Application of Comprehensive 2D Chromatography in the Anti-Doping Field: Sample Identification and Quantification

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Abstract

Anti-doping analysis requires an exceptional level of accuracy and precision given the stakes that are at play. Current methods rely on the application of chromatographic techniques linked with mass spectrometry to provide this. However, despite the effectiveness of these techniques in achieving good selectivity and specificity, some issues still exist. In order to reach the minimum required performance level as set by WADA, labs commonly use selective monitoring by quadrupole mass spectrometry. This can be potentially fooled through the use of masking agents or by moving the peaks, as often only a small portion of the spectrum is used for analysis. Further issues exist in the inability to detect new or modified compounds, or to reanalyse samples/spectra. One technique that could overcome these problems is that of comprehensive 2D chromatography. Here a second separation column is employed to generate greater separative power. Compared to conventional separation, GCxGC allows for a greater peak capacity (i.e., number of peaks that can be resolved within a given time) and greater separation of coeluting compounds, which makes the technique promising for the complex task required in anti-doping. When combined with Time of Flight Mass Spectrometry this technique demonstrates vast potential allowing for full mass range datasets to be obtained for retroactive analysis. Similarly, LCxLC provides improvements in resolving power compared to its 1D counterpart and can be used both online as part of the analysis or offline solely as a purification step. In this review we summarise the work in this field so far, how comprehensive chromatography has been applied to anti-doping studies, and discuss the future application for this technique.

Keywords: GCxGC; two-dimensional chromatography; WADA; Anabolic Androgenic Steroids; doping; anti-doping

1.1 Introduction

The World Anti-doping Agency (WADA) was set-up in 1999 with the main aim to unify the global anti-doping efforts. As part of this, each year, they publish a list of prohibited substances which is broken down into 11 sub-categories,[1] namely: anabolic agents, peptide hormones and growth factors, Beta-2-agonists, hormone and metabolic modulators, and diuretics and masking agents which are designated S1-5, respectively and banned at all times. Plus, categories S6-S9: stimulants, narcotics, cannabinoids and glucocorticoids, respectively which are banned in competition. S0 adds any non-approved substance (*e.g.* drugs in clinical trials) to the prohibited list whilst for sports such as archery, automobile racing, billiards, darts, golf, shooting, skiing and snowboarding and underwater sports, a further class of Beta-blockers (P1) are also banned, at all times for some and in-competition only for others. In addition, there are three categories of prohibited methods (M1 Manipulation of Blood and Blood Components; M2 Chemical and Physical Manipulation; M3 Gene and Cell Doping).

Current WADA protocols rely on chromatography (liquid and gas) hyphenated with mass spectrometry along with immunological methods for some of the larger molecule prohibited substances (> 500

Da).[2] WADA set minimum required performance levels (MRPLs) for the groups of compounds mostly in the ng ml^{-1} range.[3] They require the limit of detection (LOD) to be half the specified MRPL for any given compound analysis. For example, stanozolol has an MRPL of 2 ng ml^{-1} therefore LOD must be 1 ng ml^{-1}. The MRPL is not a threshold, nor a LOD, as a positive adverse finding can be below this (considering time between tests and metabolic rates). These vary between compounds, classes and sports.

Whilst other analytical techniques for antidoping applications have been reported in literature, such as surface plasmon resonance (SPR),[2] quartz crystal microbalance (QCM)[4] and pseudo-immunoassays,[5] approved antidoping analysis has remained relatively one-track with a focus on chromatography combined with mass spectrometry.

The group of Peter Van Eenoo have published several papers over the last decade, tracking the improvements in chromatography-mass spectrometry used in the Belgian WADA accredited laboratory to date (DoCoLab). Initially, they reported using two GC-MS methods in 2010; one for AASs, β-agonists, hormone antagonists and modulators, and a second for narcotics and stimulants.[6] A year later, they published one combined method for all these compound groups, by transferring to GC-MS/MS using a triple quadrupole instrument. This allowed not only for an improvement in the mass spectrometry (more compounds detected, both quantitatively and qualitatively and lower LODs) but also showed improvements to the chromatography and allowed for almost halving the run time (26 minutes down to 15 minutes).[7,8] They also published a paper comparing the use of different time of flight analysers for the detection of anabolic steroids in antidoping control as detectors on liquid chromatography systems.[9] In 2015, they reported how switching from electron ionisation (EI) to chemical ionisation (CI) improved the sensitivity.[10] The authors reported that chemical ionisation, using ammonia as positive chemical ionisation reagent, produced more selective transitions and increased intensity of the bigger fragments. As these fragments are the diagnostic ions, this equates to an increase in sensitivity, lowering the detection limits. At the time of this work, this was important as WADA had just lowered the required MRPLs.

More recently, they published on the migration to a high-resolution mass spectrometry (HRMS) analysis coupled to gas chromatography. This transition to HRMS is something that had been implemented for the liquid chromatography method several years previously.[11] The authors also illustrated that the use of low energy electron ionisation (LE-EI) demonstrated similar benefits to CI, with 18 eV being the suggested optimal (compared to standard 70 eV). LE-EI is a softer ionisation than the standard 70 eV and therefore gives rise to a higher concentration of the higher mass fragments, this is displayed as more intense peaks in the spectra, increasing the signal to noise ratio and decreasing the LOD.

This research all focused on improving the mass spectrometry, however, the authors suggest that one improvement that appears to have been overlooked by WADA-accredited labs is to the chromatography, more specifically the application of 2D chromatography.

1.1.1 Comprehensive Two-Dimensional Chromatography

GCxGC was first reported in the early 1990s by Liu and Phillips[12] and consists of two orthogonal columns directly in series with a modulator in between. Typically, the first column is non-polar and standard dimensions, with the second column often more polar and shorter, with a smaller ID and thinner film (although the polarity can be switched, known as reverse). Modulation can be split into two types: flow and thermal. Thermal modulation can be further split based on whether a refrigeration

unit (intra cooler, also known as 'cryogen-free'; minimum temperature -90 °C) or cryogen (*e.g.* liquid N_2, minimum temperature -196 °C) is used. This impacts the size of molecule that can be modulated; cryogenic modulation can modulate a three-carbon chain length and greater whereas refrigeration can only modulate from six carbons (due to difference in minimum achievable temperature).[13] Thermal modulation commonly uses a combination of cold and hot jets to trap the analytes as they exit the first column, focus, then release onto the second column in 'slices' (Figure 1). A flow modulator relies on a 6-point valve, which is filled and emptied to create a similar sliced effect. There may also be a secondary oven housed within the main GC oven to allow separate temperature control of the secondary column, typically 30 °C above the primary column. This use of two orthogonal columns increases the number of theoretical plates and therefore the number of compounds that can be separated. The peak capacity of comprehensive 2D GC is the product (not sum) of the peak capacity of the two individual columns.

Time-of-Flight mass spectrometry (ToFMS) is the favoured detector to combine with GCxGC as a high scan rate is required as a result of the sharpening of peaks provided by GCxGC. Furthermore, it enables deconvolution of spectra as it is not a scanning technique (unlike quadrupole MS), adding an extra dimension of separation, should it be required.

Further advantages of the GCxGC-ToFMS system include the ability to acquire data as a full scan (compared to selective monitoring, as with SIMs/quadrupole) allowing for the observation of any new compounds appearing in the chromatogram, thus preventing future occurrences such as the BALCO scandal,[14] as well as storage of the data for future retro-analysis for discovered compounds (compared to storage of biological samples for the same reason).[15] The Bay Area Laboratory Co-operative (BALCO) supplied steroids to athletes in the 1980s and 1990s until a US Federal investigation in 2002. It is most famous for the supply of tetrahydrogestrinone (THG), at the time, a new designer steroid that the authorities were not aware of. It remained undetected due to the necessity to use selected monitoring for known substances and was only discovered thanks to a contaminated syringe being handed over to the authorities. By using improved chromatography (*e.g.* 2D techniques), the full scan mass spectrum can be collected therefore any new / unusual substances will be detected as additional peaks for further investigation.

Two-dimensional LC was first introduced at a similar time as GCxGC and follows the same basic principles as the flow modulated GCxGC systems. It can be divided into two subcategories: comprehensive and heart-cutting. Comprehensive 2D LC (LCxLC) is where the sample travels through the first LC column then the full sample is transferred to the second column in aliquots *via* a valve system.[16] Heart-cutting is the transfer of only the portion of the sample that is of interest to the second column, the rest is either sent to waste or to a secondary detector. In gas chromatography, a Dean's switch is used to carry this out. -. Two-dimensional LC can also be divided into online and offline use. This review focusses on the application of 2D LC to the anti-doping field but the review by Stoll *et al.* illustrates that LCxLC has further been used in urine analysis for other applications.[16]

Since 2009, Thevis and co-workers have produced an annual review of the analytical advancements published in the anti-doping testing field. In several of these yearly updates, the authors have noted relevant two-dimensional GC publications, mostly in the initial screening procedures for anabolic androgenic steroids section, and are discussed in detail below. Most of these were early in these years[17–19] with the authors suggesting in the 2010 review that the "two-dimensional chromatographic approach an interesting contribution".[17] More recently they noted the use of heart-cutting (Dean's switch), this is carried out for the confirmatory testing procedures, specifically the combustion isotope ratio mass spectrometry.[20–22] Although the authors have found most 2D

chromatography literature in this category, herein is included literature from the other prohibited list categories, where found.

Further, in their review in 2015, Nicoli *et al.* suggested that GCxGC had been "scarcely employed" in the antidoping testing field due to the techniques relatively new commercial existence.[23] With further comment that 2D-LC techniques were not advanced enough at the time of publication to have been adopted.

In this critical review we consider the existing literature, discuss successes and look forward to the future of the application of 2D chromatography in the anti-doping field.

1.2 Analysis of prohibited substances by Comprehensive Chromatography

S1 – Anabolic Agents

The 'athlete biological passport' (ABP) monitors the ratios of testosterone and epitestosterone as part of the steroidal profile that also calculating ratios using androsterone, etiocholanolone (5β-androsterone), 5α-Androstane-3α,17β-diol and 5β-Androstane-3α,17β-diol. In order to calculate these ratios, WADA currently requires a limit of quantification "not greater than 2 ng ml^{-1}" for these analytes.[24] For all other AAS (endogenous and exogenous), the minimum required performance level (MRPL) is also 2 ng ml^{-1} except boldenone which is 5 ng ml^{-1}. Clenbuterol has a MRPL of 0.2 ng ml^{-1}, an exception in the 'other anabolic agents' category which is otherwise also 2 ng ml^{-1}.[3] In order to achieve accurate results, enzymatic hydrolysis of the samples is performed before analysis using β-glucoronidase from *E. coli*, as less than 3 % of total androgens in a urine sample are 'free' with most being conjugated to a glucuronide moiety.[25] This is usually followed by further extraction procedure(s).

Anabolic steroids have a core seventeen-carbon gonane (perhydrocyclopenta[a]phenanthrene) fused-ring structure with only minor differences in side groups. Furthermore, anabolic steroids commonly have an alcohol or ketone group at carbons 3 and/or 17. Estrogens are classed through having one extra carbon (C18) androgens have two (C18 and C19) with glucocorticoids a further class of compound similar in structure (see section S9). The combination of this high degree of similarity between compounds and the requirement to detect over 60 named steroids,[26] compounds of similar structure or biological effects and their metabolites simultaneously makes chromatographic separation challenging, requiring powerful chromatography or detection or, ideally, both.

Current WADA analysis protocols for the detection and quantification of androgenous anabolic steroids (AASs) use GC-MS(/MS) in SIM mode, after derivatization of analytes following extraction from urine samples.[24] However, it has also been reported that there was an increasing use of LC-MS(/MS) in the early 2000s due to advances in the technology and the advantage of being able to handle labile, non-volatile larger biomolecules than typically possible by GC, which may be useful when investigating several of the classes of prohibited substances in a single method (Figure 2). Within the last decade there has been further movement towards comprehensive 2D GC (-FID or -MS).[27]

General Screening Procedures

Little research has currently been carried out into the optimisation of GCxGC of AASs; namely by the groups of Marriot, and Brenna.

Marriot and Mitrevski demonstrated that 2D GC can meet current WADA criteria for, e.g. retention time reproducibility in both dimensions (retention time difference shall be 1 % or ±0.1 minutes, whichever is smaller[28]).[29] They reported the second dimension times also met the 1% criteria even though this is not explicitly covered by the guidelines, suggesting that this would need to be considered if GCxGC was to become routine in doping lab analysis.

Zulfiqar *et al.* reported the use of GCxGC-ToF MS to resolve 12 AASs.[30] They reported LOD of 11.7-27.0 pg (on column with a 1 µl injection, equates to 0.234-0.540 ng ml^{-1} in sample, taking into account their preconcentration) for the range of androgens studied. This meets the WADA LOD and MRPL set for this category (1 and 2 ng ml^{-1}, respectively). In this case, the pre-concentration step involved the use of solid phase extraction (SPE) specifically a purposefully synthesised molecularly imprinted polymer (MIP) used as the sorbent in the molecular imprinted solid phase extraction (MISPE). The sample preparation prior to chromatography should be given as much consideration as the detector choice post chromatography.

Most of the literature reviewed uses a relatively complex heating program (Figure 3). For example, Mitrevski *et al.* used a multi-step heating method (Figure 3, dark blue line) to obtain separation of 27 sterols, whilst reporting both a standard (non-polar followed by polar, NP-P) and a reverse phase column set as well as GCxGC-FID and coupled to ToF-MS.[31,32] The authors reported that, when using the NP-P column setup (on FID) two pairs of compounds that co-eluted in 1D could be resolved using otherwise identical chromatographical parameters. Furthermore, the peak signal is increased significantly, with peak height approximately 2. 5 times greater in GCxGC. Additionally, the sensitivity of GCxGC-FID is similar to that of GC-qMS in SIM mode, highlighting the power of the 2D separation in separating analyte peaks from other peaks, background noise, matrix, solvent and column bleed.

Interestingly, the authors noted that using the non-polar column first in the sequence led to better separation than using the polar column first in the GCxGC; although they suggested that better separation came with a trade-off of peak shape; with the reverse exhibiting better peak shape and therefore sensitivity when using a Longitudinal Modulation Cryogenic system with CO_2 as cryogen and nitrogen as flush gas.[31]

In their other paper, Mitrevski *et al.* reported that the detector was switched to ToFMS, where they suggested that a 0.2 µm film thickness on the second column demonstrated a better ‘spread’ of analytes.[32] The authors investigated the separation of 6 WADA prohibited AAS, and with the NP-P (thicker film) set-up, they reported LODs of 1 ng for 19-norandrosterone, 17α-methyl-5α-androstane-3α,17β-diol, 17α-methyl-5β-androstane-3α,17β-diol, as well as the other anabolic agent, clenbuterol, and 2 ng ml^{-1} for epimethendiol and 3’-OH-stanozolol; all meeting the WADA MRPL guidelines. Although this is a good starting point, the small analyte sample size does not truly indicate applicability to antidoping testing, therefore the authors feel further work on an increased number of analytes would be required.

Barbosa *et al.* used a similarly complex heating program to successfully separate 40 doping agents (from different pharmacological classes) and their metabolites.[33] This included AAS and their metabolites (S1, including clenbuterol, designated an ‘other anabolic agent’), β2-agonists (S3), aromatase inhibitors and selective estrogen receptor modulators (both S4, Hormone and Metabolic Modulators), diuretics & masking agents (S5), stimulants (S6), narcotics (S7) and cannabinoids (S8)

plus a drug used for the enhancement of oxygen uptake, transport or delivery (prohibited methods category 'M1'). However, the authors purposefully worked at a concentration 10 times higher than the WADA MRPL (for steroids) as their intention for this article was to highlight the improvement in resolution and sensitivity rather than detection limits, which they suggested had been demonstrated in other papers. The authors suggest that the signal to noise ratios obtained at this elevated concentration indicate that the sensitivity of the instrument set up would be suitable for antidoping analysis but do not provide any example data to support this. This is the broadest study the author has found to date, and would benefit from a study into the determination of LODs across the range of analytes in order to ascertain whether full WADA MRPL can be met.

Zulfiqar reported the use of different but relatively simple heating programs in his thesis, depending on whether the analytes were derivatized or not (Figure 3; black line for underivatized analytes, red line for derivatized steroids).[34]

Meanwhile, Zhang and Tobias used isothermal oven programs after a rapid increase in temperature at the beginning (pink and orange lines, respectively, Figure 3).[35,36] With the temperature held at 300°C for 35 minutes, this made for a long run-time (total 47.75 minutes) per sample, much longer than the currently employed 12 minutes used in anti-doping testing. Moreover, holding the column temperature at close to the maximum operational temperature will reduce the column lifetime due to degradation, increasing the maintenance downtime of the instrument. Furthermore, as is illustrated in Figure 3, all of the GCxGC literature examples found have longer duty cycles than the current WADA test method by a significant margin, an increase which will not be accepted by WADA labs if they were to implement this technology and one that the authors feel is unnecessary.

LECO have also published an application note on the use of the Pegasus 4D ToFMS instrument equipped with a two stage cryogenic modulator and secondary oven for this application.[37] This was carried out using a standard dimension first column (BPX-5) and a 20 m x 0.18 mm x 0.20 µm BPX-50 in the second dimension with a multi-step heating cycle (grey line, Figure 3). They reported S/N ratios of between 85 and 1353 for 5 WADA prohibited steroids at the MRPL of 2 ng ml^{-1}, with calculated LOD (at S/N = 10) in the range of 15 to 235 pg ml^{-1} (0.015-0.235 ng ml^{-1}); well below that required by the MRPL guidelines.

Most of the literature found follows the standard column order of non-polar in the first dimension (typically a '5' or a '1'; (1 or 5 % phenyl-methyl polysiloxane stationary phase) followed by a polar column. In most cases this is a mid-polar '50' (50 % phenyl-methyl polysiloxane stationary phase) with the other choice being a '1701' (cyanopropylmethyl phenylmethyl polysiloxane stationary phase). Whilst most literature analysis is carried out on derivatised steroids, this does not appear to affect the column choice. Furthermore, most references use a 30 m long, 0.25 mm I.D., 0.25 µm film thickness in the first dimension, the only other dimensions used is coincidentally by those that use the OV-1701 in the second dimension, whereby a 17 m, 0.2 mm I.D., 0.11 µm film thickness column is employed. The second dimension column ranges in length from 1 to 2 m.

Zhang *et al.* chose to use GCxGC-qMS in both electron and chemical ionisation (EI and CI, respectively) modes as a way to increase selectivity compared to ToF-MS.[35] In their other paper, also focusing on CI mode of qMS, they report that analysis of derivatised steroids gives superior results compared to underivatized and that although there were no chromatographical benefits to either using trimethylsilyl (TMS) or acetate derivatives, acetates were chosen as TMS is not compatible with combustion isotope ratio MS (C-IRMS).[36] More importantly, the authors reported that "the measured LOQs with full mass scan GCxGC-qMS were comparable with the LOQs measured using 1D GC-MS in SIM mode"; highlighting the advantage of 2D GC.

Stepan *et al.* reported the GCxGC-ToFMS analysis of 25 underivatized spiked steroids extracted from two different types of nutritional supplements.[38] The authors determined reporting limits for all 25 AASs between 0.007 and 0.114 mg kg^{-1}; suggesting that these are low enough to determine concentrations found in supplements. This area of research is important to athletes as the WADA code of conduct states that there is strict liability on the athletes to ensure a prohibited substance does not enter their body, *via* any means, regardless of intent.[39]

Bileck *et al.* evaluated the use of GCxGC-ToFMS using a standard mix of 40 steroids, from the 5 subgroups (androgens, estrogens, progestogens, mineralocorticoids and glucocorticoids).[40] Of these, the authors reported a LOD for testosterone of 2.7 nM (0.78 ng ml^{-1}) and 3.6 nM (1.04 ng ml^{-1}) in methanol and steroid-free urine matrix (SFUM), respectively. It was suggested the detection limits achieved were 5 to 20 times better than those achieved using GC-qMS, and are below the set MRPL but still leaves further room for improvement in order to consistently work comfortably below the required LOD, for all steroids, regardless of matrix. Furthermore, as this technique allows for untargeted analysis, an additional 30 steroidal compounds were detected from urine samples that would not be seen in the qMS method. This is vital in the progress of antidoping testing, in order to keep up with advancements in available performance enhancing substances. It would be interesting to see this pushed further to 'real' human urine samples.

Silva Jr. *et al.* reported the separation, detection, identification, and deconvolution of several AAS, clenbuterol and several important AAS metabolites (two metabolites of methyltestosterone and 3-hydroxystanazolol) from other exogenous compounds, found in urine samples, at the WADA set MRPL of 2 ng ml^{-1} using GCxGC-ToFMS.[41,42] These metabolites were focussed on as the authors claim these are the most challenging analytes in current methodologies, with particular focus on the success of deconvolution of the 3-hydroxystanozolol from an otherwise co-eluting endogenous matrix compound. In the second paper, these five compounds remained a focus but the urine was spiked with 27 AAS.[42] They used a linear regression model, based on 8 of the earliest eluting compounds, to locate the remaining compounds within the 2D chromatogram, based on their 1D retention times. The authors suggest that this work could aid the transition of all WADA labs to 2D chromatography should they choose to adopt these technologies.

GCxGC predicted retention times for steroids have been modelled based on thermodynamic properties.[43] Silva *et al.* reported the advantage of this is that these thermodynamic models are independent of the operating conditions and that the use would aid in the identification of unknowns, as the authors suggest this is still a limiting factor in GCxGC due to the "thousands of peaks". Following on from previous work, this was based on the use of a thermally modulated system. This was carried out in the hope this would assist in the detection of unknown / suspect drugs in samples. By first carrying out 1D GC-MS experiments, they were able to predict retention times in both dimensions, by running a series of temperature rates, entropy, enthalpy and adiabatic molar heat capacity were calculated which are the parameters required for the predictions using a MATLAB code. Of the steroid derivatives studied, only the methoxime-trimethylsilylated carbonyl (MO-TMS) derivative of androsterone had an error on the first-dimension retention time of greater than 10 seconds, while all second-dimension retention times were within 0.5 s of the prediction. This was further demonstrated by applying the modelling to urine samples (figure 4). These errors were higher than those reported for the prediction of a pneumatic modulation, but that is to be expected given the more complex nature of the system. The authors suggested that these errors were still acceptable and that this was a promising start in the development of a thermodynamic library and model that could aid in the detection and identification of designer steroids. Barbosa *et al.* used this model to accelerate their initial optimisation.[33]

GCxGC-MS for steroid analysis is not just confined to biological matrices. Other research reported includes wastewater[44,45] and coconut[46] / vegetable oils.[47]

Confirmatory Testing

Because testosterone is an endogenous AAS *i.e.* it is naturally produced in the body, it is not enough to simply rely on the concentration in a urine sample to determine an adverse finding. There are two further investigations:[24]

1) The ratio of testosterone to epitestosterone is calculated. This should naturally be *ca.* 1, if there is exogenous use of testosterone, the concentration of epitestosterone is unaffected, therefore the ratio changes.
2) To circumnavigate test (1) some athletes will use both testosterone and epitestosterone in order to mask their testosterone consumption. However, endogenous testosterone is synthesised in the body from the variety of plant matter consumed in the diet, where different plants discriminate against $^{13}CO_2$ in the atmosphere to differing extents, therefore endogenous testosterone is an average of the ^{13}C content of the individuals diet whereas synthetic testosterone is commonly synthesised from a single plant species, specifically soy, and therefore contains a more defined ^{13}C, with this knowledge, $\delta^{13}C$ can be examined *via* combustion isotope ratio mass spectrometry (C-IRMS).[48,49]

In 2008, Tobias *et al.* claimed the first use of GCxGC coupled to C-IRMS for the detection of steroid acetates.[50] A programmable temperature vaporization (PTV) inlet was used, with conditions optimised in order to divert solvent away from the analysis (so as to preserve the detector). This is often carried out using a back flush, but the PTV was chosen here (after its use had been published elsewhere) so as to limit the number of connections in an already complex system. The optimised conditions for the PTV inlet were 50 °C for 1 minute (0.9 minute solvent vent) then ramped to 300 °C at 600 °C min^{-1}.

This was followed by a second paper in 2011 where the authors reported the use of GCxGC coupled to C-IRMS for the detection of synthetic testosterone.[51] The authors reported that current anti-doping analysis of urine samples by GC-C-IRMS requires extensive clean-up *via* several HPLC steps / additional SPE extractions prior to analysis (on top of the SPE, deconjugation and derivatisation employed for standard GC-MS analysis); however, this was not required with the use of GCxGC whilst maintaining the ability to fulfil WADA requirements for the analyses. Essentially, the first column in this set-up acts as the clean-up step (where previously preparative HPLC was employed) then the chromatography takes place on the second, usually polar, column.

Brailsford *et al.* also used GCxGC with heart-cutting in order to gain cleaner samples for analysis *via* C-IRMS.[52] For their primary column the authors chose a 60 m 1701 column (cyanopropyl), after optimisation on a 1D system, as they reported it demonstrated the best separation of testosterone and epitestosterone. A shorter, narrower 624 column (30 m x 0.15 mm x 0.84 µm; mixed stationary phase, less polar than the 1701) was also investigated but was overloaded at the relatively high concentrations required for a good IRMS signal. They used a large volume injection (40 µl) with a PTV inlet. Unfortunately, a single run was over 160 minutes, however, as this replaces laborious, manual liquid chromatography clean-up (prior to standard GC-C-IRMS analysis) the authors suggest this is not a problem, especially considering that the use of autosamplers would allow these analyses to carry on throughout the night *etc.* when the otherwise manual sample preparation would not. The authors

believe that, even when considering the sample preparation replacement, this is an extreme length of time and could be shortened whilst maintaining the required separation.

Several years later Casilli *et al.* further reported the use of online heart-cutting as the clean-up step for IRMS and simultaneous quadrupole MS.[53] They reported a shorter run time of approximately 90 minutes.

Most recently Putz *et al.* demonstrated similar[54] with reported limits of quantification (LOQ) of 5 ng ml^{-1} for 5α-androstane-3α,17β-diol and 5β-androstane-3α,17β-diol; 10, 12, 15 and 30 ng ml^{-1} for 5α-androst-16-en-3α-ol, testosterone, pregnanediol and 11-ketoetiocholanolone, respectively, and 150 ng ml^{-1} for both etiocholanolone and androsterone.

LCxLC

In 2018, Blokland *et al.* reported the use of LCxLC-ToFMS for the detection of several of the WADA prohibited classes of compounds simultaneously from urine samples.[55] The author suggests that in order to take this multiclass analysis approach, any clean-up prior to analysis has to be broad to ensure no loss of analyte; however, this leads to a potential increase in matrix interference and thousands of compounds in such a crude extract, something that standard chromatography, or even high resolution mass spectrometry, cannot handle without the aid of comprehensive chromatography. Of the 63 compounds investigated, there were several β2-agonists (see section S3), glucocorticoids (section S9) and AASs. For the AASs, the authors reported detection limits of 2 µg l^{-1} (2 ng ml^{-1}) for nandrolone and testosterone but 10 µg l^{-1} (10 ng ml^{-1}) for boldenone and hydroxystanozolol (metabolite of stanazolol). For the 'other anabolic agent' clenbuterol, a LOD of 1 µg l^{-1} (1 ng ml^{-1}) was reported. The LOD for nandrolone and testosterone meet the WADA MRPL, but further work is required in the case of boldenone, hydroxystanozolol and clenbuterol (MRPL of 5, 2 and 0.2 ng ml^{-1}, respectively).[3] Interestingly, this work was not carried out with the specific WADA criteria in mind, suggesting that these could be improved as required. The authors believe that this ability to analyse multiple classes of compounds simultaneously by using comprehensive chromatography to remove the additional matrix *etc.* resulting from the broader sample preparation is hugely beneficial to antidoping screening.

Further work by Baglai *et al.* illustrated improvements in comprehensive 2D LC by using 'active' (rather than passive) modulation to separate 14 anabolic steroids (figure 4).[56] This is the use of 'trap columns' as part of the otherwise simple loop system, so as to reduce the volume of the fractions and thereby reduce the dilution effect often observed in 2D LC. The authors suggested this gave an increase in signal height by a factor of 2.4-7.6, and therefore a factor of 7 increase in signal-to-noise, which would lead to a lower LOD. However, the authors do not report the LODs therefore it is difficult to assess the applicability of this method based on WADA MRPL requirements.

More recently LCxLC has been employed as an online purification procedure prior to carbon isotope analysis by Wen *et al.* and Lalonde *et al.*.[57,58] Wen *et al.* used two HPLC columns in series in order to purify 19-norandrosterone (19-NA, a metabolite of several steroids including nandrolone) online with GC-C-IRMS in order to increase efficiency compared to previously reported offline methods. They reported the successful analysis of urine samples containing 2 ng ml^{-1} 19-NA, in line with WADA MRPL expectations.

Meanwhile, Lalonde *et al.* reported the use of 2D-LC to purify eight underivatized steroids, including testosterone, dehydroepiandrosterone (DHEA) and metabolites, prior to isotope analysis. They demonstrated improved purity with reduced analysis time compared to a standard 1D HPLC procedure.

S3 – Beta-2 Agonists

For the analysis of Beta-2-agonists, WADA currently requires a MRPL of 20 ng ml^{-1}, however, salmeterol and higenamine should not be reported at levels below 50 % MRPL (10 ng ml^{-1}).[3] Furthermore, for salbutamol and formoterol there is a minimum required threshold value before an adverse analytical finding (AAF) is reported; in this case those values are 1.0 µg ml^{-1} and 40 ng ml^{-1}, respectively,. These are known as 'threshold substances'.[59]

GCxGC has reportedly been used for the analysis of samples for another class of the WADA banned substances, β-agonists, by McGuire and Marriot in 2013.[60] Unusually, this work employed an FID detector rather than MS with a standard dimension DB-5MS first dimension column, as seen in the steroid analysis, but a mid-polar trifluoropropyl VF200ms^2D (2 m x 0.15 mm x 0.15 µm) as second dimension column. They reported LOD (S/N = 3) of 4.49, 2.53 and 4.09 µg ml^{-1} (4490, 2530 and 4090 ng ml^{-1}) for clenbuterol, salbutamol and terbutaline, respectively. These relatively high detection limits, which were above the WADA MRPL for this class of 20 ng ml^{-1},[3] illustrate that a more sensitive detector, such as mass spectrometry, is still necessary even with the improvements provided by two dimensional GC. It would be interesting to see mass spectrometry applied to this work.

By LCxLC-MS, Blokland *et al.* reported detection limits of 2 µg l^{-1} (2 ng ml^{-1}) for salbutamol and fenoterol and 1 µg l^{-1} (1 ng ml^{-1}) for tulobuterol and salmeterol.[55] These are below the 20 ng ml^{-1} MRPL (10 ng ml^{-1} LOD) for this class.

S6 & S7 – Stimulants and Narcotics

For the analysis of stimulants, WADA mostly requires a MRPL of 100 ng ml^{-1}, with the exception being octopamine which has an MRPL of 1000 ng ml^{-1}. Several stimulants are also considered threshold substances: Cathine, Ephedrine, Methylephedrine and Pseudoephedrine where the threshold limits for these is 5, 10, 10 and 150 ug ml^{-1}, respectively (5000, 10000 and 150000 ng ml^{-1}). The general MRPL set by WADA for the analysis of narcotics is 50 ng ml^{-1}, with the exceptions of buprenorphine (5 ng ml^{-1}) and fentanyl and its derivatives (2 ng ml^{-1}).[3] Moreover, morphine is listed as a threshold substance, here the threshold is set at 1 µg ml^{-1}.

Comprehensive 2D GC has also been reported for use in the detection of other illicit drugs as first reported in 2003 by Kueh *et al.*[61]

In 2009 Heim and Pugh reported the use of GCxGC to separate 11 non-derivatised drugs (mostly WADA prohibited stimulants).[62] They reported relative limits of detection of 0.212, 0.145, 0.038, 0.065, 0.277, 0.082 and 9.830 ng ml^{-1} for methamphetamine, ecstasy, cocaine, codeine, oxycodone, heroin and LSD 25 respectively. These are several magnitudes more sensitive than the required MRPL of 100 ng ml^{-1} outlined by WADA.[3]

At the same time, Guthery *et al.* published two papers using comprehensive GCxGC-ToFMS for the detection of various narcotics and drugs of abuse. In the first paper, biological serum was spiked with several benzodiazepines and 'street heroin' (a mixture of heroin, 6-MAM, morphine, acetylcodeine, codeine and papaverine) which the authors reported were all separated from each other as well as from the serum matrix interference (over 1700 peaks identified by the software, in total, most of

which could be attributed to the serum matrix). With further identification of many of the endogenous compounds from the serum which are present in higher concentrations than the analytes of interest. They reported detection limits of 1.6 and 2.5 ng ml^{-1} for flunitrazepam and 7-aminoflunitrazepam, respectively. The authors suggested that the modulation displayed an order of magnitude increase in S/N compared to using the same two-column system in a 1D mode (without modulation).[63] Although it is often suggested that a high scan rate (500 spectra/s) is required due to the small peak widths in GCxGC, the authors suggested that 50 spectra/s was sufficient in this analysis, creating smaller data files, the size of which, Van Eenoo suggested is an obstacle to be overcome in order for WADA testing to switch to ToFMS.[64] It is also suggested that the lower scan rate increases sensitivity.

In the second paper, the authors reported the qualitative detection of opiates in hair samples (figure 6).[65] Whilst hair samples are useful, as they have an extended detection window compared to other biological samples such as urine, currently it is not an approved matrix with WADA. For two hair samples suspected to be from illicit drug users, 25 and 18 compounds, respectively, were identified that indicated that this was the case. Not only were two major metabolites of heroin, morphine and 6-monoacetylmorphine, but the use of 'street heroin', rather than pharmaceutical grade, was also confirmed by the presence of other opiate alkaloids such as codeine, papaverine, thebaine, meconin and narcotine. It is useful to see evidence of the broad range of (biological) samples that 2D chromatography can be applied to, especially in the context of extending the detection windows and the use of samples for which collection may be less invasive than the current urine collection; two areas that are currently at the forefront of antidoping innovation.

S8 – Cannabinoids

The required MRPL for cannabimimetics is 1 ng ml^{-1},[3] with 11-nor-Δ9-tetrahydrocannabinol-9-carboxylic acid (carboxy-THC) listed as a threshold substance where the threshold is set at 150 ng ml^{-1}.

In 2010, Milman *et al.* reported the use of 2D-GCMS for quantification of three cannabinoids and two of their metabolites from oral fluid.[66] Oral fluid is not currently a WADA approved sample matrix, however, there is an ongoing pilot study being carried out by USADA with UFC athletes into its use. The authors reported the use of a Dean's switch to split the effluent between an FID and a secondary column followed by MS. However, even though they reported a single extraction, this required two different solvents (collected and derivatised separately) followed by 2 different ionisation methods; EI and negative CI, on two separate MS instruments.

They reported detection limits of 0.5, 0.5, 1.0 and 0.4 ng ml^{-1} for CBD, THC, CBN and 11-OH-THC, respectively, using their 2D-GCMS system with electron ionisation plus an LOD of 0.006 ng ml^{-1} for THCCOOH using negative chemical ionisation. These all meet the WADA MRPL of 1 ng ml^{-1} for cannabimimetics.[3] Once again it is useful to see research using other biological matrices, especially as this is in keeping with a pilot study into its viability, however, this sample preparation program may be an increase on current procedures therefore would not be favourable for WADA labs.

More recently, Eckberg *et al.* reported the use of 2D liquid chromatography to overcome otherwise difficult separation of structurally similar synthetic cannabinoid mixtures, however, this paper is proof of concept, with no detection limits reported.[67] In order for the success to be assessed against WADA MRPL, further work would be required with information such as LODs reported.

S9 – Glucocorticoids

Glucocorticoids, like anabolic steroids, have a fused ring backbone with an additional ethyl hydroxyl ketone side group located at carbon-17. This makes analysis more complex and challenging as there will be an increasing number of compounds with similar structures. For these compounds, WADA set the MRPL at 30 ng ml^{-1}.[3]

Blockland *et al.* reported LODs of 5 µg l^{-1} (5 ng ml^{-1}) for prednisolone and methylprednisolone and 10 µg l^{-1} (10 ng ml^{-1}) for dexamethasone and betamethasone within their LCxLC method.[55] Again, these meet the WADA MRPL for this class (30 ng ml^{-1}).[3] In all three classes investigated within this research, the authors reportedly met the relevant MRPL suggesting this broad method could be further widened to include further classes that are currently detected using LC for a complete antidoping screening method.

P1 – Beta-Blockers

Beta-blockers are prohibited at all times in archery and shooting and prohibited in-competition only for a further handful of sports. For WADA analyses, the MRPL is set at 100 ng ml^{-1}.[3]

Gonçalves *et al.* reported the use of two-dimensional liquid chromatography for the quantification of two beta-blockers in human plasma.[68] By using a restricted access material (RAM) column as the first column, followed by an analytical column in the second, a combined pre-treatment and analysis method with lower limit of quantification (LLOQ) for alprenolol and propranolol of 5 ng ml^{-1} was demonstrated. Whilst the use of 2D chromatography in this manner does not increase the peak capacity as is the advantage of comprehensive 2D chromatography, it does allow for a fully automated method, with the possibility of running 24/7, as highlighted by the replacement of manual chromatography with comprehensive chromatography in previous discussions of heart-cutting in steroid confirmatory analysis. It also still allows for effective sensitivity as the reported LLOQ is significantly lower than the 100 ng ml^{-1} MRPL set b WADA for this category.[3]

1.3 Summary and Future Outlook

Analytical momentum to move towards 2D chromatography has been surprisingly slow given the advantages. With thirty WADA-accredited labs globally, the need for consistency plays a big part in the reluctance to change, and that is something that will need to be overcome if analysts want to stay ahead of the game. As with any change in technology, there is a cost involved with the addition of modulators and either an intracooler or a continuous source of liquid nitrogen. Peter Van Eenoo also suggested in a recent seminar, that although storing data files (rather than biological samples) for retro-analysis was something that was being investigated, currently the data files were too big for this to be practical for 10 years, when considering the number of samples that are run. Current physical storage presents freeze-thaw issues whereas external data storage may present security concerns. The gains made within the chromatography by this technique allow the reliance on the detector for sensitivity to be lessened and therefore there is wider scope for the detector of choice. By removing the necessity for targeted mass spectral analysis, full data can be collected, unknow compounds

detected and future scandals avoided, something that the sporting community is undoubtably keen to avoid.

Where analytical duty cycles have been increased compared to the one dimensional method, it is because it has replaced other preparative steps that were used prior to analysis, not because two-dimensional chromatography automatically requires a longer run time. The modulation periods are under 10 seconds each, and the additional column is typically relatively short and thin, meaning analyses can be carried out in similar time to a traditional 1D analysis. The ability to separate otherwise undetected analyte peaks from the overwhelming matrix response by using comprehensive 2D chromatography opens up analysis to a wider range of samples that previously it may not have been possible to analyse. This does not mean that sample preparation should be forgotten; by retaining clean-up and pre-concentration steps or even adding innovation here, prior to analysis, the benefits will only be further amplified, with further decreases in LOD and increases in sensitivity.

Within two-dimensional chromatography, spectra display patterned behaviour where similar compounds can be grouped in areas of the spectra. This predictability allows for extra structural information when using a non-identifying detector (*e.g.* FID) or the use of cheminformatics to predict retention times of new / unknown analytes. This can only aid in the fight against the evolution of doping.

A lack of literature utilising comprehensive chromatography for the detection/quantification of peptide hormones, growth factors, related substances and mimetics (category S2), hormones and metabolic modulators (S4) or diuretics and masking agents (S5) highlights the need for further research in this field in order to illustrate its potential for use in anti-doping analysis. The authors believe that the examples discussed highlight the scope of comprehensive chromatography which they believe will translate to these other prohibited groups.

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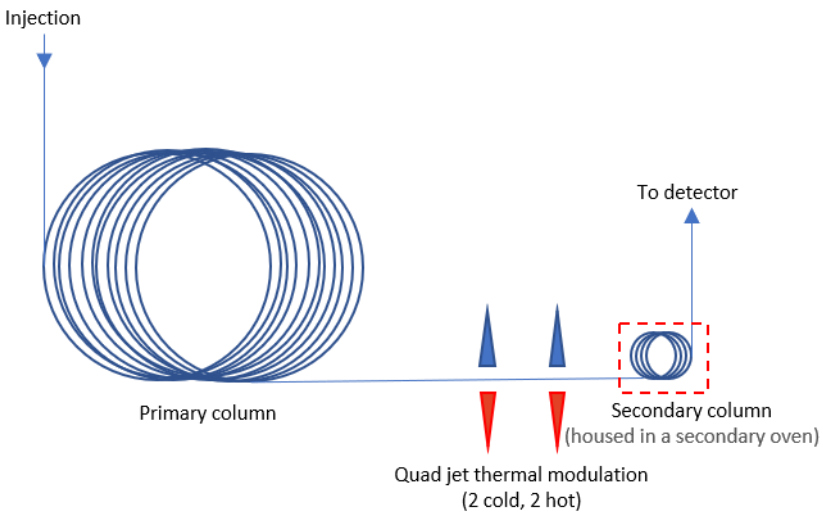
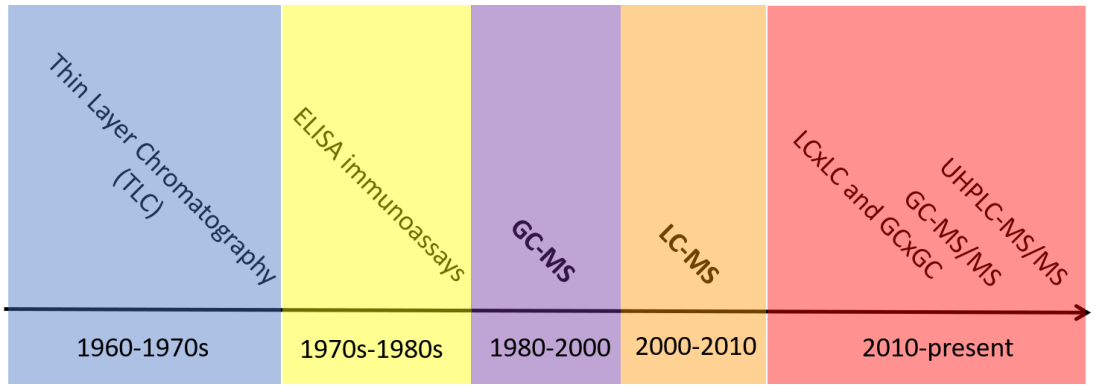
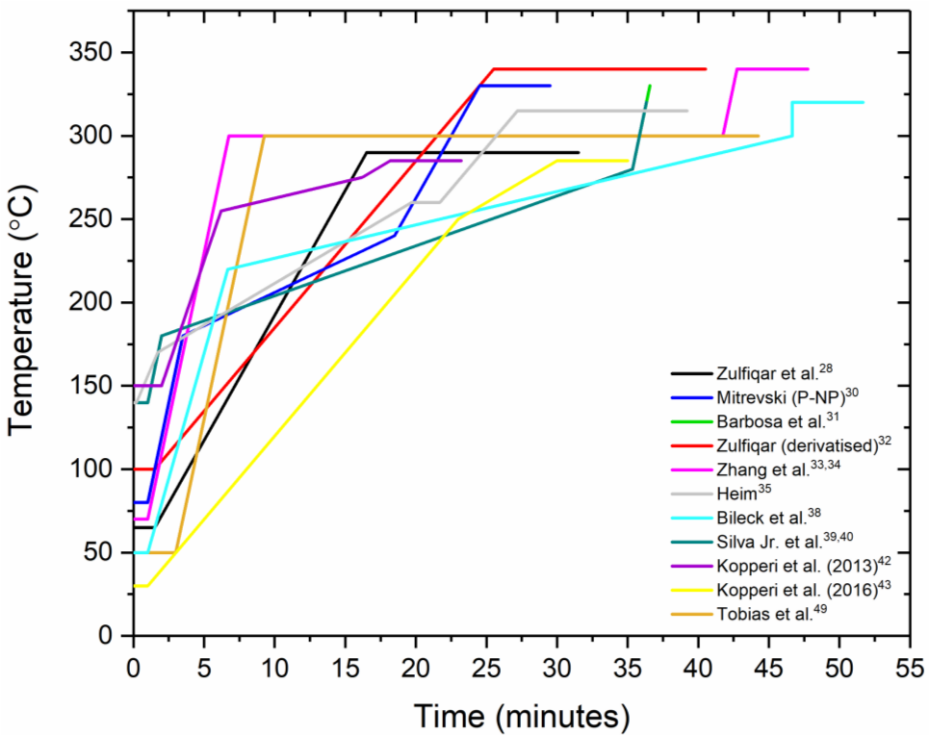
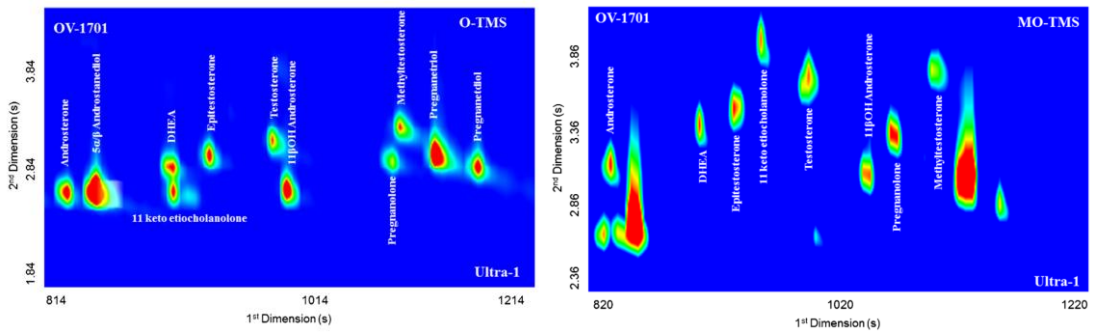
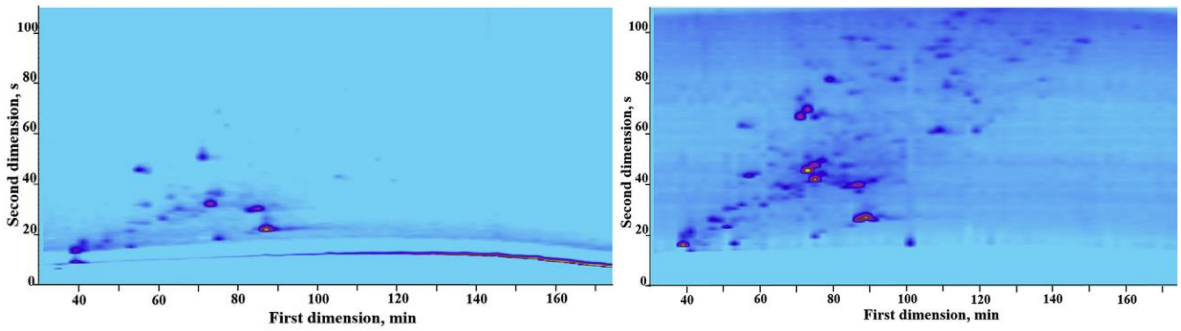

Injection
To detector
Primary column
Secondary column
(housed in a secondary oven)
Quad jet thermal modulation
(2 cold, 2 hot)


Thin Layer Chromatography (TLC)
ELISA immunoassays
GC-MS
LC-MS
LCxLC and GCxGC
GC-MS/MS
UHPLC-MS/MS
1960-1970s
1970s-1980s
1980-2000
2000-2010
2010-present


Temperature (°C)
Time (minutes)
0
50
100
150
200
250
300
350
0
5
10
15
20
25
30
35
40
45
50
55
Zulfiqar et al.[28]
Mitrevski (P-NP)[30]
Barbosa et al.[31]
Zulfiqar (derivatised)[32]
Zhang et al.[33,34]
Heim[35]
Bileck et al.[38]
Silva Jr. et al.[39,40]
Kopperi et al. (2013)[42]
Kopperi et al. (2016)[43]
Tobias et al.[49]





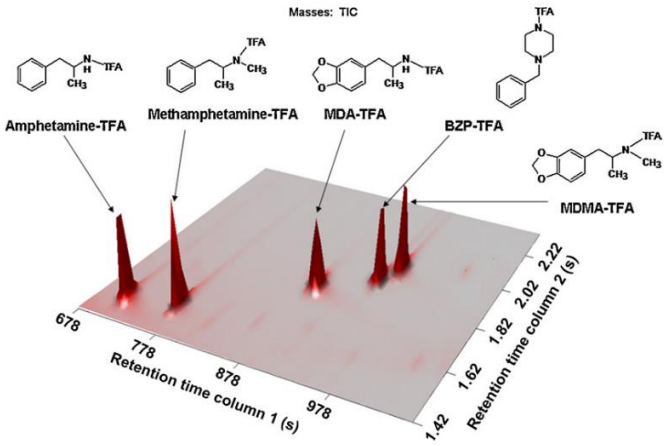


Figure 1: Schematic representation of quad jet thermal modulation comprehensive GCxGC

Figure 2: Timeline of steroidal analysis to date, adapted from Mass Spectrometry for the Detection of Endogenous Steroids and Steroid Abuse in (Race) Horses and Human Athletes.[27]

Figure 3: Comparison of GC oven heating programs reported in the literature.

Figure 4: Contour plots of a selection of derivatised steroids (O-TMS and MO-TMS) from Silva et al..[43]

Figure 5: Comparison of TIC from bovine urine sample LCxLC-MS separation (positive-ionization ESI) with passive modulation (LHS) and active-modulation (RHS). Adapted from Baglai et al..[56]

Figure 6: Example of a 2D surface plot (TIC) reproduced from Guthery et al..[65]