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Substance Identification in Anti-Doping Control by Means of Mass Spectrometry. Data Reduction and Decision Criteria

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ABSTRACT

A real doping case for which the national-level reviewing body deemed it probable that a misidentification of the national-level athlete's sample occurred at the WADA accredited laboratory, thus making the athlete in this case strictly anonymous, is used to discuss criteria for data reduction and tolerance windows in GC-MS and LC-MS/MS. Stricter criteria for data reduction would remedy the present ambiguities.

Keywords*: *Substance Identification, Anti-Doping, Mass Spectrometry, Data Reduction

1. Introduction

A high-profile Swedish female athlete, winner of an Olympic gold medal and several World and European championships, expressed a few years ago in a TV-programme how the fear of the presence of a prohibited substance in her sample during a doping control had made her almost paranoid; leaving a water bottle out of sight for only a few seconds could be enough for its being replaced with a contaminated bottle. She has never tested positive, but the strict liability imposed on athletes by the World Anti-Doping Code (WADC) leaves an extremely narrow margin of mistakes for athletes. The bar for an athlete to bear no fault or negligence for the presence of a prohibited substance in an athlete's sample is set extremely high; even to bear no significant fault or negligence require extraordinary circumstances.

When an athlete is convicted for an anti-doping rule violation according to WADC 2.1 §, usually the only evidence available to the anti-doping organisation is the presence of a prohibited substance, metabolite or marker in the athlete's sample, as determined by a laboratory accredited by the World Anti-Doping Agency (WADA). Provided that the analysis has been performed according to WADA's International Standard for Laboratories (ISL), the analysis cannot be challenged by the athlete. Under the current WADC, the athlete must not only show that the laboratory made a departure from the ISL but also that this departure reasonably could have caused the adverse analytical finding; this makes the laboratory result in practise unchallengeable for an athlete.

Given the enormous consequences for an athlete to be found guilty of an anti-doping rule violation, and the singular importance attributed to the laboratory results, it is of course imperative that the science underpinning substance identification is impeccable. There are views expressed in the scientific literature that the underpinning science and the application of guidelines in substance identification have shortcomings, and I will briefly review this literature. However, in the present paper the data reducetion and confirmation criteria will be in focus. In particular, I will use a concrete case where the national-level reviewing body determined that there was a significant probability that the laboratory had mixed up the samples. Thus, in the example I will use, the athlete is strictly anonymous.

2. Brief Overview of the Doping Literature

The literature on doping with a focus on analytical chemistry by means of chromatography and mass spectrometry falls broadly into three categories. In the first category are the original research articles and review articles concerning detection of prohibited substances by various mass spectrometric techniques. The reviews by Thevis and Schänzer [1,2] give a good overview of this literature and the implementation of new techniques. The WADA International Standard for Laboratories (ISL) [3] encourages the accredited laboratories to publish the results of

their research in peer-reviewed journals, something which accounts for part of the papers referenced in [1]. The conventional gas chromatography-mass spectrometric techniques (GC-MS) have been the work horses in the laboratory anti-doping fight, but the trend is that liquid chromatography-(tandem) mass spectrometry (LC-MS/MS) is emerging as the most powerful technique in doping control analysis [2]. One can note, however, that according to the WADA International Standard for Laboratories [3], the GC-MS or LC-MS technique is "··· the analytical technique of choice for confirmation of *Prohibited Substances*, *Metabolite*(*s*) of a *Prohibited Substance*, or *Marker* (*s*) of the *Use* of a *Prohibited Substance* or *Prohibited Method*." (5.2.4.3.1.2 in ISL).

The second category concerns criteria in chromatography and mass spectrometry which should ensure that the unambiguous presence of a substance in a biological specimen has been established. The classic paper in this category is the one by Sphon [4], and it is surprising how little the confirmation criteria has changed since this pioneering work. The present methodology as used by WADA and other organisations such as the European Council (EC), Food and Drug Administration (FDA, USA), etc., which is not uniform, has been reviewed by River [5] and Van Eenoo and Delbeke [6].

To the third category belongs papers that critically point out weaknesses in substance identification [7,8] by mass spectrometry, or other possible weaknesses in doping control such as calculation of decision limits (with papers also appearing rebutting the criticism) [9-16], the application of decision criteria [17-19], validation of specificity [20], and flawed laboratory data in a specific doping case (and rebuttal) [21-23].

The most severe critique of the doping control system, however, was levelled in a *Nature* Editorial [24]:

"*Nature* believes that accepting 'legal limits' of specific metabolites without such rigorous verification goes against the foundational standards of modern science, and results in an arbitrary test for which the rate of false positive and false negatives can never be known".

The same issue of *Nature* contained a commentary by Berry [25], which used the Floyd Landis doping case (see also [21-23]) as starting point for a critical scrutiny of the science of doping, in particular from the point of view of statistics and logic. Naturally these critical views [24,25] in one of the leading scientific journals in the natural sciences did not go unchallenged. Scientists associated with doping laboratories [26] and WADA [27] delivered rebuttals, but *Nature* also opened for a few further critical remarks [28,29].

3. Experiment and Data Reduction

The data to be discussed here have the advantage that despite coming from an actual doping case, they have never been scrutinized by an anti-doping reviewing body for the reason that the anti-doping agency and the WADA accredited laboratory failed to convince the national-level reviewing body to their comfortable satisfaction that there had not been a misidentification of samples, and the case was dismissed on these grounds only. According to the first page of the Laboratory Documentation Package (LDP) from the Doping Control Laboratory, Karolinska University Hospital, signed by the laboratory director in accordance with [30], a sample number different from the one of the accused athlete, but belonging to the same batch, was assigned to the internal code used by the laboratory during the analysis. This means that the athlete whose analyzed sample will be discussed is strictly anonymous despite deriving from a real case. Thus, we completely avoid the discussions whether a decision by a disciplinary court can be taken as "proof" of the scientific correctness of the statements brought forward by expert witnesses [10,13], and the strong emotions stirred in the Floyd Landis case.

The sample of the anonymous athlete was claimed to containing 4.9 ng/mL of 3'-OH-Stanozolol, a metabolite of the anabolic androgenic steroid Stanozolol. The level is about a factor of two higher than the so-called Minimum Required Performance Level (MRPL) [31] of 2 ng/mL. This level is sometimes misunderstood to mean a level below which the presence of 3'-OH-Stanozolol does not constitute an adverse analytical finding. This is wrong, and the level has been introduced to ensure that all WADA accredited laboratories have the capacity to report the presence of forbidden substances and metabolites in a uniform way. Some laboratories are able to report lower concentrations than others, and concerning the specific metabolite 3'-OH-Stanozolol, it is not a threshold substance.

The identification criteria for substances in urine samples combine chromatographic separation and mass spectrometry. The chromatographic step is of no concern here and will not be discussed further. Mass spectrometry uses the sequence ionization, fragmentation, and detection. Electron impact ionization at 70 eV, where the ionization cross section typically peaks for many small molecules, is a "hard" ionization method, and the molecular ion will undergo unimolecular decomposition on the μs timescale. The fragmentation pattern reveals information about the parent ion, but the pattern can differ depending on what system is used for mass separation of the ionized fragments, and a reference standard is required for compareson. When "soft" ionization is used, such as electrospray ionization, leading to less unimolecular decay, collision induced dissociation (CID) is used (MS/MS), and also in this case it is well known that the CID fragmentation pattern can depend significantly on the employed MS/MS technique [32]. The criteria for substance identification

are discussed in [5,6] for different organisations (includeing WADA), and the implementation of the specific WADA criteria are outlined in technical documents TD2003IDCR [33] (prior to September 1, 2010) and TD2010IDCR [34] (effective date September 1, 2010).

Table 1 shows the results for 3'-OH-Stanozolol from the anonymous sample and a comparison with the standard, both taken from the LDP. The parent ion [M] is 3'-OH-Stanozolol-3TMS$^+$ with mass 560 Da. One can make several observations using **Table 1**. The WADA Technical Document TD2003IDCR [33] (which was the document in force when the analysis was made) requires three diagnostic ions to be used, yet the standard operating procedure of the WADA accredited laboratory relaxes this requirement to only two ions; the peak at 254.1 Da hardly qualifies as a diagnostic ion since it is normalized to 100% for both the standard and the sample. The mass difference between the two diagnostic ions is 15 Da, and it is very likely that the peak at 545.3 Da corresponds to $[M\text{-}CH_3]^+$. de Zeeuw [7] specifically points out that this ion does not provide much additional diagnostic information, however, there is nothing in WADA's technical documentations preventing the analyst from using it. It is common, de Zeeuw [7] notes, to not paying attention to the diagnostic value of the fragments. Most striking with the table is that the two diagnostic ions only marginally fall within the required acceptance range [33,34]. This requires the peak at 545.3 Da to be further scrutinized. **Figure 1** shows a selected ion monitoring chromatogram taken from the LDP.

Table 1. Relative ion abundances in a GC-MS analysis of 3'-OH-Stanozolol at the Doping Control Laboratory, Karolinska University Hospital.

Standard (m/z in Da)	Relative abundance (%)	Acceptance range (%)	Sample (%)
254.1	100.0	-	100.0
545.3	60	50 - 70	51
560.3	58	48 - 68	49

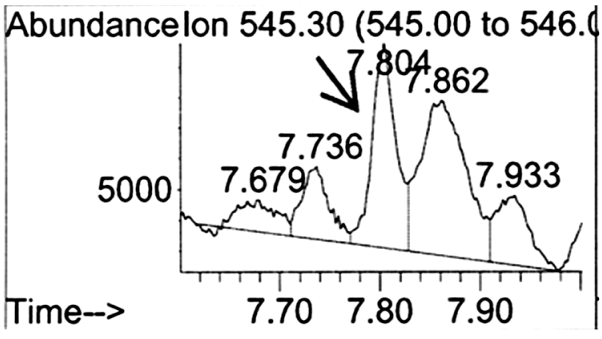

Figure 1. Chromatogram from selected ion monitoring at retention times 7.60 - 8.00 min recorded at the Doping Control Laboratory, Karolinska University Hospital (reproduced from the LDP) by means of GC-MS.

The relative abundance of the 545.3 peak "[···] shall preferably be determined from the peak area or height of integrated selected ion chromatograms" [33]. In this particular case, the standard operating procedure required the peak area to be integrated from "valley to valley", *i.e.* from retention time 7.77 min to 7.83 min. It is obvious from a simple visual inspection that the broad peak at retention time 7.862 min will make a larger contribution to the area in the interval 7.77 - 7.83 min than the fraction of peak 7.804 min (545.3 Da) that falls outside of this region. Taking this into account, the relative abundance of ion 545.3 Da is estimated to be 46% and not 51%. The relative abundance thus falls outside of the acceptance range, and the sample does not fulfil the WADA criteria to constitute an adverse analytical finding.

There is a caveat to this reasoning, and this is the following. The technical document TD2003IDCR [33] and the standard operating procedure do not require the analyst to proceed as just outlined, *i.e.* using the correct peak area, and it is only in TD2010IDCR [34] the analyst is permitted (but not required) to use computer-assisted peak-resolving software. Thus, a procedure which would not be tolerated in my department's course in experimental physics for freshman physics students is part of the standard operating procedure of a WADA accredited laboratory and accepted by the most recent WADA technical document for mass spectrometry [34].

WADA accredited laboratories use more than one method to determine whether a forbidden substance or metabolite is present in a sample, and in the present case the laboratory also used LC-MS/MS, see **Figure 2**. **Table 2** shows the results. Four diagnostic ions with a relative abundance different from 100% are now within the acceptance range. Does this mean that the presence of 3'-OH-Stanozolol in the sample now has been unambiguously established? No, it does not, on the contrary. In **Table 1** in ref. [34] WADA has tightened the maximum tolerance windows for relative ion intensities. It is not explained in detail the rationale for this, however, it is clear from TD 2010IDCR [34] that a paper by Stein and Heller [35] has been deemed important.

We now apply the new acceptance ranges in force since September 1, 2010 [34] to the present case, see **Table 3**. Now only one out of four diagnostic ions falls within the acceptance range and the results do not fulfil WADA's requirements for being reported as an adverse analytical finding.

The technique of LC-MS/MS is widely used by WADA accredited laboratories in the anti-doping fight. It is known only to WADA how many athletes that have been convicted based on the old rules [33], which with the new rules would have been acquitted. Since many thousand doping controls are performed all over the world each year,

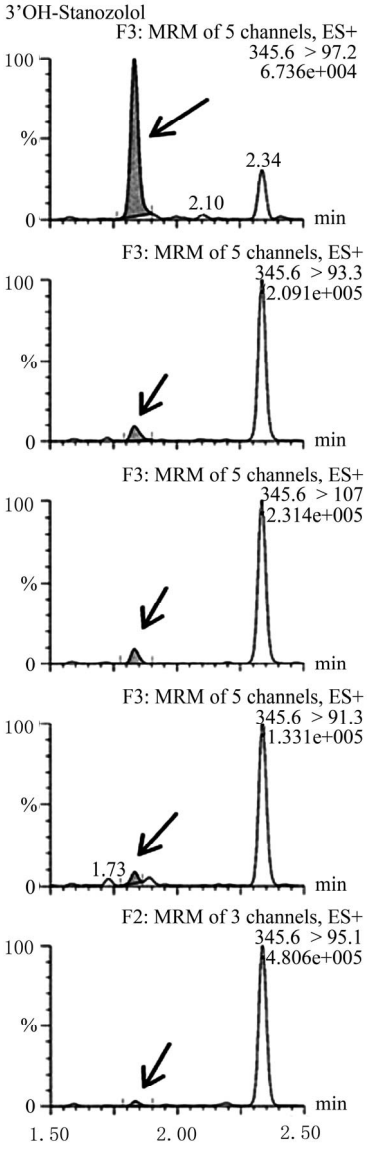

Figure 2. Chromatogram from selected ion monitoring at retention times 1.50 - 2.50 min recorded at the Doping Control Laboratory, Karolinska University Hospital by means of LC-MS/MS (reproduced from the LDP). The arrows show the peaks given in the left columns of Tables 2 and 3.

Table 2. Relative ion abundances in an LC-MS/MS analysis of 3'-OH-Stanozolol at the Doping Control Laboratory, Karolinska University Hospital.

Standard (m/z in Da)	Relative abundance (%)	Acceptance range (%) according to TD2003IDCR [33]	Sample (%)
97.2	100.0	100.0	100.0
93.3	26.8	20.0 - 33.5	31.5
107	25.9	19.4 - 32.4	31.6
91.3	17.6	7.6 - 27.6	11.7
95.1	18.4	8.4 - 28.4	24.1

Table 3. Relative ion abundances in an LC-MS/MS analysis of 3'-OH-Stanozolol at the Doping Control Laboratory, Karolinska University Hospital.

Standard (m/z in Da)	Relative abundance (%)	Acceptance range (%) according to TD2010IDCR [34]	Sample (%)
97.2	100.0	100.0	100.0
93.3	26.8	21.4 - 32.2	31.5
107	25.9	20.6 - 31.1	31.6
91.3	17.6	14.1 - 21.1	11.7
95.1	18.4	14.7 - 22.1	24.1

it would seem unlikely that the author has happened to come across the one and only case.

4. Conclusions

In this paper I have demonstrated that the method of data reduction in a doping control case can make the difference between an adverse analytical finding and acquittance. This is highly undesirable, in particular since the more rigorous data reduction is the scientifically sound one and leads to a non-fulfilment of the WADA criteria for substance identification in a particular case. The new technical document TD2010IDCR represents a step forward [34], but as long as the use of peak-resolving software is only permitted, not mandatory, ambiguities will prevail. As long as the text in the technical document is permissive, an athlete can never challenge the laboratory result at the Court of Arbitration for Sport (CAS).

The importance of the *exact* phrasing in WADA's technical documents is made apparent in a recent award delivered by CAS [36] in which the technical documents are referred to no less than 37 times, and every "t" crossed and "i" dotted in CAS reading of the documents. It is beyond the scope of the present article, and beyond the competence of the author, to review all technical documents of WADA, however, leaving open to the analyst how to derive an ion's relative intensity is a flagrant loop hole in anti-doping science.

The new, sharpened tolerance windows imposed by WADA [34] are welcome but leaves open the uneasy question concerning how many adverse analytical findings that have been reported using the old criteria, which would appear as negatives using the new criteria. Or to rephrase the question: how many false-positives were reported prior to September 1, 2010?

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