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Abstract

Nowadays routine residue monitoring involves the analysis of many compounds from different classes, mainly in urine. In the past two decades, developments heavily focused on the mass spectrometers (MS) and faster and more sensitive MS detectors have reached the market. However, chromatographic separation (CS) was rather ignored and the cognate developments in CS were not in line. As a result residue analysis did not improve to the extend as anticipated. Chromatographic separation by LCxLC is a promising technique and will enable a further increase in the range of compounds and compound classes that can be detected in a single run. In the present study a self-built LCxLC system, using a 10 port valve, was connected to a single quadrupole mass spectrometer with electrospray interface. Standards containing a mixture of sulfonamides, β-agonists and (steroid)hormones, 53 compounds in total, were analyzed. Results demonstrated that these compounds were well separated and could be detected at low levels in urine, i.e. LOD from 1 µg L^{-1} for most β-agonists to 10 µg L^{-1} for some sulfonamides and most hormones. To enhance the sensitivity, optimization was performed on an advanced commercial LCxLC system connected to a full scan accurate mass spectrometer. This ultimately resulted in a fast high through-put untargeted method, including a simple sample clean-up in a 96 well format, for the analysis of urine samples.

Keywords: antibiotics, comprehensive 2D-LC, growth promotors, LCxLC, mass spectrometry, residues

Introduction

The number of residues and contaminants, such as antimicrobials, steroid hormones, dioxins, growth promoters and natural toxins, that are routinely analyzed for enforcement purposes tends to grow every year. There are several reasons for this trend, *e.g.* because of new legislation. For example when a new risk is identified and regulated based on the toxic properties of the responsible compound (Tareke et al. 2002). Or when EFSA is asking for levels of (not yet regulated) contaminants in products in order to calculate human exposure for risk assessment purposes (Mulder et al. 2016). But also when new compounds are produced and being used, *e.g.* designer steroids, β-agonists and other growth promoters (Fragkaki et al. 2013, Nielen et al. 2006, Nielen et al. 2003, van den Broek et al. 2015). Moreover, when looking at supplements and food safety, it should be emphasized that more and more pharmaceuticals are being added to so-called "natural" (food)supplements, *e.g.* DES and isopropyloctopamine (Bovee et al. 2016, Toorians et al. 2010).

Sometimes new compounds or matrices can be included in existing methods, but in general the increasing number of compounds in different types of matrices is generating an ever growing number of methods. As a result, costs for food residue analysis have increased, which contradicts with the often shrinking budgets of the responsible authorities. There are two main options to improve the situation. One option is to make use of broad and cheap bio-based high through put screening assays, *i.e.* bioassays and biosensors (Bovee et al. 2009). However, samples screened as suspect or positive by these assay types need chemical analytical confirmation in order to identify the compounds responsible for the observed effects (Peters et al. 2010). This approach is thus only efficient in fields were most samples are compliant. Thereby reducing the amount of negative samples that otherwise would have been measured unnecessarily by expensive chemical analytical methods. The other option is to develop chemical analytical methods that enable the detection of more compounds and more compound classes in a single run. This will lead to new challenges, as the sample clean-up has to be less specific, *i.e.* in order to make sure that all compounds are extracted, the extracts will also contain much more matrix compounds and will thus result in more matrix effects. Based on experience, a crude extract will contain about 8,000 to 30,000 different compounds. A typical liquid chromatographic system is capable to baseline separate 50 to 300 compounds. Slower elution and longer columns can be applied to increase the number of peaks separated (Wren 2005), but in general 300 to 1,000 is considered the maximum without ending in unrealistic long run times (Wren 2005). Thus in crude extracts, chromatography alone is not capable to separate the compounds of interest from the matrix compounds. For a high resolution accurate mass spectrometer (hrMS) with a theoretical resolution of 10,000 (10% valley), the real resolution is estimated a factor four lower due to isotopes, adducts and fragments present in the mass spectrum. An hrMS alone is thus also not capable to analyze crude extracts. When a normal chromatographic system is coupled to an hrMS, the peak capacity can be estimated by multiplying the resolution with the peak capacity, *i.e.* approximately 750,000 (2,500 x 300 for an LC-hrMS (Gethings et al. 2014). In theory, such peak capacity is high enough to detect all compounds in crude extracts. However, in real practice LC is not truly orthogonal, *i.e.* in the middle part of the chromatogram hundreds of compounds are introduced into the hrMS at the same time. In case of electrospray it will influence the spray characteristics and efficiency which thereby results in signal suppression (Annesley 2003). Moreover, the masses are not equally distributed over the mass window, they will cluster around the ^{12}C masses since most compounds contain carbon. To detect all compounds by MS, the peak capacity of the chromatographic system has to be increased.

Optimization of column material and mobile phases is probably not the best option to increase the peak capacity, since the combination of the packing material of a LC column and a mobile phase is not universal applicable to all compounds or compound classes. Comprehensive LCxLC is a far more promising way to increase the peak capacity in order to widen the range of compounds and compound classes that can be detected in a single run. In case of full orthogonality, the peak capacity is the amplified product of the two columns used (Vivo-Truyols et al. 2010). For example, when two columns with a peak capacity of 100 and 300 are coupled, this will result in a peak capacity of 30,000 (100 x 300). In practice this will be lower since true orthogonality is impossible (Gilar et al. 2005). However, it demonstrates that a massive increase in peak capacity can be achieved when two different liquid chromatographic systems are combined, *i.e.* when different stationary phases are used (Vivo-Truyols et al. 2010). Until now there are only a few studies describing the use of LCxLC in combination with high resolution accurate mass MS (hrMS) for food residue analysis (Cacciola et al. 2017, van de Schans et al. 2017). However, these studies mainly focused on the hardware capabilities and not on the use for daily routine analysis.

In the present study the use of LCxLC-hrMS for routine applicability was explored. First with a self-built LCxLC system in which different columns and stationary phases were connected to a single quadrupole mass spectrometer via an electrospray interface (ESI). As a test case, antimicrobials (sulfonamides), β-agonists and (steroid)hormones were selected in order to detect residues with different physical chemical properties. As the results with the self-built system were already very promising, additional studies were performed on a commercial connected LCxLC-time of flight mass spectrometer. Using urine samples containing residues of these three compound classes, both spiked and from real practice, demonstrated that it is possible to analyze many residues of different classes with one method in a single run.

Materials and Methods

Materials

Formic acid (FA), methanol (MeOH), water, and acetonitrile (ACN) (all ULC/MS grade) were purchased from Aktu-All (the Netherlands), β-glucuronidase from *E Coli* K12, 140 U/mg, from Roche (the Netherlands) and disodium hydrogen phosphate dehydrate and potassium dihydrogen phosphate from Merck (the Netherlands). The 96 wells SPE OASIS-HLB (30 mg) were obtained from Waters (the Netherlands).

Analytical standards of betamethasone, 17β-boldenone, 17α-methyltestosterone, 17α-nortestosterone, 17β-nortestosterone, 17α-testosterone, 17β-testosterone, and 17β-trenbolone were purchased from Steraloids (Newport, US), brombuterol, cimaterol, cimbuterol, clencyclohexerol, clenhexyl, clenpenterol, clenproperol, mabuterol, mapenterol, tulobuterol, chlorbrombuterol, and hydroxymethylclenbuterol from Witega (Berlin, Germany) and carbuterol, reproterol, and zilpaterol were kindly provided by EURL (Berlin, Germany. Dexamethasone, flumethasone, prednisolone, clenbuterol, fenoterol, isoxsuprine, procaterol, salbutamol, salmeterol, terbutaline, ritodrine, metaproterenol, ractopamine, sulfadiazine, sulfamerazine, sulfamethizole, sulfamethoxazole, sulfamethoxypyridazine, sulfapyridine, sulfaquinoxaline, sulfathiazole, dapsone, sulfisoxazole, sulfamonomethoxine, sulfadimethoxine, sulfamoxole, sulfacetamide, sulfaphenazole, trimethoprim, and the isotope labeled sulfadimethoxine-d6 were purchased from Sigma-Aldrich (St. Louis, US), 17α-trenbolone, 16β-OH-stanozolol, and the isotope labeled 17β-testosterone-d3 from NMI (Sydney, Australia), sulfachloropyridazine, sulfadimidine, and sulfadoxine from Riedel de Haen (Seelze, Germany), and the isotope labelled dapsone-d8 and fenoterol-d6 from Toronto Research (Toronto, ON, Canada). The 17α-boldenone was kindly provided by the EURL (Wageningen, the Netherlands).

Urine samples used in this study were obtained from routine control programs in the Netherlands. The urines are from bovine animals of different ages and gender.

Standard solutions for self-build and commercials LCxLC

Phosphate buffer pH 7.4 was prepared by dissolving 2.86 g disodium hydrogen phosphate dihydrate and 0.208 g potassium dihydrogen phosphate in 100 mL milli-Q water. For deconjugation, a working solution was freshly prepared by adding 500 µL β-glucuronidase to 50 mL phosphate buffer 7.4. Stock solutions containing 1 mg mL^{-1} of all compounds were prepared in ethanol from which working solutions were made by 10 times dilution steps.

Sample clean-up for LCxLC-ToF-MS analysis

To 500 µL urine sample, 10 µg L^{-1} of (isotope labeled) internal standard mixture (Table 1) and 500 µL phosphate buffer pH 7.4 was added. A matrix matched calibration curve was prepared by spiking urine with standards at concentrations of 0, 1, 2.5, 5, 7.5 and 10 µg L^{-1}. Deconjugation was performed by adding 10 µL working solution ß-glucuronidase (Gomes et al. 2009), followed by incubation at 37 °C for 16 h. Sample clean-up was performed with a 96-wells plate (Oasis HLB sorbent) preconditioned in succession with 1 mL methanol and 500 µL water. The sample was applied and passed through. The Oasis HLB plate was then washed with respectively 1 mL water and 1 mL 10% acetonitrile and elution was performed with 1 mL 90 % acetonitrile. To the extract was 10 µL DMSO added as a keeper after which the sample was evaporated at 55 °C under a stream of nitrogen till around 10 µL DMSO remains after which 100 µl 20:80 v/v-% acetonitrile:water. Ten µL was injected directly into the LCxLC-ToF-MS system.

Instrumental set-up, chromatographic-and MS conditions

Self-built LCxLC system

Explorative LCxLC experiments were performed on a self-built system consisting of a Waters ACQUITY UPLC® I-Class System and an H-Class system and a Waters single quadrupole mass spectrometer QDa with ESI interface. Both LC systems were connected via a Rheodyne 10 port valve consisting of two 100 µL loops.

Several combinations of columns, mobile phases and conditions were tested. For the first dimension the following columns were tested: Waters HSS Cyano (1.8 um, 1x150 mm), Waters BEH C_{18} (1.8 um, 1x150 mm), Waters Phenyl (1.8 um, 1x150 mm) and a Phenomenex Kinetix (2.6 um, 1.0x150 mm) column. The column oven temperature was set at 50 °C, the injection volume was 10 µL and the flowrate was set at 40 µL min^{-1}. Mobile phase A consisted of water:acetonitrile (90:10) containing 0.1% formic acid and mobile phase B consisted of water:acetonitrile (10:90) containing 0.1% formic acid. The initial solvent was 0% mobile phase B and the gradient increased to 80% mobile phase B in 27 minutes, remained one minute at 80% mobile phase B after which the mobile phase B was decreased to 0% in 1 minute and remained at this condition for 13 minutes.

Second dimensional separation was performed on a Waters Phenyl column (1.7 µm, 2.1×50 mm) while the column oven was set at 75 °C and the flowrate at 800 µL min^{-1}. Mobile phase A was water:acetonitrile (90:10) containing 0.1% formic acid and mobile phase B was water:acetonitrile (10:90) containing 0.1% formic acid. A shifted gradient was used in which the percentage A was decreased with 5% for each modulation. The initial solvent was 0% mobile phase B and the gradient increased to 40% mobile phase B in 45 seconds after which the mobile phase B was increased to 100% in 5 seconds and remained at this condition for 15 seconds.

Mass spectrometric (MS) data were obtained by analyzing the samples on a Waters single quadrupole mass spectrometer QDa. Selected Ion Recording (SIR) mode was used whereby the unit mass of each compound was measured in positive ionization mode. The mass spectrometric conditions were: capillary voltage: 0.8 kV, cone voltage: 15 V, probe temperature: 450°C, sample frequency: 5 Hz. Analysis of MS data was performed with Waters Masslynxx v4.0.

Commercial LCxLC system

Further experimentation was performed on an Agilent 1290 infinity LCxLC system (Waldbronn, Germany) equipped with a quaternary pump in the first dimension and a binary pump in the second dimension, two DAD detectors, a sample manager and column oven compartment. The interface valve to create the two-dimensional system contained two 80 µL sample loops, connected via an ESI source with an Agilent 6540 time of flight (ToF) MS (Waldbronn, Germany).

For the first dimension a Waters Phenyl (1.8 um, 1x150 mm) column was used. The column oven temperature was set at 50 °C, the injection volume was 10 µL and the flowrate was set at 60 µL min^{-1}. Mobile phase A consisted of water:acetonitrile (90:10) containing 0.1% formic acid and mobile phase B consisted of water:acetonitrile (10:90) containing 0.1% formic acid. The initial solvent was 0% mobile phase B and the gradient increased to 80% mobile phase B in 27 minutes, after which the mobile phase B was increased to 100% in 1 minute and remained at this condition for 3 minutes after which the mobile B was decreased to 0% and remained 7 minutes at this condition.

Second dimensional separation was performed on a Waters Phenyl column (1.7 µm, 1×50 mm) while the column oven was set at 75 °C and the flowrate at 350 µL min^{-1}. Mobile phase A was water:acetonitrile (90:10) containing 0.1% formic acid and mobile phase B was water:acetonitrile (10:90) containing 0.1% formic acid. A shifted gradient was used in which the percentage A was decreased with 5% for each modulation. The initial solvent was 0% mobile phase B and the gradient increased to 50% mobile phase B in 45 seconds after which the mobile phase B was increased to 100% in 5 seconds and remained at this condition for 15 seconds.

MS data were collected over a scan range of 100-500 amu in positive ionization mode. The MS parameters were set as follow: nebulizer gas pressure: 45 psi, sheath gas temperature: 350 °C, sheath gas flow rate: 11 L/min, drying gas flow rate: 16 L/min, capillary voltage: 3500 V, fragmentor voltage: 360 V, acquisition rate: 5 spectra/s. For mass reference, a solution containing purine (m/z 121.05) and Hexakis(1H,1H,3H-perfluoropropoxy)phosphazene (m/z 922.01) was used and analysis of MS data was performed with MassHunter B.07.00.

Calculation of resolution and peak capacity

The peak capacity (n_c) is calculated using equation (1).

$$n_c = 1 + \left(\frac{t_g}{\bar{w}}\right) \quad (1)$$

I.e., t_g is the total gradient time and $\bar{w}$ is the average peak width at the base of the peak. The chromatographic resolution (Rs) is calculated by equation (2).

$$Rs = \frac{2\left[(t_R)_B - (t_R)_A\right]}{W_B + W_A} \quad (2)$$

I.e., $(t_r)_b$ and $(t_r)_a$ are the retention times of two isomers and w_b and w_a are the peak widths at the base of the peak.

Results and Discussion

Explorative LCxLC

An in-house LCxLC system was build using a standard 10 port valve to connect two LC systems. Detection was performed on a single quad mass spectrometer. Several column combinations were tested and the best combination was connected via the 10 port valve to build the operational LCxLC system. Only the most informative options and the final best option are described in the present study.

The following considerations were taken into account. In general, using LCxLC, a low flow is chosen in the first dimension compared to the second dimension, primary because the second dimension flow should physically be able to deal with the flow in the first dimension, but higher second dimension flows are also preferred in order to obtain fast chromatographic separation within the fill time of the sample loops (Li et al. 2015, Vivo-Truyols et al. 2010). However, due to the low flow, the eluting peaks in the first dimension are relatively broad and should be refocused in the second dimension. Refocusing is also necessary to deal with the large injection volume from the 10 port valve (100 µL in this self-built case). For the classes of test compounds selected (sulfonamides, β-agonists, (steroid)hormones) in the present study, HILIC and normal phase are not a good option due to the non-polar properties of most of these compounds. Therefore reversed phase columns with different retention mechanisms were selected to either serve for the first or second dimension, *i.e.* columns possessing: a) strong dipolar and ionic interactions (Cyano columns); b) strong hydrophobic interactions (C_{18} and C_8 columns); and c) strong pi-pi and planar shape interactions (phenyl columns). The Waters HSS Cyano, Waters BEH C_{18}, Waters Phenyl, and Phenomenex Kinetix columns were tested with a selection of compounds of the three classes.

Compounds eluted at low percentages of the organic modifier from the cyano column, while the compounds were most retained on a phenyl column. The cyano and phenyl columns were therefore considered as a suitable combination for LCxLC, using the cyano column in the first dimension and phenyl column in the second dimension. To obtain optimal chromatographic separation at low flowrates, a 1 mm i.d. 150 mm cyano column was used in the first dimension. This column was coupled to a 2.1 mm i.d 50 mm phenyl column in the second dimension. The flow in the first dimension was 0.04 mL min^{-1}, with a loop size of 100 µL. The second dimension gradient was performed within 1 minute and a shifted gradient compared to the first dimension was used in order to gain optimal separation (orthogonality) (Jandera 2012, Vivo-Truyols et al. 2010). Although the self-built LC hardware setup was relative simple and coupled via ESI to a single quadrupole mass spectrometer, which was operated in single reaction monitoring, a reasonable two dimensional separation was achieved. As figure 1 shows that the obtained orthogonality is already quite good, since the peaks (compounds) are nicely distributed over the space.

However, there are still a few shortcomings. The flow in the second dimension was rather high (1 mL min^{-1}), resulting in reduced sensitivity compared to lower flows (data not shown) and the sensitivity of the single quad mass spectrometer used is limited compared to medium and high end triple quadrupole mass spectrometers. Moreover, the compounds measured contain isomeric compounds and although most of these structure isomeric compounds can be separated already in this self-built system, two are still very difficult to separate, *i.e.* dexamethasone and betamethasone, a problem that even mass spectrometers with higher resolutions cannot solve. Further improvement of the self-built LCxLC-MS setup was therefore considered necessary, both on the LCxLC part and the MS used.

Optimization of LCxLC using an advanced commercial LCxLC system connected to ToF-MS

Further optimization to achieve better separation and sensitivity was carried out on a more advanced LCxLC system obtained from Agilent (Waldbronn, Germany). This system is equipped with a dedicated LCxLC valve that optimizes the modulation and was connected to a full-scan accurate mass spectrometer, which makes the detection of different classes of compounds more versatile compared to a single quadrupole mass spectrometer. The isomeric compounds dexamethasone and betamethasone were used as test compounds for this further optimization, as a kind of worst case example.

The same cyano column (i.d. 1 mm, L 150 mm) was used in first dimension on the Agilent system as was used in the home-built system, as the latter already gave quite good results. The flow rate which had to be used on this column was calculated in order to obtain an optimal number of modulations over a peak. The optimal number of modulations over a peak is considered to be 2.4 (Vivo-Truyols et al. 2010). This number of modulations is based

on maintaining the first dimension LC resolution (avoiding under sampling) without sacrificing to much sensitivity due to oversampling of the first dimension peak. By optimizing the flow rate in order to obtain the optimal number of modulations, peaks can be split over two modulations which result in 2 peaks in the second dimension at the same retention time, while they originate from one peak in the first dimension. This optimum number of modulations results in a flow in the first dimension of 0.06 mL min^{-1}.
As mentioned before, it is of utmost importance to optimize the flow rate in the second dimension. In general high flow rates are needed for fast second dimension chromatography (fast elution and peak separation). However, high flow rates reduce sensitivity when connected to a MS, while sensitivity is needed for detection of low residue levels. Moreover, not only the flow rate will affect the sensitivity, also the composition of the eluent. As the composition changes over time, due to the fact that a shifted gradient is used, it is difficult to influence the sensitivity by adjusting the shifted gradient, since it is needed in order to separate the many compounds in this multiclass detection method. The focus to obtain better sensitivity was therefore on the optimization of the flow rate for the second dimension. The flow optimization experiments were performed in single LC mode. Figure 2 shows the effect of the flow rate on the sensitivity and separation of the isomers dexamethasone and betamethasone.

As expected, higher flow rates will result in a dilution effect and thereby reduce the overall sensitivity (peak height) (figure 2). An option to increase the sensitivity and maintain fast separation is to use columns with smaller diameters (Vissers 1999). From a theoretical point of view, there is no reason not to reduce the column diameter, as a reduced column diameter will give the same linear flow velocity at much lower volumetric flow rates. However, the packing of the column becomes more critical, especially with regard to dead volumes throughout the system. To assess the effect of the flow rate and the use of a column with a smaller diameter on the resolution and peak capacity, a similar experiment was performed by using a phenyl i.d. 1 mm column instead of 2.1 mm. Figure 3 shows that the peak capacity (n_c) is higher at higher flow rates and that the peak capacity is even higher on the i.d. 1 mm column then on the 2.1 mm column. The resolution (Rs) is hardly affected and similar when using the 1 mm or 2.1 mm column.

Figure 4 shows the chromatograms at the different flow rates used on the i.d. 1 mm phenyl column. It shows that the negative effect of an increased flow on the sensitivity is far less pronounced on this phenyl i.d. 1 mm column then on the phenyl i.d. 2.1 mm column (figure 2). This is most likely the result of a more efficient ionization in the ESI source due to the relative low flow rates. Overall the phenyl i.d. 1 mm column is preferred for the second dimension. Although the retention time is longer, i.e. around 4 minutes instead of 2 minutes (figures 2 and 4), the resolution is slightly lower, the peak capacity is higher, and the sensitivity is much more stable at different flow rates compared to the 2.1 mm column. Moreover, smaller diameters are a green choice, as less solvent is used, i.e. around 0.15 mL min^{-1} using the 1mm column instead of 1.5 mL min^{-1} when using the 2.1 mm column.

Finally, the temperature was optimized to reduce the retention time in the second dimension. In general the equilibrium between the compound, eluent and stationary phase is faster at higher temperatures (Edge et al. 2006), which will result in faster chromatography. It was indeed found that increasing temperatures resulted in faster chromatography, i.e. faster analysis due to lower retention times, without loss of chromatographic resolution (figure 3).

Evaluation of the performance of the LCxLC-ToF-MS system

Figure 5 shows an example of an LCxLC run, in which 80 µL fractions were directed from the first column to the second column. It shows that these conditions resulted in multiple modulations of sulfacetamide and clenbuterol. Such splitting has some advantages and disadvantages. One of the disadvantages is the loss of sensitivity, since the peak is divided in two, another disadvantage is that the data processing will be rather troublesome, since there is no software that can integrate peaks that are split over two modulations. However, there might also be an advantage, *e.g.* if a matrix interference is present in one of the modulations it might be possible that the interference will not appear in the other modulation. Also different classes will group together over the 2D chromatogram (figure 6). From figure 6 it can be depicted that the classes of compounds are partly separated in case of sulfonamides and β-agonists and are fully separated for the steroids. This grouping can be helpful if only certain classes of compounds are of interest, but the grouping can also be helpful in case the identity of a certain or unknown compound needs to be elucidated. As besides the mass-spectrometric data, the grouping gives additional structural information about the compound.

Performance of the method in a routine setting analyzing urine samples

To evaluate the newly developed and optimized LCxLC method, bovine urine samples from routine control programs were analyzed using a simple high through put 96 well sample clean-up. Samples originating from both male and females calves and cows were analyzed in different series. Each set of samples contains a matrix matched standard (MMS) line with different concentrations of sulfonamides, beta-agonists and (steroid)hormones. To each sample, an isotope labeled standard was added containing compounds of each class to monitor the performance during the sample clean-up and the LCxLC-MS analysis. Figure 5 shows some examples of chromatograms of spiked urine samples for the three classes of compounds and demonstrates that these three classes can be detected in this matrix without interfering peaks. Although difficult to quantify, it is obvious that the separation power of LCxLC helps to obtain clean chromatograms. At the end, the limit of detection (LOD) was determined for each compound based on the MMS line and it turned out that the sensitivity differed between the classes of compounds. Table 1 gives a summary of the determined LODs. Detection of sulfonamides and beta-agonists was possible between 1 and 10 $\mu g\ L^{-1}$. For (steroid)hormones the detection levels were higher, between 2 and 10 $\mu g\ L^{-1}$ for the 15 compounds shown in Table 1. The detection limits for the (steroid)hormones are thus higher than the required minimum performance limits of 1-2 $\mu g\ L^{-1}$ as advised by the European Union Reference Laboratory. Using a triple-quad detector could be a solution, as this detector is more sensitive for steroids than full-scan mass-spectrometers (Anumol et al. 2017, Berendsen et al. 2017).

Subsequently, the LCxLC-ToF-MS method was used to analyze 53 samples of bovine urine from routine control programs in the Netherlands. Despite the simple clean-up, the whole method was found to perform well based on the presence of the internal standards in all samples and because no shift in retention times (<1%) were observed over time for these isotope labeled standards, i.e. during the whole analysis with series over 70 injections. The recovery for the internal standards was on average 59% for testoterone-D3, 17% for fenoterol-D6 and 78% for dapson-D8. The recovery for fenoterol-D6 was relative low due to the polar nature of this compound, i.e. containing a secondary amine and four hydroxyl groups, resulting in a lower affinity for the material used in the SPE column and thus also in a lower recovery. As the method aimed to use a simple clean-up for broad detection of compounds from different classes, it is unavoidable that the recovery will be sub-optimal for some compounds. Compounds were identified by matching there retention time and exact mass, and concentrations were determined by use of the matrix matched calibration lines. Most of the samples contained testosterone, probably as a natural endogenous occurring steroid. In some samples 17α-nortestosterone was detected. The determined concentrations of 17α-nortestosterone were low, but above the LOD of 2 $\mu g\ L^{-1}$. It has been shown that this compound can occur naturally at low levels in urine too, for example when cows are pregnant (Sterk et al. 1998). The detection of these natural compounds demonstrates that the method is capable to detect residues in bovine samples of urine. However, the occurrence of these natural hormones in urine do not fully exclude an illegal treatment. Other methods are needed to confirm the real origin of these natural hormones, i.e. synthetic or true endogenous naturals, such as GC-c-IRMS analysis (Ferchaud et al. 1998, Sterk et al. 1998). No other (steroid)hormones or beta-agonists were detected, while a large number of samples contained sulfonamides. In all cases the use of these sulfonamides was registered. Thus overall, no other compounds then natural or registered veterinary compounds were detected.

Conclusions

An LCxLC-ToF-MS method was developed capable of analyzing different classes of compounds in urine in one run, using only a minimal high through-put sample clean-up in a 96 well format. As far as the authors know this is the first published LCxLC-MS method capable of analyzing different classes of forbidden or regulated compounds in urine with a simple clean-up in a single run. A critical note is the use of urine for regulated compounds. This is limited as there are no official MRL's set for urine. Thus other matrices, i.e. for which an official MRL is established, should be analyzed after a positive screening result of a urine sample. Anyway, it is foreseen that the current LCxLC method can be extended to other compound classes as well and when combined with more sensitive HRAM mass spectrometers, the LODs will improve and the scope of the method can be extended, i.e. to fulfill also the requirements for steroids.

The current LCxLC method was applied to a large number of urine samples from routine control programs. During the analysis the retention time was found to be stable. Natural occurring steroids and registered sulfonamides were detected, showing that the method can be used in practice, thus enabling the fast high through-put untargeted analysis of urine samples for the presence of sulfonamides, beta-agonists and (steroid)hormones in one single run.

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Figures

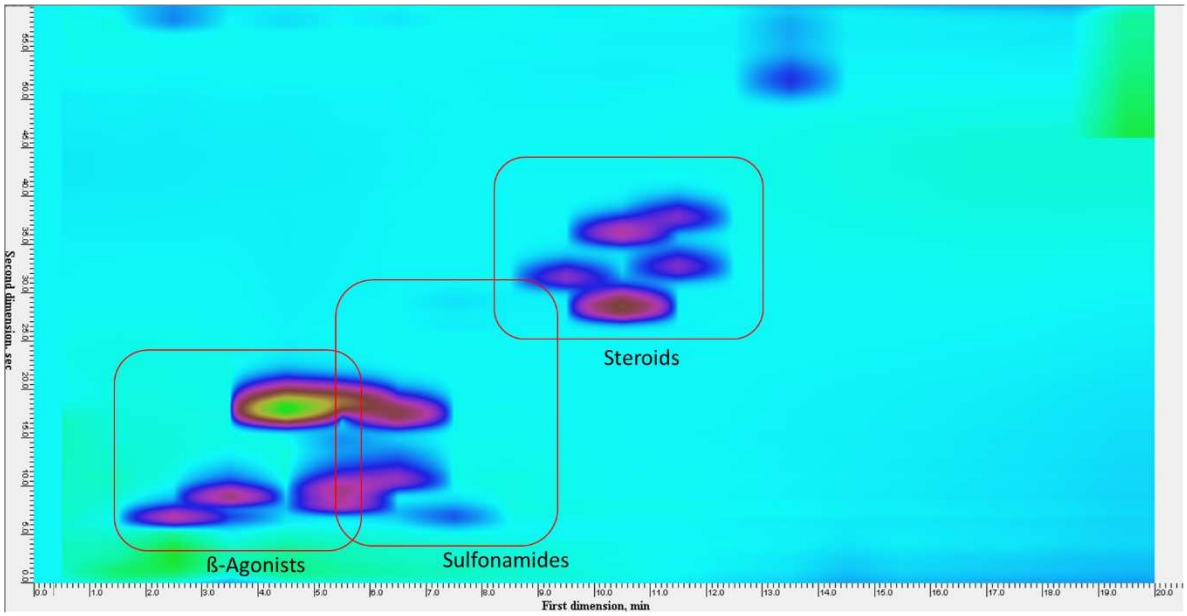

Figure 1. Contour plot of a selection of compounds from different classes of compounds (TIC of all SIR), i.e. sulfonamides, β-agonists and (steroid)hormones, as obtained in the self-built LCxLC-MS system operated in SIR, using a cyano column in the first dimension (i.d. 1 mm, L 150mm, flow 100 µL min^{-1}) and a phenyl column in the second dimension (i.d. 2.1 mm, L 50mm, flow 1 mL min^{-1}).

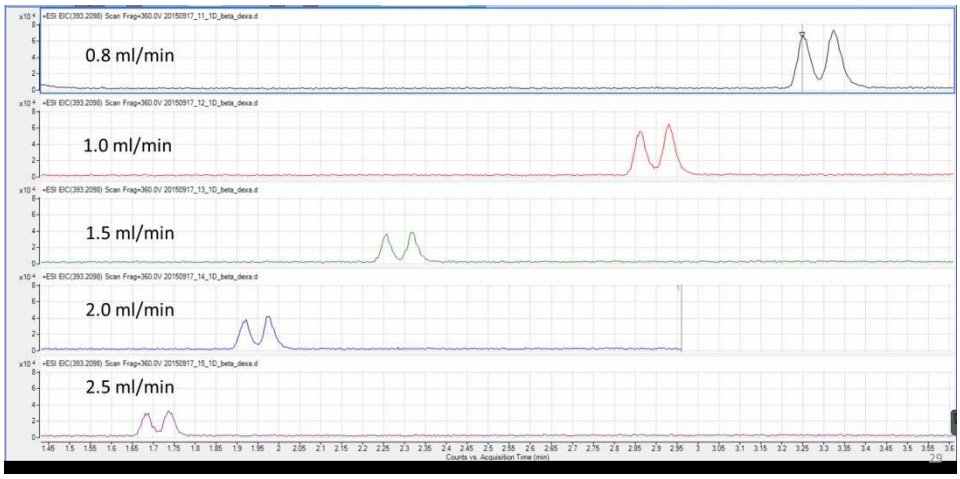

Figure 2. Influence of the flow rate in the second dimension on the sensitivity, retention time and peak separation of dexamethasone and betamethasone. First dimension: cyano i.d. 1 mm, L 150 mm column and a flow of 0.6 mL min^{-1}. Second dimension: phenyl (i.d. 2.1 mm, L 50 mm) connected via an ESI with an Agilent 6540 ToF for mass spectrometric analysis. Y-ax scale is fixed at the highest intensity run (0.8 mL min^{-1}).

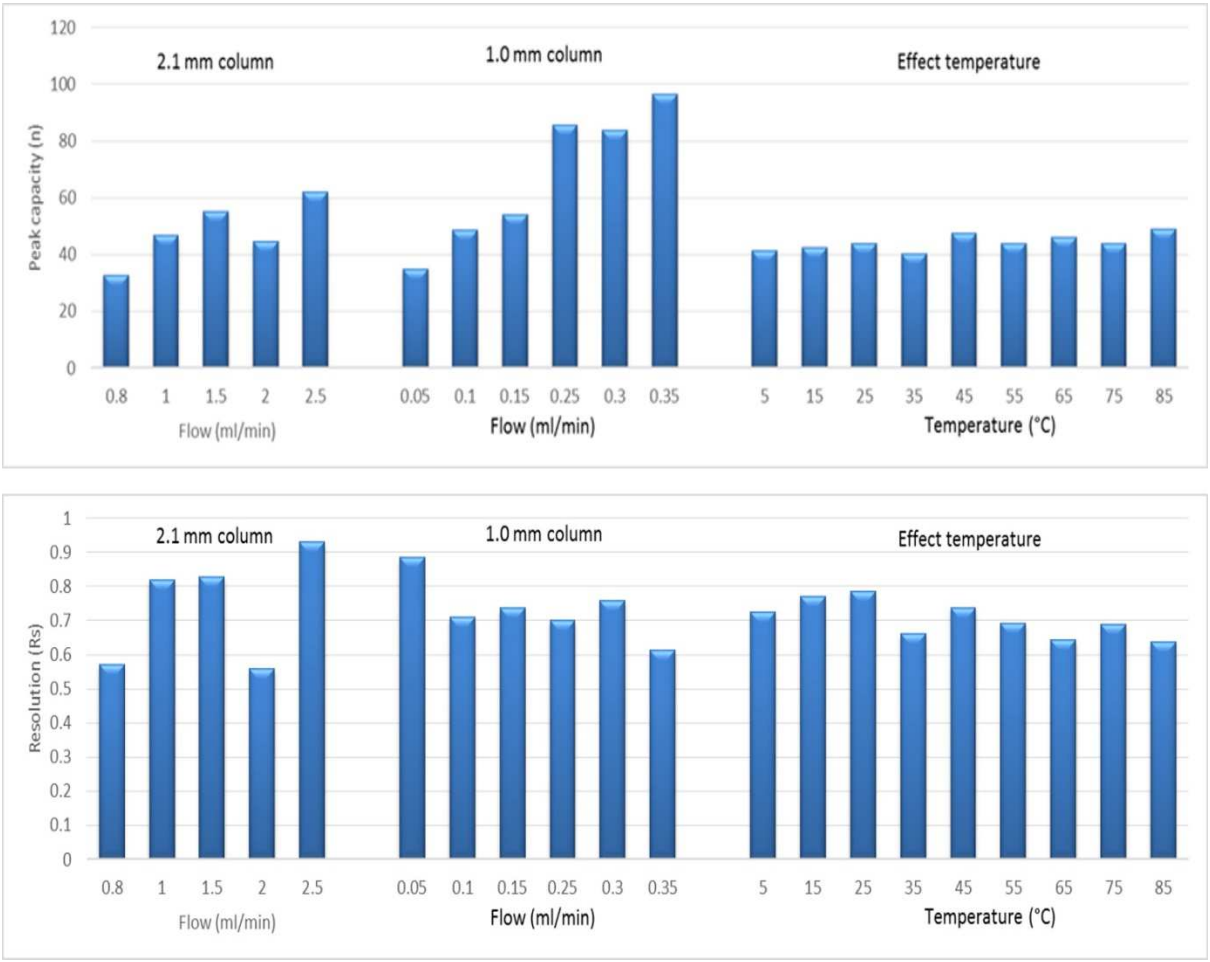

Figure 3. Effect of column diameter, flow and temperature on (A) peak capacity (n_c) and (B) resolution (Rs). The effect of the temperature was only measured with the phenyl i.d. 1.0 mm, L 50 mm column.

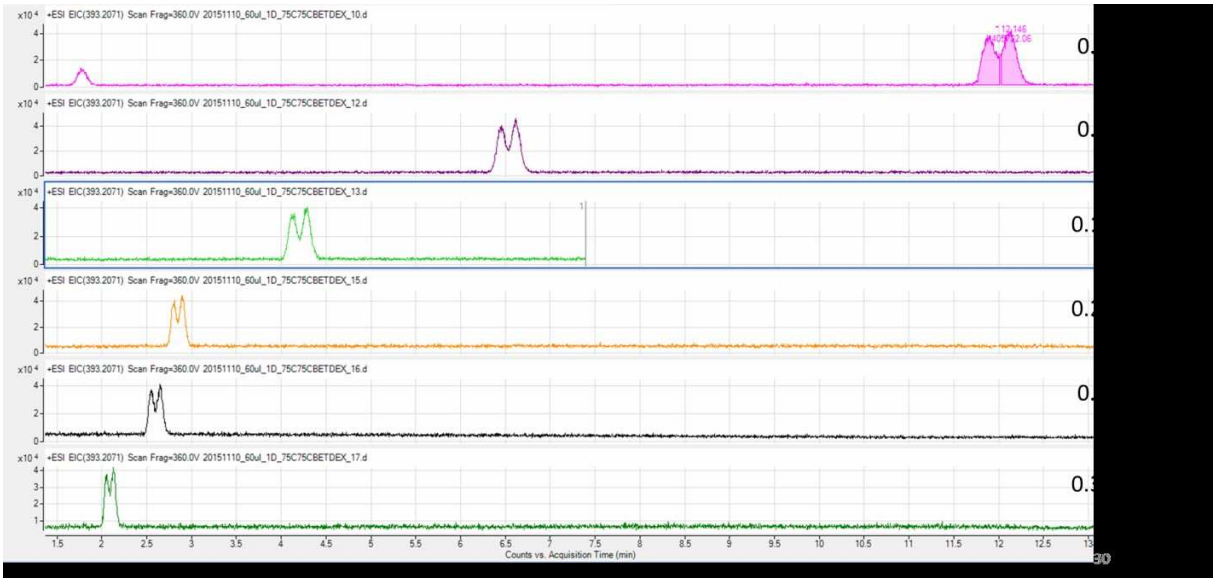

Figure 4. Influence of the flow rate on the sensitivity, retention time and peak separation of dexamethasone and betamethasone. Column used in the second dimension is a phenyl i.d. 1 mm, L 50 mm connected via an ESI with an Agilent 6540 ToF for mass spectrometric analysis.

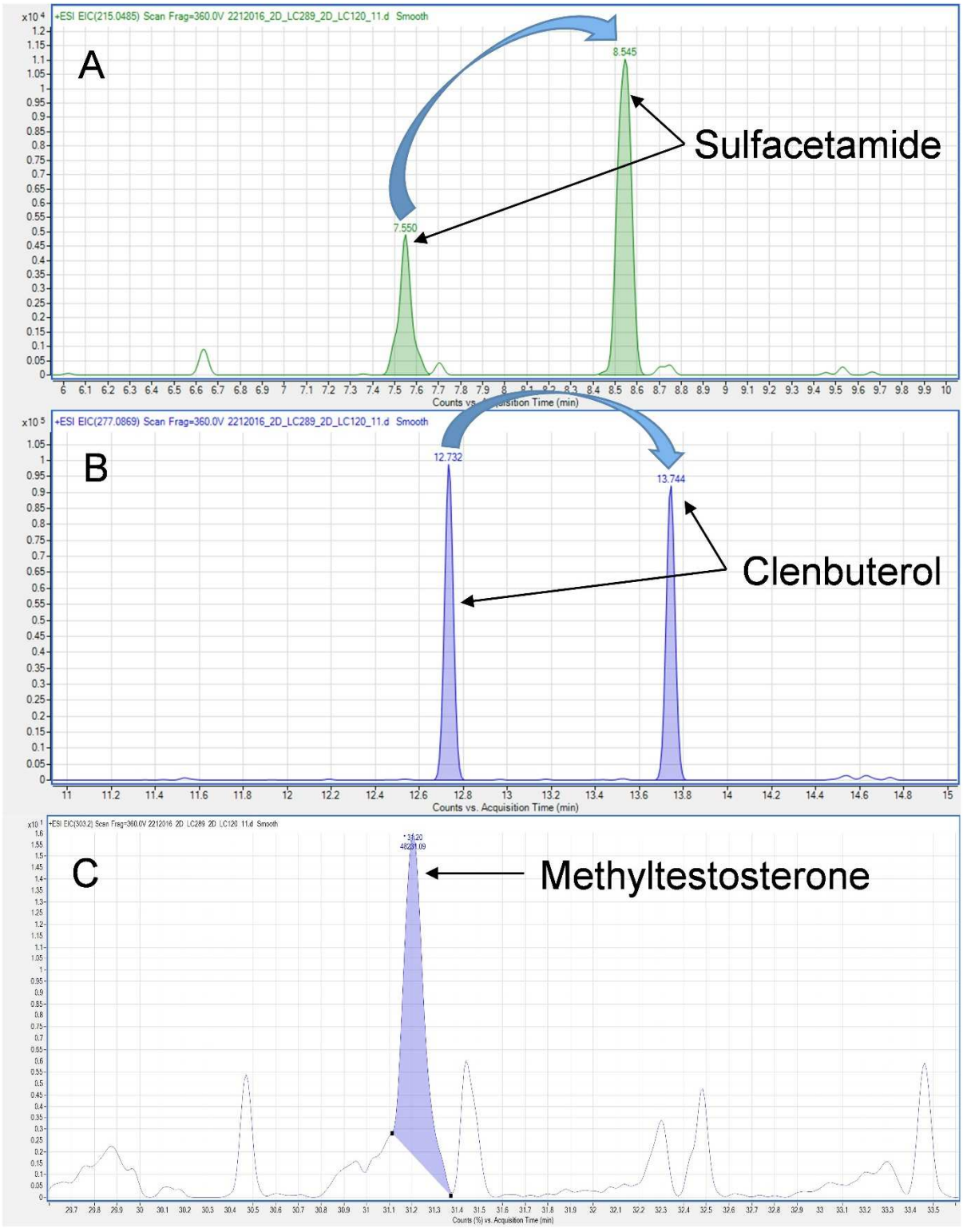

Figure 5. Examples of EIC chromatograms of spiked bovine urine samples, the arrows indicates that a peak is modulated over two cuts: A) sulfonamide: sulfacetamide, 2 ng mL^{-1}; B) beta-agonist: clenbuterol, 2 ng mL^{-1}; and C) steroid: testosterone, 10 ng mL^{-1}.

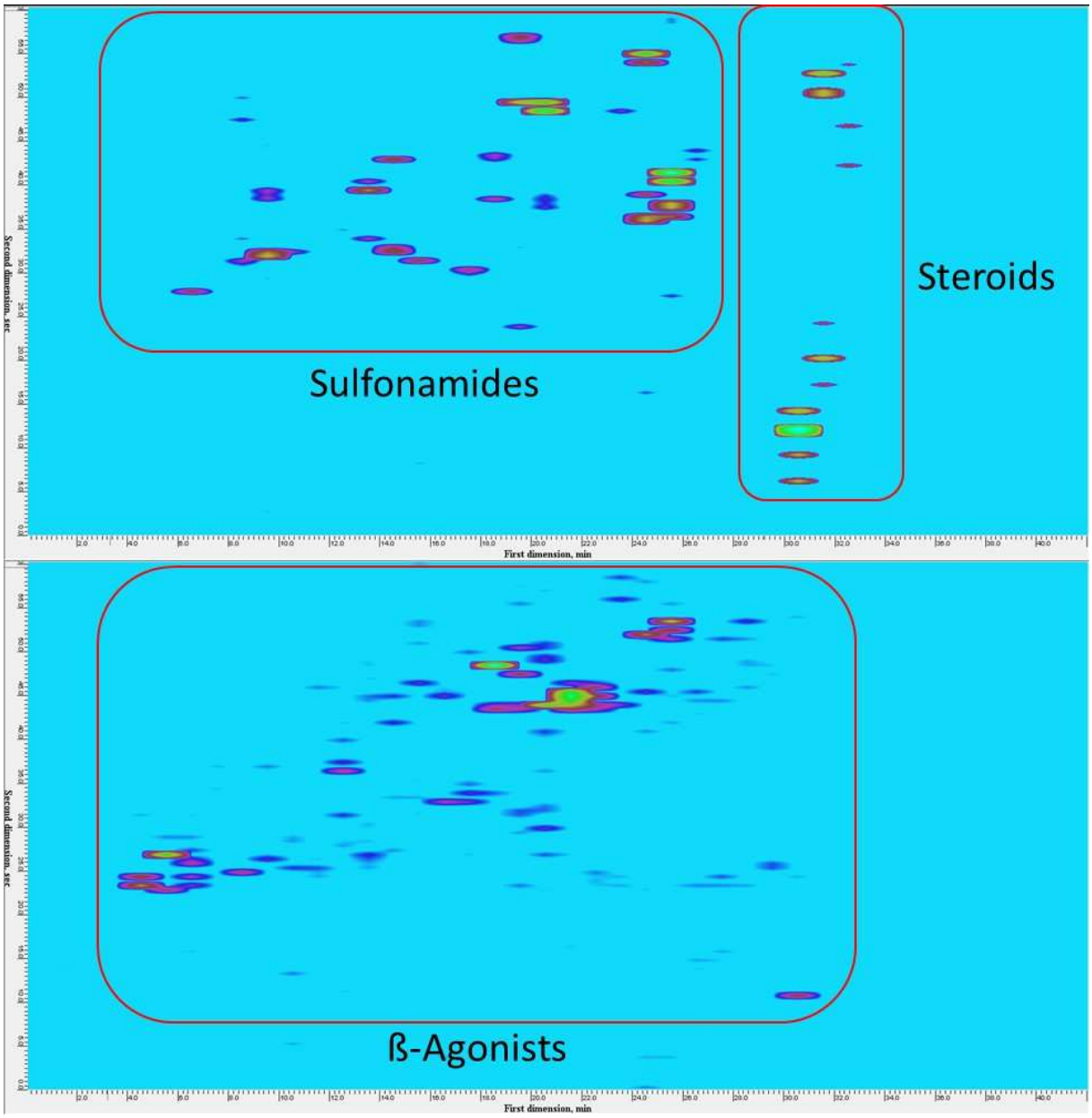

Figure 6. Contour plot presenting all compounds present in the standard mixture containing sulfonamides, β-agonists and steroids. First dimension: cyano i.d. 1 mm, L 150mm column and a flow of 60 uL min^{-1}), second dimension: phenyl i.d. 1 mm, L 50 mm colums and a flow of 350 µL min^{-1}. Note that there are more peaks visible than compounds analyzed this is due to the modulation effect.

508 *Table 1. Compounds analyzed: elemental composition, theoretical exact mass and limit of detection (µg L^{-1})*

Nr	Compound	Elemental composition	Exact mass $[M+H]^+$	Retention time (Min)	LOD (µg L^{-1})
	Sulfonamides				
1	Sulfacetamide	$C_8H_{10}N_2O_3S$	215.0485	7.54 / 8.53	2
2	Trimethoprim*	$C_{14}H_{18}N_4O_3$	291.1452	7.56 / 8.57	2
3	Sulfapyridine	$C_{11}H_{11}N_3O_2S$	250.0644	7.59 / 8.61	2
4	Sulfadiazine	$C_{10}H_{10}N_4O_2S$	251.0603	8.56/ 9.56	2
5	Sulfathiazole	$C_9H_9N_3O_2S_2$	256.0209	8.57 / 9.56	10
6	Sulfamerazine	$C_{11}H_{12}N_4O_2S$	265.0753	9.64 / 10.65	2
7	Sulfamoxole	$C_{11}H_{13}N_3O_3S$	268.0750	9.68	2
8	Sulfisoxazole	$C_{11}H_{13}N_3O_3S$	268.0750	9.68	10
9	Sulfaquinoxaline	$C_{14}H_{12}N_4O_2S$	301.0754	9.73 / 10.75	2
10	Sulfadimidine	$C_{12}H_{14}N_4O_2S$	279.0910	9.74 / 10.75	2
11	Sulfamethizole	$C_9H_{10}N_4O_2S_2$	271.0318	11.70 / 12.68	10
12	Sulfamethoxypyridazine	$C_{11}H_{12}N_4O_3S$	281.0703	10.70 / 11.71	2
13	Sulfamonomethoxine	$C_{11}H_{12}N_4O_3S$	281.0703	12.70	1
14	Sulfachloropyridazine	$C_{10}H_9ClN_4O_2S$	285.0207	14.73	10
15	Sulfamethoxazole	$C_{10}H_{11}N_3O_3S$	254.0594	16.80 / 17.81	10
16	Sulfadoxine	$C_{12}H_{14}N_4O_4S$	311.0808	17.86 / 18.86	5
17	Dapson	$C_{12}H_{12}N_2O_2S$	249.0692	18.69	2
18	Dapson-D8	$C_{12}H_4D_8N_2O_2S$	257.1194	18.71	not applicable
19	Sulfadimethoxine	$C_{12}H_{14}N_4O_4S$	311.0808	23.98 / 24.97	10
20	Sulfadimethoxine-D6	$C_{12}H_8D_6N_4O_4S$	317.1186	23.96	not applicable
21	Sulfaphenazole	$C_{15}H_{14}N_4O_2S$	315.0910	24.75	10
	β-Agonists				
22	Metaproterenol	$C_{11}H_{17}NO_3$	212.1281	4.44	1
23	Carbuterol	$C_{13}H_{21}N_3O_3$	268.1661	4.44 / 5.45	2
24	Salbutamol	$C_{13}H_{21}NO_3$	240.1594	4.46 / 5.45	2
25	Cimaterol	$C_{12}H_{17}N_3O$	220.1444	5.45 / 6.46	1
26	Terbutaline	$C_{12}H_{19}NO_3$	226.1438	5.46	1
27	Zilpaterol	$C_{14}H_{19}N_3O_2$	262.1550	6.51	1
28	Fenoterol	$C_{17}H_{21}NO_4$	304.1543	6.45	2
29	Fenoterol-D6	$C_{17}H_{15}D6NO_4$	310.1920	6.45	not applicable
30	Procaterol	$C_{16}H_{22}N_2O_3$	291.1703	6.45 / 7.46	2
31	Cimbuterol	$C_{13}H_{19}N_3O$	220.1444	6.50	1
32	Ritodrine	$C_{17}H_{21}NO_3$	288.1594	8.49 /9.50	1
33	Clencyclohexerol	$C_{14}H_{20}Cl_2N_2O_2$	319.0974	8.55	1
34	hydroxymethylclenbuterol	$C_{12}H_{18}Cl_2N_2O_2$	293.0818	8.60 / 9.62	1
35	Tulobuterol	$C_{12}H_{18}ClNO$	228.1149	11.73	1
36	Clenbuterol	$C_{12}H_{18}Cl_2N_2O$	277.0869	12.73 / 13.75	1
37	Isoxsuprine	$C_{18}H_{23}NO_3$	302.1750	12.58 / 13.59	1
38	Clenproperol	$C_{11}H_{16}Cl_2N_2O$	263.0712	14.74	10
39	Chlorbrombuterol	$C_{12}H_{18}BrClN_2O$	321.0364	14.76 / 15.76	1

40	Brombuterol	$C_{12}H_{18}Br_2N_2O$	364.9858	16.79 / 17.79	1
41	Mabuterol	$C_{13}H_{18}ClF_3N_2O$	311.1133	18.84	1
42	Clenpenterol	$C_{13}H_{20}Cl_2N_2O$	291.1026	18.93	1
43	Ractopamine	$C_{18}H_{23}NO_3$	302.1751	19.79	1
44	Reproterol	$C_{18}H_{23}N_5O_5$	390.1772	19.98	10
45	Clenhexyl	$C_{14}H_{20}N_2Cl_2O$	303.1025	21.89	1
46	Mapenterol	$C_{14}H_{20}ClF_3N_2O$	325.1289	23.93	1
47	Salmeterol	$C_{25}H_{37}NO_4$	416.2795	29.21	1
	Hormones (steroids)				
48	Prednisolone	$C_{21}H_{28}O_5$	359.1853	26.18	5
49	Methylprednisolone	$C_{22}H_{30}O_5$	375.2166	28.26	5
50	Dexamethasone	$C_{22}H_{29}FO_5$	393.2071	28.22	10
51	Betamethasone	$C_{22}H_{29}FO_5$	393.2071	28.32	10
52	Flumethasone	$C_{22}H_{28}F_2O_5$	411.1977	28.24	5
53	α-Nortestosterone	$C_{18}H_{26}O_2$	275.2005	29.28	2
54	β-Nortestosterone	$C_{18}H_{26}O_2$	275.2005	29.46	2
55	α-Trenbolone	$C_{18}H_{22}O_2$	271.1692	30.09	2
56	β-Trenbolone	$C_{18}H_{22}O_2$	271.1692	31.31	2
57	α-Testosterone	$C_{19}H_{28}O_2$	289.2162	29.09/30.09	2
58	β-Testosterone	$C_{19}H_{28}O_2$	289.2162	30.49	2
59	β-Testosterone-D3	$C_{19}H_{25}D_3O_2$	292.2350	30.49	not applicable
60	Methyltestosterone	$C_{20}H_{30}O_2$	303.2318	31.21	10
61	α-Boldenone	$C_{19}H_{26}O_2$	287.2006	30.24	10
62	β-Boldenone	$C_{19}H_{26}O_2$	287.2006	31.16	10
63	16β-hydroxystanozolol	$C_{21}H_{32}N_2O_2$	345.2537	35.32	10

509 ** Does not belong to the class of sulfonamides, but is often co-administrated with sulfonamides.*
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