



ALMA MATER STUDIORUM  
UNIVERSITÀ DI BOLOGNA

DOTTORATO DI RICERCA IN  
SALUTE, SICUREZZA E SISTEMI DEL VERDE  
Ciclo 37

**Settore Concorsuale:** 03/D1 – CHIMICA E TECNOLOGIE FARMACEUTICHE, TOSSICOLOGICHE E NUTRACEUTICO-ALIMENTARI

**Settore Scientifico Disciplinare:** CHIM/08 – CHIMICA FARMACEUTICA

ANTI-DOPING ANALYSIS IN SPORT: NEW METHODS ON  
BIOLOGICAL SAMPLES TO PROTECT ATHLETE HEALTH AND  
SAFETY.

**Presentata da:** Giusy Tespio

**Coordinatore Dottorato:**

Prof. Daniele Torreggiani

**Supervisore:**

Dr. Guido Pelletti

**Co-supervisori:**

Dr. Michele Protti

Prof. David Neil Manners

Esame finale anno 2025

## Index

Abstract .....	6
<b>CHAPTER I-THESIS INTRODUCTION .....</b>	<b>7</b>
<b>1. Doping in sport.....</b>	<b>8</b>
<b>2. Anti-doping controls .....</b>	<b>9</b>
<b>2.1 Anti-doping laboratories .....</b>	<b>10</b>
<b>3. Illicit substances of interest .....</b>	<b>11</b>
<b>3.1 Anabolic Androgenic Steroids.....</b>	<b>12</b>
<b>3.2 Peptide and glycoprotein hormones .....</b>	<b>15</b>
<b>4. Matrices and samples .....</b>	<b>17</b>
<b>5. References.....</b>	<b>20</b>
<b>CHAPTER II-WADA INTERNATIONAL STANDARDS .....</b>	<b>27</b>
<b>1. International Standards for Testing and Investigations (ISTI) .....</b>	<b>28</b>
<b>1.1 Testing Requirements.....</b>	<b>28</b>
<b>1.2 Athlete Whereabouts .....</b>	<b>28</b>
<b>1.3 Sample Collection Process .....</b>	<b>28</b>
<b>1.4 Selection of Athletes for Testing.....</b>	<b>28</b>
<b>1.5 Investigations .....</b>	<b>29</b>
<b>1.6 Therapeutic Use Exemptions (TUEs) .....</b>	<b>29</b>
<b>1.7 Sample Storage and Reanalysis.....</b>	<b>29</b>
<b>1.8 Results Management and Reporting.....</b>	<b>29</b>
<b>1.9 Retesting and Longitudinal Profiling .....</b>	<b>30</b>
<b>1.10 Anti-Doping Education and Awareness .....</b>	<b>30</b>
<b>2. International Standards for Laboratories (ISL).....</b>	<b>30</b>
<b>2.1 Accreditation and Compliance .....</b>	<b>30</b>
<b>2.2 Prohibited Substances and Methods.....</b>	<b>30</b>
<b>2.3 Analytical Methods.....</b>	<b>31</b>
<b>2.4 Quality Control and Assurance.....</b>	<b>31</b>
<b>2.5 Chain of Custody .....</b>	<b>31</b>
<b>2.6 Reporting Results and Measurement Uncertainty .....</b>	<b>31</b>
<b>2.7 Confirmatory Testing.....</b>	<b>32</b>
<b>2.8 Long-Term Storage and Retesting.....</b>	<b>32</b>

2.9 Athlete Biological Passport (ABP) .....	32
2.10 Data Management and Security .....	32
2.11 Laboratory Ethics and Independence.....	32
<b>3. International Standards for Results Management .....</b>	<b>33</b>
3.1 Initiation of Results Management .....	33
3.2 Provisional Suspension.....	33
3.3 Results Management Process .....	34
3.4 Hearing Process .....	34
3.5 Decision-Making and Sanctions .....	34
3.6 Appeals Process.....	34
3.7 Multiple Violations .....	35
3.8 Confidentiality and Public Disclosure .....	35
3.9 Results Management for Non-Analytical Violations.....	35
3.10 Statute of Limitations.....	35
3.11 Handling of Multiple Organizations .....	35
3.12 Reanalysis of Samples .....	36
<b>4. International Standards in this study .....</b>	<b>36</b>
<b>5. References.....</b>	<b>37</b>
<b>CHAPTER III-ANALYTICAL METHODS .....</b>	<b>38</b>
1. Aim of the research .....	39
2. Analytical approaches .....	40
2.1 Innovative biological matrices: miniaturized samples.....	40
2.2 Sample pretreatment.....	41
2.3 Instrumental methods .....	42
3. Method validation .....	44
3.1 Analytical measurement range and linearity.....	44
3.2 Analytical specificity .....	44
3.3 Analytical sensitivity .....	45
3.4 Accuracy and precision.....	45
3.5 Recovery.....	46
3.6 Stability .....	46
4. References.....	47

<b>Chapter IV-DEVELOPMENT OF ANALYTICAL METHODS .....</b>	<b>50</b>
<b>1.Dried blood spot coupled to liquid chromatography-tandem mass spectrometry for the analysis of anabolic androgenic steroids.....</b>	<b>51</b>
<b>1.1Introduction .....</b>	<b>51</b>
<b>1.2 Materials and Methods .....</b>	<b>54</b>
<b>1.2.1 Chemicals and solutions .....</b>	<b>54</b>
<b>1.2.2 LC–MS/MS system .....</b>	<b>55</b>
<b>1.2.3 Microsample Collection and Pre-treatment .....</b>	<b>56</b>
<b>1.2.4 DBS .....</b>	<b>57</b>
<b>1.3 Method Validation.....</b>	<b>57</b>
<b>1.3.1 Calibration Curves .....</b>	<b>57</b>
<b>1.3.2 Recovery and Precision Assays.....</b>	<b>58</b>
<b>1.3.3 Selectivity.....</b>	<b>58</b>
<b>1.3.4 Stability.....</b>	<b>58</b>
<b>1.4 Results and Discussion .....</b>	<b>58</b>
<b>1.4.1 LC–MS/MS conditions .....</b>	<b>58</b>
<b>1.4.2 DBS development.....</b>	<b>60</b>
<b>1.4.3 Calibration curves .....</b>	<b>61</b>
<b>1.4.4 Recovery and Precision Assays.....</b>	<b>62</b>
<b>1.4.5 Selectivity.....</b>	<b>65</b>
<b>1.4.6 Stability.....</b>	<b>65</b>
<b>1.5. Conclusion.....</b>	<b>66</b>
<b>1.6 References .....</b>	<b>68</b>
<b>2. Identification and UHPLC-MS/MS analysis of hCG and IGF-I from Dried Blood Spots .....</b>	<b>71</b>
<b>2.1. Introduction .....</b>	<b>71</b>
<b>2.2 Materials and Methods .....</b>	<b>72</b>
<b>2.2.1 Materials .....</b>	<b>72</b>
<b>2.2.2 Microsample collection and pre-treatment .....</b>	<b>73</b>
<b>2.2.3 Extraction procedure from DBS by testing different solvents.....</b>	<b>73</b>
<b>2.2.4 Digestion and reduction of final volume.....</b>	<b>75</b>
<b>2.2.5 Liquid Chromatography and Mass Spectrometry .....</b>	<b>75</b>
<b>2.2.6 Application of the best extraction protocols on DBS fortified with hCG and IGF-I.....</b>	<b>76</b>
<b>2.2.7 Simultaneously detect IGF-I and hCG with the best and reproducible extraction protocol..</b>	<b>77</b>
.....	77

2.2.8 Data analysis .....	77
2.3 Results .....	77
2.3.1 Extraction solvent optimization .....	77
2.3.2 Digestion and reduction .....	78
2.3.3 The best extraction protocols on DBS fortified with hCG and IGF-I .....	79
2.3.4 The best reproducible extraction protocol to detect hCG and IGF-I simultaneously.....	81
2.3.5 Data analysis .....	82
2.4 Discussion.....	83
2.5 Limitations and Future Perspectives.....	86
2.6 Conclusion.....	87
2.7 References .....	88
<b>Chapter V-KEY FINDINGS AND ADVANCEMENTS OF STUDIES .....</b>	<b>91</b>
<b>1. Introduction.....</b>	<b>92</b>
<b>2 Key Findings and advancements from the two studies. ....</b>	<b>92</b>
2.1 Development and validation of an LC-MS/MS method for AAS detection using DBS.....	93
2.2 Extraction and detection of hCG and IGF-I from DBS using UHPLC-MS/MS .....	94
<b>3 Impact of research on anti-doping science. ....</b>	<b>95</b>
3.1 Addressing sample collection and handling challenges .....	95
3.2 Enhancing analytical sensitivity and specificity .....	96
3.3 Facilitating widespread and routine testing.....	96
3.4 Enabling longitudinal monitoring and retrospective analysis .....	97
3.5 Contributing to a more ethical and transparent anti-doping regime .....	97
<b>4 Innovation and future perspectives .....</b>	<b>98</b>
4.1 Refinement and automation of DBS analytical protocols .....	98
4.2 Expanding the range of detectable substances .....	98
4.3 Development of individualized doping detection models.....	99
4.4 Integrating DBS with other biological matrices for a multi-omics approach .....	99
4.5 Enhancing global anti-doping efforts with DBS .....	100
4.6 Development of portable analytical devices for on-site testing.....	100
4.7 Legal and regulatory considerations for implementing DBS.....	101
4.8 Educational and awareness initiatives for athletes and stakeholders .....	101
4.9 Collaborative research and development initiatives .....	102
<b>Chapter VI-THESIS CONCLUSION.....</b>	<b>103</b>

## **Abstract**

In this PhD project, original and innovative approaches for the qualitative-quantitative analysis of doping substances have been studied, developed and validated for applications in various fields such as forensic, clinical and pharmaceutical. All parameters involved in the developed analytical workflows have been adequately and accurately optimized, from sample collection to sample pretreatment to instrumental analysis. Advanced dry blood microsampling technologies have been developed, which can bring several advantages to the method as a whole, such as a significant reduction in the use of solvents, feasible storage and transport conditions and improvement of the stability of the analyte. At the same time, the use of capillary blood allows to increase the compliance of the subject and the applicability of the global method by exploiting such innovative technologies. The biological samples involved in this project were subjected to optimized pretreatment techniques developed ad hoc for each target analyte, also making use of advanced microextraction techniques. Finally, original and advanced instrumental analytical methods based on high-performance liquid chromatography and nano liquid chromatography (HPLC, Nano UHPLC) coupled to mass spectrometry were developed. In addition, a pharmacovigilance study coupled with statistical analysis was carried out to assess the health risk of certain specific doping substances. Each method has been designed to achieve highly selective, sensitive but sustainable systems and has been validated according to international guidelines. All methods developed here have proven to be suitable for the analysis of test compounds and may be useful tools in pharmaceutical chemistry, pharmaceutical analysis, clinical studies and forensic investigations.

# **CHAPTER I**

## **THESIS INTRODUCTION**

## **1. Doping in sport**

Athletes have long used substances to improve their athletic performance [1]. There is evidence of the use of performance enhancing drugs in different parts of the ancient world, and some of these practices are still present today. Examples of doping are found among the Greeks in the third century BC, athletes of the ancient Olympics, the Egyptians, gladiators of the Roman era and medieval knights, among others [2]. For example, athletes who participated in the ancient Olympic Games often ingested substances such as mushroom extracts and plant seeds, as well as following special diets to win competitions, Roman gladiators used stimulants to make the races more intense and engage the public [3]. As knowledge of the physiological dynamics of the organism has advanced, new ways have emerged to achieve improved performance, culminating in the ability to manipulate human genetic material and regulate gene expression, Increasing or reducing the production of certain enzymes and other proteins.

Contemporary sport in the 21st century is characterized by the development of various sports products, which aim to achieve the maximum possible result. However, it should be constantly taken into account that any improvements should be implemented according to the "spirit of sport", which is defined as the pursuit of human excellence through the refinement of each person's natural talents [4]. In this regard, monitoring drug abuse and substance abuse and methods that potentially or evidently improve athletic performance through analytical chemistry strategies is one of the main pillars of modern anti-doping efforts [5]. Thanks to the continuous growth of knowledge in medicine, pharmacology and (bio)chemistry, new chemical entities are often created and developed, many of which are a temptation for sportsmen because of the supposed/ attributed beneficial effects of such substances and preparations on, for example, endurance, strength and regeneration [6]. Thanks to new technologies, the extension of existing test protocols, new knowledge on metabolism, distribution and elimination of compounds banned by the World Anti-Doping Agency (WADA), analytical tests have been further improved [7]. The World Anti-Doping Agency (WADA) was established in 1999 as an independent international agency composed and financed in equal measure by the sports movement and governments [8]. The WADA Code (The Code) is the key document that harmonizes anti-doping policies, rules and regulations within sports organizations and between public authorities around the world. The code is in conjunction with eight other documents (international standards) that aim to promote consistency between anti-doping organizations in various areas [9]. International Standards include the Forbidden List (The List), an annually updated list that lists all those

substances and methods prohibited in sport. The listed compounds and methods are classified in categories S0 - S9 and M1 - M3 respectively, while  $\beta$ -blockers (P1) are prohibited in selected sports. The compounds could be divided into two classes: non-threshold substances and prohibited threshold substances above a certain level [8]. According to WADA, doping is contrary to the "sporting spirit", which must be cultivated in a natural way, following the rules, without artificial improvements. Doping remains contrary to the ethical principles of sport and medicine [10].

## **2. Anti-doping controls**

The earliest scientific anti-doping tests were likely not performed on humans but on racehorses by chemist Bukowsky in Vienna in 1910 [11]. When it comes to humans, it wasn't until the 1984 Los Angeles Olympic Games that specific tests were introduced to identify banned doping substances in athletes. These tests were done through urine analysis using chromatographic methods: gas chromatography (GC) and gas chromatography combined with mass spectrometry (GC/MS) [12]. Even today, GC/MS analysis is regarded by chemists and toxicologists as the leading method for detecting the presence of specific chemical substances in a biological fluid, even at extremely low concentrations (some ng/ml) [13]. The International Olympic Committee (IOC) Medical Commission and the 1988 World Conference in Ottawa set the rule that athletes who test positive for doping should face a penalty of two years' suspension for the first violation and a lifetime ban for a second offense. The Italian National Olympic Committee (CONI) has adopted the IOC regulations regarding the list of banned substances, the certification of anti-doping laboratories, and the implementation of strict guidelines for urine collection, such as conducting surprise sample collections during training [14]. As for urine sampling in athletes for anti-doping tests, the IOC determined that tests should be conducted on the top four finishers in the final standings, along with additional athletes selected randomly. Based on the regulations set by the International Olympic Committee (IOC), athletes must provide a urine sample immediately following the conclusion of a competition. The athlete is required to report without delay, accompanied if needed by a trusted individual such as a coach or medical professional. Standard urine testing protocols require collecting at least 75 ml of urine in a designated container, while a control officer supervises the process. The officer then checks the urine's specific gravity and pH levels, ensuring the density exceeds 1.010 g/ml and that the pH falls

between 5.0 and 7.0. Should the sample not meet these criteria, the IOC representative may ask for additional samples. The athlete verifies that their urine is divided into two containers, labeled as samples A and B. These are both sent to an IOC laboratory, where they are stored in a locked freezer until testing. Only the lab director has access to the key. The freezer must be equipped with a device that continuously monitors the internal temperature and connected to an emergency power source to guarantee a constant energy supply. Sample A is tested first, while sample B remains available for a potential reanalysis, should the athlete request it if found positive for any banned substances. Should the analysis of sample A identify any prohibited substances (which are also quantified for certain substances), the athlete may face penalties. These sanctions, as intended by the IOC, are meant to be consistent across all sports and nations affiliated with the committee [15]. Since 2002, the procedures for conducting anti-doping checks have been defined by the IOC through a specific WADA anti-doping programme: "International Standard for Testing" [16].

## **2.1 Anti-doping laboratories**

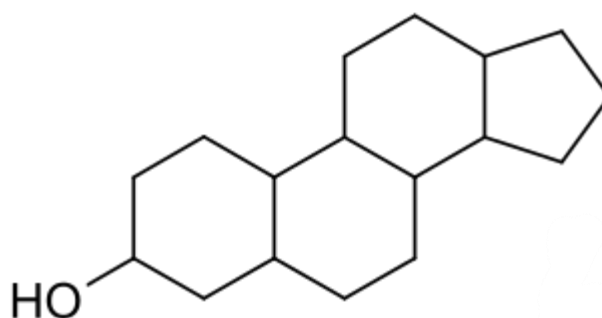
The laboratory responsible for conducting anti-doping tests plays a crucial role in the overall work of the IOC Anti-Doping Commission. Given the serious consequences for athletes who test positive, these laboratories must be highly reliable [17]. Since January 1, 2004, WADA has been the agency in charge of accrediting anti-doping laboratories in the countries affiliated with the IOC. The accreditation process follows guidelines outlined in a specific WADA anti-doping program called the "INTERNATIONAL STANDARD FOR LABORATORIES" [18]. These labs (one or two per country) must be well-equipped, and the staff must possess a high level of expertise in analytical chemistry, particularly in chromatographic techniques such as gas chromatography (GC) and high-performance liquid chromatography (HPLC), as well as gas chromatography/mass spectrometry (GC/MS) and liquid chromatography/mass spectrometry (LC/MS). The final analysis of doping substances in urine (both in their original form or as metabolites) is performed using GC/MS or LC/MS techniques. Much like testing for drugs of abuse in biological samples, the detection of doping substances involves two types of chemical analysis: a) "screening" methods and b) "confirmation" methods.

### **3. Illicit substances of interest**

In the list of banned substances, the two main categories of abused compounds are anabolic steroids and peptide hormones. Anabolic androgenic steroids (AAS), are synthetic derivatives of testosterone that have attracted attention as doping substances because of their potential to increase protein synthesis, decrease protein breakdown and increase muscle growth by activating the androgen receptor [19]. They are used by athletes to increase strength and muscle mass and to increase tolerance to challenging training loads. Their use is particularly widespread among weightlifters, athletes engaged in speed and power competitions, soccer players and especially fans of "body building" [20]. To achieve positive results in sports, AAS users accompany the drug with intense training and a high-protein diet. The side effects of high doses of AAS are important and depend on three main factors: dosage, duration of intake and cumulative dose [21]. They are the growth arrest in young people and the disorders of the sexual and reproductive sphere, both in men (reduction of spermatogenesis and testicular hypotrophy), and in women (virilization, reduction or cessation of menstruation); in addition, cardiovascular alterations and liver and prostate cancers may develop [22]. The peptide hormones are a category of compounds widely used among athletes, for this reason they are in fact prohibited in competition and out of competition [23]. Some peptide and glycoprotein hormones belong to this group; the most commonly used are: corticotropin (ACTH), chorionic gonadotropin (hCG), luteinizing hormone (LH), insulin, insulin-like growth factor-1 (IGF-1), growth hormone (hGH) and erythropoietin (EPO) [24]. These hormones have an anabolic effect similar to that of AAS, therefore, they can promote the development of muscle mass. In the past we were used hormones of extractive origin (extracted from the pituitary of the cadavers), with serious risk of contracting diseases [25]. Currently, growth hormones are used prepared by the DNA-recombinant technique and therefore no longer present such risk. The abuse of peptide hormones can cause, however: hypertension, diabetes and acromegaly. Athletes tend to use them in the vicinity of races, instead of AAS as, unlike androgenic steroids (easily detectable at a doping control), they escape the controls [26,27].

### 3.1 Anabolic Androgenic Steroids

Anabolic androgenic steroids (AAS) are synthetic derivatives of testosterone, a hormone that is produced endogenously in our body from cholesterol, from which all steroid hormones derive [28]. These compounds have a cyclic closed-loop chemical structure, typical of the steroid class: in fact, AAS are characterized by the presence of four rings of carbon atoms arranged to form a structure called perihedron-1,2-cyclopentenfenantrene shown in Figure 1. Most natural steroids present, at the rings, one or two methyl groups located in different positions on the structure [29].



**Figure 1.** Structure formula of anabolic androgenic steroids

Together with testosterone, the main steroid hormone, small amounts of epitestosterone, its isomer, and dihydrotestosterone (DHT) are produced.

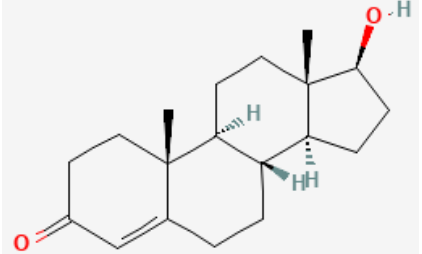
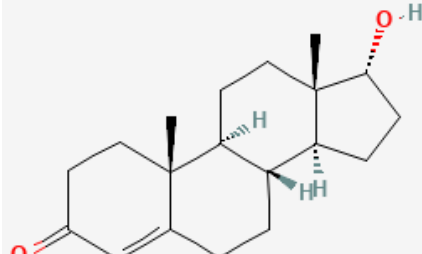
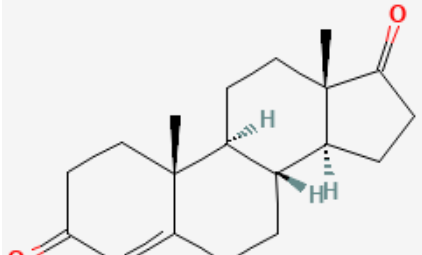
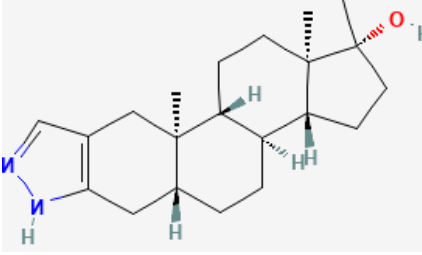
Steroid hormones are considered biologically active substances, as they play important roles in sexual development, sugar metabolism and the maintenance of homeostasis. In addition, they are also used as biomarkers because of their endogenous concentration, which indicates physiological and pathological conditions [30]. In the literature it is reported that plasma levels of testosterone are 0.6 mg/dl in the male and 0.3 mg/dl in the female: these threshold values, in fact, allow you to evaluate the intake of steroids and any metabolic dysfunctions [31]. An increasingly frequent practice is to resort to the consumption of synthetic anabolic steroids, to bring benefits both in terms of appearance and performance. Taking AAS leads to increased muscle strength: a double-blind study of 43 men randomized in 4 different groups found that the administration of testosterone resulted in an increase in muscle strength and lean mass in all recipients, with particular emphasis on those who have been involved in sports [32]. In a second double-blind study, carried out five years later, they demonstrated a

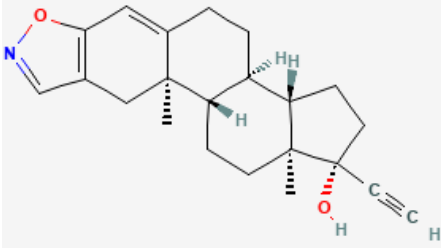
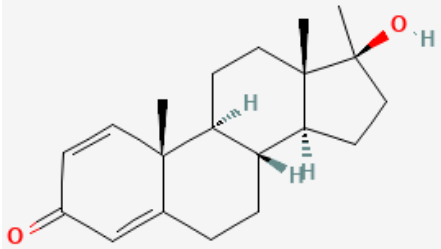
dose-dependent relationship between increased strength and AAS intake [33] with results similar to the previous analysis. It was tested in 61 males distributed according to 5 different doses of testosterone (ranging between 25-600 mg) in combination with a gonadotropin releasing hormone agonist, to cancel the secretion of endogenous testosterone. Potential positive effects on physical performance, due to the use of AAS, may also concern the increase in haemoglobin and haematocrit, reduction of reaction times and improved tolerance to intense training.

The use of AAS has numerous side effects. Higher doses than the physiological dose are associated with cardiovascular complications, with recent and sudden cardiac deaths in young healthy athletes who have abused testosterone for several years [34,35]. A study of causes of death among those who reported androgen use, reported that about 35% suffered from chronic cardiac alterations, among the most common pathologies: concentric cardiac hypertrophy, dilatative cardiomyopathy, fibrosis and myocytolysis, significantly reduced left ventricular ejection fraction and diastolic dysfunction [36,37]. Myocardial hypertrophy may be enhanced by concomitant use of GH, left ventricular hypertrophy may persist even after cessation of steroid intake, and their abuse may be related to acute and fatal myocardial infarction. Other side effects include the suppression of the hypothalamus-hypophysis-testicular axis. Although suppression of pituitary secretion of gonadotropins is potentially reversible, most, depending on the period of AAS use, present hypogonadism that may persist for prolonged periods after androgen intake has been discontinued [38]. Special attention should be paid to psychiatric side effects. Many studies have reported an association between AAS use and aggression, violence, mood swings [39] and manic behaviours [40]. In AAS-dependent subjects, psychological-psychiatric symptoms are more common and severe, with a double incidence of anxiety and depression crises than those who use AAS occasionally [41]. There is also an increased risk of other drug abuse, alcohol, suicide and murder. Use is associated with antisocial, violent behaviour closely related to criminal activity.

Testosterone is the most widely used steroid for this purpose, but its quantification does not provide sufficient evidence of external intake, as it is also produced by the body endogenously. This has led to the need to detect and quantify in the urinary matrix its precursor, dehydroepiandrosterone (DHEA), its epimer, epitestosterone (E) and its metabolites such as dihydrotestosterone (DHT), androstenedione and etiocholanolone [42].

By examining and consulting the list of prohibited substances provided by the World Anti-Doping Agency (WADA) [8], considering the athlete's biological passport [43] and carrying out literature research on the most frequent use of AAS, in this experimental thesis work, some of the most common AAS were chosen as analytes of interest: testosterone, epitestosterone, androstenedione, stanozolol, danazole and methandrostenolone. Table 1 shows the structure formula of each analyte and its molecular weight

AAS	MW (g/mol)	STRUCTURAL FORMULA
TESTOSTERONE	288.42	 <p>The image shows the chemical structure of testosterone, a steroid hormone. It consists of four fused rings (three six-membered and one five-membered). There is a double bond in the first ring and a ketone group at the 3-position. The side chain at the 17-position is a methyl group at C-13 and a hydroxyl group at C-17. Stereochemistry is indicated with wedges and dashes.</p>
EPITESTOSTERONE	288.42	 <p>The image shows the chemical structure of epitestosterone, which is identical to testosterone. It consists of four fused rings (three six-membered and one five-membered). There is a double bond in the first ring and a ketone group at the 3-position. The side chain at the 17-position is a methyl group at C-13 and a hydroxyl group at C-17. Stereochemistry is indicated with wedges and dashes.</p>
ANDROSTENEDIONE	286.41	 <p>The image shows the chemical structure of androstenedione, a steroid hormone. It consists of four fused rings (three six-membered and one five-membered). There is a double bond in the first ring and a ketone group at the 3-position. The side chain at the 17-position is a methyl group at C-13 and a ketone group at C-17. Stereochemistry is indicated with wedges and dashes.</p>
STANOZOLOL	328.49	 <p>The image shows the chemical structure of stanozolol, an anabolic steroid. It consists of four fused rings (three six-membered and one five-membered). There is a double bond in the first ring and a ketone group at the 3-position. The side chain at the 17-position is a methyl group at C-13 and a methyl group at C-17. Stereochemistry is indicated with wedges and dashes.</p>

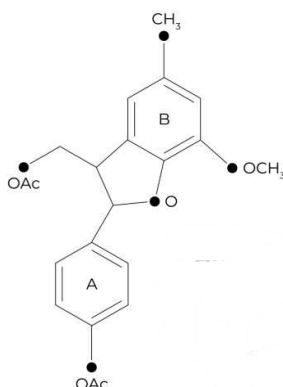
DANAZOL	337.46	
METHANDROSTENOLONE	300.44	

**Table 1:** Structure formula and molecular weight of analytes

### 3.2 Peptide and glycoprotein hormones

Among the substances prohibited by the World Anti-Doping Agency, “peptide hormones, growth factors, related substances, and mimetics” are classified as prohibited both in- and out-of-competition in section S2 [44]. Among them, growth hormone releasing peptides and secretagogues (GHRPs and GHSs, respectively) have the ability to increase natural growth hormone (hGH) plasma levels and potentially mask illicit use of recombinant growth hormone (rGH) by elevating the derived suppressed levels of hGH, thus providing clear performance enhancement [45,46,47]. Gonadotropin releasing hormone (GnRH) and its analogues are claimed to raise endogenous luteinizing hormone (LH), follicle stimulating hormone (FSH) and testosterone levels, among other potential benefits for doping purposes [48,49]. Anti-diuretic small hormones such as desmopressin can be misused in sports environments due to their masking properties related to their impact on the athlete’s biological passport (ABP) hematic parameters [50]. HCG (Fig.4) belongs to the glycoprotein hormone (GPH) family that also comprises LH, follicle stimulating hormone (FSH) and thyroid stimulating hormone (TSH) [51]. The GPHs are heterodimers consisting of an  $\alpha$  (GPH $\alpha$ ) and a  $\beta$  subunit. The  $\alpha$  subunit contains 92 amino acids and is identical in all GPHs whereas the  $\beta$  subunits are different and confer biological specificity. The  $\beta$  subunits display considerable homology; that between hCG and LH being about 80% whereas that with FSH $\beta$

and TSH $\beta$  is lower. HCG $\beta$  contains 145 and LH $\beta$  121 amino acids, the difference being due to a 24 amino acid extension on hCG $\beta$ , the so-called C-terminal peptide (CTP) [52].

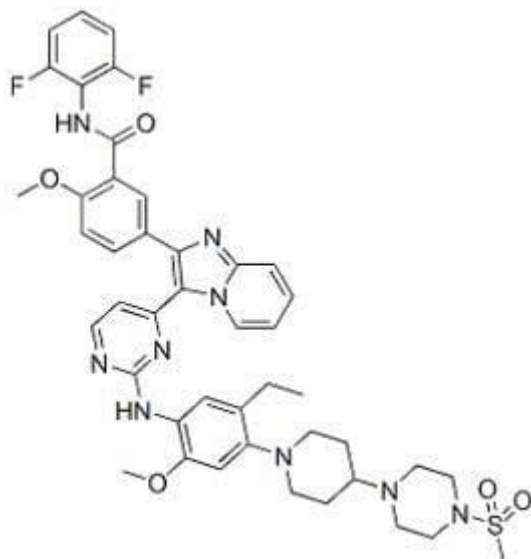


**Figure 4.** Structure formula of hCG

HCG is highly glycosylated, one third of the mass being attributed to eight carbohydrate moieties. Each subunit contains two N-linked and the CTP on hCG $\beta$  four O-linked oligosaccharides [53,54,55]. Owing to variation in the content of terminal sialic acid on the carbohydrate chains, hCG displays extensive charge heterogeneity with pI values ranging from three to seven. HCG produced in early pregnancy and by trophoblastic cancer contains more complex carbohydrates than that produced later in pregnancy [56,57]. The proportion of this so-called hyperglycosylated hCG (hCG<sub>h</sub>) decreases to a few per cent after 10 weeks of pregnancy [58]. Thus, little hCG occurs in commercial hCG preparations prepared from pregnancy urine, which are commonly used for doping.

IGF-1 (Fig.5) is a peptide containing 70 amino acids with three disulfide bridges; it is the principal mediator of GH's actions, though not all of them. The bioavailability of IGF-1 is affected by its binding to transporter proteins (IGFBP); six subtypes bind 98% of the circulating product. In doping, IGF-1 is available on the black market with commercial names such as Increlex® (mecasermin) and Iplex® (mecasermin rinfabate) [59]. There are truncated forms with missing amino acids, such as DES (1-3) IGF-1, which increases its potency ten-fold, and modified analogs that prolong its half-life. Figures on its use range from 6% to 7% to up to 16% [60]. The doses reported in weightlifters are 50–75  $\mu$ g/day for nine weeks up to a total of 2,200–8,600  $\mu$ g [61]. It has been prohibited by the WADA since 2003. IGF-1 has a lesser effect on collagen proteins than GH. The secondary effects are in

part similar to those arising from GH, although it can cause hypoglycaemia and thus, it is normally injected with meals. It is detected by directly measuring it through a radioimmunoassay or liquid chromatography–mass spectrometry together with the determination of its transporter proteins. There are variations, mainly according to sex.



**Figure 5.** Structure formula of IGF-1

#### **4. Matrices and samples**

The selection of the sample, to be analysed in the anti-doping analysis, very often depends on the purpose of the analysis. In particular, it is possible to refer to two different types of samples: biological and non-biological samples. The main purpose of the analysis of biological samples is to verify the intake of the test substances and then to confirm their possible use. The main biological samples used in the analysis of the substances used are fluids such as plasma [62], serum [63], urine [64] or oral liquid [65] which may provide information on a recent intake of substances. Urine, and in some cases also blood matrices (whole blood, serum, plasma), are considered the biological samples of choice for routine doping testing using a common workflow, starting with an initial screening that it must be quick, selective and sensitive, followed by a confirmation procedure [66]. A sample of urine, blood, breath, saliva, sweat or hair may be analysed. Urine testing is non-invasive and rapid and can detect a large number of substances taken over 1-4 days, sometimes for longer periods depending on the type of substance [67]. Testing blood is invasive, but it determines whether the athlete is under the influence of doping substances at that precise moment [68].

The requirements of high standards and quality requirements for modern doping testing pose many challenges to laboratories involved in such testing. In this regard, the project involves the use of dried blood matrices on special supports in the form of "spots" instead of "in test tubes", such as Dried Blood Spot (DBS), as an alternative and highly innovative approach that allows for the most reliable monitoring to be performed, ensuring the stability of biological samples [69]. In particular, contamination during the sampling of test pieces may cause degradation of the compounds under investigation, resulting in false negative results. Although the techniques of freezing and/or cooling blood samples in test tubes and the use of preservatives attempt to reduce some of these metabolic phenomena, they are not as effective as a storage and drying strategy on special media, this means that any enzymatic activity can be stopped immediately [70]. In addition to these obvious advantages of micro sampling with DBS, the ease and speed of sampling without specific treatment, storage and transport requirements should be emphasized. The DBS approach is therefore an innovative strategy capable of processing a larger number of samples in a short time (high throughput screening); all pre-analytical steps (from spot collection to analysis) are simplified; the stability of the compounds is guaranteed; doping control is maximised at all stages, from sample collection to delivery of the analysis result [71]. The spot sampling strategy can also extend to other biological matrices, such as urine, with the possibility of obtaining valuable additional information on athletes' doping positivity. The blood analysis alone hardly provides complete information, which can be supported by the use of additional samples such as Dried Urine Spot (DUS), saliva and sweat [72]. The project represents a significant advance in scientific knowledge of analytical methodology, allowing for a substantial strengthening of both key aspects of anti-doping control strategies, The deterrence of illegal practices in sport and the protection of athletes.

## 5. References

- [1] De Rose EH. Doping in athletes--an update. *Clin Sports Med.* 2008 Jan;27(1):107-30, viii-ix. doi: 10.1016/j.csm.2007.10.001.
- [2] Verroken M. Drug use and abuse in sport. *Baillieres Best Pract Res Clin Endocrinol Metab.* 2000 Mar;14(1):1-23. doi: 10.1053/beem.2000.0050.
- [3] Watson CJ, Stone GL, Overbeek DL, Chiba T, Burns MM. Performance-enhancing drugs and the Olympics. *J Intern Med.* 2022 Feb;291(2):181-196. doi: 10.1111/joim.13431. Epub 2022 Jan 10.
- [4] Catlin, D. H., & Murray, T. H. (2016). Performance-enhancing drugs, fair competition, and Olympic sport. *JAMA*, 315(11), 1103-1104.
- [5] Gerrard D. Drug misuse in sport: a historical perspective. *N Z Med J.* 2015 Dec 4;128(1426):16-8.
- [6] Reardon, C. L., & Creado, S. (2014). Drug abuse in athletes. *Substance Abuse and Rehabilitation*, 5, 95-105.
- [7] Cowan DA. Antidoping analysis: a special focus. *Bioanalysis.* 2020 Jun;12(11):707-709.
- [8] World Anti-Doping Agency. (2024). Prohibited List January 2024. Link al documento.
- [9] World Anti-Doping Agency (WADA). (2023). *The World Anti-Doping Code 2024*. Montreal, Canada: WADA. Disponibile su [https://www.wada-ama.org/sites/default/files/2023-09/2024list\\_en\\_final\\_22\\_september\\_2023.pdf](https://www.wada-ama.org/sites/default/files/2023-09/2024list_en_final_22_september_2023.pdf).
- [10] Young R. The Development of the World Anti-Doping Code. *Med Sport Sci.* 2017;62:11-21. doi: 10.1159/000460681. Epub 2017 Jun 1.
- [11] Waller CC, McLeod MD. A review of designer anabolic steroids in equine sports. *Drug Test Anal.* 2017 Sep;9(9):1304-1319. doi: 10.1002/dta.2112. Epub 2016 Nov 18. PMID: 27732767.
- [12] Catlin DH, Kammerer RC, Hatton CK, Sekera MH, Merdink JL. Analytical chemistry at the Games of the XXIIIrd Olympiad in Los Angeles, 1984. *Clin Chem.* 1987 Feb;33(2 Pt 1):319-27. PMID: 2879653.
- [13] Cowan DA. Drug testing. *Essays Biochem.* 2008;44:139-48. doi: 10.1042/BSE0440139. PMID: 18384288.
- [14] Regulation CONI available on: <https://www.coni.it/it/impianti/norme-e-regolamenti.html>

[15] Regulation International Olympic Committee available on: <https://olympics.com/ioc/documents/international-olympic-committee>

[16] World Anti-Doping Agency (WADA). (2023). International Standards for Testing and Investigations available on: <https://www.wada-ama.org/en/resources/world-anti-doping-code-and-international-standards/international-standard-testing-and>

[17] International Olympic Committee Centers available on: <https://olympics.com/ioc/medical-research/research-centres>

[18] World Anti-Doping Agency (WADA). (2023). International Standards for Laboratories available on: <https://www.wada-ama.org/en/resources/world-anti-doping-code-and-international-standards/international-standard-laboratories>

[19] Geyer H, Schänzer W, Thevis M. Anabolic agents: recent strategies for their detection and protection from inadvertent doping. *Br J Sports Med.* 2014 May;48(10):820-6. doi: 10.1136/bjsports-2014-093526. Epub 2014 Mar 14.

[20] Hartgens F, Kuipers H. (2024). Effects of androgenic-anabolic steroids in athletes. *Sports Medicine*, 34(8):513-54.

[21] García-Arnés JA, García-Casares N. (2022). Doping and sports endocrinology: anabolic-androgenic steroids. *Rev Clin Esp (Barc)*, Dec;222(10):612-620.

[22] Bond P, Smit DL, de Ronde W. (2022). Anabolic-androgenic steroids: How do they work and what are the risks? *Front Endocrinol (Lausanne)*, Dec 19;13:1059473.

[23] Sonksen PH, Cowan D, Holt RI. Use and misuse of hormones in sport. *Lancet Diabetes Endocrinol.* 2016 Nov;4(11):882-883. doi: 10.1016/S2213-8587(16)30218-2. Epub 2016 Aug 16.

[24] Kicman AT, Cowan DA. Peptide hormones and sport: misuse and detection. *Br Med Bull.* 1992 Jul;48(3):496-517. doi: 10.1093/oxfordjournals.bmb.a072559.

[25] Barroso O, Schamasch P, Rabin O. Detection of GH abuse in sport: Past, present and future. *Growth Horm IGF Res.* 2009 Aug;19(4):369-74. doi: 10.1016/j.ghir.2009.04.021. Epub 2009 May 30.

[26] De Santi M, Baldelli G, Brandi G; GSMS-SItI, Working Group on Movement Sciences for Health, Italian Society of Hygiene preventive Medicine and Public Health; WDPP,

Working Group Doping Prevention Project. Use of hormones in doping and cancer risk. *Ann Ig*. 2019 Nov-Dec;31(6):590-594. doi: 10.7416/ai.2019.2319.

[27] Barroso O, Handelsman DJ, Strasburger C, Thevis M. Analytical challenges in the detection of peptide hormones for anti-doping purposes. *Bioanalysis*. 2012 Jul;4(13):1577-90. doi: 10.4155/bio.12.128.

[28] D.R. Mottram, A.J. George, *Anabolic steroids*, Best Practice and Research Clinical Endocrinology and Metabolism, vol. 14, cap. 1, (2000), pp 55-69.

[29] K. Shimada, K. Mitamura, T. Higashi, *Gas chromatography and high-performance liquid chromatography of natural steroids*, *Journal of Chromatography A*, 935 (2001), pp 141–172.

[30] Y. Hobo, J. Nishikawa, Y. Miyashiro, A. Fujikata, *Measurement of steroid hormones by liquid chromatography-tandem mass spectrometry with small amounts of hair*, *Steroids* 164 (2020) 108732.

[31] de Wit AE, Bosker FJ, Giltay EJ, de Kloet CS, Roelofs K, van Pelt J, Penninx BWJH, Schoevers RA. Testosterone in human studies: Modest associations between plasma and salivary measurements. *Andrologia*. 2018 Feb;50(1). doi: 10.1111/and.12779. Epub 2017 Mar 7.

[32] Bhasin S, Storer TW, Berman N, et al. The effects of supraphysiologic doses of testosterone on muscle size and strength in normal men. *N Engl J Med*. 1996; 335: 1-7.

[33] Bhasin S, Woodhouse L, Casaburi R, Singh AB, Bhasin D, Berman N, et al. Testosterone dose-response relationships in healthy young men. *Am J Physiol Endocrinol Metab*. 2001; 281: E1172-81.

[34] Montisci M, El Mazloum R, Cecchetto G, Terranova C, Ferrara SD, Thiene G, et al. Anabolic androgenic steroids abuse and cardiac death in athletes: morphological and toxicological findings in four fatal cases. *Forensic science international*. 2012; 217: 13-8. 34.

[35] Fineschi V, Riezzo I, Centini, F, Silingardi E, Licata M, Beduschi G, et al. Sudden cardiac death during anabolic steroid abuse: morphologic and toxicologic findings in two fatal cases of bodybuilders. *International journal of legal medicine*. 2007; 121: 48-53.

- [36] Far HR, Agren G, Thiblin I. Cardiac Hypertrophy in deceased users of anabolic androgenic steroids: an investigation of autopsy findings. *Cardiovascular pathology: the official journal of the Society for Cardiovascular Pathology*. 2012; 21: 312-6.
- [37] Baggish AL, Weiner RB, Kanayama G, Hudson JI, Picard MH, Hutter AM, Jr., et al. Long-term anabolic-androgenic steroid use is associated with left ventricular dysfunction. *Circulation Heart failure*. 2010; 3: 472-6
- [38] Boregowda K, Joels L, Stephens JW, Price DE. Persistent primary hypogonadism associated with anabolic steroid abuse. *Fertility and sterility*. 2011; 96: 7-8.
- [39] Teale P, Scart J, Judson S. Impact of the emergence of designer drugs upon sports doping testing. *Bioanalysis*. 2012;4:71-88.
- [40] Kanayama G, Pope HG, Jr. Illicit use of androgens and other hormones: recent advances. *Current opinion in endocrinology, diabetes, and obesity*. 2012; 19: 211-9.
- [41] Ip EJ, Lu DH, Barnett MJ, Tenerowicz MJ, Vo JC, Perry PJ. Psychological and physical impact of anabolic- androgenic steroid dependence. *Pharmacotherapy*. 2012; 32: 910-9.
- [42] H. Elmongy, M. Masquelier, M. Ericsson, *Development and validation of a UHPLC-HRMS method for the simultaneous determination of the endogenous anabolic androgenic steroids in human serum*, *Journal of Chromatography A* 1613 (2020) 460686.
- [43] World AntiDoping Agency (WADA), *Athlete Biological Passport Operating Guidelines*, (2021).
- [44] García-Arnés JA, García-Casares N. Doping and sports endocrinology: growth hormone, IGF-1, insulin, and erythropoietin. *Rev Clin Esp (Barc)*. 2023 Mar;223(3):181-187. doi: 10.1016/j.rceng.2023.01.005. Epub 2023 Feb 1.
- [45] M. Okano, Y. Nishitani, M. Sato, A. Ikekita, S. Kageyama Influence of intravenous administration of growth hormone releasing peptide-2 (GHRP-2) on detection of growth hormone doping: growth hormone isoform profiles in Japanese male subjects. *Drug Test. Anal.*, 2 (2010), pp. 548-556
- [46] G.P. Baumann. Growth hormone doping in sports: a critical review of use and detection strategies. *Endocr. Rev.*, 33 (2012), pp. 155-186
- [47] P. Ferro, G. Krotov, I. Zvereva, G. Rodchenkov, J. Segura. Structure-activity relationship for peptidic growth hormone secretagogues. *Drug Test. Anal.*, 9 (2017), pp. 87-95

- [48] E. van Breda, H.A. Keizer, H. Kuipers, B.H.R. Wolffenbuttel. Androgenic anabolic steroid use and severe hypothalamic-pituitary dysfunction: a case study. *Int. J. Sports Med.*, 24 (2003), pp. 195-196
- [49] D.J. Handelsman, A. Idan, J. Grainger, C. Goebel, L. Turner, A.J. Conway. Detection and effects on serum and urine steroid and LH of repeated GnRH analog (leuprolide) stimulation. *J. Steroid Biochem. Mol. Biol.*, 141 (2014), pp. 113-120
- [50] F. Sanchís-Gomar, V.E. Martínez-Bello, A.L. Nascimento, C. Pérez-Quilis, J.L. García-Giménez, J. Viña, M. Gómez-Cabrera. Desmopressin and hemodilution: implications in doping. *Int. J. Sports Med.*, 31 (2010), pp. 5-9
- [51] Stenman UH, Hotakainen K, Alfthan H. Gonadotropins in doping: pharmacological basis and detection of illicit use. *Br J Pharmacol.* 2008 Jun;154(3):569-83. doi: 10.1038/bjp.2008.102. Epub 2008 Apr 14.
- [52] Cole LA. New discoveries on the biology and detection of human chorionic gonadotropin. *Reprod Biol Endocrinol.* 2009 Jan 26;7:8. doi: 10.1186/1477-7827-7-8.
- [53] Kessler MJ, Mise T, Ghai RD, Bahl OP. Structure and location of the *O*-glycosidic carbohydrate units of human chorionic gonadotropin. *J Biol Chem.* 1979a; 254:7909–7914.
- [54] Kicman AT, Brooks RV, Collyer SC, Cowan DA, Nanjee MN, Southan GJ, et al. Criteria to indicate testosterone administration. *Br J Sports Med.* 1990; 24:253–264.
- [55] Elliott MM, Kardana A, Lustbader JW, Cole LA. Carbohydrate and peptide structure of the alpha- and beta-subunits of human chorionic gonadotropin from normal and aberrant pregnancy and choriocarcinoma. *Endocrine.* 1997; 7:15–32.
- [56] Mizuochi T, Nishimura R, Derappe C, Taniguchi T, Hamamoto T, Mochizuki M, et al. Structures of the asparagine-linked sugar chains of human chorionic gonadotropin produced in choriocarcinoma. Appearance of triantennary sugar chains and unique biantennary sugar chains. *J Biol Chem.* 1983;258:14126–14129.
- [57] Birken S. Specific measurement of o-linked core 2 sugar-containing isoforms of hyperglycosylated human chorionic gonadotropin by antibody b152. *Tumour Biol.* 2005;26:131–141.
- [58] Kovalevskaya G, Birken S, Kakuma T, Ozaki N, Sauer M, Lindheim S, et al. Differential expression of human chorionic gonadotropin (hCG) glycosylation isoforms in failing and continuing pregnancies: preliminary characterization of the hyperglycosylated hCG epitope. *J Endocrinol.* 2002; 172:497–506.
- [59] Adams, P. T., & Lee, C. R. (2024). *Regulatory Approaches to IGF-I Use in Sports: Policies and Enforcement.* International Journal of Sport Policy and Politics.
- [60] J. Bailes, M. Soloviev Insulin-like growth factor-1 (IGF-1) and its monitoring in medical diagnostic and in sports Biomolecules, 11 (2021), pp. 1-1

[61] B.P. Brennan, G. Kanayama, H.G. Hudson Jil Pope Human growth abuse in male weightlifters Am J Addict, 20 (2011), pp. 9-13

[62] Thomas A, Krombholz S, Wolf C, Thevis M. Determination of ghrelin and desacyl ghrelin in human plasma and urine by means of LC-MS/MS for doping controls. Drug Test Anal. 2021 Nov;13(11-12):1862-1870. doi: 10.1002/dta.3176. Epub 2021 Oct 26.

[63] Miller GD, Cox HD, Nair V, Eichner D. Anti-doping analytes in serum: A comparison of SST and SST-II Advance blood collection tubes. Drug Test Anal. 2019 Jul;11(7):931-936. doi: 10.1002/dta.2592. Epub 2019 May 8.

[64] K. Labuz, P. Adamowicz, M. Kała, K. Pyrc, E. Reszke, P. Mielczarek, J. Silberring, M. Smoluch. “*Detection of legal highs in the urine of methadone-treated patient by LC-MS*”, Basic Clin Pharmacol Toxicol., 2019, 125, 253-258.

[65] C.A. Coulter, C.M. Moore. “*Analysis of Drugs in Oral Fluid Using LC-MS/MS*”, Methods Mol Biol., 2019, 1872, 237-259.

[66] Rabin O, Uiba V, Miroshnikova Y, Zabelin M, Samoylov A, Karkischenko V, Semyonov S, Astrelina T, Razinkin S. Meldonium long-term excretion period and pharmacokinetics in blood and urine of healthy athlete volunteers. Drug Test Anal. 2019 Apr;11(4):554-566. doi: 10.1002/dta.2521. Epub 2018 Nov 12. PMID: 30328291.

[67] Abushareeda W, Vonaparti A, Saad KA, Almansoori M, Meloug M, Saleh A, Aguilera R, Angelis Y, Horvatovich PL, Lommen A, Alsayrafi M, Georgakopoulos C. High resolution full scan liquid chromatography mass spectrometry comprehensive screening in sports antidoping urine analysis. J Pharm Biomed Anal. 2018 Mar 20;151:10-24. doi: 10.1016/j.jpba.2017.12.025. Epub 2017 Dec 21. PMID: 29291455.

[68] Lewis L, Goodrum J, Cai C, Muir T, Boutard K, Capdevielle T, Longo S, Fedoruk M, Miller G. Application of micro capillary blood sampling in an anti-doping setting. Drug Test Anal. 2024 Aug;16(8):835-840. doi: 10.1002/dta.3603. Epub 2023 Nov 20. PMID: 37986710.

[69] Thevis M, Walpurgis K, Thomas A. DropWise: current role and future perspectives of dried blood spots (DBS), blood microsampling, and their analysis in sports drug testing. Crit Rev Clin Lab Sci. 2023 Jan;60(1):41-62. doi: 10.1080/10408363.2022.2103085. Epub 2022 Aug 7. PMID: 35938300.

[70] Thevis M, Kuuranne T, Dib J, Thomas A, Geyer H. Do dried blood spots (DBS) have the potential to support result management processes in routine sports drug testing? Drug

Test Anal. 2020 Jun;12(6):704-710. doi: 10.1002/dta.2790. Epub 2020 Apr 7. PMID: 32180361.

[71] Solheim SA, Thomas A, Ringsted TK, Thevis M, Breenfeldt Andersen A, Holm-Sørensen H, Nordsborg NB, Mørkeberg J. Analysis of dried blood spots is a feasible alternative for detecting ephedrine in doping control. *Drug Test Anal.* 2022 Oct;14(10):1685-1695. doi: 10.1002/dta.3338. Epub 2022 Jul 1. PMID: 35738840.

[72] Grignani P, Manfredi A, Monti MC, Moretti M, Morini L, Visonà SD, Fattorini P, Previderè C. Genetic individual identification from dried urine spots: A complementary tool to drug monitoring and anti-doping testing. *Drug Test Anal.* 2022 Jul;14(7):1234-1243. doi: 10.1002/dta.3243. Epub 2022 Feb 28. PMID: 35195361; PMCID: PMC9540579.

# **CHAPTER II**

## **WADA INTERNATIONAL STANDARDS**

## **1. International Standards for Testing and Investigations (ISTI)**

The **International Standard for Testing and Investigations (ISTI)** under the World Anti-Doping Agency (WADA) Anti-Doping Code provides guidelines and rules for doping control, including testing athletes and conducting investigations into potential anti-doping rule violations (ADRVs). Here are the key components.

### **1.1 Testing Requirements**

**-In-Competition and Out-of-Competition Testing:** Testing can occur during competition or at any time outside of competition. Out-of-competition testing is essential to detect substances that might not be found during events.

**-Random and Targeted Testing:** Athletes are selected for testing either randomly or based on specific targeting criteria, such as suspicious performance patterns, injury recovery, or previous anti-doping violations.

### **1.2 Athlete Whereabouts**

**- Athlete's Responsibility:** Athletes must provide accurate and up-to-date information on their location (whereabouts) to enable out-of-competition testing. This includes specifying a 60-minute daily time slot when they are available for testing.

**- Missed Tests:** Failure to provide whereabouts information or missing a test can result in a violation if it occurs three times within 12 months.

### **1.3 Sample Collection Process**

**-Doping Control Officers (DCOs):** Trained officials collect urine and/or blood samples following strict protocols to ensure the integrity of the sample. They must document the process, ensure athlete privacy, and maintain the chain of custody.

**- Athlete Rights and Responsibilities:** Athletes have the right to be informed about the procedure, request modifications for disabilities, and have a representative present. However, they are required to comply with sample collection once notified.

## 1.4 Selection of Athletes for Testing

- **Test Distribution Planning (TDP):** Anti-doping organizations (ADOs) must create a risk-based test distribution plan targeting high-risk athletes, sports, or periods of doping risk. They should also balance different types of testing (urine, blood, etc.) and ensure proportional testing across gender and sports.
- **Intelligence-Led Targeting:** This focuses on collecting and analyzing data (such as suspicious behaviors, reports, or other evidence) to guide targeted testing and investigations.

## 1.5 Investigations

- **Intelligence Gathering:** ADOs must establish mechanisms for gathering intelligence, including tips from whistleblowers, media reports, or suspicious trends in biological data.
- **Investigation of ADRVs:** Investigations can be initiated based on information suggesting an anti-doping rule violation (e.g., possession, trafficking, tampering, etc.). ADOs must follow due process, respecting confidentiality and legal rights.
- **Collaboration with Other Organizations:** ADOs often work with law enforcement, customs, or other organizations to gather evidence for doping-related violations like trafficking or administration of banned substances.

## 1.6 Therapeutic Use Exemptions (TUEs)

Athletes can apply for a TUE if they need to use a prohibited substance for legitimate medical reasons. Testing and investigations must account for these exemptions, ensuring that any positive test result is assessed in light of an approved TUE.

## 1.7 Sample Storage and Reanalysis

- Long-Term Storage:** Samples can be stored for up to 10 years to allow for reanalysis as detection technologies improve. This ensures that athletes cannot evade detection through emerging substances or techniques.
- Reanalysis Protocols:** WADA provides guidelines on reanalyzing stored samples if new intelligence arises, or new detection methods become available.

## **1.8 Results Management and Reporting**

**-Violations and Sanctions:** ADOs must promptly report any potential violations, initiate results management procedures, and ensure due process for athletes, including the opportunity for hearings.

**-Confidentiality and Reporting:** All testing and investigation processes must respect confidentiality, with results only shared with relevant parties.

## **1.9 Retesting and Longitudinal Profiling**

**-Athlete Biological Passport (ABP):** This involves long-term monitoring of biomarkers related to blood and steroid profiles. Any abnormal variations may trigger targeted testing or further investigation.

## **1.10 Anti-Doping Education and Awareness**

-ISTI emphasizes education, ensuring that athletes and support personnel are fully aware of their rights, responsibilities, and the consequences of doping violations.

The ISTI ensures a fair, consistent, and robust approach to both testing and investigations, enabling ADOs to detect and deter doping while respecting athletes' rights. It plays a critical role in maintaining the integrity of sport and ensuring a level playing field. In summary, the ISTI provides the foundation for how athletes are tested, how investigations into potential doping violations are conducted, and how data and intelligence are used to enforce the WADA Anti-Doping Code globally [1].

## **2. International Standards for Laboratories (ISL)**

WADA-accredited laboratories must adhere to stringent rules at the analytical level to ensure that anti-doping analyses are consistent, reliable, and scientifically valid. These rules are primarily outlined in the **International Standard for Laboratories (ISL)** and related technical documents. Here are the key rules.

### **2.1 Accreditation and Compliance**

-Laboratories must be accredited by WADA and follow guidelines set by the **International Standard ISO/IEC 17025**, which covers general requirements for laboratory competence.

-Regular proficiency testing and external quality assessments are required to demonstrate competence in detecting prohibited substances.

## **2.2 Prohibited Substances and Methods**

-**WADA Prohibited List:** Laboratories must detect and analyze substances and methods specified in the WADA Prohibited List, which is updated annually.

-**Testing Sensitivity:** They must use analytical methods that are sensitive enough to detect even trace amounts of banned substances, including thresholds for certain substances (e.g., anabolic agents, stimulants).

## **2.3 Analytical Methods**

-**Validated Procedures:** All analytical methods must be validated and scientifically justified. This includes the use of advanced techniques such as **mass spectrometry (MS)**, **liquid chromatography (LC)**, and **gas chromatography (GC)** for detecting various classes of substances.

-**Method Suitability:** The chosen methods must be appropriate for the specific sample matrix (e.g., urine, blood) and the nature of the prohibited substance being tested.

## **2.4 Quality Control and Assurance**

-**Internal Quality Control (IQC):** Laboratories are required to maintain continuous quality control procedures, such as running blank and control samples alongside athlete samples to ensure accuracy and reliability.

-**External Quality Assessments (EQA):** Laboratories must participate in external quality assessments organized by WADA, which involve testing unknown samples provided by WADA to ensure consistent performance across labs.

## **2.5 Chain of Custody**

**-Sample Integrity:** The chain of custody for each sample must be maintained throughout the testing process, ensuring that the sample is handled securely and without tampering from the moment it is collected to final analysis.

**-Documentation:** Detailed records of all steps in the sample analysis, including who handled the sample and what procedures were performed, must be meticulously kept.

## **2.6 Reporting Results and Measurement Uncertainty**

**-Adverse Analytical Findings (AAFs):** When prohibited substances are detected, laboratories must report an Adverse Analytical Finding (AAF) to the relevant anti-doping organization (ADO). Results must be backed by sufficient scientific evidence.

**-Measurement Uncertainty:** Laboratories must quantify and report the measurement uncertainty, especially for substances with defined thresholds, like testosterone or ephedrine, to ensure accurate results in borderline cases.

## **2.7 Confirmatory Testing**

**-A-Sample and B-Sample Testing:** If the A-sample tests positive, laboratories are required to perform confirmatory tests using the B-sample to verify the result. This involves applying more specific or advanced techniques, ensuring no false positives.

**-Blind Testing and Reanalysis:** In cases of doubt or disputes, laboratories may be asked to conduct blind testing or reanalyse stored samples using new methods or technology.

## **2.8 Long-Term Storage and Retesting**

**-Sample Storage:** WADA mandates that laboratories store samples for up to 10 years, allowing for future retesting using more advanced methods if new intelligence or techniques become available.

**-Retesting Protocols:** When reanalysing stored samples, laboratories must follow strict protocols to ensure consistency with the original analysis.

## **2.9 Athlete Biological Passport (ABP)**

**-Longitudinal Profiling:** Laboratories must participate in the Athlete Biological Passport (ABP) program, where they monitor biomarkers in an athlete's blood or steroid profile over time. Significant deviations in these profiles may trigger additional testing or investigations.

## **2.10 Data Management and Security**

**-LIMS (Laboratory Information Management System):** Laboratories must use secure systems to manage and store all data related to doping analysis, ensuring that results and documentation are protected from unauthorized access or tampering.

**-Confidentiality:** Laboratories are required to maintain confidentiality, sharing results only with authorized anti-doping organizations or stakeholders in line with WADA protocols.

## **2.11 Laboratory Ethics and Independence**

**-Scientific Integrity:** Laboratories must uphold the highest scientific and ethical standards, ensuring that their work is impartial, transparent, and free from external influence.

**-Independence from Sports Organizations:** WADA-accredited laboratories must operate independently from any sport organization, ensuring that results are not biased or influenced by sporting interests.

WADA-accredited laboratories must comply with a rigorous set of rules at the analytical level to ensure accurate, reliable, and scientifically valid doping analysis. These rules cover every aspect of the testing process, including method validation, quality control, sample integrity, reporting, and long-term storage. The primary goals are to uphold scientific integrity, ensure consistent detection of prohibited substances, and maintain the fairness of sport by preventing doping [2].

## **3. International Standards for Results Management (ISRM)**

The **International Standard for Results Management (ISRM)** under the World Anti-Doping Agency (WADA) Anti-Doping Code establishes a framework for the fair, efficient, and consistent handling of potential anti-doping rule violations (ADRVs). The ISRM outlines how cases should be managed from the initial report of a violation through to its resolution, ensuring due process for all parties involved. Here are the key components:

### **3.1 Initiation of Results Management**

**-Triggering Events:** Results management begins when a potential ADRV is identified, such as a positive result from a doping test (Adverse Analytical Finding, AAF), missed test, refusal to provide a sample, or intelligence-based evidence (e.g., trafficking or tampering).

**Notification:** The athlete or person involved must be promptly notified of the potential violation, along with details of the violation, evidence, and rights.

### **3.2 Provisional Suspension**

**-Immediate Suspension:** Upon notification of certain violations, such as a positive test for a prohibited substance, a provisional suspension can be imposed while the case is being reviewed.

**-Right to Appeal:** The athlete has the right to challenge or request a hearing regarding the provisional suspension.

### **3.3 Results Management Process**

**-Initial Review:** The Anti-Doping Organization (ADO) conducts an initial review of the case to ensure there has been no departure from established protocols that could invalidate the result.

**-TUE Consideration:** The ADO checks whether the athlete has a valid Therapeutic Use Exemption (TUE) for any prohibited substance detected.

### **3.4 Hearing Process**

**-Right to a Fair Hearing:** Athletes or individuals accused of ADRVs have the right to a timely, fair, and impartial hearing. The hearing body must be independent and impartial.

**-Representation and Evidence:** Athletes have the right to legal representation, present evidence, and cross-examine witnesses during the hearing.

**-Burden of Proof:** The burden of proof rests on the ADO to establish that an ADRV occurred. However, the athlete may bear the burden of proof in certain defenses (e.g., establishing that ingestion was unintentional).

### **3.5 Decision-Making and Sanctions**

**-Sanctions for Violations:** If a violation is confirmed, the hearing panel will determine appropriate sanctions. These can range from a warning to a period of ineligibility, depending on the severity of the violation, the substance involved, and the athlete's degree of fault.

**-Aggravating and Mitigating Circumstances:** The panel may consider factors such as previous violations (aggravating) or unintentional use (mitigating) when deciding on the sanction.

### **3.6 Appeals Process**

**-Right to Appeal:** Both the athlete and the ADO have the right to appeal the decision to the Court of Arbitration for Sport (CAS) or other designated bodies. Appeals must be filed within specified deadlines.

**-Appealable Decisions:** Not just the final ruling but also specific elements of the decision, such as the sanction length, can be appealed.

### **3.7 Multiple Violations**

**-Handling of Second Violations:** If an athlete commits another ADRV, sanctions will be more severe. The results management process for multiple violations follows specific guidelines in the ISRM to determine the appropriate cumulative sanctions.

### **3.8 Confidentiality and Public Disclosure**

**-Confidential Process:** The results management process remains confidential until a final decision is made. Information may only be disclosed to relevant parties (e.g., the athlete, ADO, and WADA)

**-Public Disclosure:** After a final decision, the ADO must publicly disclose the outcome, including the violation, the athlete's name, and the sanction imposed. However, if the athlete is exonerated, no public disclosure is required unless the athlete requests it.

### **3.9 Results Management for Non-Analytical Violations**

**-Intelligence-Led Violations:** For non-analytical cases (e.g., possession, trafficking, or tampering), results management still applies. These cases often arise from investigations, whistleblower reports, or intelligence data rather than a positive drug test.

**-Investigations:** ADOs must follow due process, gather evidence, and manage the results in the same systematic way as for analytical violations.

### **3.10 Statute of Limitations**

**-Time Limits:** The ISRM provides a 10-year statute of limitations for initiating results management procedures for ADRVs. This means an athlete can be prosecuted for violations up to 10 years after the event occurs, allowing for retesting or new evidence to emerge.

### **3.11 Handling of Multiple Organizations**

**-Coordination Among Organizations:** In cases where multiple ADOs are involved (e.g., WADA, National Anti-Doping Organizations, International Federations), the ISRM ensures coordination between these bodies to prevent duplication and ensure fair handling.

### **3.12 Reanalysis of Samples**

**-Long-Term Sample Retesting:** If new evidence or technology becomes available, stored samples may be reanalyzed. This can trigger a new results management process if an ADRV is discovered through retesting.

The ISRM ensures that anti-doping rule violations are handled in a structured, fair, and transparent manner. It outlines clear procedures for each stage of the process, from initial notification through to hearings and appeals. This standard helps maintain the integrity of anti-doping efforts, ensures athlete rights are respected, and supports consistency in how sanctions are applied globally. In summary, the ISRM provides a comprehensive framework

for managing potential ADRVs, ensuring due process, protecting athlete rights, and promoting consistency across anti-doping organizations worldwide [3].

#### **4. International Standards in this study**

The study does not fully comply with all existing WADA standards. This is a conscious decision, taken to allow for the exploration and evaluation of innovative protocols that may not yet be in compliance with current anti-doping regulations. The main aim of this research is to study new methodologies and experimental techniques which could revolutionise the field, making testing and monitoring more effective and efficient in the future. While recognising the importance of following international standards set by WADA, the study intentionally pushes these boundaries to obtain information that could pave the way for the evolution of anti-doping practices.

This research aims to collect preliminary data which can be adapted to WADA standards. It is essential to note that, although the study differs from these, such deviations are undertaken with a rigorous scientific approach and ethical considerations. The aim is not to undermine existing regulation, but to develop more advanced and comprehensive anti-doping measures in the long term. The results of this research could potentially support a more dynamic and responsive approach to emerging challenges in the fight against doping.

## 5. References

[1] Downloaded from; <https://www.wada-ama.org/en/resources/world-anti-doping-code-and-international-standards/international-standard-testing-and>

[2] Downloaded from: <https://www.wada-ama.org/en/resources/world-anti-doping-code-and-international-standards/international-standard-laboratories>

[3] Downloaded from: <https://www.wada-ama.org/en/resources/world-anti-doping-code-and-international-standards/international-standard-results>

# **CHAPTER III**

## **ANALYTICAL METHODS**

## **1. Aim of the research**

The aim of the research work carried out during this Ph.D. project has been the study, design and development of innovative and original analytical approaches for the identification and quantification of doping substances in biological samples. In the approaches herein developed, advanced techniques have been used for all phases, starting from the sample collection and preparation to the instrumental analysis. In particular, for the sampling of biological matrices, especially whole blood, different miniaturised sample collection procedures were developed. Microsampling technologies have been studied for several of the methodologies designed within this project, in order to bring a significant number of advantages, among which a less invasive sampling procedure, higher stability of the analytes, feasible storage and transportation conditions and reduction of the solvents and materials used for sample preparation. With regard to sample pretreatment phases, original procedures were designed, aimed at reducing the number of steps needed, analysis time and use of solvents. With this aim, microextraction techniques have also been investigated, able of efficiently extracting the compounds of interest from the biological matrix. In addition, each strategy herein developed has made use of instrumental analytical techniques based on high-performance liquid chromatography (HPLC) coupled to different detection means, such as mass spectrometry (MS).

Each phase of the research project has been carefully studied, optimized and validated (following international guidelines) in order to obtain innovative, high-performance and reliable analytical methods. The research carried out during this Ph.D. was aimed at obtaining innovative strategies to be exploited for the identification and quantification of doping substances, improve current analytical practices and have new strategies to improve knowledge about illicit substances in sport.

## **2. Analytical approaches**

### **2.1 Innovative biological matrices: miniaturized samples**

Among the different types of biological samples, the ones of choice are usually haematic matrices (blood, plasma and serum) which provide important information both on the consumption of doping substances and on the pharmacokinetics and metabolism. Despite this, the use of macro-volumes of fluid samples, presents several issues [1]. First of all, the sample collection is highly invasive, (e.g., venepuncture for whole blood collection); moreover, once collected, the sample must be stored at low temperatures and often analysed in a short time frame. Furthermore, since the matrices are aqueous, the compounds present in the samples are often subjected to degradation phenomena.

The study of innovative miniaturised dried sample collection techniques can be a valid alternative to the traditional sampling procedure in test tubes. Microsampling refers to all those techniques able to collect small volumes of sample (less than 50  $\mu\text{L}$ ) of biological fluids. Usually, this volume is collected by means of minimally invasive procedures, that can make use of microcapillaries or microfluidic systems. Once collected, the matrix can be stored in small containers or deposited on a suitable substrate to create a dried sample. These new microsampling approaches are becoming a promising alternative sampling strategy, especially in bioanalysis, as they have been developed to overcome the obstacles represented by traditional sampling, storage and transportation of liquid samples (e.g., invasiveness, pain, sample stability).

Dried microsample techniques are probably the most widespread miniaturised sample collection approaches. These techniques basically consist of collecting a small volume of a biological fluid (e.g. blood, plasma, serum, urine) [2] on a porous cellulosic or polymeric substrate and allowing it to dry, most often at room temperature (RT). When the sample is dried, it can be directly analysed or it can also be stored for prolonged times, usually without stability issues for the analytes [3] and with the possibility to analyse the samples even after a long time. Microsampling techniques have shown several advantages over traditional sampling. One of the most important is the minimal invasiveness of sample collection procedures [4], with less pain and stress for the subject. Another advantage is related to cost

decrease, since collection, storage and shipping can be performed at RT, without the need for special logistic requirements or to establish a cold chain. This could be a great advantage, particularly for those developing countries or other places with limited resources. Microsampling techniques are often characterised by feasible procedures for which the presence of a specialised operator is not necessary, thus allowing both home and self-sampling [5, 6]. In addition, the use of these approaches reduces in turn the use of solvents and materials required for sample pretreatment, which often consists of fast and cost-effective procedures. Thanks to all these advantages, microsampling technologies are gaining more and more approval from the scientific community to the point that in 2018, the U.S. Food and Drug Administration (FDA) introduced guidelines for the development of methods based on Dried Blood Spots (DBS) [7]. DBS testing is the pioneering technique and the most studied approach to dried blood microsampling. More than a century ago, Ivar Bang had already recognised the potential of using DBS for the monitoring of blood glucose. However, only after fifty years DBS began to gain popularity in bioanalysis, when Guthrie and Susie in 1963 proposed and applied this technique for phenylalanine determination [8] in the blood of new-borns for phenylketonuria diagnosis. Today, DBS methods are studied in different fields of research, such as pharmacokinetic studies [9], therapeutic drug monitoring (TDM) [10], for the management of infectious diseases [11], in the bioanalysis of antibodies and therapeutic proteins [12] and, of course, for forensic purposes [13]. Several techniques based on the generation of DBS have recently been proposed for the identification and quantification of drug abuse in possible addicted subjects [14].

## **2.2 Sample pretreatment**

The sample preparation phase has been carefully designed and optimised for each sample used in the different strategies developed. The goal was to obtain feasible, fast and low environmental impacting pretreatment protocols able of providing a suitable sample for instrumental analysis and efficiently extracting the compounds of interest from the matrix. Several preparatory technologies and techniques have been tested, and the protocols that have been shown to be the most feasible and performing have been chosen for the final method. Among the various techniques investigated, ultrasonic-assisted extraction (UAE), protein precipitation (PP) and a polyimide-base microextraction technology have shown to be among the most promising. In particular, the last one consists of an advanced technique

of microextraction able to selectively extract the compounds of interest directly from the biological matrix thanks to a suitable polymer substrate. Unlike traditional pretreatment techniques, some miniaturised extraction procedures do not extract quantitatively the analyte from the sample, but an equilibrium is established between the concentration of the analyte on the material of the microextraction system and the matrix, usually a biological fluid [15, 16]. The achievement of a stable equilibrium allows to have a high reproducible and repeatable extraction procedure. Moreover, the substrates are produced and synthesized in order to be selective towards the analytes of interest, therefore obtaining a purified final extract, without the need for further sample cleaning steps. Microextraction technologies are also very sustainable, as they make use of small amounts of organic solvents, and often the starting materials for devices can be common materials such as filter paper [17], wooden toothpicks [18], computer fans [19] or magnetic materials [20].

### **2.3 Instrumental methods**

Once collected and pretreated, the samples are then subjected to instrumental analysis. For this purpose, original instrumental analytical methods have been developed, based on innovative techniques and technologies. The main technique exploited was based on separation by high performance liquid chromatography (HPLC), that allows to separate the compounds of interest, even in very complex matrices. Chromatographic methods are widely used for the determination of doping substances in biological [21, 22, 23] samples [24, 25, 26]. The method here developed were reverse-phase liquid chromatography systems in which the separation is provided by the affinity that a substance has with a stationary phase (SP), usually silica-base and functionalised with lipophilic functional groups. The passage of an eluent (called mobile phase, MP) moves the analytes at different speeds along the SP depending on their affinity for SP and MP, thus leading to the separation of the compounds, that will elute in different times (retention times,  $t_R$ ). In order to allow the detection of eluted compounds, the chromatographic systems were combined with different detection systems, including mass spectrometry, electrochemical detection, UV-Vis spectrophotometry and spectrofluorimetry. These techniques exploit the different chemical-physical characteristics of the molecules (such as the ability to absorb light, to emit light when hit by a wavelength or to oxidise/reduce under fixed current potentials) to allow their quantitative determination. In particular, mass spectrometry (MS) is able to qualify and quantify a substance by the generation of ions after an ionization step. Depending on the type of MS technology and the

parameters set, it is possible to identify the molecular ion (the ionised molecule of the substance) or a fragment of it, generated following the breakdown of the molecule. This strategy allows to obtain highly sensitive and selective methods, reducing the possibility of incurring in false positive errors. Each condition of the chromatographic systems and of the detection means have been designed and optimised in a careful manner, obtaining original analytical methods.

### **3. Method validation**

One of the main steps in developing new analytical approaches is the method validation process. This is performed by evaluating different parameters such as the linearity of the method, precision (*intraday* and *interday*), accuracy, sensitivity and selectivity. To this end, international organisations have drawn up a series of guidelines to be used as a reference. The two main guidelines for the development of analytical methods in the pharmaceutical field are those by the U.S. FDA [7] and the European Medicines Agency [27]. These documents describe the procedures to be carried out for the evaluation of the different validation parameters and the threshold values that must not be exceeded in order to consider the developed method reliable. Each of the approaches developed in this Ph.D. project has been validated by considering several parameters.

#### **3.1 Analytical measurement range and linearity**

To start validating a method, you must first have familiarity with the operating procedures, evaluate the measurement range, calibration and sensitivity limit. The quantification range of the method and then the concentrations of the calibration standards are chosen on the basis of the concentration range of the analyte expected in the patient samples. When the method is validated, the calibration curve shall be continuous and reproducible. Each calibration curve is characterised by the lower limit of quantification (LLOQ) and the upper limit of quantification (ULOQ) [7]. Each analytical session in LC-MS/MS requires that the calibration curve should give a determination coefficient ( $r^2$ ) of at least 0.990.

#### **3.2 Analytical specificity**

Analytical specificity is the property of the method to measure only the analyte of interest, without interference from other substances present in the sample. The specificity of a LCMS/MS method is ensured at detector level with multiple reaction monitoring (MRM) and at chromatography level (by successfully separating isobaric compounds) [7].

#### **3.3 Analytical sensitivity**

Analytical sensitivity is the ability to distinguish small differences in analyte concentrations. The detection limit (LOD) is the concentration at which the detector returns a signal of the analyte at least 5 times higher than the base signal of a blank sample [28]. The limit of quantification (LOQ) is the lowest concentration of an analyte that can be measured accurately. It is the minimum referable concentration and corresponds to the LLOQ. The limit of quantification is the parameter used in LC-MS/MS to quantify the sensitivity of the method and to determine it, average, standard deviation (SD) and coefficient of variation % (CV%) of 10 intra-replicates are calculated series around a low-concentration biological sample, pushing until the LOQ imprecision is no more than 20% [7].

### **3.4 Accuracy and precision**

In the validation of the method, the parameter of greatest interest is the accuracy, the degree of concordance between the result of a single measurement and the true value of the measured. Accuracy is the degree of agreement between the average value obtained from different determinations of the same measurement and an accepted reference value. It is expressed as bias which is the systematic deviation from true value. Accuracy will depend on how accurate the calibration materials are, their concentration and especially on the choice of matrix which should be as switchable as possible with that of the real samples. Calibrators with a defined degree of uncertainty, using certified reference material (CRM) [29] are preferred. Accuracy may be assessed by analysing for at least five measurements replicated in at least three different analytical sessions per concentration, using at least three samples at known concentrations (with reference measurement system, matrix certified reference materials). The accuracy is evaluated as a percentage bias with the following formula:

$$\text{Bias\%} = [(\text{mean value of measurements} - \text{target value}) / \text{target value}] * 100.$$

Where known concentration materials are not available, the misstatement may be estimated by recovery tests and dilution tests [7].

### **3.5 Recovery**

Recovery is the amount of analyte determined by a method of analysis in relation to the expected quantity, expressed as a percentage of the difference between measured concentrations and endogenous concentration and the added concentration. The difference between the optimum recovery (100%) and the value obtained determines the bias % value that can be used to assess the accuracy of the method.

$$\text{Recovery} = \frac{[(\text{measured concentration} - \text{endogenous concentration}) / \text{added concentration}] * 100}{100}$$

Recovery must be within 100 ± 15% [7].

### **3.6 Stability**

Once prepared, samples may not be analysed immediately. This may be due to waiting time in the autosampler, or to an instrument block. The same samples may also need to be retested the following day [7]. The stability study of processed samples is usually conducted by simulating situations that may occur in routine. The significance of the values obtained in subsequent injections compared to the first injection shall be evaluated. The sample is considered stable at the specific condition under assessment if the average concentration of each level falls within 15% of the concentration determined at time zero [7]. The purpose of the pre-analytical stability study is to evaluate the storage of the sample under different temperature conditions, so that its concentration does not deviate significantly from the initial sampling value. The stability study is an important part of the validation method to ensure proper management of biological samples throughout the analytical process. The parameters and conditions to be evaluated should be exceeded to cover the normal timing of sampling and analysis procedures (as estimated by normal laboratory management).

#### 4. References

- [1] B.B. Chaudhari, P. Sridhar, S. Moorkoth, L.E. Lewis, S. Mallayasamy. “*Validation of an HPLC method for estimation of cefotaxime from dried blood spot: alternative to plasma-based PK evaluation in neonates*”, *Bioanalysis.*, 2021, 13, 16.
- [2] A.L. Fabris, M. Yonamine. “*Dried matrix spots in forensic toxicology*”, *Bioanalysis.*, 2021, 13, 18.
- [3] V. Londhe, M. Rajadhyaksha. “*Opportunities and obstacles for microsampling techniques in bioanalysis: Special focus on DBS and VAMS*”, *J Pharm Biomed Anal.*, 2020, 182, 113102.
- [4] Y.C. Guerra Valero, S.C. Wallis, J. Lipman, C. Stove, J.A. Roberts, S.L. Parker. “*Clinical application of microsampling versus conventional sampling techniques in the quantitative bioanalysis of antibiotics: a systematic review*”, *Bioanalysis*, 2018, 10, 407-423.
- [5] K. Van Uytvanghe, L. Heughebaert, C.P. Stove., “*Self-sampling at home using volumetric absorptive microsampling: coupling analytical evaluation to volunteers' perception in the context of a large-scale study*”, *Clin Chem Lab Med.*, 2020, 59, e185-e187.
- [6] B.U.W. Lei, T.W. Prow. “*A review of microsampling techniques and their social impact*”, *Biomed Microdevices.* 2019, 21, 81.
- [7] “*Bioanalytical Method Validation – Guidance for Industry*”, U.S. Food and Drug Administration (FDA), 2018.
- [8] P. Sok, P.J. Lupo, M.A. Richard, K.R. Rabin, E.A. Ehli, N.A Kallsen, G.E. Davies, M.E. Scheurer, A.L. Brown. “*Utilization of archived neonatal dried blood spots for genome-wide genotyping*”, *PLoS One.* 2020,15, e0229352.
- [9] G.S. Moorthy, C. Vedar, K.J. Downes, J.C. Fitzgerald, M.H. Scheetz, A.F. Zuppa. “*Microsampling Assays for Pharmacokinetic Analysis and Therapeutic Drug Monitoring of Antimicrobial Drugs in Children: A Critical Review*”, *Ther Drug Monit.*, 2021, 43, 335-345.
- [10] P.E. Morgan. “*Microsampling Devices for Routine Therapeutic Drug Monitoring-Are We There Yet?*”, *Ther Drug Monit.* 2021, 43, 322-334.
- [11] J. Rudge, S. Kushon. “*Volumetric absorptive microsampling: its use in COVID-19 research and testing*”, *Bioanalysis.*, 2021, 13, 1851-1863.

- [12] P.D. Mingas, J. Zdovc, I. Grabnar, T. Vovk. “*The Evolving Role of Microsampling in Therapeutic Drug Monitoring of Monoclonal Antibodies in Inflammatory Diseases*”, *Molecules*, 2021, 26, 1787.
- [13] L. Mercolini, R. Mandrioli, V. Sorella, L. Somaini, D. Giocondi, G. Serpelloni, M.A. Raggi. “*Dried blood spots: liquid chromatography-mass spectrometry analysis of  $\Delta^9$ -tetrahydrocannabinol and its main metabolites*”, *J Chromatogr A.*, 2013, 1271, 33-40.
- [14] R. Mandrioli, L. Mercolini, M. Protti. “*Blood and Plasma Volumetric Absorptive Microsampling (VAMS) Coupled to LC-MS/MS for the Forensic Assessment of Cocaine Consumption*”, *Molecules*. 2020, 25, 1046.
- [15] M. Abdel-Rehim, S. Pedersen-Bjergaard, A. Abdel-Rehim, R. Lucena, M.M. Moein, S. Cárdenas, M. Miró. “*Microextraction approaches for bioanalytical applications: An overview*”, *J Chromatogr An.*, 2020, 1616, 460790.
- [16] B.R.B.D. Costa, W.J.R. Santos Júnior, I.F. Maximiano, N.C. Gomes, B.T. Freitas, B.S. De Martinis. “*Application of microextraction techniques in alternative biological matrices with focus on forensic toxicology: a review*”, *Bioanalysis*, 2021, 13, 45-64.
- [17] M.C. Díaz-Liñán, M.T. García-Valverde, R. Lucena, S. Cárdenas, A.I. López-Lorente. “*Paper-based sorptive phases for microextraction and sensing*”, *Anal Methods*, 2020, 12, 3074-3091.
- [18] J. Millán-Santiago, R. Lucena, S. Cárdenas. “*Wooden-based materials: Eco-friendly materials for direct mass spectrometric analysis and microextraction*”, *J Sep Sci.*, 2022, 45, 223-232.
- [19] F.A. Casado-Carmona, G. Lasarte-Aragónés, A. Kabir, K.G. Furton, R. Lucena, S. Cárdenas. “*Fan-based device for integrated air sampling and microextraction*”, *Talanta.*, 2021, 230, 122290.
- [20] F.A. Casado-Carmona, R. Lucena, S. Cárdenas. “*Magnetic paper-based sorptive phase for enhanced mass transference in stir membrane environmental samplers*”, *Talanta.*, 2021, 228, 122217.

- [21] L. Mercolini, M. Protti, M.C. Catapano, J. Rudge, A.E. Sberna. “*LC-MS/MS and volumetric absorptive microsampling for quantitative bioanalysis of cathinone analogues in dried urine, plasma and oral fluid samples*”, *J Pharm Biomed Anal.*, 2016, 123, 186-94.
- [22] A.A. Al-Qurain, D.B. Williams, L. Mackenzie, M.S. Roberts, W.D. Wiese. “*Simultaneous LC-MS/MS quantification of oxycodone, tramadol and fentanyl and their metabolites (noroxycodone, oxymorphone, O-desmethyltramadol, N-desmethyltramadol, and norfentanyl) in human plasma and whole blood collected via venepuncture and volumetric absorptive micro sampling*”, *J Pharm Biomed Anal.*, 2021, 203, 114171.
- [23] M.M. Madry, T. Kraemer, M.R. Baumgartner. “*Cocaine adulteration with the anthelmintic tetramisole (levamisole/dexamisole): Long-term monitoring of its intake by chiral LC-MS/MS analysis of cocaine-positive hair samples*”, *Drug Test Anal.* 2019, 11, 472-478.
- [24] M. Protti, V. Brighenti, M.R. Battaglia, L. Anceschi, F. Pellati, L. Mercolini. “*Cannabinoids from Cannabis sativa L.: A New Tool Based on HPLC-DAD-MS/MS for a Rational Use in Medicinal Chemistry*”, *ACS Med Chem Lett.* 2019, 10, 539-544.
- [25] T.R. Fiorentin, M. Fogarty, R.P. Limberger, B.K. Logan. “*Determination of cutting agents in seized cocaine samples using GC-MS, GC-TMS and LC-MS/MS*”, *Forensic Sci Int.* 2019, 295, 199-206.
- [26] S. Münster-Müller, S. Hansen, T. Opatz, R. Zimmermann, M. Pütz. “*Chemical profiling of the synthetic cannabinoid MDMB-CHMICA: Identification, assessment, and stability study of synthesis-related impurities in seized and synthesized samples*”, *Drug Test Anal.* 2019, 11, 1192-1206.
- [27] “*Guideline on bioanalytical method validation*”, European Medicinal Agency (EMA), 2011.
- [28] D'Avolio A., Cantù M., Gervasoni J., Artusi C., Marinova M., Nonnato A., Cangemi G., Persichilli S. Validation of quantitative bioanalytical methods in mass spectrometry. *Clinical Biochemistry*; 2018. 42(1), 51-61.
- [29] CLSI C62 Liquid Chromatography-Mass Spectrometry Methods. Clinical and Laboratory Standards Institute; 2022.

# **Chapter IV**

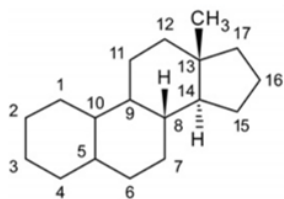
## **DEVELOPMENT OF ANALYTICAL METHODS**

## **1. Dried blood spot coupled to liquid chromatography-tandem mass spectrometry for the analysis of anabolic androgenic steroids.**

### **1.1 Introduction**

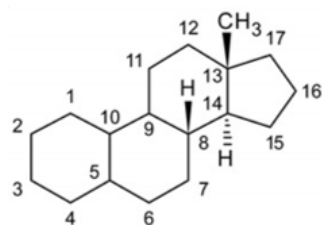
The increasingly frequent use of anabolic androgenic steroids (AAS) in the sports community has led analysis laboratories to search for methods characterized by high sensitivity and selectivity, to identify and quantify these compounds in biological matrices [1]. The use of AAS is very common among athletes, as these compounds modify muscle strength and size, increasing the ratio of lean mass to fat mass and thus amplifying the results of exercise [2]. It is not only professional athletes who use banned substances; amateurs also use of AAS extensively, and this could lead to a complex public health problem, as taking doping substances can cause serious side effects [3]. The side effects or allergic reactions resulting from the use of anabolic steroids are very serious. They affect the cardiovascular system, the reproductive system, the endocrine system, the liver, the osteoarticular system and the neuropsychic system [4,5]. People who use AAS can experience strokes, heart attacks, a significant decrease in testosterone production by the testes, the appearance of female characteristics such as breast growth in men, the body's own hormonal regulation is disturbed and, to some extent, inhibited, so that the body produces less endogenous hormones, liver cell damage can occur and, in some cases, tumours [6,7]. In people who use these substances, an increase in the fragility of the insertions resulting from a reduction in their elasticity has been observed, and it has been ascertained that increased muscle strength associated with reduced elasticity facilitates the occurrence of sprains or ruptures [8]. It has been showed that individuals who abuse AAS are more likely to incur neuropsychiatric disorders such as anxiety, paranoia, and depression. Although the possible role of AAS in causing neurotoxicity is still unclear, evidence of prevalent neurotoxic effects after administration of synthetic AAS is accumulating [9]. Therefore, AAS have been included in the World Anti-Doping Agency (WADA) list of banned substances, both in and outside of competitions, and why it is of utmost importance to develop and update analytical tools to reliably detect their use and possible abuse [10]. In the recent past, gas chromatography (GC) coupled with mass spectrometry (MS) has been routinely applied for screening and confirmatory analysis of AAS and metabolites



[11,12,13,14]. However, the analysis of AAS by GC-MS requires lengthy derivatisation steps and still suffers from sensitivity and selectivity problems for some steroids present at very low levels. Liquid chromatography (LC) coupled with tandem MS (MS/MS) is increasingly used as an alternative analytical tool to GC-MS procedures. There are several reports on the analysis of AAS using tandem liquid chromatography-mass spectrometry (LC-MS/MS) in human samples [15,16]. This method is often applied in routine antidoping analyses on urine samples, because this matrix contains relatively high concentrations of drugs, and its sampling is non-invasive. However, traditional urine sampling and handling have disadvantages: they require storage and transport at controlled temperatures, increasing the overall cost of analysis and the complexity of the pre-analysis phases. In addition, the possible presence of microorganisms in urine samples can alter AAS profiles, as they can trigger metabolism in stored samples [17,18]. Indeed, the stability of the sample over time is a crucial aspect, especially in doping activities, where reanalysis of the sample is required for confirmation, as in the case of "sample B", reanalysed to ascertain any positivity resulting from the first analysis [19]. The purpose of this work is the development of an original blood sampling and pre-treatment technique coupled to a fully validated LC-MS/MS method for AAS analysis, in order to overcome the current limitations arising from the use of large volumes of fluid samples and to develop new simplified procedures while maintaining high reliability and robustness of the resulting analytical data [20]. Six representative AAS were considered, namely, Androstenedione, Testosterone, Epitestosterone, which are endogenous in nature, Methandrostenolone, Stanozolol and Danazol, which are exogenous in nature (Table 1a,1b).



Carbon N.	Androstenedione	Testosterone	Epitestosterone	Methandrostenolone
1	-H	-H <sub>2</sub>	-H <sub>2</sub>	-H
2	-H	-H <sub>2</sub>	-H <sub>2</sub>	-H
1,2	-C=C-			-C=C-
3	=O	=O	=O	=O
2,3				
4	-H <sub>2</sub>	-H	-H	-H
5	-H α			
4,5		-C=C-	-C=C-	-C=C-
6	-H <sub>2</sub>	-H <sub>2</sub>	-H <sub>2</sub>	-H <sub>2</sub>
5,6				
9	-H α	-H α	-H α	-H α
10	-CH <sub>3</sub> β	-CH <sub>3</sub> β	-CH <sub>3</sub> β	-CH <sub>3</sub> β
11	-H <sub>2</sub>	-H <sub>2</sub>	-H <sub>2</sub>	-H <sub>2</sub>
17	=O	-OH β	-OH α	-OH β, -CH <sub>3</sub> α

**Table 1a.** Chemical structures of the analytes (first part)



Carbon N.	Stanozolol	Danazolol
1	-H <sub>2</sub>	-H <sub>2</sub>
2		
3		
2,3		
4	-H <sub>2</sub>	-H
5	-Hα	
4,5		-C=C-
6	-H <sub>2</sub>	-H <sub>2</sub>
9	-Hα	-Hα
10	-CH <sub>3</sub> β	-CH <sub>3</sub> β
11	-H <sub>2</sub>	-H <sub>2</sub>
17	-CH <sub>3</sub> α	-C=CHα

**Table 1b.** Chemical structures of the analytes (second part)

The Dried Blood Spot (DBS) technique is an innovative and promising alternative to traditional in-tube sampling [21]. DBS has numerous advantages over traditional urine sampling: simplified sample collection, increased stability of compounds, no requirement for transport and storage at controlled temperature, overall cost savings [22]. In addition, compared with traditional venous blood sampling, DBS sampling is non-invasive and feasible in a non-hospital setting without the need for specialized personnel. Due to these advantages, the DBS technique also provides a reduction of matrix effect and interference [23,24] The analytical approach for dried blood microsamples presented here aims to provide useful data for the implementation of rapid and simple analytical protocols, with potential immediate applicability in sports doping test scenarios and neurotoxicity studies. DBS has the potential to add to the current global antidoping program by integrating existing blood and urine tests to expand program test coverage and the stability to better reveal doping practices [25]. The study is extremely innovative as there are few studies in the literature on DBS applied to AAS for anti-doping analysis [26].

## **1.2 Materials and Methods**

### **1.2.1 Chemicals and solutions**

The analyte standards Testosterone (17-hydroxyandrost-4-en-3-one), Epitestosterone (17 hydroxyandrost-4-en-3-one), Androstenedione (androst-4-ene-3,17-Dione), Stanozolol (17 $\beta$ -hydroxy-17 $\alpha$ -methyl-5; -androstan[3,2-c] pirazole), Danazol (17 $\alpha$ -ethylene-17 $\beta$ -hydroxy-4-androsten-[2,3-d] isoxazole) (98%) and Metandrostenolone (17 $\beta$ -Hydroxy-17-methylandrosta-1,4-dien-3-one) (97%), were purchased as well powders from Sigma-Aldrich (Saint Louis, MI, US). Methanol for HPLC (99.9%), acetonitrile for HPLC (99.9%), formic acid 85% (w/w) and dibasic sodium phosphate were purchased by Sigma-Aldrich (Milan, Italy). The ultrapure water (18.2 M $\Omega$  cm) was obtained thanks to the use of a milliq apparatus of the company Millipore (Milford, MA, US) MilliQ® apparatus. Analytes working solutions were obtained by diluting stock solutions with a mixture of 0.1% FA in acetonitrile and 0.1% FA in water (50/50). Stock solutions were stored at -20 °C until used and all solutions were used and conserved in amber glass vials from Waters (Milford, MA, USA). The DBS

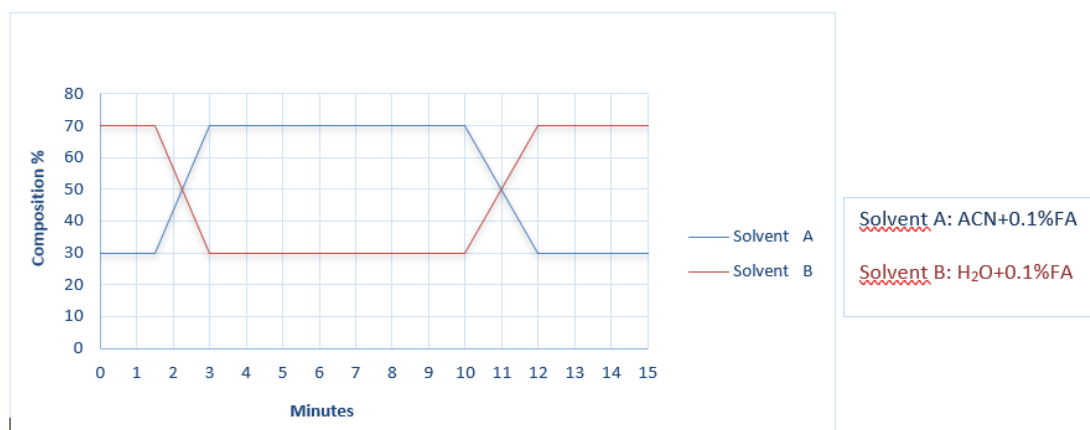
(Protein Saver<sup>TM</sup> 903<sup>TM</sup> Card) cards were provided by Whatman<sup>TM</sup> GE Healthcare Bio-Sciences Corp (Italy).

### 1.2.2 LC–MS/MS system

The LC equipment is a Waters Alliance e2695 system equipped with an autosampler. Chromatographic separations were performed using an Agilent Zorbax SB-C18 column (50 mm x 2.1 mm, I.D. 3.5 m), maintained at room temperature and preceded by a Phenomenex C18 guard column (4x2 mm). The mobile phase consisted of 0.1% AF formic acid in acetonitrile ACN (solvent A) and 0.1% AF formic acid in ultrapure water H<sub>2</sub>O (solvent B). The gradient pattern of the mobile phase composition is shown in Table 2 and in the graph in Figure 1.

Minutes	Solvent A % (ACN+0.1% FA)	Solvent B % (H <sub>2</sub> O+ 0.1% FA)
0.00	30	70
1.50	30	70
3.00	70	30
10.0	70	30
12.0	30	70
15.0	30	70

**Table 2.** Gradient scheme of mobile phase composition



**Figure 1.** Gradient pattern of the mobile phase composition

Mass spectrometry acquisition was performed on a Waters Micromass Quattro Micro triple quadrupole, using an electrospray ionization source operating in

positive mode (ESI+) with ionization polarity switching, with the following optimized settings: ion source voltage, 3.6 kV; ion source temperature, 120 °C; desolvation temperature, 100 °C; desolvation gas flow (nitrogen), 750 L/h; argon was used as the collision gas. Dwell times per channel were set at 300 ms for each analyte. The precursor and product ions, with cone voltage and collision energy values for each analyte, are shown in Table 3.

Compound	MW	MS/MS				
		Parent ion	Main charge state	Daughter ions <sup>a</sup>	Cone Voltage (V)	Collision Energy (eV)
Testosterone	288.42	289.36	[M+H] <sup>+</sup>	109.20	33.00	19.00
				<i>97.10</i>	33.00	17.00
Androstenedione	286.41	287.52	[M+H] <sup>+</sup>	109.20	29.00	21.00
				<i>97.10</i>	29.00	25.00
Epiandrosterone	288.42	289.36	[M+H] <sup>+</sup>	97.10	33.00	19.00
				<i>109.20</i>	33.00	17.00
Danazol	337.46	338.43	[M+H] <sup>+</sup>	120.20	37.00	27.00
				<i>148.00</i>	37.00	27.00
<del>Stanozolol</del>	328.49	329.57	[M+H] <sup>+</sup>	81.95	55.00	31.00
				<i>95.00</i>	55.00	31.00

<sup>a</sup> *In italics*, confirmatory product ions.

**Table 3.** Analyte-dependent MS/MS parameters

Data were acquired in multiple reaction monitoring mode (MRM) and processed using Waters MassLynx 4.1 software.

### 1.2.3 Microsample Collection and Pre-treatment

The miniaturized sampling strategy and pre-treatment protocol developed during this experimental work was tested by using whole blood drawn from six healthy volunteers. Following a puncture using a disposable lancet, 10 µL of blood was drawn from the fingertips of the volunteers via a glass capillary, and then deposited on a DBS card. The blood was left to dry for 75 min at room temperature. When the blood was dry, a 10-mm-diameter spot was taken from the card with a perforator and placed in an amber vial, into which 500 µL of methanol was added. The vial containing the sample, was subjected to the ultrasonic bath for 5 minutes and then centrifuged at 4000 rpm for 5 minutes at 4°C. The supernatant was brought to dryness under a flow of N<sub>2</sub> and redissolved with 100 µL of methanol. To perform analysis on spiked samples, DBS cards were fortified with 10 µL of a standard solution containing the analytes at known

concentrations. The obtained spiked blood samples were then subjected to pre-treatment and LC-MS/MS analysis.

#### **1.2.4 DBS**

Whatman® Protein Saver™ 903™ Card (Italy) cards were used for DBS collection, where 10 µL of whole blood were transferred into the centre of pre-marked circles. DBS were left to dry for 75 min at RT and then stored in a sealed plastic bag containing a suitable desiccant amount (i.e., silica gel) until pre-treatment and analysis.

### **1.3 Method Validation**

#### **1.3.1 Calibration Curves**

Aliquots of 10 µL of standard solutions of analytes at seven different concentrations, were added to the blank matrices before sampling. The resulting DBS samples were subjected to the pre-treatment procedures previously described and finally injected into the LC-MS/MS system. The ratios between the peak area of the analyte and standards obtained were plotted against the corresponding analyte concentrations (expressed in ng/mL), and the calibration curves were set by the method of least squares. The limit of quantification (LOQ) and limit of detection (LOD) values were calculated according to the International Conference on Harmonization (ICH) [27], which defines them as the analyte concentration whose signal corresponds to 10 and 3 times the signal of the background noise, respectively. Method detection limits (MDLs) were calculated on 7 replicates according to Environmental Protection Agency (EPA) guidelines [28].

#### **1.3.2 Recovery and Precision Assays**

Absolute recovery and precision were evaluated by adding known amounts of the analytes (at three different concentrations representative of each calibration curve) to blank DBS, then subjecting the mixtures to the sample pre-treatment and injecting them into the LC-MS/MS system. The analyte peak areas were compared to those obtained by injecting standard solutions at the same theoretical concentrations and absolute recovery was calculated. The assays described above were repeated five times within the same day to obtain intraday precision (repeatability) and five times over five different days to obtain interday precision (intermediate precision), both expressed as relative standard deviation (RSD%).

### **1.3.3 Selectivity**

Blank samples from six different volunteers were subjected to the pretreatment procedure and injected into the HPLC-MS/MS system. The resulting chromatograms were checked for possible interference from endogenous compounds. The acceptance criterion was no interfering peak higher than an analyte peak corresponding to its LOD.

### **1.3.4 Stability**

The short- and medium-term stability of the analytes in the matrix was tested. After sample collection, DBS was fortified with a standard mixture of the analytes at a given concentration, and then, once sample pre-treatment was performed, LC-MS/MS analysis was carried out at time zero and after precise time intervals. To assess the short- and medium-term stability of the analytes in matrix, the areas of each analyte at time zero, after 7 days and after 14 days were measured and subsequently compared. During these time intervals, the fortified samples were stored at room temperature away from light and heat sources.

## **1.4 Results and Discussion**

### **1.4.1 LC-MS/MS conditions**

MS and MS/MS spectra of the analytes were acquired by direct infusion of 1 g/mL solutions at 20 L/min, using a mixture of 0.1% FA in acetonitrile and 0.1% FA in water (50/50) for dilutions. All spectra were acquired using both ESI+ and ESI- ionization mode, in order to choose the best MS/MS conditions for each analyte. A suitable chromatographic setup was then chosen for the simultaneous analysis of the six AAS, chosen for this study. After screening some RP columns with C8 and C18 sorbents, normal bore (4.6-3.0 mm) and medium resolution (5-m diameter) particles, with the aim of total analysis time reduction, a special C18 column with both narrow bore (2.1 mm) and higher-resolution particles (2.5  $\mu$ m) was selected. It dealt with Zorbax SB C18 (2.1 mm  $\times$  50 mm), an inorganic-organic hybrid sorbent coupled to enhanced end capping, specifically developed for MS applications. To be used with this C18 column, three different mobile phase acidic additives were tested: FA, acetic acid and trifluoroacetic acid at the concentrations of 0.05%, 0.1% and 0.2%, both with acetonitrile and methanol as the organic modifier. The use of a mixture composed of 0.1% FA in acetonitrile

and 0.1% FA in water, under composition gradient conditions at a flow rate of 0.35 mL/min, produced sharp, symmetric and baseline resolved peaks for all analytes, with a total chromatographic run of 15 min. The MRM chromatograms obtained are shown in Figure 2.

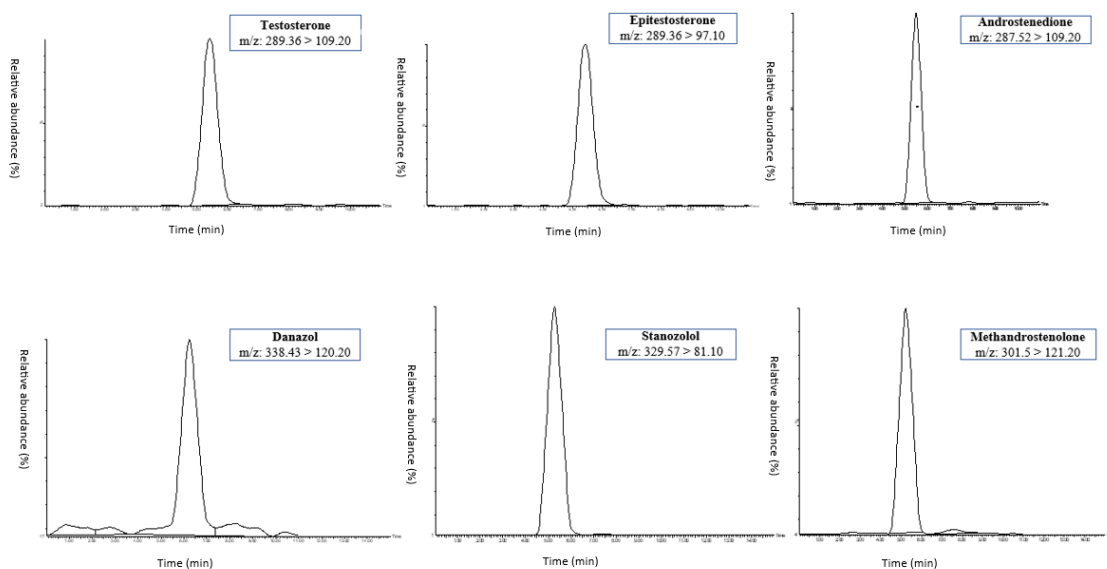
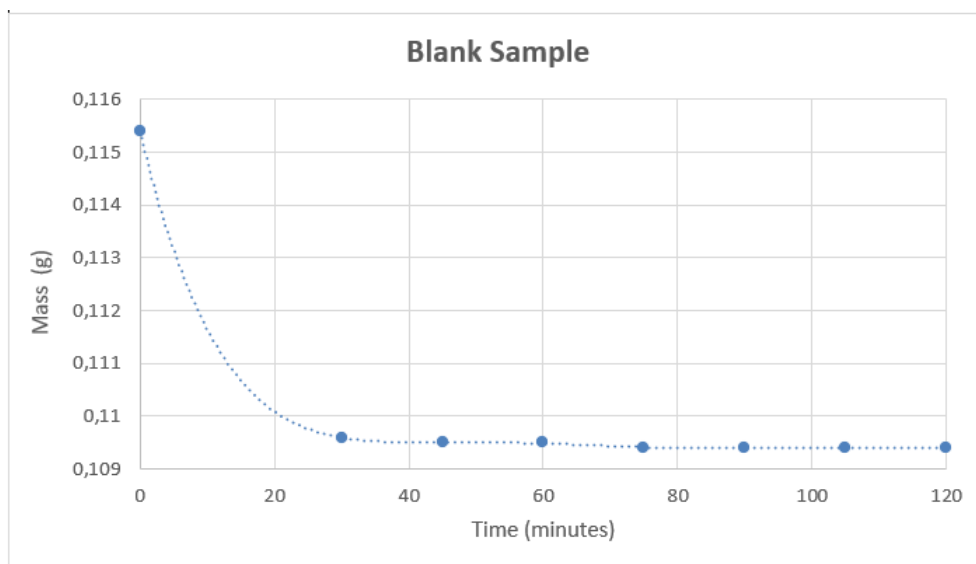


Figure 2. LC-MS/MS chromatograms of six studied AAS

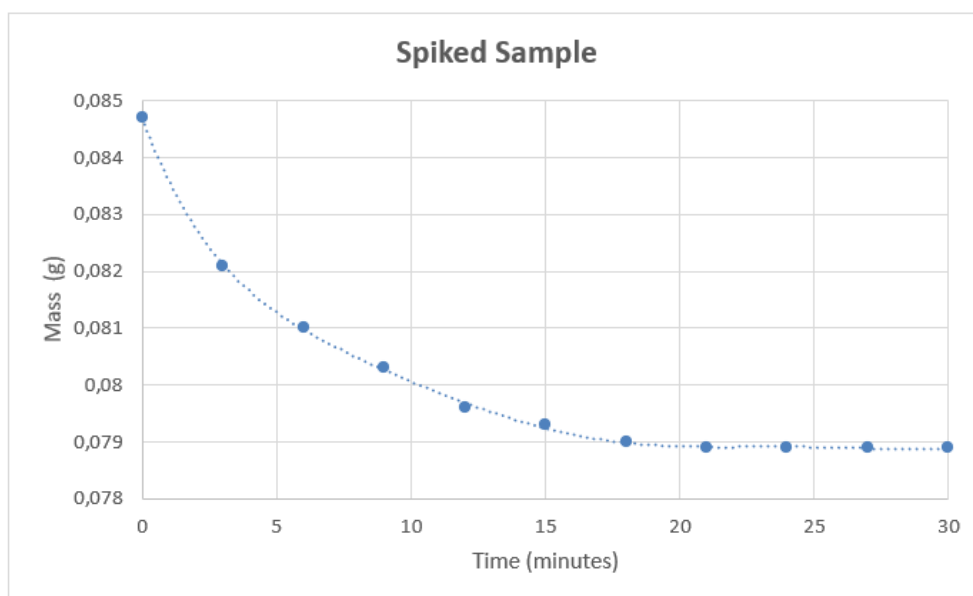
### 1.4.2 DBS development

Since a single blood drop from a finger prick can vary in volume and the spread of blood on the card can vary depending on density (HCT), potential volumetric bias can be circumvented through the accurate sampling and spotting of 10 L of blood by using end-to-end glass capillaries followed by a whole-spot analysis instead of spot sub-punching. In this way those issues concerning blood spread can be avoided. The cellulose-based cards were tested (Whatman 903 Protein Saver). Blood adsorption onto the paper can be considered itself a first step of pretreatment, which can retain several matrix components in the paper. After gravimetric analysis, we could see that drying of 10  $\mu$ L of blood occurs after 75 min at RT (Figure 3).



**Figure 3.** Gravimetric analysis on blank sample

Then the entire dried spot was fortified 10  $\mu\text{L}$  of a mix containing the analytes of interest, that took 20 minutes to be absorbed by the card. Again, a gravimetric test was performed (Figure 4).



**Figure 4.** Gravimetric analysis on spiked sample

Different extraction solvents and volumes were tested: methanol, acetonitrile, and phosphate buffer (50 mM, pH 7.4), as well as mixtures of these solvents. An aliquot of 500  $\mu\text{L}$  of pure methanol resulted in very satisfactory extraction yield values, so, no further cleaning procedure was necessary. The extraction

supernatant was then collected, brought to dryness under a gentle stream of nitrogen, redissolved in 100  $\mu$ L of MeOH, and injected into the LC-MS/MS system.

### **1.4.3 Calibration curves**

To determine linearity, calibration curves were constructed, by the method of least squares, of all analytes at seven different concentrations by injecting standard mixtures in triplicate. Good results were obtained because the linearity coefficients ( $r^2$ ) were always greater than or equal to 0.9981. LODs and LOQs for individual analytes were determined; since LODs are less than 2 ng/mL and LOQs less than 5 ng/mL, the method enjoys good sensitivity. These LODs also meet the MRPLs required by WADA for antidoping analysis of exogenous AAS. Linearity on the spiked microsamples was determined on miniature blood samples containing known concentrations of analytes; thus, it was possible to construct calibration curves, calculate the linear correlation coefficient ( $r^2$ ), and determine values regarding the LOD and at the LOQ for each individual compound. Spiked blood samples were obtained by adding aliquots of standard solutions of the analytes at seven different concentrations. The spiked matrix was sampled through the above devices and subjected to the pre-treatment procedure described above before being injected into the LCMS/MS system. The data are satisfactory: we obtain linear correlation coefficient values greater than or equal to 0.9981 for each analyte. The limits of detection (LOD) are less than 2 ng/mL, and the limits of quantification (LOQ) less than 5 ng/mL. These results give the method a high sensitivity. All results are shown in Table 4.

Compound	Linearity range (ng/mL) <sup>a</sup>	$r^2$	LOD (ng/mL)	LOQ (ng/mL)
Testosterone	5-500	0.9993 0.9992	1.4	4.6
Androstenedione	5-500	0.9981 0.9980	1.4	4.6
Epitestosterone	5-500	0.9989 0.9990	1.1	3.5
Danazol	5-500	0.9997 0.9994	0.4	1.2
Stanozolol	5-500	0.9991 0.9994	1.5	4.9
Methandrostenolone	5-500	0.9997 0.9983	1.3	4.2


<sup>a</sup>  $n = 6$ .

**Table 4.** Linearity, limit of quantification (LOQ), limit of detection (LOD)

#### 1.4.4 Recovery and Precision Assays

For tests regarding intraday and interday precision, three different concentrations were examined, representative of the entire linearity ranges: 50 ng/mL, 75 ng/mL, and 100 ng/mL. Intraday precision was obtained by injecting standard mixtures of the analytes six times within the same day; while for interday precision data, the mixtures were injected on six different days while maintaining the same concentrations and conditions as for intraday precision. The results obtained from the intraday and interday precision tests are expressed as percent relative standard deviation (RSD%) and are satisfactory being always less than 5.4%, as shown in Table 5.

Compounds	(ng/mL)	RSD%	RSD%
		<i>interday</i>	<i>intraday</i>
Testosterone	50	3.8	4.2
	75	2.2	2.9
	100	1.8	2.1
Epitestosterone	50	3.5	3.9
	75	2.3	2.6
	100	2.0	2.3
Androstenedione	50	5.0	5.3
	75	4.2	4.5
	100	2.5	2.9
Stanozolol	50	3.8	4.4
	75	3.0	3.8
	100	1.8	2.2
Danazol	50	4.0	4.2
	75	3.3	3.5
	100	1.9	2.1
Methandrostenolone	50	4.0	4.4
	75	2.5	2.8
	100	2.0	2.4

**Table 5.** Interday e intraday values obtained on standard mixtures  (CTRL) ▾

Following the standard tests, intraday and interday precision on blood microsamples were performed in the same manner, analysing three different concentrations representative of the respective linearity ranges. Intraday precision for spiked DBS was obtained by injecting the mixtures six times during the same day at the three concentrations of interest, whereas for interday precision data, the mixtures were injected on six different days maintaining the same concentrations and conditions as intraday precision. The results obtained from the intraday and interday precision tests for spiked DBS are expressed as a percentage relative standard deviation (RSD%) and are satisfactory, being less than 9.5%, as shown in Table 6.

<b>Compounds</b>	<b>(ng/mL)</b>	<b>RSD%</b> <i>interday</i>	<b>RSD%</b> <i>intraday</i>
<b>Testosterone</b>	50	9.5	7.4
	100	8.8	11.2
	500	6.9	9.3
<b>Epitestosterone</b>	50	9.0	5.8
	100	9.1	7.6
	500	3.7	7.3
<b>Androstenedione</b>	50	7.7	8.6
	100	7.0	9.2
	500	8.9	6.5
<b>Stanozolol</b>	50	5.7	9.5
	100	9.3	9.4
	500	3.2	9.4
<b>Danazol</b>	50	4.3	9.5
	100	8.9	9.0
	500	9.3	9.5
<b>Methandrostenolone</b>	50	9.1	8.6
	100	8.5	8.6
	500	9.0	8.9

**Table 6.** Interday e intraday values obtained on spiked DBS

From the DBS analysis, the average values of the extraction yields are shown in Table 7.

<b>Compounds</b>	<b>(ng/mL)</b>	<b>Recovery %</b>
<b>Testosterone</b>	50	75
	75	72
	100	70
<b>Epitestosterone</b>	50	81
	75	79
	100	83
<b>Androstenedione</b>	50	85
	75	77
	100	74
<b>Stanozolol</b>	50	87
	75	83
	100	72
<b>Danazol</b>	50	92
	75	90
	100	96
<b>Methandrostenolone</b>	50	85
	75	78
	100	90

**Table 7. Recovery obtained for three different concentrations**

These values are the average of three independent analyses at three different concentration levels.

#### **1.4.5 Selectivity**

Blank samples from six different volunteers were subjected to the pre-treatment procedure and injected into the HPLC-MS/MS system. The resulting chromatograms were checked for possible interference from endogenous compounds. The acceptance criterion was no interfering peak higher than an analyte peak corresponding to its LOD.

#### **1.4.6 Stability**

Stability was tested in methanolic stock solutions for each analyte stored for 14 days at -20°C for comparison with freshly purchased solutions. To assess the stability of the analytes in the dried matrices, blank samples were spiked with the analytes and then analysed at regular intervals up to 14 days after sampling. During this period, the DBSs were stored at RT, in a dark and dry place. The

results obtained were compared with those of spiked samples analysed immediately after staining and drying. From the observation of the values shown in Table 8, we can state that the short-to-medium-term stability of the analytes in matrix is acceptable.

Compounds	Recovery %		
	t 0	7 days	14 days
Testosterone	80	80	72
Epitestosterone	78	72	79
1-Androstenedione	85	81	61
Stanozolol	72	64	63
Danazol	75	68	67
Methandrostenolone	75	72	66

**Table 8. Recovery obtained at different times**

### 1.5. Conclusion

An innovative method of microsampling, based on DBS, has been developed for the purpose of antidoping analysis. A dried sample has been shown to be much more practical than traditional urine: it can be stored for up to 14 days at room temperature without losing more than 9% of its original analyte content. In addition, because they are miniaturized samples, they take up much less shelf space and storage equipment and require only minimal amounts of solvents for extraction. Finally, validation and comparison assays have provided ample assurance that this micromachine sampling approach provides comparable, and often even better, performance than the corresponding fluid urine matrix in terms of absolute recovery (>70%), precision (RSD<7.6%), matrix effect () and accuracy (range 89-97%). Comparison with existing methods shows that most of them require lengthy pre-treatment steps, which often include derivatization of the sample. Moreover, many methods are not fully validated, and those that are validated have similar or worse analytical performance than the proposed method in terms of absolute recovery and matrix effect. Some methods achieve exceptional sensitivity, but naturally using higher sample volumes; however, they still have the drawback of dealing with fluid urine samples in terms of handling, shipping, storage and related expenses. The dry matrix approach can be said to be

significantly better than the classical approach because it can be customized and used according to the needs of the moment, available equipment and personnel, and local laws. The results obtained lay the groundwork for the forthcoming development and optimization of standardized sampling and extraction protocols with immediate applicability, both for preliminary screening and confirmatory analysis that can be easily implemented by both accredited laboratories deputed to monitor professional athletes and regional laboratories for the monitoring and safeguarding of amateur athletes.

## 1.6 References

- [1] Protti M.; Marasca C.; Cirrincione M.; Sberna A.E.; Mandrioli R.; Mercolini L. *Dried Urine Microsampling Coupled to Liquid Chromatography—Tandem Mass Spectrometry (LC–MS/MS) for the Analysis of Unconjugated Anabolic Androgenic Steroids*, Boguslaw Buszewski. 2020
- [2] Hartgens, F.; Kuipers, H. Effects of androgenic-anabolic steroids in athletes. *Sports Med.* 2004, 34, 513–554.
- [3] Piacentino, D.; Kotzalidis, G.D.; Del Casale, A.; Aromatario, M.R.; Pomara, C.; Girardi, P.; Sani, G. Anabolic-androgenic steroid use and psychopathology in athletes. A systematic review. *Curr. Neuropharmacol.* 2015, 13, 101–121.
- [4] Pope, H.G., Jr.; Kanayama, G.; Hudson, J.I. Risk factors for illicit anabolic androgenic steroid use in male weightlifters: A cross-sectional cohort study. *Biol. Psychiatry* 2012, 71, 254–261.
- [5] Bruno M. Gli steroidi anabolizzanti e il loro abuso nella pratica sportiva [Anabolic steroids and their abuse in sports]. *Clin Ter.* 1990 Nov 15;135(3):159-72.
- [6] Nieschlag, E.; Vorona, E. Doping with anabolic androgenic steroids (AAS): Adverse effects on non-reproductive organs and functions. *Rev. Endocr. Metab. Disord.* 2015, 16, 199–211.
- [7] Kibble MW, Ross MB. Adverse effects of anabolic steroids in athletes. *Clin Pharm.* 1987 Sep;6(9):686-92.
- [8] Hartgens F, Kuipers H. Effects of androgenic-anabolic steroids in athletes. *Sports Med.* 2004;34(8):513-54.
- [9] Pomara, C.; Neri, M.; Bello, S.; Fiore, C.; Riezzo, I.; Turillazzi, E. Neurotoxicity by synthetic androgen steroids: Oxidative stress, apoptosis, and neuropathology: A review. *Curr. Neuropharmacol.* 2015, 13, 132–145.
- [10] World Anti-Doping Agency, the 2022 Prohibited List. Available online: [https://www.wada-ama.org/sites/default/files/wada\\_2022\\_english\\_prohibited\\_list\\_0.pdf](https://www.wada-ama.org/sites/default/files/wada_2022_english_prohibited_list_0.pdf) (accessed on 9 November 2022).
- [11] Wang, J.; Wu, M.; Liu, X.; Xu, Y. Profiling of urinary steroids by gas chromatography-mass spectrometry detection and confirmation of androstenedione administration using isotope ratio mass spectrometry. *Steroids* 2011, 76, 1560–1565.
- [12] Van Eenoo, P.; Van Gansbeke, W.; De Brabanter, N.; Deventer, K.; Delbeke, F.T. A fast, comprehensive screening method for doping agents in urine by gas chromatography-triple quadrupole mass spectrometry. *J. Chromatogr. A* 2011, 1218, 3306–3316.

- [13] Raro, M.; Portolés, T.; Pitarch, E.; Sancho, J.V.; Hernández, F.; Garrosta, L.; Marcos, J.; Ventura, R.; Segura, J.; Pozo, O.J. Potential of atmospheric pressure chemical ionization source in gas chromatography tandem mass spectrometry for the screening of urinary exogenous androgenic anabolic steroids. *Anal. Chim. Acta* 2016, 906, 128–138.
- [14] Cha, E.; Kim, S.; Kim, H.J.; Lee, K.M.; Kim, K.H.; Kwon, O.S.; Lee, J. Sensitivity of GC-EI/MS, GC-EI/MS/MS, LC-ESI/MS/MS, LC-Ag (+) CIS/MS/MS, and GC-ESI/MS/MS for analysis of anabolic steroids in doping control. *Drug Test. Anal.* 2015, 7, 1040–1049.
- [15] Guo, F.; Shao, J.; Liu, Q.; Shi, J.-B.; Jiang, G.-B. Automated and sensitive determination of four anabolic androgenic steroids in urine by online turbulent flow solid-phase extraction coupled with liquid chromatography-tandem mass spectrometry: A novel approach for clinical monitoring and doping control. *Talanta* 2014, 125, 432–438.
- [16] Fragkaki, A.G.; Petropoulou, G.; Athanasiadou, I.; Kiouisi, P.; Kioukia-Fougia, N.; Archontaki, H.; Bakeas, E.; Angelis, Y.S. Determination of anabolic androgenic steroids as imidazole carbamate derivatives in human urine using liquid chromatography–tandem mass spectrometry. *J. Sep. Sci.* 2020, 43, 2154–2161.
- [17] Mazzarino, M.; Abate, M.G.; Alocci, R.; Rossi, F.; Stinchelli, R.; Molaioni, F.; de la Torre, X.; Botrè, F. Urine stability and steroid profile: Towards a screening index of urine sample degradation for antidoping purpose. *Anal. Chim. Acta* 2011, 683, 221–226.
- [18] Ojanperä, S.; Leinonen, A.; Apajalahti, J.; Lauraeus, M.; Alaja, S.; Moisander, T.; Kettunen, A. Characterization of microbial contaminants in urine. *Drug Test. Anal.* 2010, 2, 576–581.
- [19] The World Anti-Doping Code. International Standard for Testing and Investigations (ISTI). Available online: [https://www.wada-ama.org/sites/default/files/resources/files/isti\\_november2022\\_0.pdf](https://www.wada-ama.org/sites/default/files/resources/files/isti_november2022_0.pdf)
- [20] Mercolini L, Mandrioli R, Protti M, Conca A, Albers LJ, Raggi MA. Dried blood spot testing: a novel approach for the therapeutic drug monitoring of ziprasidone-treated patients. *Bioanalysis*. 2014 Jun;6(11):1487-95.
- [21] Protti M, Marasca C, Cirrincione M, Sberna AE, Mandrioli R, Mercolini L. Dried Urine Microsampling Coupled to Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) for the Analysis of Unconjugated Anabolic Androgenic Steroids. *Molecules*. 2020 Jul 14;25(14):3210.
- [22] T. Joye, J. Sidi, J. Déglon, A. Karmime, F. Sporkert, C. Widmer, B. Favrat, P. Lescuyer, M. Augsburg, A. Thomas, *Liquid chromatography-high resolution mass spectrometry for broad spectrum drug screening of dried blood spot as microsampling procedure*, *Anal. Chim. Acta* 1063 (2019), pp. 110-116.

- [23] L. Tretzela, A. Thomasa, H. Geyera, G. Gmeinerb, G. Forsdahlb, V. Popc, W. Schänzera, M. Thevisa, *Use of dried blood spots in doping control analysis of anabolic steroid esters*, Journal of Pharmaceutical and Biomedical Analysis 96 (2014), pp. 21–30.
- [24] V. Londhe, M. Rajadhyaksha, *Opportunities and obstacles for microsampling techniques in bioanalysis: Special focus on DBS and VAMS*, Journal of Pharmaceutical and Biomedical Analysis 182 (2020) 113102.
- [25] World AntiDoping Agency (WADA), “*DRIED BLOOD SPOTS (DBS) FOR DOPING CONTROL Requirements and Procedures for Collection, Transport, Analytical Testing and Storage*”, WADA Technical Document – TD2021DBS (2021).
- [26] Tretzel L, Thomas A, Geyer H, Gmeiner G, Forsdahl G, Pop V, Schänzer W, Thevis M. Use of dried blood spots in doping control analysis of anabolic steroid esters. J Pharm Biomed Anal. 2014 Aug 5; 96:21-30.
- [27] International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use, Validation of Analytical Procedures: Text and Methodology Q2(R1). Available online: [https://www.ich.org/fileadmin/Public\\_Web\\_Site/ICH\\_Products/Guidelines/Quality/Q2\\_R1/Step4/Q2\\_R1\\_Guideline.pdf](https://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q2_R1/Step4/Q2_R1_Guideline.pdf) (accessed on 9 November 2022)
- [28] Jarabek AM, Farland WH. The U.S. Environmental Protection Agency's risk assessment guidelines. Toxicol Ind Health. 1990 Oct;6(5):199-216.

## **2. Identification and UHPLC-MS/MS analysis of hCG and IGF-I from Dried Blood Spots**

### **2.1 Introduction**

In recent years, the World Anti-Doping Agency (WADA) has increasingly focused its efforts on detecting not only small, banned molecules, but also larger endogenous molecules such as hormones, with the aim of incorporating an endocrinological module into the athlete's biological passport [1]. The challenges faced by modern anti-doping analytical science are becoming more complex, as drug targets expand, the pharmaceutical industry introduces new therapeutic compounds, and the internet provides access to designer drugs designed to enhance performance. These technical challenges are numerous, including the need for advanced instrumentation that enables faster analyses with higher sensitivity, specific techniques that can distinguish between endogenous and exogenous metabolites, and biological assays for detecting peptide hormones or their markers [2]. One of the most commonly misused hormones in sports, and thus included in the WADA list, is Human Growth Hormone (GH), as well as GH-related growth factors such as insulin-like growth factor-1 (IGF-1), which athletes use to enhance performance [3]. The main action of this hormone is to promote muscle cell growth [4,5]. In addition to IGF-I usage, some athletes turn to pharmaceutical preparations of human chorionic gonadotropin (hCG) to stimulate testosterone production before competition and to prevent testicular arrest and atrophy during and after prolonged androgen administration cycles [6].

For this reason, it is crucial to develop methods that provide a molecular profile of the athlete, incorporating proteomic and metabolomic analysis. Identifying changes in protein and metabolite levels is essential for establishing threshold values, which can serve as the basis for new anti-doping methods based on proteomics. Dry matrix microsampling, and specifically dried blood spots (DBS), represents a promising strategy to enhance current doping control efforts. This method could significantly improve the stability of compounds and extend the post-sampling detection window [7]. Miniaturization and automation are pivotal opportunities for future doping strategies, as they address issues related to compound instability in aqueous samples, which could lead to false negatives, limited detection intervals, delays in retests, and challenges in detecting

pharmacologically relevant concentrations of non-prohibited substances. Furthermore, these methods can enable fast, reliable, and easily automated protocols with high throughput [8].

In the case of DBS, the absorption onto the matrix or device itself plays a crucial role in the sample pretreatment, as it helps retain many matrix-interfering components, reducing the need for additional purification steps after extraction [9]. DBS techniques have been implemented to collect blood microvolumes with the goal of improving the stability of labile compounds, simplifying sample collection, facilitating the automation of pre-analysis stages, minimizing risks of contamination and tampering, and reducing biological risks for operators due to minimal sample handling. Additionally, these methods offer reduced analytical costs thanks to the ability to store and transport samples without requiring special precautions, such as freezing or dry ice. New microsampling methods also enable the detection of unconventional doping systems, which could potentially involve studies of genetic factors.

This paper presents a preliminary study aimed at developing an effective extraction protocol for IGF-I and hCG from DBS samples using UHPLC-MS/MS. However, further validation steps are necessary to confirm the robustness and reliability of the proposed method. According to the WADA guidelines [10], the method will need to undergo rigorous validation procedures, including the determination of sensitivity, specificity, and reproducibility, as well as the establishment of reliable threshold values for these compounds. Additionally, further research is needed to assess the potential interference from endogenous substances and to evaluate the method's applicability in real-world anti-doping testing scenarios.

## **2.2 Materials and Methods**

### **2.2.1. Materials**

The standard analytes IGF-I (Insulin-like Growth Factor-I human) and hCG (Human Chorionic Gonadotropine) and the human blood were purchased as well powders from Sigma-Aldrich (Saint Louis, MI, US). The DBS (Protein Saver™ 903™ Card) cards were provided by Whatman™ GE Healthcare Bio-Sciences Corp (Italy). Sodium deoxycholate (SDOX), ammonium bicarbonate (AMBIC),

phosphate-buffered saline solution (PBS), lysis buffer (urea 6 M, Tris 150 mM pH 8,5,  $\beta$ -ottil-glucopiranoside 1%), iodoacetamide (IAM) and dithiothreitol (DTT) were obtained from Sigma-Aldrich (St. Quentin Fallavier, France). Acetonitrile (ACN), methanol absolute (MeOH) and trifluoroacetic acid (TFA) (all LC-MS grade) were purchased from Biosolve Chimie (Dieuze, France). Water was purified using a Milli-Q system (Millipore, MA, USA). Trypsin Gold (MS grade) was obtained from Promega (Madison, USA).

### **2.2.2 Microsample collection and pre-treatment**

Whole blood was used for the miniaturized sampling strategy and pre-treatment protocol. Ten  $\mu$ l of blood were deposited on a DBS board made of cellulose composed by five circles of 13 mm, each capable of holding from 75 to 80  $\mu$ l of sample. The blood was left to dry for 75 minutes at room temperature. Dried blood was added with 5  $\mu$ l of hCG and 5  $\mu$ l of IGF-I (concentrates 100ng/ml) and left to dry for another 30 minutes. After the drying phase, a 10 mm diameter stain was cut and deposited in an Eppendorf Protein LoBind Tube.

### **2.2.3 Extraction procedure from DBS by testing different solvents**

The selection of the solvents for protein extraction from dried blood on paper was performed according to previously published paper [11,12,13] and validated extraction protocols [14,15]. The solvents tested were methanol, acetonitrile, PBS, ammonium bicarbonate, sodium deoxycholate and lysis buffers. Twelve different tubes were prepared, six containing one DBS and one of the selected solvents. The other six tubes were prepared in the same way but with the addition of the steps of reduction and alkylation, to understand if they were necessary for the extraction of blood proteins from paper. The twelve tubes with a final volume of 250  $\mu$ l were analysed at the Qubit fluorometer. To select the most appropriate sample pre-treatment procedure, we have evaluated different extraction.

- **Methanol, acetonitrile [11] and PBS [12] extraction:** 100  $\mu$ l of solvent were added to the DBS stowed in the tube. Then ultrasonic bath for 10 minutes, vortex for 30s and ultrasonic bath for 10 minutes were performed. Steps of reduction and alkylation: DTT 10mM was added, and the sample was put to the Thermomixer for 30 minutes at 60 °C - 1000rpm, then IAM 50mm was added and the sample left 30 minutes at room temperature. Subsequently trypsin was added, followed

by an overnight digestion at 37 °C. In supernatant digestion, DBS was removed before trypsin was added. Digestion was stopped by adding TFA 10% (v/v).

- **Ammonium bicarbonate extraction [13]:** 100 µl of AMBIC were added to the DBS stored in the tube, then the sample incubated at the Thermomixer for 30 minutes at 50 °C - 1000rpm. An ultrasonic bath was performed for 10 minutes. Steps of reduction and alkylation: DTT 10mm was added and the sample put into the Thermomixer for 30 minutes at 50 °C - 1000rpm. Then, IAM 50mm was added and the sample left 20 minutes at room temperature. Trypsin was added to carry out a digestion overnight at 37 ° C. In supernatant digestion, DBS was removed before trypsin was added. Digestion was stopped by adding TFA 10% (v/v).
- **Sodium deoxycholate extraction [14]:** 50 µl of MeOH were added to the DBS stored in the tube. Ultrasonic bath for 10 minutes, vortex for 30s and ultrasonic bath for 10 minutes were performed. The DBS was removed from the tube and dried in the Speedvac for 5 minutes. The DBS was returned to the tube and SDOX 1% (v/v) was added. The sample was shaken for 5 minutes at 95°C - 1000rpm and again subjected to the ultrasonic bath for 10 minutes. Step of reduction and alkylation: DTT 10mm was added, and the sample was put in the Thermomixer for 60 minutes at 60 °C - 1000rpm. IAM 50mm was added and left 60 minutes at room temperature. The sample was diluted to 0.2% (v/v) with AMBIC. Subsequently trypsin was added, to carry out a digestion overnight at 37 ° C. In supernatant digestion, DBS was removed before trypsin was added. Digestion was stopped by adding TFA 10% (v/v). The sample was centrifuged at 4 °C - 14000rpm.
- **Lysis buffer extraction [15]:** 50 µl of MeOH were added to the DBS stored in the tube, an ultrasonic bath for 10 minutes, vortex for 30s and ultrasonic bath for another 10 minutes were performed. 200 µl of lysis buffers were added. The content is then transferred to Microcon-30 kDa (Merck) centrifugal filter units and centrifuged at 13.000 g for 30 minutes. The reduction was carried out with DTT 10 mM at 57 °C for 30 minutes and alkylation was performed with IAM 50 mM for 30 minutes at room temperature. Four washes with 500 µl AMBIC 50 mM were carried out to remove the reagents. Digestion on paper was carried out with trypsin in AMBIC buffer 50 mm overnight at 37°C. Digestion on supernatant took place by eliminating DBS before the addition of trypsin. Digestion was stopped by adding TFA 10% (v/v). The elution of the peptides was carried out by

centrifugation once at 13.000 g for 30 minutes, once with TFA 0.1% (v/v) and twice with ACN+TFA 0.1% (v/v). At each pass the recovered filtrate was dried in the Speedvac and finally re-distilled into AMBIC 50 mM.

The choice of the best solvent was made by an initial analysis with a qubit fluorometer to see which solvent extracted more protein from the blood from the paper. A fluorometric quantification of qubit (ThermoFisher Scientific) was performed using a kit consisting of Protein BR Assay Reagent, Protein BR Assay Buffer, Standard 1 (0 mg/mL BSA) and Standard 2 (10 mg/mL BSA). The test is accurate for sample concentrations from 100 µg/mL to 20 mg/mL. The Protein BR Assay Buffer is added to the samples and subsequently the reagent is also added. The test is carried out by incubation for 10 to 15 minutes and then reading. The four best solvents have been identified from this initial analysis. The analysis was repeated on two consecutive days three times, to have a good reproducibility of the data.

#### **2.2.4 Digestion and reduction of final volume**

Reduction, alkylation, and digestion steps were tested on the solvents, providing higher recovery to improve the extraction process. Finally, volumes were also optimized. The tubes containing the best four solvents were reduced and alkylated by also adding the digestion on supernatant step, reaching a final volume of 250 µL. In addition, eight other samples with a final volume of 100µL were prepared (to verify whether less solvent use would give the same or better extraction result) four of these were digested by directly adding trypsin on the DBS, and the other four were digested by adding trypsin to the supernatant, after removing the DBS. These twelve different samples were analyzed at the UHPLC-MS/MS.

#### **2.2.5 Liquid Chromatography and Mass Spectrometry**

Samples were analyzed by nanoLC–MS/MS using an RSLCnano UltiMate 3000 System coupled to a nanoESI Q-Exactive or Q-Exactive HF mass spectrometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA) in the data-dependent acquisition (DDA) high-energy c-trap dissociation (HCD) mode. The peptides recovered in the trap column were separated on a capillary reverse-phase C18 T3 column 75 µm × 250 mm length (Waters) at 45 °C with a linear 130 min gradient

elution from 2.5 to 90% of buffer B [water/ACN/formic acid 10%:90%:0.1% (v/v/v)] in buffer A [water/ACN/formic acid 98%: 2%: 0.1% (v/v/v)] at a fixed flow rate of 220 nL/min. Full MS survey scans were recorded over the  $m/z$  range of 375–1500 with a resolution of 60,000 using an automatic gain control target value (AGC) of 3E6 with a maximum injection time of 60 ms. Up to 20 intense  $2^+$ – $5^+$  charged ions were selected for HCD with a normalized collision energy of 28%, with a precursor isolation window at 2  $m/z$ , a resolution of 15,000, and an AGC value of 1E5 with a maximum injection time of 60 ms. The minimum MS<sup>2</sup> target value was set at 1E3, and the dynamic exclusion was for 20 s.

### **2.2.6 Application of the best extraction protocols on DBS fortified with hCG and IGF-I**

After the UHPLC-MS/MS analysis, the two best solvents of the four previously selected were identified, also identifying the best final volume but not clearly the best digestion method. Having identified the two best procedures for extracting blood proteins from DBS, work began on IGF-I and hCG enriched DBS to see which extraction procedure was best for these specific proteins and the best digestion method.

Analyses were performed on white DBS to check the extraction performance, so in addition to the DBS with dried blood added and subsequently enriched, also the white device with only the analytes of interest were analysed. DBS were enriched with 5  $\mu$ l hCG and 5  $\mu$ l IGF-I at concentrations of 100 ng/ml. Four samples were prepared by extraction in lysis buffer, two white samples, one with digestion on paper and the other digested on supernatant, and two blood samples, with both digestion methods. The same was done for sodium deoxycolate extraction. The eight samples were analysed at UHPLC-MS/MS and replicated.

### **2.2.7 Simultaneously detect IGF-I and hCG with the best and reproducible extraction protocol**

It has been verified by the literature that a pre-extraction in methanol is necessary to extract hCG, so the pre-extraction was carried out to extract both proteins, since the aim was to have a single extraction for both analytes. Eight samples, four added samples and four blood samples were analysed. After analysis, the best solvent for extraction was selected and further analyses were carried out to verify

the best digestion method. Six samples were analysed, three spiked, two with digestion on paper and one with digestion on supernatant and three DBS samples, two with digestion on paper and one with digestion on supernatant. After this last analysis, the best final protocol for both proteins was assembled, and the extraction was repeated three times for good reproducibility.

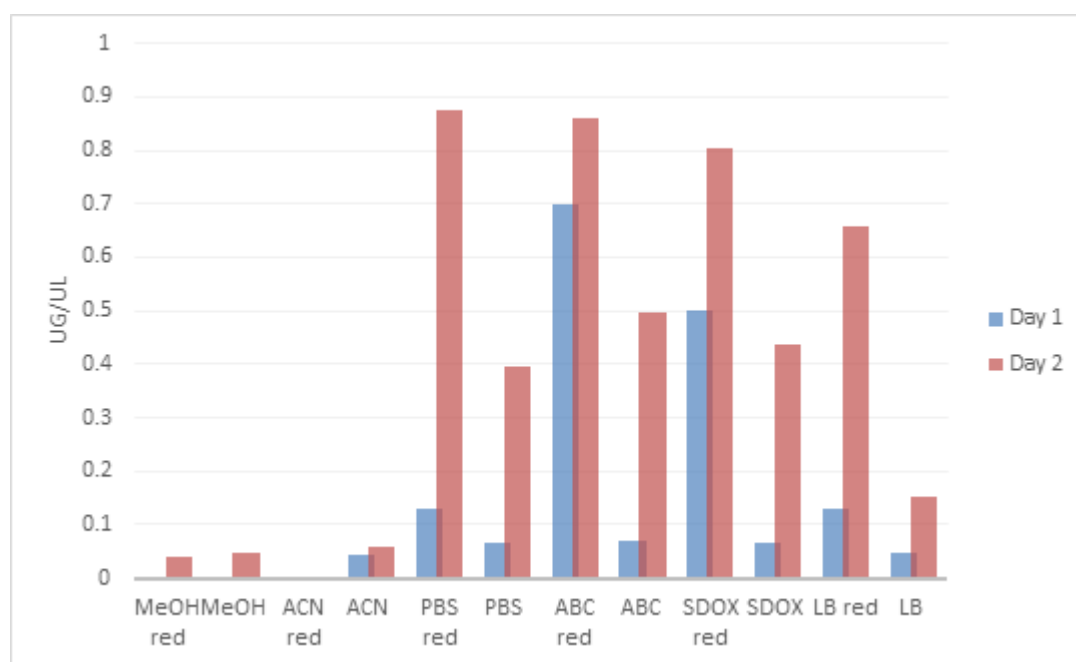
### 2.2.8 Data analysis

Spectra were processed using Proteome Discoverer v2.4 (Thermo Fisher Scientific). The database search was performed with the following parameters: MS and MS/MS mass tolerance of 10 ppm and 0.02 Da, respectively, trypsin specificity with up to 2 missed cleavages, and fixed carbamidomethylation (C), deamidation (NQ) and oxidation (M). Proteins with at least one high-confidence peptide and six amino acids were validated. The target FDR was set at 0.01. The abundance value obtained was used to make the chart.

## 2.3. Results

### 2.3.1 Extraction solvent optimization

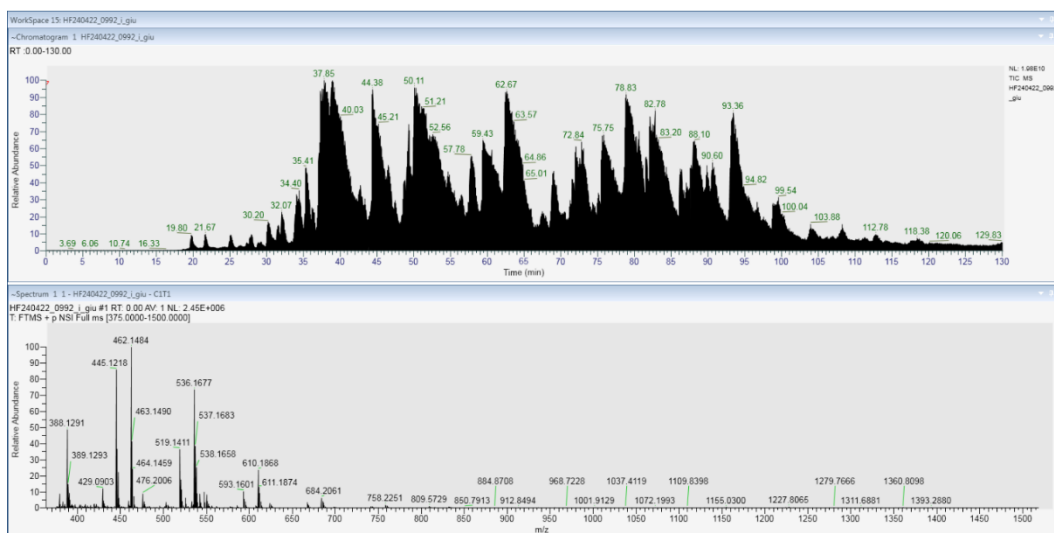
The solvents providing best extraction recoveries from dried blood were PBS, ammonium bicarbonate, sodium deoxycholate and lysis buffer, with the addition of reduction and alkylation steps (Figure1).



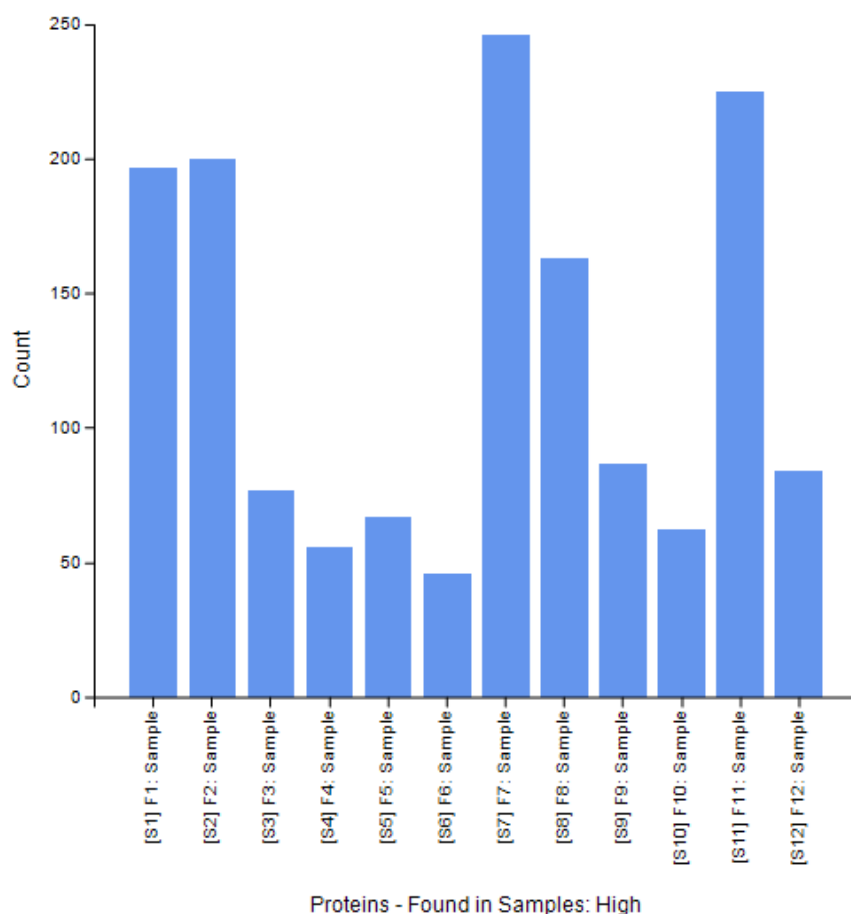
**Figure 1.** Qubit fluorometer quantification of proteins extracted from blood with different solvents

### 2.3.2 Digestion and reduction

After digesting with trypsin and reducing the final volume to 100  $\mu$ l, the samples were analysed with UHPLC-MS/MS (Figure 2). The analysis showed that the four best extractions were the two with lysis buffer and both digestion methods with a final volume of 100  $\mu$ l and the two with sodium deoxycholate and both digestion methods with a final volume of 100  $\mu$ l (Figure 3).



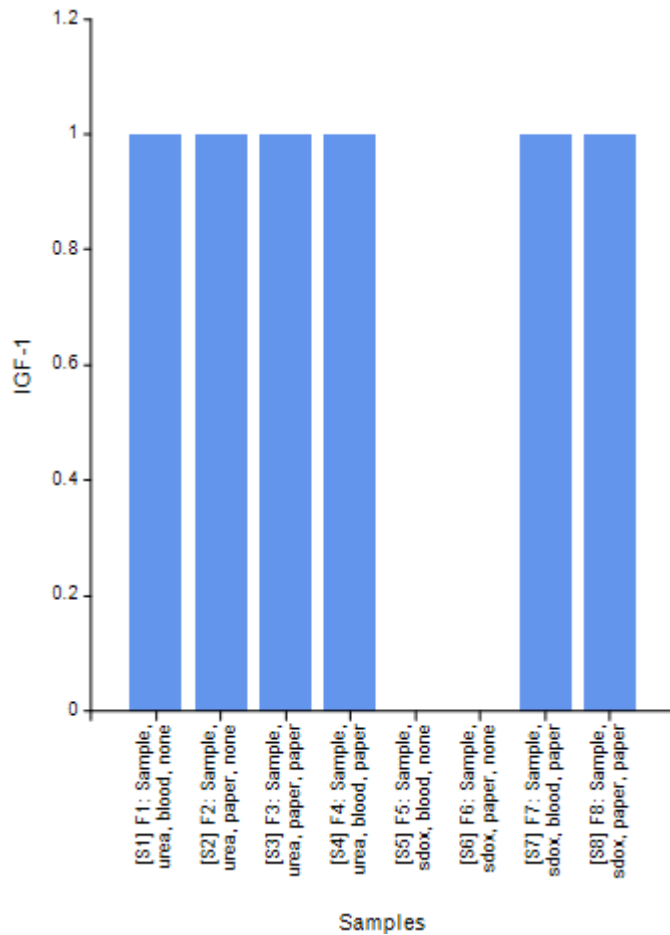
*Figure 2. UHPLC-MS/MS chromatogram*



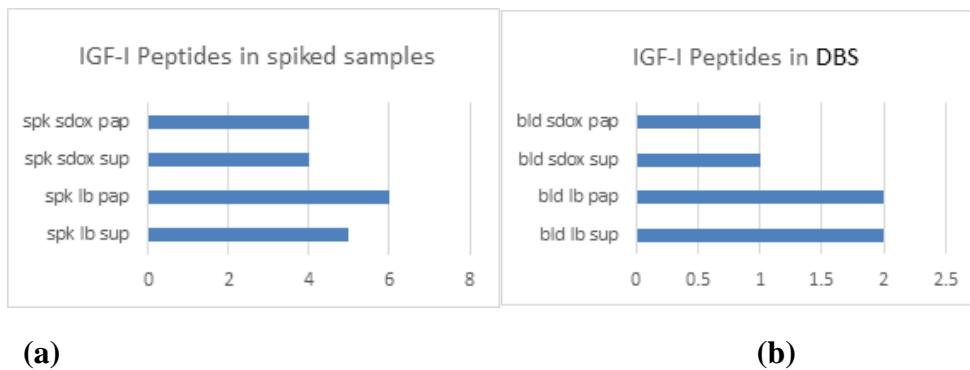
**Figure 3.** *The highest amount of proteins extracted from DBS for each solvent used, digestion method and final volume: [S1] PBS digestion supernatant 250  $\mu$ l; [S2] AMBIC digestion supernatant 250  $\mu$ l; [S3] SDOC digestion supernatant 250  $\mu$ l; [S4] LB digestion supernatant 250  $\mu$ l; [S5] PBS digestion paper 100  $\mu$ l; [S6] AMBIC digestion paper 100  $\mu$ l; [S7] LB digestion paper 100  $\mu$ l; [S8] SDOC digestion paper 100  $\mu$ l; [S9] PBS digestion supernatant 100  $\mu$ l; [S10] AMBIC digestion supernatant 100  $\mu$ l; [S11] LB digestion supernatant 100  $\mu$ l; [S12] SDOC digestion supernatant 100  $\mu$ l.*

### 2.3.3 The best extraction protocols on DBS fortified with hCG and IGF-I

The eight samples analysed with UHPLC-MS/MS showed that in all samples, except for the SDOX-spiked sample extracted and digested on supernatant and the corresponding blood sample, IGF-I was detected (Figure 4). The analyses were also repeated to verify that the analytes were detected again and in all samples. Lysis buffer extraction was the best, as more peptide sequences of IGF-I were detected (Figure 5 a,b).



**Figure 4.** Peptide sequences detected for IGF-I with different extraction and digestion procedures



**Figure 5.** (a) Number of peptide sequences detected for IGF-I spiked samples with different extraction and digestion procedures; (b) number of peptide sequences detected for IGF-I blood samples with different extraction and digestion procedures.

### 2.3.4 The best reproducible extraction protocol to detect hCG and IGF-I simultaneously

The solvent that extracted the greatest number of peptide sequences for both analytes was the lysis buffer that showed the best extraction yield in DBS samples. (Figure 7 a,b).

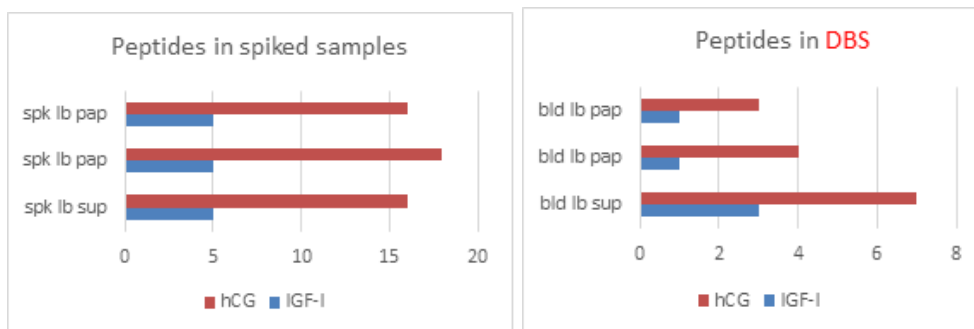


(a)

(b)

**Figure 6.** (a) Number of hCG and IGF-I peptide sequences in spiked samples extracted with different digestion procedures; (b) number of hCG and IGF-I peptide sequences in blood samples extracted with different digestion procedures.

After demonstrating that the lysis buffer is the best solvent to extract hCG and IGF-I from DBS, another analysis was carried out to understand which was the best digestion procedure. Six samples were prepared with lysis buffer extraction, three spiked samples, two with digestion on paper and one with supernatant digestion, and three blood samples two digested on paper and one with supernatant digestion. The analysis showed a better extraction yield for the digested sample on supernatant (Figure 8 a,b).



(a)

(b)

**Figure 7.** (a) Number of peptide sequences detected for hCG and IGF-I spiked samples with different digestion procedures; (b) number of peptide sequences detected for hCG and IGF-I blood samples with different digestion procedures.

After obtaining the best extraction protocol for hCG and IGF-I, three spiked samples and three blood samples extracted with lysis buffer and digested on supernatants were analysed to see if the outlined extraction protocol had a good reproducibility (Figure 9 a,b). Twelve of the fifteen hCG peptide sequences were

extracted in DBS samples and instead for IGF-I peptide sequences all five of those detected in spiked samples were extracted in DBS samples, for each of the three samples analysed (Figure 10 a,b).



(a)

(b)

**Figure 8.** (a) Number of peptide sequences detected for hCG and IGF-I spiked samples with lysis buffer extraction and digestion on supernatant; (b) number of peptide sequences detected for hCG and IGF-I blood samples with lysis buffer extraction and digestion on supernatant.



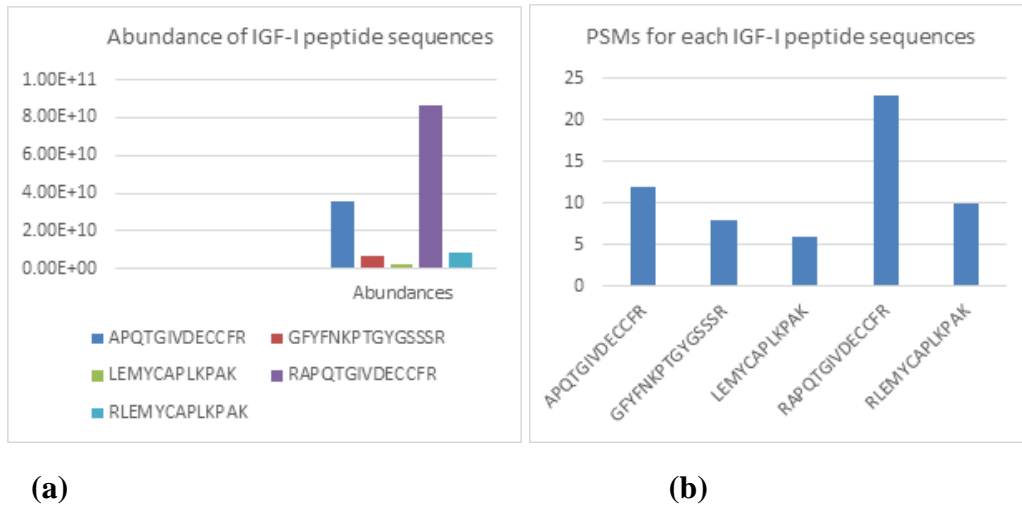
(a)

(b)

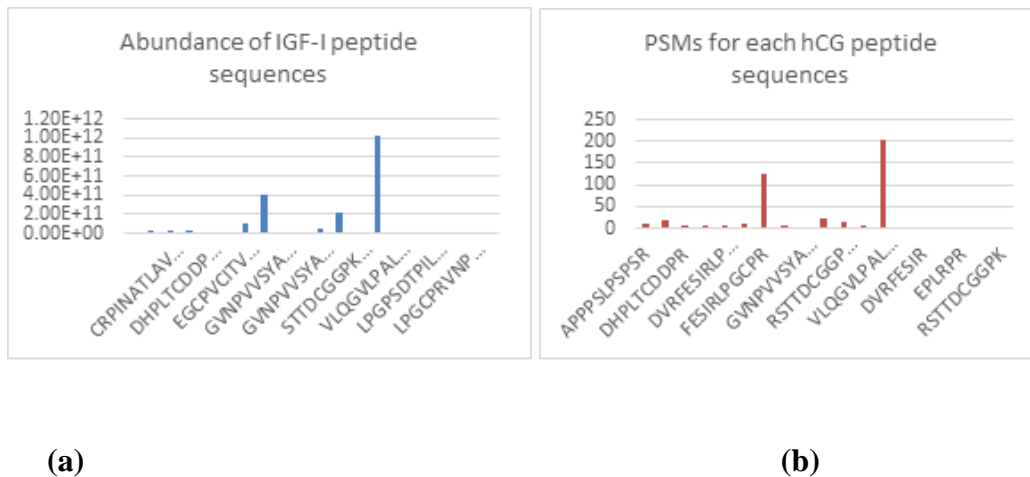
**Figure 9.** (a) peptide sequences of IGF-I detected in DBS samples; (b) peptide sequences of hCG detected in DBS samples.

### 2.3.5 Data analysis

Abundance has been calculated for each peptide sequence, and it was found that the most abundant sequence for IGF-I is RAPQTGIVDECCFR, which also corresponds to the largest number of PSMs performed for each peptide sequences (Figure 11 a,b). The same has been done for hCG peptide sequences, showing that the most abundant sequence is VLQGVLPALPQVVCNYR to which the highest number of PSMs correspond (Figure 12 a,b).



**Figure 10.** (a) abundance of IGF-I peptide sequences; (b) PSMs performed for each IGF-I peptide sequence.



**Figure 11.** (a) abundance of hCG peptide sequences; (b) PSMs performed for each hCG peptide sequence.

## 2.4. Discussion

In this paper, following a review of the literature on the potential applications of DBS in doping analysis [16,17,18] and specifically in the extraction of peptides [19,20,21] present in the prohibited WADA list, a method for the extracting hCG and IGF-I from DBS [22,23] using UHPLC-MS/MS analysis has been developed.

As a first step, the most suitable extraction solvents for recovering blood proteins from paper were identified based on literature and previously established protocols. To optimise the extraction protocol, the blood samples were analysed in duplicate: one with the solvent of interest and another with the same solvent

plus reduction and alkylation steps, to determine the conditions that yielded the highest protein recovery. For the preliminary analysis, a Qubit fluorometer was employed, as it is a precise and overly sensitive fluorescence-based quantification tool [24]. The final volume was reduced to make the extraction more environmentally friendly and cost-effective, and the necessity of a digestion step was evaluated. UHPLC-MS/MS analysis was then conducted to identify the optimal extraction solvents and verify the best protein recovery from DBS, considering factors such as volume reduction, and the inclusion of a digestion step.

After identifying the two most effective solvents for extracting blood proteins from paper, extractions were carried out on DBS samples spiked with the two target analytes to determine which solvent was more efficient for specifically extracting hCG and IGF-I. The decision to spike the samples with a concentration of 100 ng/ml for each analyte was based on prior studies, which indicate that this is among the lowest detectable concentrations in blood [25,26]. The spiked samples were prepared without the presence of blood, simulating DBS conditions, to assess the extraction yield from the blood matrix. Furthermore, two different digestion methods were tested: one directly on the paper and the other on the supernatant. This comparison aimed to determine whether applying trypsin directly onto the paper would enhance the digestion of dried proteins, being more hydrophilic, would be extracted more efficiently from the paper and then digested in the supernatant.

Previous studies had observed that hCG had been successfully extracted from DBS using methanol [11], so the goal was to achieve a single extraction method for both analytes. Thus, pre-extraction in methanol was also employed to detect both IGF-I and hCG.

Samples extracted using both solvents and digestion techniques were analysed, and the lysis buffer was identified as the most effective solvent, as the protein recovery from DBS was superior compared to sodium deoxycholate, particularly in the case of DBS samples subjected to supernatant digestion. Based on the preliminary results regarding digestion methods, the analysis was repeated to evaluate whether digestion on the supernatant offered higher efficiency than digestion directly on the paper matrix. Two digested samples were prepared on

paper, as digestion on paper was considered more practical, given that there is no need to remove the DBS from the FASP filter, a step necessary in digestion with supernatants. Subsequent analysis confirmed that digestion in the supernatant was the most effective method.

After establishing the most efficient extraction protocol for IGF-I and hCG from DBS, the procedure was repeated in triplicate to ensure data reproducibility, thereby validating the chosen protocol. Following protocol development, data analysis was essential to determine which peptide sequences were successfully extracted in spiked samples and subsequently detected in DBS samples. All five IGF-I peptide sequences identified in spiked samples were also detected in DBS samples, demonstrating high extraction efficiency. However, for hCG only nine peptide sequences were detected in DBS samples compared to 14/15 identified in spiked samples. The relative abundance of each peptide sequence found in the DBS samples was calculated to understand which sequence was consistently detected. This data is crucial, as identifying even a single sequence can confirm the presence of IGF-I and hCG in the blood. Additionally, the number of PSMs (peptide-spectrum matches), or the acquired spectra for each peptide sequence, was considered, as a higher number suggests greater abundance. This data was further confirmed by observing the relative abundance of each sequence. By identifying the most abundant peptide sequence for each protein, valuable information is provided for future studies, as identifying this sequence can confirm the presence of these prohibited substances in dried blood samples.

Following this preliminary study on the development of an extraction protocol, a future goal would be to evaluate the individual fluctuations of IGF-I and hCG in blood samples from volunteers to define normality intervals. Given the large inter-individual variability in IGF-I and hCG blood levels, it is not possible to define universal limits for the entire population. However, establishing individual normal ranges could be an effective tool for detecting abnormal fluctuations of IGF-I and hCG resulting from the use of drugs or food supplements containing these substances.

Key innovations include: the identification of the most effective solvents for protein recovery which improves extraction yields, helping to provide valuable information to doping analysis methodologies; the evaluation of digestion

methods, directly on the DBS versus the supernatant, demonstrates that supernatant digestion improves protein recovery, an area not extensively explored in existing literature; The use of DBS to detect banned peptides provides a practical and non-invasive sampling method, addressing the challenges associated with traditional blood sampling; The focus on relative abundance of peptide sequences establishes a basis for confirming the presence of IGF-I and hCG, facilitating more effective doping screening. This study is considered preliminary, and further validation steps are essential to confirm the robustness and reliability of the proposed method. According to WADA guidelines [10], the method must undergo rigorous validation procedures, including assessing sensitivity, specificity, and reproducibility. It will also be crucial to establish reliable threshold values for these compounds, along with an evaluation of potential interference from endogenous substances. Additionally, the method's applicability in real-world anti-doping testing scenarios needs to be further evaluated.

In conclusion, this study not only makes a significant contribution to the existing literature on detecting hCG and IGF-I in the context of doping, but also proposes methodologies that can be adapted and applied to other areas of biomedical research. The integration of advanced analytical techniques and the consideration of individual variability may have a lasting impact on sports medicine and pharmacovigilance, with future applications potentially enhancing the precision of doping control.

## **2.5 Limitations and Future Perspectives**

Although this study provides promising preliminary results in developing an extraction protocol for IGF-I and hCG from dried blood spots (DBS), several limitations need to be addressed in future work. Firstly, the study relied on spiked DBS samples, which may not fully replicate the complexity of real biological samples, and thus the extraction efficiency and peptide recovery may differ in actual doping cases. The choice of solvents and digestion methods, while optimized in this study, may still need refinement to ensure maximum reproducibility and applicability across a wider range of substances. Additionally, the validation of the method was limited to a small number of analytes, and further studies are needed to assess its robustness in detecting other banned substances on the WADA list. Another limitation is the lack of comprehensive validation of

the proposed protocol under the strict criteria set by the World Anti-Doping Agency (WADA), particularly in terms of sensitivity, specificity, and accuracy in real-world doping control settings. Future research should focus on expanding the method's validation, including its ability to detect low concentrations of target compounds in various matrices and evaluating potential matrix effects or interference from endogenous substances. A critical next step will also involve establishing individual reference ranges for IGF-I and hCG, considering the significant inter-individual variability in hormone levels, which will help set thresholds for abnormal fluctuations caused by doping or the use of performance-enhancing supplements. Ultimately, the integration of this method into routine doping testing could greatly enhance the accuracy and efficiency of anti-doping controls. Future studies will need to focus on validating this approach with larger sample sizes, different athlete populations, and various doping contexts to establish a universally applicable and reliable method for detecting IGF-I, hCG, and other prohibited substances in sport.

## **2.6 Conclusion**

In conclusion, this study successfully developed and optimized a robust extraction protocol for hCG and IGF-I from dried blood spots (DBS) using UHPLC-MS/MS analysis. By systematically evaluating various extraction solvents and digestion methods, we identified lysis buffer as the most effective solvent, significantly enhancing the extraction yields of both analytes. The results demonstrated the feasibility of detecting key peptide sequences associated with hCG and IGF-I in DBS samples, providing a solid foundation for future doping analyses. Additionally, the identification of specific peptide sequences can facilitate the confirmation of the presence of these prohibited substances in athletes' blood. Looking ahead, further research will focus on establishing individual normal ranges for IGF-I and hCG in healthy populations. This will be crucial for distinguishing between normal physiological fluctuations and potential doping, ultimately contributing to more effective monitoring strategies in sports anti-doping efforts.

## 2.6 References

- [1] Ponzetto F, Giraud S, Leuenberger N, Boccard J, Nicoli R, Baume N, Rudaz S, Saugy M. Methods for Doping Detection. *Front Horm Res.* 2016; 47:153-67. doi: 10.1159/000445177. Epub 2016 Jun 27. PMID: 27348309.
- [2] Ayotte C, Miller J, Thevis M. Challenges in Modern Anti-Doping Analytical Science. *Med Sport Sci.* 2017; 62:68-76. doi: 10.1159/000460701. Epub 2017 Jun 1. PMID: 28571012.
- [3] Bidlingmaier M. New detection methods of growth hormone and growth factors. *Endocr Dev.* 2012; 23:52-9. doi: 10.1159/000341748. Epub 2012 Nov 23. PMID: 23182820.
- [4] Mongongu C, Moussa EM, Semence F, Roulland I, Ericsson M, Coudoré F, Marchand A, Buisson C. Use of capillary dried blood for quantification of intact IGF-I by LC-HRMS for antidoping analysis. *Bioanalysis.* 2020 Jun;12(11):737-752. doi: 10.4155/bio-2020-0013. Epub 2020 Jun 1. PMID: 32479742.
- [5] Velloso CP. Regulation of muscle mass by growth hormone and IGF-I. *Br J Pharmacol.* 2008 Jun;154(3):557-68. doi: 10.1038/bjp.2008.153. PMID: 18500379; PMCID: PMC2439518.
- [6] Kicman AT, Brooks RV, Cowan DA. Human chorionic gonadotrophin and sport. *Br J Sports Med.* 1991 Jun;25(2):73-80. doi: 10.1136/bjism.25.2.73. PMID: 1751893; PMCID: PMC1478834.
- [7] Ververi C, Vincenti M, Salomone A. Recent advances in the detection of drugs of abuse by dried blood spots. *Biomed Chromatogr.* 2023 Jul;37(7):e5555. doi: 10.1002/bmc.5555. Epub 2022 Dec 4. PMID: 36417316.
- [8] L. Tretzel, A. Thomas, H. Geyer, V. Pop, W. Schänzer, M. Thevis Dried blood spots (DBS) in doping controls: a complementary matrix for improved in- and out-of-competition sports drug testing strategies. *Anal. Meth.*, 7 (2015), pp. 7596-7605
- [9] Protti M, Rudge J, Sberna AE, Gerra G, Mercolini L. Dried haematic microsamples and LC-MS/MS for the analysis of natural and synthetic cannabinoids. *J Chromatogr B Analyt Technol Biomed Life Sci.* 2017 Feb 15;1044-1045:77-86. doi: 10.1016/j.jchromb.2016.12.038. Epub 2016 Dec 30. PMID: 28088044.
- [10] Protti M, Sberna PM, Sberna AE, Ferrante R, Mandrioli R, Mercolini L. Enhanced urinary stability of peptide hormones and growth factors by dried urine

- microsampling. *J Pharm Biomed Anal.* 2021 Sep 10; 204:114234. doi: 10.1016/j.jpba.2021.114234. Epub 2021 Jun 29. PMID: 34246878.
- [11] Rosting C, Gjelstad A, Halvorsen TG. Water-Soluble Dried Blood Spot in Protein Analysis: A Proof-of-Concept Study. *Anal Chem.* 2015 Aug 4;87(15):7918-24. doi: 10.1021/acs.analchem.5b01735. Epub 2015 Jul 15. PMID: 26100949.
- [12] Martin NJ, Bunch J, Cooper HJ. Dried blood spot proteomics: surface extraction of endogenous proteins coupled with automated sample preparation and mass spectrometry analysis. *J Am Soc Mass Spectrom.* 2013 Aug;24(8):1242-9. doi: 10.1007/s13361-013-0658-1. Epub 2013 Jun 1. PMID: 23728546; PMCID: PMC3713260.
- [13] Huynh HH, Bœuf A, Derbez-Morin M, Dupuy AM, Lalere B, Delatour V, Vinh J. Development of an antibody-free ID-LC MS method for the quantification of procalcitonin in human serum at sub-microgram per liter level using a peptide-based calibration. *Anal Bioanal Chem.* 2021 Aug;413(19):4707-4725. doi: 10.1007/s00216-021-03361-0. Epub 2021 May 14. PMID: 33987701.
- [14] Ravi Kumar RK, Ndiaye MM, Haddad I, Vinh J, Verdier Y. ChipFilter: Microfluidic-Based Comprehensive Sample Preparation Methodology for Microbial Consortia. *J Proteome Res.* 2024 Mar 1;23(3):869-880. doi: 10.1021/acs.jproteome.3c00288. Epub 2024 Feb 14. PMID: 38353246; PMCID: PMC10913871.
- [15] Thevis M. Broadening the Horizon of Antidoping Analytical Approaches Using Dried Blood Spots. *Clin Chem.* 2021 Aug 5;67(8):1041-1043. doi: 10.1093/clinchem/hvab074. PMID: 34060612.
- [16] Yuan Y, Xu Y, Lu J. Dried blood spots in doping analysis. *Bioanalysis.* 2021 Apr;13(7):587-604. doi: 10.4155/bio-2021-0019. Epub 2021 Mar 17. PMID: 33728984.
- [17] Garzinsky AM, Thomas A, Guddat S, Görgens C, Dib J, Thevis M. Dried blood spots for doping controls-Development of a comprehensive initial testing procedure with fully automated sample preparation. *Biomed Chromatogr.* 2023 Aug;37(8):e5633. doi: 10.1002/bmc.5633. Epub 2023 Apr 23. PMID: 36974028.
- [18] Reverter-Branchat G, Segura J, Pozo OJ. On the road of dried blood spot sampling for antidoping tests: Detection of GHRP-2 abuse. *Drug Test Anal.* 2021

Mar;13(3):510-522. doi: 10.1002/dta.2975. Epub 2020 Nov 25. PMID: 33197153.

[19] Reverter-Branchat G, Bosch J, Vall J, Farré M, Papaseit E, Pichini S, Segura J. Determination of Recent Growth Hormone Abuse Using a Single Dried Blood Spot. *Clin Chem*. 2016 Oct;62(10):1353-60. doi: 10.1373/clinchem.2016.257592. Epub 2016 Aug 9. PMID: 27507700.

[20] Thevis M, Geyer H, Tretzel L, Schänzer W. Sports drug testing using complementary matrices: Advantages and limitations. *J Pharm Biomed Anal*. 2016 Oct 25; 130:220-230. doi: 10.1016/j.jpba.2016.03.055. Epub 2016 Mar 28. PMID: 27040951.

[21] Cox HD, Rampton J, Eichner D. Quantification of insulin-like growth factor-1 in dried blood spots for detection of growth hormone abuse in sport. *Anal Bioanal Chem*. 2013 Feb;405(6):1949-58. doi: 10.1007/s00216-012-6626-y. Epub 2012 Dec 22. PMID: 23263515.

[22] Lange T, Thomas A, Walpurgis K, Thevis M. Fully automated dried blood spot sample preparation enables the detection of lower molecular mass peptide and non-peptide doping agents by means of LC-HRMS. *Anal Bioanal Chem*. 2020 Jun;412(15):3765-3777. doi: 10.1007/s00216-020-02634-4. Epub 2020 Apr 16. PMID: 32300840; PMCID: PMC7220872.

[23] Mardis E, McCombie WR. Library Quantification: Fluorometric Quantitation of Double-Stranded or Single-Stranded DNA Samples Using the Qubit System. *Cold Spring Harb Protoc*. 2017 Jun 1;2017(6):pdb.prot094730. doi: 10.1101/pdb.prot094730. PMID: 27803271.

[24] Gatti R, De Palo EF, Antonelli G, Spinella P. IGF-I/IGFBP system: metabolism outline and physical exercise. *J Endocrinol Invest*. 2012 Jul;35(7):699-707. doi: 10.3275/8456. Epub 2012 Jun 18. PMID: 22714057.

[25] Klett D, Combarous Y. Highly sensitive *in vitro* bioassay for luteinizing hormone and chorionic gonadotropin allowing their measurement in plasma. *Reprod Fertil*. 2021 Nov 11;2(4):300-307. doi: 10.1530/RAF-21-0045. PMID: 35118407; PMCID: PMC878858

# **Chapter V**

## **KEY FINDINGS AND ADVANCEMENTS OF STUDIES**

## **1. Introduction to the Innovation of DBS in Anti-Doping Analysis**

Anti-doping analysis is a crucial aspect of sports integrity, aimed at ensuring a fair playing field for all athletes by detecting the use of prohibited substances. Traditional approaches predominantly rely on liquid blood and urine samples, which, although effective, present several challenges. These methods are invasive, requiring trained personnel for sample collection, and pose logistical challenges, including the need for rapid processing, cold chain maintenance, and the risk of sample degradation over time.

The adoption of Dried Blood Spot (DBS) microsampling introduces a transformative approach to anti-doping testing. DBS involves collecting a small drop of blood from a finger prick onto a cellulose card, which is then dried and stored at room temperature. This technique offers multiple advantages, such as minimal invasiveness, enhanced analyte stability, ease of storage and transport, and the potential for retrospective analysis. These features make DBS particularly suitable for large-scale, decentralized, and longitudinal monitoring in both competitive and out-of-competition settings.

This chapter synthesizes the key findings from two original research studies included in this thesis, both of which utilize DBS to address specific challenges in the detection of prohibited substances. The first study focuses on the analysis of anabolic androgenic steroids (AAS), while the second investigates the extraction and quantification of peptide hormones, specifically human chorionic gonadotropin (hCG) and insulin-like growth factor I (IGF-I), which are also banned by the World Anti-Doping Agency (WADA). Through these studies, this work demonstrates the significant potential of DBS as a reliable, efficient, and innovative tool in the advancement of anti-doping science.

## **2. Key Findings and Advancements from the Two Studies**

### **2.1. Development and Validation of an LC-MS/MS Method for AAS Detection Using DBS**

The first study aimed to establish a validated analytical method for the simultaneous detection of six different AAS using DBS and liquid chromatography-tandem mass spectrometry (LC-MS/MS). The focus was on optimizing both the chromatographic conditions and the DBS sample preparation to achieve high sensitivity and precision, compliant with the stringent standards required for anti-doping testing.

- **Chromatographic Optimization:** The choice of an appropriate chromatographic setup was critical for achieving the desired separation and sensitivity. After screening various reversed-phase (RP) columns, a special C18 column with an inorganic-organic hybrid sorbent was selected. This column, coupled with enhanced end capping, was specifically designed for high-performance liquid chromatography (HPLC) applications and provided superior separation efficiency. Different acidic additives (formic acid, acetic acid, trifluoroacetic acid) and their concentrations were tested to optimize peak resolution and shape. The selected method, using 0.1% formic acid in acetonitrile and water as the mobile phase, provided sharp, symmetric, and baseline-separated peaks within a 15-minute total run time.
- **Addressing DBS-Specific Challenges:** One of the inherent challenges of DBS is the variability in blood drop volume and spread on the cellulose card, which can lead to inconsistencies in analyte concentration and recovery. This issue was addressed by using end-to-end glass capillaries to ensure accurate spotting of a consistent 10  $\mu$ L blood volume, followed by whole-spot analysis. This approach mitigated volumetric biases and improved the reliability of the analytical results.
- **Sensitivity and Specificity:** The method demonstrated excellent linearity across a wide concentration range, with correlation coefficients ( $r^2$ ) of  $\geq 0.9981$  for all analytes. The limits of detection (LOD) were below 2 ng/mL, and the limits of quantification (LOQ) were less than 5 ng/mL, surpassing the minimum required performance levels (MRPLs) stipulated by WADA. These sensitivity levels are crucial for detecting trace amounts of AAS in athletes' blood, even long after their administration.
- **Precision and Accuracy:** Both intraday and interday precision were evaluated at three different concentrations representative of the entire linearity range. The method exhibited excellent precision, with relative standard deviations (RSD%)

below 5.4% for liquid samples and below 9.5% for DBS samples. This high level of reproducibility confirms the method's robustness and suitability for routine anti-doping testing.

The findings from this study indicate that DBS, coupled with an optimized LC-MS/MS method, is a viable alternative to traditional liquid blood analysis for AAS detection. The method's high sensitivity, precision, and ability to handle the unique challenges of DBS make it a promising tool for more accessible and reliable anti-doping testing.

## **2.2. Extraction and Detection of hCG and IGF-I from DBS Using UHPLC-MS/MS**

The second study extends the application of DBS to the detection of larger biomolecules, specifically the peptide hormones hCG and IGF-I. These hormones, often used for their performance-enhancing properties, pose a significant analytical challenge due to their complex structure and low concentrations in blood. The study aimed to develop a robust extraction and detection protocol for these analytes using ultra-high-performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS).

- **Optimization of Extraction Protocols:** A comprehensive review of existing literature and preliminary experiments were conducted to identify the most effective solvents for protein extraction from DBS. Various solvents, including methanol, acetonitrile, and lysis buffers, were tested, with lysis buffer emerging as the most efficient for protein recovery. The study also evaluated the impact of reduction and alkylation steps on extraction efficiency. These steps were found to enhance protein solubilization, resulting in higher yields.
- **Digestion Strategies:** Protein digestion is a critical step in the analysis of peptide hormones. The study compared two digestion approaches: on-paper digestion and digestion of the extracted supernatant. On-paper digestion, while practical, proved less effective than supernatant digestion due to the incomplete release of peptides from the paper matrix. The optimized protocol, which included reduction and alkylation followed by supernatant digestion, achieved the best recovery rates and reproducibility for both hCG and IGF-I.

- **Peptide Identification and Quantification:** The method successfully identified all five targeted IGF-I peptide sequences in both spiked and DBS samples, demonstrating the protocol's efficiency. For hCG, nine peptide sequences were consistently detected in DBS, compared to 14/15 in spiked samples, indicating some loss during the drying process. However, the presence of key peptide sequences in DBS confirmed the method's suitability for detecting these hormones in a dried matrix. The study also quantified the relative abundance of each peptide sequence, providing critical data for future method refinement and validation.
- **Implications for Individualized Doping Detection:** The study suggests that individualized monitoring of IGF-I and hCG levels could be a powerful tool in anti-doping efforts, given the substantial inter-individual variability in these hormones' baseline concentrations. Establishing personal reference ranges could help distinguish between natural fluctuations and exogenous administration, potentially reducing false positives and enhancing the specificity of anti-doping testing.

### **3. Impact of Research on Anti-Doping Science**

The integration of Dried Blood Spot (DBS) microsampling into anti-doping analysis presents a substantial advancement in the field, addressing several longstanding challenges inherent to traditional sampling methods. The research presented in this thesis has demonstrated the viability of DBS as an innovative and practical solution for the detection of prohibited substances, offering numerous benefits that could revolutionize anti-doping protocols.

#### **3.1. Addressing Sample Collection and Handling Challenges**

Traditional anti-doping testing typically involves the collection of liquid blood or urine samples, which necessitates trained personnel, secure collection environments, and immediate processing to ensure sample integrity. This process is not only logistically demanding but also invasive and can be distressing for athletes. DBS microsampling offers a simpler, less invasive alternative that can be performed with minimal training and equipment. A single drop of blood collected from a finger prick onto a cellulose card can be stored and transported

at room temperature without the need for a cold chain, significantly reducing logistical complexity.

Furthermore, DBS minimizes the risk of sample contamination and degradation, as analytes remain stable on the dried matrix for extended periods. This characteristic is particularly valuable for anti-doping testing, where samples may need to be transported over long distances or stored for retrospective analysis in the event of new analytical methods being developed or when re-analysis is warranted due to emerging intelligence.

### **3.2. Enhancing Analytical Sensitivity and Specificity**

The development and validation of high-performance liquid chromatography-tandem mass spectrometry (LC-MS/MS) methods optimized for DBS, as demonstrated in the studies included in this thesis, significantly enhance the analytical sensitivity and specificity required for anti-doping analysis. The research has shown that DBS can be effectively used to detect both small molecules, such as anabolic androgenic steroids (AAS), and larger biomolecules, such as peptide hormones (hCG and IGF-I), with limits of detection and quantification that meet or exceed the minimum required performance levels (MRPLs) set by WADA.

This capability is crucial in the fight against doping, where detecting even trace amounts of banned substances can make the difference between a negative and a positive test result. By expanding the range of detectable analytes and improving the robustness of the analysis, DBS-based methods can potentially lead to more accurate and reliable anti-doping testing outcomes, reducing the likelihood of false negatives and enhancing the deterrence effect of doping controls.

### **3.3. Facilitating Widespread and Routine Testing**

The ease of DBS collection and the minimal logistical requirements for sample storage and transport make this technique particularly well-suited for widespread and routine testing. This could be transformative for out-of-competition testing, where logistical and financial constraints often limit the frequency and coverage of doping controls. With DBS, testing can be conducted more frequently and in a

wider range of settings, including remote or underserved regions, without the need for specialized facilities or trained phlebotomists.

Additionally, the non-invasive nature of DBS collection could increase athlete compliance and willingness to participate in testing programs. This is especially relevant in sports with younger athletes or those who may be more apprehensive about traditional blood collection methods. The greater acceptability and accessibility of DBS could ultimately lead to more comprehensive and effective anti-doping surveillance.

### **3.4. Enabling Longitudinal Monitoring and Retrospective Analysis**

One of the most promising applications of DBS in anti-doping is its potential for longitudinal monitoring. By collecting and storing small blood samples at regular intervals, it is possible to create a biological passport that tracks an athlete's biomarkers over time. This approach allows for the detection of abnormal variations that may indicate doping, even when direct detection of a banned substance is not possible.

Furthermore, the stability of DBS samples enables retrospective analysis, which can be particularly valuable in cases where new analytical techniques are developed after samples have been collected. The ability to re-analyze stored DBS samples using these improved methods could lead to the identification of previously undetectable doping cases, thereby enhancing the overall effectiveness and credibility of anti-doping programs.

### **3.5. Contributing to a More Ethical and Transparent Anti-Doping Regime**

The adoption of DBS microsampling also has ethical implications. By reducing the invasiveness of sample collection and minimizing the potential for privacy breaches, DBS contributes to a more athlete-friendly and transparent anti-doping regime. This is important for maintaining the trust and cooperation of athletes, which are essential for the success of any anti-doping program. The research presented in this thesis, therefore, not only advances scientific methodologies but also aligns with the broader goal of ensuring that anti-doping efforts are conducted in a manner that respects athlete rights and well-being.

## **4. Innovation and Future Perspectives**

The research findings presented in this thesis highlight the transformative potential of DBS microsampling in anti-doping science, laying a foundation for future innovations and broader applications. Several promising avenues for further development and implementation are discussed below.

### **4.1. Refinement and Automation of DBS Analytical Protocols**

While the studies conducted demonstrate the efficacy of DBS in detecting both AAS and peptide hormones, there is considerable scope for refining these analytical protocols to enhance their performance further. Automation of the DBS sample preparation process, including automated punching, extraction, and analysis, could significantly reduce variability and improve throughput. Automated systems would not only streamline the workflow but also increase reproducibility and precision, making DBS a more practical option for routine anti-doping testing.

Additionally, advancements in high-resolution mass spectrometry and novel chromatographic techniques could further improve the sensitivity and selectivity of DBS-based methods. For instance, the development of more targeted and selective extraction protocols for specific analytes, as well as the use of ion mobility spectrometry, could enhance the resolution and accuracy of detection, especially for complex biological matrices like dried blood.

### **4.2. Expanding the Range of Detectable Substances**

While the current research has focused on AAS and peptide hormones, there is potential to expand DBS applications to a wider array of prohibited substances, including stimulants, narcotics, and cannabinoids. This would involve developing and validating DBS-based methods for these substances, ensuring that they meet the same rigorous standards of sensitivity and specificity required for traditional liquid samples.

Moreover, the integration of omics technologies, such as proteomics and metabolomics, with DBS could enable the detection of novel biomarkers of

doping, providing additional tools for indirect detection. These biomarkers could include metabolic by-products or protein expression changes associated with the use of certain substances, offering a complementary approach to direct analyte detection.

### **4.3. Development of Individualized Doping Detection Models**

One of the major challenges in anti-doping science is accounting for inter-individual variability in biomarkers. The individualized nature of biological responses means that standard population-based thresholds may not always be

sufficient to detect doping effectively. To address this, future research could focus on developing personalized doping detection models using longitudinal data collected via DBS. By creating individualized biomarker profiles for each athlete, these models could help distinguish between natural physiological fluctuations and changes indicative of doping.

This approach would involve collecting DBS samples from athletes at regular intervals over time, establishing a baseline for each individual. Deviations from this baseline, rather than population norms, would then trigger further investigation. Such a model could significantly reduce false positives and false negatives, providing a more nuanced and accurate tool for anti-doping authorities.

Moreover, the use of advanced statistical and machine learning techniques could enhance the predictive power of these individualized models. By analyzing large datasets of longitudinal DBS samples, it may be possible to identify subtle patterns and correlations that are not apparent with traditional methods. This could lead to the development of more sophisticated algorithms capable of detecting even the most covert doping strategies.

### **4.4. Integrating DBS with Other Biological Matrices for a Multi-Omics Approach**

Future anti-doping research could benefit from integrating DBS analysis with other biological matrices, such as saliva, urine, and hair, to provide a comprehensive view of an athlete's physiological state. Each matrix offers unique

insights and complementary information, and combining them could significantly enhance the sensitivity and specificity of doping detection.

For instance, while DBS is excellent for detecting substances present in the bloodstream, urine may be more suitable for detecting metabolites or substances excreted in higher concentrations. Similarly, hair analysis can provide a long-term record of substance use, which could complement the more immediate snapshot provided by DBS. By employing a multi-omics approach that leverages the strengths of each matrix, anti-doping authorities could achieve a more robust and reliable detection system.

#### **4.5. Enhancing Global Anti-Doping Efforts with DBS**

The portability and stability of DBS make it an ideal tool for expanding anti-doping efforts globally, particularly in regions with limited resources or infrastructure. By reducing the need for specialized collection and storage facilities, DBS could enable more widespread testing, helping to ensure a level playing field for athletes worldwide.

This could involve deploying DBS collection kits to remote locations, allowing athletes to provide samples without the need for travel or specialized personnel. The samples could then be mailed to central laboratories for analysis, significantly reducing logistical barriers. Additionally, partnerships with local healthcare providers could facilitate the integration of DBS into existing healthcare systems, providing a sustainable model for ongoing anti-doping surveillance.

#### **4.6. Development of Portable Analytical Devices for On-Site Testing**

One of the most exciting future prospects for DBS in anti-doping is the development of portable analytical devices capable of on-site testing. Such devices, utilizing technologies like paper-based microfluidics or point-of-care mass spectrometry, could allow for rapid screening of DBS samples directly at sporting events or training sites. This would provide immediate results, enabling quicker response times and reducing the window of opportunity for athletes to evade detection.

While significant technological challenges remain, including miniaturization of analytical equipment and ensuring sufficient sensitivity and specificity, ongoing research in the field of portable diagnostics suggests that this vision may become a reality in the not-too-distant future. The successful development of such devices could revolutionize anti-doping testing, making it more efficient, responsive, and accessible.

#### **4.7. Legal and Regulatory Considerations for Implementing DBS**

For the widespread adoption of DBS in anti-doping testing, it is crucial to address the legal and regulatory considerations associated with this new sampling method. This includes ensuring that DBS collection and analysis methods meet the rigorous standards set by organizations such as WADA and national anti-doping agencies. Developing standardized protocols for DBS collection, storage, and analysis will be essential to ensure the validity and comparability of results across different laboratories and testing contexts.

Additionally, the legal framework surrounding the use of DBS samples, particularly concerning issues of athlete consent, privacy, and data security, must be carefully considered. The non-invasive nature of DBS may raise new ethical questions, such as the potential for inadvertent collection of additional health information not related to doping. Clear guidelines and robust safeguards will be necessary to address these concerns and protect athletes' rights.

#### **4.8. Educational and Awareness Initiatives for Athletes and Stakeholders**

To facilitate the adoption of DBS in anti-doping testing, it is important to conduct educational and awareness initiatives targeting athletes, coaches, and other stakeholders. This will help build trust in the new methodology and ensure that all parties understand its benefits and limitations. Educational campaigns should focus on the science behind DBS, its advantages over traditional methods, and how it can contribute to fair play in sports.

In addition, training programs for anti-doping personnel will be essential to ensure the proper implementation of DBS collection and analysis protocols. This will

involve not only technical training but also updates to existing anti-doping guidelines and procedures to accommodate the unique aspects of DBS.

#### **4.9. Collaborative Research and Development Initiatives**

Finally, continued collaboration between academia, anti-doping agencies, and technology developers will be critical for advancing DBS methodologies and integrating them into routine anti-doping testing. Collaborative research initiatives could focus on addressing current limitations of DBS, such as improving the sensitivity of detection for certain substances, developing better extraction and quantification techniques, and exploring new applications of the technology.

Moreover, partnerships with technology companies could accelerate the development of portable testing devices and automated DBS processing systems. By fostering a multidisciplinary approach that combines expertise from various fields, it will be possible to overcome the current challenges and fully realize the potential of DBS in anti-doping science.

In conclusion, the research presented in this thesis not only demonstrates the feasibility and effectiveness of DBS microsampling for anti-doping analysis but also opens up a wide range of future possibilities for innovation and improvement. As the field continues to evolve, DBS has the potential to become a cornerstone of anti-doping testing, contributing to a more effective, ethical, and sustainable approach to protecting the integrity of sport.

# **Chapter VI**

## **THESIS CONCLUSION**

The research activities carried out in this Ph.D. project concerned the design and development of new approaches to the detection and determination of doping compounds in medicinal analytical chemistry, within biological matrices. The study deepened knowledge about anabolic androgenic steroids and proteins, through different approaches and advanced technologies for each stage of the analytical process, from sampling, through pretreatment to instrumental analysis.

With regard to the analysis of anabolic androgenic steroids, the first approach involved the design of an original UHPLC-MS method for the identification and quantification of six major traditional (blood) and innovative (micro blood samples). For this research, all MS parameters and chromatographic conditions have been adequately tested and optimized. The final method was then validated on standard analyte mixtures with satisfactory results. After the sample was pretreated, the extracts were analysed and the extraction yields evaluated with satisfactory results. In addition, the performance of micro sampling technology was evaluated, obtaining excellent results in terms of volumetric accuracy of sampling and drying time, thus proving to be a promising alternative to classic test tube samples.

In the second study, an optimized extraction method for hCG and IGF-I from dried blood spots (DBS) was developed using UHPLC-MS/MS analysis.

Through a comprehensive review of the literature, effective extraction solvents have been identified, with buffer lysis emerging as the most efficient for maximizing protein yields. Various extraction protocols and digestion methods have been rigorously tested, revealing that digestion on supernatants significantly improves extraction results compared to traditional methods.

The success of detection of key peptide sequences related to hCG and IGF-I in DBS samples demonstrates the reliability of the method. The relative abundance of these sequences increases the potential to confirm the presence of these banned substances in athletes' blood.

Looking ahead, the study highlights the importance of establishing individual normal ranges for IGF-I and hCG levels. This progress will help differentiate between normal and potential physiological variations, thus improving the effectiveness of anti-doping monitoring strategies and further validating the innovative use of DBS in this context.

In conclusion, this Ph.D. project has developed new approaches for the detection of doping compounds in biological matrices. The successful design of a UHPLC-MS method for anabolic androgen steroids, combined with the optimization of extraction techniques for hCG and IGF-I from dried blood spots, underlines the potential of these methodologies to improve anti-doping analysis. Validation of extraction protocols and promising results from microsampling demonstrate a shift towards more efficient and practical test methods. As we move forward, establishing individual normal intervals for IGF-I and hCG will be crucial in distinguishing between physiological variations and doping practices, ultimately contributing to more effective anti-doping strategies. This research not only highlights the innovative use of DBS, but also lays the foundation for future studies to improve the integrity of sport.