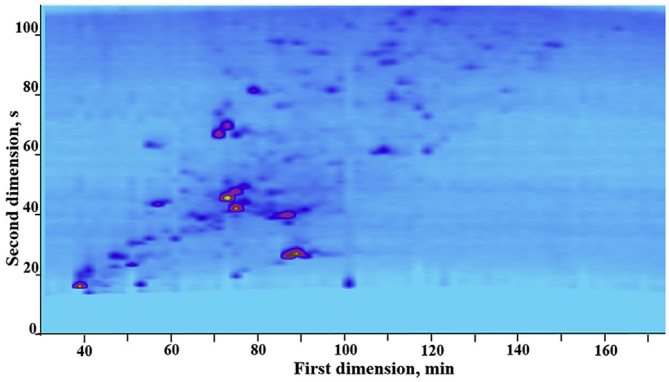



**Fig. 9.** Total-ion chromatogram obtained after HPLC × UPLC separation of bovine urine sample using the active-modulation interface. Chromatographic conditions are reported in Experimental section. This figure may be compared with Fig. 6b (non-modulated separation of the same sample under the same conditions).

the trap column used. In our case the injection volume amount to about 4% of the volume of the $^{2}$D column.

To further enhance the performance of the setup, the retention factors on the $^{2}$D materials should exceed those on the trap column, in order to re-focus the analytes at the head of the $^{2}$D column. However, a strong elution solvent should be used to ensure the full and rapid removal of compounds from the traps and to achieve the maximum focusing effect [72]. This strong solvent should not be injected on the $^{2}$D column. One solution may be to implement an additional make-up flow prior to the $^{2}$D separation. However, an additional construction may diminish the compression effect and the $^{2}$D flow rate will be increased, which will affect the compatibility of the system with MS detection. Therefore, the same packing materials were chosen as for the $^{2}$D column (*i.e.* cyano-modified silica). This allowed us to use the same mobile-phase composition without any compatibility or recovery issues.

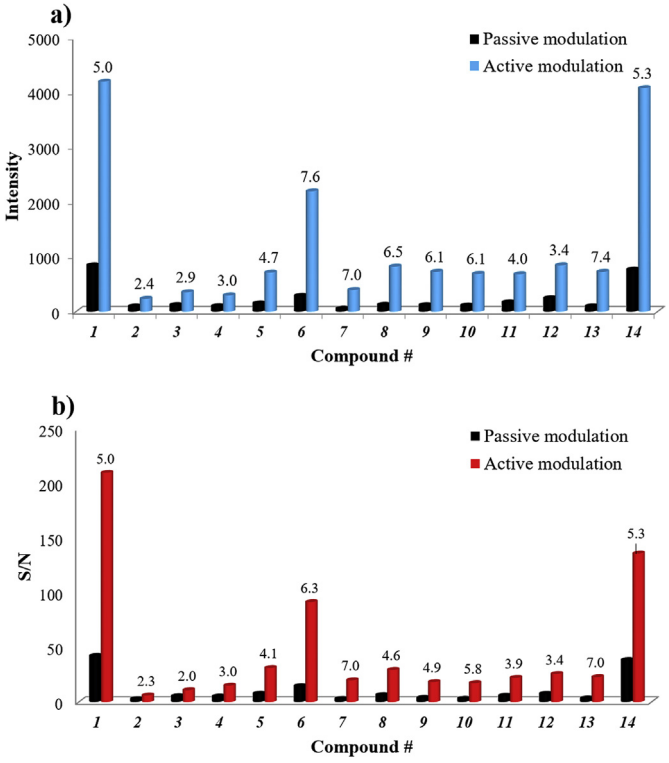



**Fig. 10.** Peak intensity enhancement (**a**) and signal-to-noise (S/N) enhancement (**b**) for steroid compounds achieved by implementing the active-modulation approach. The values achieved from the calculation are also listed in Table S2.

**Table 2**
Summary of the results achieved by LC × LC and LC/a × m/LC separations of the bovine-urine sample.

| | "Passively-modulated" ($p \times m$) | "Actively-modulated" ($a \times m$) |
|---|---|---|
| S/N[a)] | 2.6–42 | 5.9–210 |
| DF[b)] | 13 | 3 |
| $^{1}n_c \times {}^{2}n_c$[c)] | 660 | 860 |
| Detected peaks | 36 | 76 |
| Solvent consumption, mL | 520 | 190 |

a) The range of the signal-to-noise values determined for 14 steroid standards spiked into the urine sample (details see in Table S2 in Supplementary material); b) calculated according to eq. (2); c) calculated according to eq. (1). using the experimentally obtained 2D peak widths.

The results obtained by this active-modulation approach are clearly observed in Fig. 7c. The peak shape was drastically improved and the peak height greatly increased in comparison with the performance of the loop-type interface. Good resolution of steroid isomers in the two-dimensional separation space was maintained when a $^{2}$D column with a narrower diameter was used,.

The modulated LC × LC strategy shows clear advantages when applied for the analysis of a urine sample (Fig. 9) in comparison with a passively-modulated LC × LC separation of the same sample (Fig. 6b). The sensitivity is greatly improved, with a maximum peak enhancement for steroid standards, added to the urine samples at a concentration 8.3 μg L$^{-1}$, by a factor of 7.6 (Fig. 10 and Table S2 in Supplementary material) accompanied by a decrease in dilution by a factor of 4.5. In order to demonstrate the benefits of the active-modulation approach, the numbers of peaks detected in non-modulated and modulated TIC's were considered in this study. The peak picking algorithm was applied with identical integration parameters (see Experimental) for both separations, allowing us to generally compare the separation performances. The a × m strategy was found to result in a greater number (76 peaks *vs.* 36) of detected peaks in TIC of bovine-urine sample with a signal-to-noise ratio exceeding three. This does not represent the total number of molecular features or separated compounds, revealed by multidimensional separations of the complex sample, but it illustrates the benefits that can be obtained. Moreover, the solvent consumption was reduced by a factor of three (160 mL for modulated *vs.* 500 mL for non-actively-modulated LC × LC analysis, respectively). A summary of the advantages achieved by the active modulation is provided in Table 2. The increased sensitivity opens possibilities for the accurate identification of additional compounds found in urine based on MS/MS experiments, but such an investigation was beyond the scope of this study.

## 4. Conclusions

In this study we proposed an active-modulation approach for improving the sensitivity of a comprehensive two-dimensional liquid chromatography method for the analysis of anabolic steroids in bovine-urine samples. A satisfactory selectivity and orthogonality were obtained by using different types of reversed-phase stationary phases in combination with mobile phases of different nature (*i.e.* ACN and MeOH) in the two dimensions. Optimization of the column diameters, flow rates and modulation times was described, with emphasis on maintaining good resolution and MS sensitivity and compatibility. By applying this strategy the volume of the fractions collected from the $^{1}$D column was decreased, along with a change in solvent composition. This

allowed more-narrow $^{2}$D columns to be used, while avoiding band broadening and splitting and greatly reducing the overall dilution of the analytes. Active modulation resulted in clear gains in signal intensities by a factor of 2.4–7.6, increased signal-to-noise ratios for steroid compounds by up to a factor of 7, decreased dilution factors and a greater number of detected compounds in the TIC (76 *vs.* 36) of a bovine-urine sample.

The current setup can be further improved by more careful optimization of the $^{1}$D separation, for example to reduce the analysis time. In the present study numerous components were removed during the sample preparation steps, following the standard sample preparation procedure. Potentially, LC × LC may be used for the analysis of more-complex urine samples, simplifying the sample-preparation procedure and reducing the time and effort required. The developed method shows potential for untargeted screening of residues in bioanalysis of drugs and toxicants. After careful validation it may possibly be used on a routine basis for regulatory monitoring and doping control.

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## Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.aca.2017.12.043.

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