FACULTY OF MEDICINE AND HEALTH SCIENCES

Detection of doping agents in dried blood spots via liquid chromatography – mass spectrometry

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Preface

First and foremost, I would like to thank Prof. Dr. Ir. Peter Van Eenoo for the opportunity to conduct this research at DoCoLab. His prompt responses to my emails and thorough answers to my questions have been invaluable throughout this process.

Special thanks to Monica Mazzarino for her excellent guidance and support, which have significantly contributed to the quality of this thesis.

I am deeply grateful to my family for their unwavering support and encouragement.

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Thank you all.

Lisa

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Summary

This dissertation investigates the use of dried blood spots (DBS), Tasso, and Volumetric Absorptive Microsampling (VAMS) for anti-doping testing. The research focuses on developing and validating a method to detect 196 doping compounds using liquid chromatography-mass spectrometry (LC-MS). This approach aims to improve anti-doping measures by offering a minimally invasive, convenient, and effective alternative to traditional urine tests.

Key findings include the high sensitivity and specificity of the method, with satisfactory limits of detection and quantification for various substances such as anabolic agents and stimulants. Minimal carry over and minimal impact from hematocrit levels emphasize the method's robustness and reliability. The study demonstrates that DBS can enhance doping control by simplifying sample collection and transport while ensuring accurate and reliable analysis.

The research concludes that the use of DBS, Tasso and VAMS in combination with LC-MS provides significant advantages in the fight against doping, promoting fair play in sports and protecting public health by discouraging the use of performance-enhancing drugs. This advancement in testing methodology could lead to more widespread and effective anti-doping practices.

Layman summary with societal impact

Developing a screening method for 196 doping compounds using dried blood spots (DBS), Tasso, and Volumetric Absorptive Microsampling (VAMS) instead of traditional urine testing has significant societal impacts. This advancement addresses a critical gap in anti-doping measures, where athletes can misuse pharmaceutical drugs as performance enhancers without immediate detection. Effective doping control is essential for maintaining fairness in sports, allowing everyone from amateurs to Olympians a fair chance to compete and win based on natural talent and hard work.

Firstly, these methods offer enhanced convenience and compliance due to their less invasive nature and ease of collection, increasing athlete willingness to participate in doping tests. They are portable and practical, allowing for sample collection in various settings without the need for specialized facilities.

From a public health perspective, better detection methods protect athletes from the harmful effects of doping and deter young athletes from using performance-enhancing drugs. Economically, these methods are cost-effective, reducing the overall costs of doping control programs due to simpler collection and storage requirements, and streamlining logistics, particularly in large-scale events like the Olympics. Ethically, effective doping control supports fair play and integrity in sports, while educational initiatives can highlight the dangers of doping and the importance of clean sports.

In conclusion, switching to DBS, Tasso, and VAMS for doping screening enhances convenience, sensitivity, and reliability, promoting public health, fair competition, technological advancement, and ethical standards in sports.

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1. Introduction

The primary objective of the World Anti-Doping Agency (WADA) is to promote an international, cooperative movement towards doping-free sports. The organization's operations are centered around the obligations delegated by the World Anti-Doping Code. One of these duties is to release an annual Prohibited List listing the drugs and techniques that are forbidden both before and after competition, with a focus on specific sports. For a substance or method to be added to the List, it must be determined that it meets at least two of the following three criteria: 1) It has the potential to enhance or enhances sport performance, 2) It represents an actual or potential health risk to the athletes, 3) It violates the spirit of sport [1].

1.1 WADA prohibited list

The WADA Prohibited List categorizes banned substances and methods into diverse classes *(Table 1).* Each class exerts its distinct impact on the human body and may influence athletes' performance in multifaceted ways. A comprehensive grasp of these classes is imperative for athletes and anti-doping entities alike to ensure equitable and healthful competition [1].

Table 1: WADA prohibited substance classes and examples

PROHIBITED AT ALL TIMES	
S0 (non-approved substances)	BPC-157
S1 (anabolic agents)	Testosterone, clenbuterol
S2 (peptide hormones, growth factors, related substances, and mimetics)	Erythropoietin, growth hormone
S3 (beta-2 agonists)	Fenoterol, salbutamol
S4 (hormone and metabolic modulators)	Tamoxifen, meldonium
S5 (diuretics and masking agents)	Acetazolamide, thiazides
PROHIBITED IN-COMPETITION	
S6 (stimulants)	Amfetamine, cocaine
S7 (narcotics)	Diamorphine (heroin), morphine
S8 (cannabinoids)	Canabis
S9 (glucocorticoids)	Cortisone, deflazacort
PROHIBITED IN PARTICULAR SPORTS	
P1 (beta-blockers)	Metoprolol, propranolol

1.1.1 S0 Non-Approved Substances

The S0 class of the WADA Prohibited List includes substances that are not approved for human use. These substances may pose unknown risks to health and safety and have not undergone the necessary regulatory approval processes. Athletes are strictly prohibited from using substances in the S0 class, regardless of whether they have performance-enhancing effects or not [2].

1.1.2 S1 Anabolic Agents

Anabolic agents are substances that promote muscle growth and strength. These include anabolic steroids, synthetic derivatives of testosterone, which are commonly abused by athletes seeking to enhance their physical performance. Anabolic agents can lead to increased muscle mass, improved recovery times, and enhanced athletic performance [3].

1.1.3 S2 Peptide Hormones, Growth Factors, Related Substances and mimetics

This class encompasses a wide range of substances that regulate various physiological processes in the body, including growth, metabolism, and tissue repair. Examples include growth hormone (GH), insulin-like growth factor 1 (IGF-1), and erythropoietin (EPO). Athletes

may abuse these substances to increase muscle mass, improve endurance, or enhance recovery [4].

1.1.4 S3 Beta-2 Agonists

Beta-2 agonists are bronchodilators commonly used to treat asthma and other respiratory conditions [1]. However, they also have the potential to enhance athletic performance by increasing aerobic capacity and reducing fatigue [5]. Examples include salbutamol and formoterol. While some beta-2 agonists are permitted with a Therapeutic Use Exemption (TUE), others are prohibited in competition due to their potential for abuse and performance enhancement [1].

1.1.5 S4 Hormone and Metabolic Modulators

This class includes substances that regulate hormone production and metabolism in the body. Examples include selective estrogen receptor modulators (SERMs) and aromatase inhibitors. These substances may be used to increase muscle mass, reduce fat, or alter hormone levels to improve athletic performance [4].

1.1.6 S5 Diuretics and Masking Agents

Diuretics are substances that increase urine production and can be used to rapidly lose weight or flush out other banned substances from the body. Masking agents are substances that interfere with drug tests by diluting or masking the presence of banned substances in urine samples. Both diuretics and masking agents are prohibited by WADA due to their potential for abuse and ability to conceal doping violations [6].

1.1.7 S6 Stimulants

Stimulants are substances that increase alertness, attention, and energy levels. They include both legal substances such as caffeine and prohibited substances such as amphetamines and cocaine. Athletes may abuse stimulants to improve focus, delay fatigue, and enhance performance [7].

1.1.8 S7 Narcotics

Narcotics are drugs that relieve pain and induce sleep. They include opioids such as morphine, codeine, and heroin. While narcotics can be used legitimately to manage pain from injuries or medical conditions, their abuse by athletes is strictly prohibited by WADA due to the potential for addiction, impairment, and unfair competitive advantages [1].

1.1.9 S8 Cannabinoids

Cannabinoids are substances derived from the cannabis plant or synthetic equivalents that act on cannabinoid receptors to reduce anxiety [8]. These substances include delta-9-tetrahydrocannabinol (THC), the psychoactive component of cannabis, as well as other cannabinoids such as cannabidiol (CBD) [1]. While cannabinoids may have various therapeutic properties and are increasingly being legalized for medical and recreational use in some jurisdictions, they are prohibited in sport by WADA [1].

1.1.10 S9 Glucocorticoids

Glucocorticoids are anti-inflammatory medications commonly used to treat conditions such as asthma, allergies, and autoimmune disorders [1]. However, they also have the potential to enhance athletic performance by reducing inflammation, pain, and swelling. Athletes may abuse glucocorticoids to mask injuries or enhance recovery [9]. Therefore, their use is restricted by WADA, and athletes require a Therapeutic Use Exemption (TUE) to use them legitimately for medical purposes [1].

1.1.11 P1 Beta Blockers

Beta blockers are medications commonly used to treat conditions such as high blood pressure, heart disease, and anxiety. They work by blocking the effects of adrenaline on the body's beta receptors, leading to reduced heart rate and blood pressure. In sports, beta blockers can be abused to calm nerves, improve focus, and enhance precision in sports that require steady hands and precise movements, such as shooting and archery. However, their use is banned

by WADA in certain sports due to their potential to mask physical symptoms of anxiety or stress and provide an unfair advantage to athletes. Beta-blockers are prohibited in-competition only for sports like darts and golf, and also prohibited out-of-competition in disciplines like archery and underwater sports [1].

1.2 Urine Screening Methods

Urine screening methods play a crucial role in doping testing, aiming to detect the presence of prohibited substances or their metabolites in athletes' bodies. In doping control, examinations to determine the existence of a prohibited substance in urine are consistently conducted through a standard procedure, commencing with an initial screening and then proceeding to a confirmation process. The screening phase needs to be rapid, discriminating, and sufficiently sensitive to prevent both false negatives and false positives. Upon obtaining a positive screening outcome, verification is essential by specifically targeting the identified compound and its metabolites [10].

In order to implement this process effectively across the extensive array of compounds listed in the WADA prohibited list, accredited anti-doping laboratories must employ multiple analytical techniques, with mass spectrometry-based methods (GC–MS/MS and LC–MS/MS) being paramount. These methods are regarded as reference standards because of their superior selectivity and sensitivity [11].

1.2.1 Liquid chromatography

In this research, a liquid chromatography (LC) technique is used, which is a method for isolating a specific molecule from a sample. In LC, a column containing a stationary phase is utilized. After injecting the sample, the mobile phase is pushed through the column at a set flow rate, creating pressure within the chromatographic system. The target analyte binds to the stationary phase based on its affinity for the functional groups, which is polarity-dependent. In normal-phase LC, the stationary phase is polar while the mobile phase is non-polar. In reverse-phase LC (RP LC), the polarities are reversed. Separation occurs due to differences in affinity between the target analyte and other compounds for the stationary phase. As the mobile phase flows through the column, it carries substances present in the sample. Compounds with low affinity for the stationary phase exit the column quickly, while those with high affinity spend more time in the stationary phase. The time between injection and exit, termed retention time, is characteristic of a molecule but can be adjusted by modifying LC parameters. The workflow of a LC device is shown in *Figure 1*. As LC only separates molecules and lacks inherent detection, a detector such as a mass spectrometer is often connected to the chromatographic system [12].

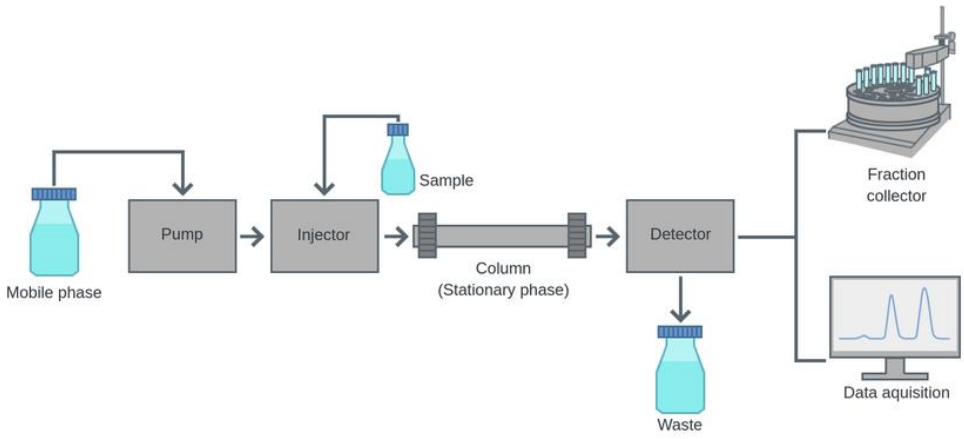


Figure 1: Schematic representation of a LC device. (https://www.sciencedirect.com/topics/chemical-engineering/liquid-chromatography)

1.2.2 Mass spectrometry

Mass spectrometry (MS) is an analytical technique that involves generating ionized molecules or atoms, separating these ions based on their mass-to-charge ratio, and measuring the (relative) abundance of each ion. This process provides information that determines the nature, composition, and structure of the analyte. MS is known for its high specificity, sensitivity, and flexibility, often requiring only a minimal amount (sometimes less than a picogram) of the target analyte [12].

A small amount of sample in the gas phase (or another suitable form) is ionized, and the resulting charged particles are analyzed in a magnetic and/or electric field, depending on the type of instrument. By analysis of the path followed by the ions in the analysis tube, which is under a high vacuum (10^{-4} Pa), the mass-to-charge ratio (m/z) of the ions and potentially their nature can be determined. This highly sensitive method is destructive [12].

During mass spectrometric analysis, the sample undergoes several successive processes:

Ionization: The sample is vaporized and ionized in the ion source of the instrument. Various ionization techniques exist, often forming singly charged ions (z=+1). For larger molecules, such as proteins, multiple charges (z>1) can result in multiply charged cations. The ion formed by removing an electron from a molecule is called the molecular ion. These molecular ions (radical cations with an odd number of electrons) fragment, leading to a statistical distribution of fragment ions, often involving stable carbocations [12].

Acceleration: Immediately after formation, the ions are extracted from the ion source, focused by a series of electronic lenses, and accelerated by an applied voltage difference (or magnetic field) to increase their kinetic energy [12].

Separation: The mass analyzer filters the ions according to their m/z ratio. Some instruments use multiple mass analyzers in series for enhanced separation [12].

Detection: After separation, the ions strike the detector at the end of their path through the mass spectrometer. The detector measures the electric charge and amplifies the weak ion current [12].

After converting the recorded detector signals, a mass spectrum is obtained. The most intense peak in the mass spectrum, with its intensity set to 100%, is called the base peak. *Figure 2* shows a schematic representation of an MS device [12].

All mass spectrometers include a vacuum system to maintain the low pressure (high vacuum) necessary for proper operation. This high vacuum minimizes ion-molecule reactions,

scattering, and neutralization of the ions formed during the ionization and fragmentation process [12].

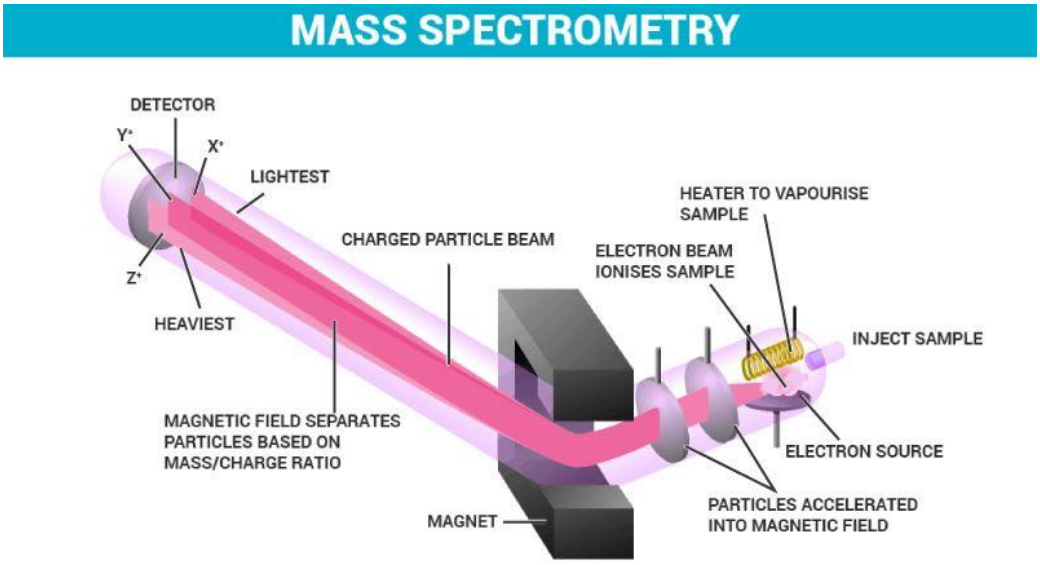


Figure 2: Schematic representation of a MS device (https://byjus.com/chemistry/mass-spectrometry/)

1.2.2.1 ESI

As the ionization source, electrospray ionization (ESI) was utilized, which operates at atmospheric pressure. In ESI, ions are produced by applying an electric field to the tip of the column-MS interface outlet. The charged droplets generated in the spray are then heated and collide with a dry gas, such as N_2. This process causes evaporation and desolvation of the solvent molecules surrounding the analyte. Consequently, the internal repulsion force among the ions within the droplets increases, leading to the droplets exploding into smaller ones. This cascade of shrinking and exploding droplets continues until it results in non-fragmented ions. These ions are either protonated or cationized and carry a variable number of charges, typically around one charge per 1000 Da. The analyte's mass can then be easily calculated from the resulting molecular ion cluster $(M+nH)^{n+}$ [12].

1.2.2.2 Orbitrap

In our study, an orbitrap mass analyzer was used. It consists of an outer electrode shaped like a barrel and an inner electrode shaped like a spindle. These electrodes capture the ions in a rotational motion around the spindle. Unlike traditional ion traps, where ions are sequentially ejected by changing electrode potentials, the orbitrap traps ions in a dynamic electrostatic field, causing them to oscillate coherently [12].

Fourier transformations measure the frequency of these coherent oscillations and convert them into a mass spectrum. This technique allows for the precise determination of the mass-to-charge (m/z) ratios of the ions [12].

Orbitrap mass spectrometers are known for their high sensitivity, high resolution, and excellent mass accuracy. They can detect low-abundance ions, making them crucial for detailed analytical work. The high resolution enables the precise separation of ions with very similar m/z ratios, facilitating the detailed analysis of complex mixtures. Furthermore, the mass accuracy ensures that the measured mass of ions closely matches their true mass, which is essential for reliable compound identification [12].

1.3 Blood analysis

While extensive discussions on the advantages and disadvantages of various doping control matrices have persisted for decades, there is a consensus on the indispensability of blood

sampling, which significantly complements urine-based doping controls [13-15]. The following Prohibited Substances can be analyzed in blood: Erythropoietin receptor agonists (EPOs); Growth Hormone (GH) analysis using either the Isoforms or the Biomarkers method; The haematological module of the Athlete Biological Passport (ABP); Blood Transfusions (BT); Hemoglobin-based oxygen carriers (HBOCs); and Steroid esters. Other Prohibited Substances which can also be analyzed in a blood sample (serum/plasma) but which may have limited availability at some Laboratories includes the following non-exhaustive list: Xenon; Insulin analogues; Desmopressin; and Insulin Growth Factors (IGF-1) analogues [16].

In addition to these predominantly high molecular mass compounds, blood analysis offers significant advantages over urinalysis, especially when temporal information is crucial, such as in cases of substances prohibited solely during competition periods. Consequently, substantial efforts have been recently directed towards enhancing analytical assays for blood/serum/plasma samples in sports drug testing laboratories [17].

1.4 Microsampling

Microsampling refers to the collection of very small sample volumes (µl) from both animal and human matrices to evaluate drug and chemical exposure within these biological contexts [18]. Alternative sampling methods with reduced volumes have predominantly emerged from scientific and ethical considerations in preclinical investigations and clinical trials involving pediatric subjects. Microsampling facilitates comprehensive sampling across study animals, enhancing the quality of scientific data and enabling a direct correlation between exposure and toxic effects. Ethical considerations inherent in microsampling also contribute to subject recruitment and retention in clinical trials involving pediatric and elderly populations. Additionally, microsampling has been shown to contribute to improved animal welfare by minimizing stress on study animals and reducing the overall number required for experimentation [19]. Of course, similar benefits can be expected in humans. Moreover, microsampling offers practical advantages such as streamlined sample storage, shipment, and analysis processes [20,21]. Given the multitude of benefits, these techniques are swiftly gaining prominence.

1.4.1 Introduction of DBS

The rigorous standards and quality demands of modern sports drug testing pose significant challenges for doping control authorities and accredited laboratories. Addressing these challenges requires innovative approaches, such as the collection and testing of additional or complementary specimens like dried blood spots (DBS) [22].

First, a crucial aspect of effective doping controls involves the frequent and unpredictable sampling of athletes, particularly out-of-competition and concerning substances like anabolic agents and erythropoiesis-stimulating agents. However, the costs associated with sample collection and analysis present significant constraints [22].

Next, in in-competition controls, it's crucial to differentiate whether substances such as stimulants or cannabinoids, which are not prohibited at all times, were present in athletes' blood at pharmacologically relevant concentrations. Drawing conclusions about blood concentrations from urine analyses is complex and has been the subject of numerous studies and discussions [22].

Finally, the challenge of unstable compounds, exemplified by substances like Synacthen involved in the Spanish Fuentes scandal, underscores the need for improved analytical methods. Despite existing methods, the compound's limited stability in blood and urine hampers analysis. DBS collection and analysis offer a promising solution, addressing cost constraints, enabling determination of drug concentrations at competition time, and conserving unstable analytes [22].

1.4.2 Dried Blood Spots

DBS sampling is a microsampling technique used to collect small blood volumes, reducing steps like freezing and plasma harvesting [23]. The use of DBS dates back to the 1960s when Dr. Robert Guthrie utilized them to measure phenylalanine for phenylketonuria detection [24]. Dried blood spot collection involves obtaining blood spots via a finger or heel prick and placing them directly onto filter paper *(Figure 3).* This paper is then left to air dry for 2-3 hours at 22°C on a non-absorbent surface, though drying time may vary. Once dried, DBS samples can be stored with desiccant to prevent moisture damage, and sent to labs for testing. Factors like sunlight, heat, and moisture can degrade analytes. Samples are punched to provide volumetric measurements, with both treated and untreated papers available commercially [23].

Major advantages of DBS include reduced blood volume requirements, minimal risk of contamination or hemolysis, easy collection, and long-term analyte preservation [25]. Concerns with DBS include hematocrit effects, homogeneity, and volume-dependent results. Parameters like selectivity, analyte stability, and extraction recovery can vary in aged matrices. Hematocrit influences blood viscosity and spreading on cards. Slow acceptance of DBS is due to sample manipulation challenges, including card punching and handling before analysis [23,26-29].

DBS analysis emerges as the newest instrument aimed at fortifying the entire system, safeguarding athletes, and promoting the equitable competition they merit. Although certain components of this innovative testing approach were already utilized during the Olympic and Paralympic Games in Tokyo, Japan, in 2020, the method was fully implemented at the Winter Games in Beijing, China in 2022 [22]. However, it's noteworthy that WADA's current technical document outlines the requirements solely for analytical testing procedures applied to DBS samples for detecting Non-Threshold Substances without Minimum Reporting Levels (MRL). Presently, WADA confines DBS testing to Out-of-Competition (OOC) prohibited classes. Nevertheless, in this thesis, we extend our analysis to include In-Competition (IC) prohibited classes, preparing for potential updates and ensuring thorough coverage of doping detection protocols [30].

Figure 3: Dried blood spot card. (https://www.umcg.nl/-/dried-blood-spot)

1.4.3 Volumetric Absorptive Microsampling (VAMS)

The VAMS system offers a consistent blood volume irrespective of hematocrit levels. This device comprises an absorbent polymeric tip that facilitates the collection of a fixed, small blood volume through capillary action. Human samples can be obtained via finger or heel prick. During collection, the device is filled by tilting the handle at a 45° angle and immersing only the tip into the blood drop to allow it to fill. It's important not to fully submerge the sampler tip to avoid sample overfilling. The device is self-indicating; once the tip is filled, it changes color to red. Additionally, the tip is affixed to a handle designed to prevent contact with surfaces during storage and shipping [28,29].

Samples can be stored or shipped at ambient temperature. The VAMS device guarantees sample homogeneity by absorbing a precise volume onto its tip. During sample preparation,

either the tip is detached from the handle or the entire device is utilized [31]. This device simplifies sample pretreatment by eliminating the need for centrifugation of the liquid matrix and the sub-punching step required for DBS [29].

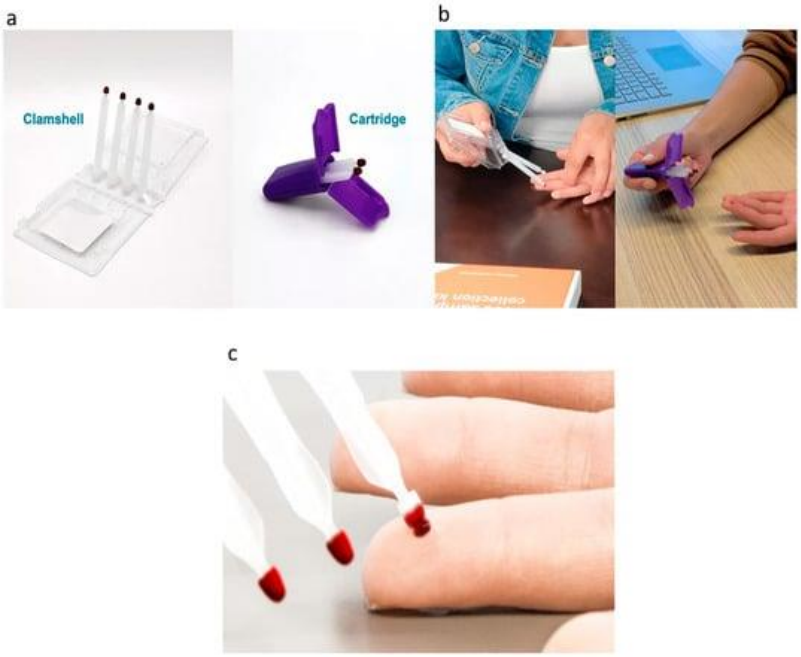


Figure 4: VAMS procedure: (a) VAMS tips are attached to a clamshell or cartridge; (b) tips are held at an angle to the blood, then removed after they are completely filled; (c) closeup of the blood absorption. (https://www.mdpi.com/2218-1989/13/10/1038)

1.4.4 TASSO

The Tasso-device is an automated capillary blood collection tool typically positioned on the skin of the upper arm, which is shown in *Figure 5*. Upon pressing a button, a lancet pierces the skin, and blood is drawn into the sample pod from the capillaries in the skin under a vacuum. The Tasso device collect four 20 mL dried whole blood samples. The sample pod can be removed from the button and sent to the laboratory. The samples can be further processed by removal of the dried blood samples from the sample pod [32].

The Tasso device provides a more comfortable sampling experience, with athletes reporting lower pain and a better overall experience compared to traditional fingerprick methods. The upper-arm collection, in particular, is favored for its reduced pain and simpler procedure. Both athletes and doping control officers (DCOs) prefer the upper-arm DBS collection over the fingerprick method. However, factors such as cost may influence the choice of device anti-doping organizations will use in real life [33].

Figure 5: Tasso M-20 device.
(https://www.google.com/url?sa=i&url=https%3A%2F%2Fdigitaltrials.scripps.edu%2Fthe-inner-workings-of-site-less-digital-clinical-trials-part-2-the-at-home-blood-collection-kit%2F&psig=AOvVaw26QqloepeJ6fySwE0xwpGL&ust=1715161565581000&source=images&cd=vfe&opi=89978449&ved=0CBQQjhxqFwoTCMjTqPSg-4UDFQAAAAAdAAAAABAR)

2 Materials and Methods;

2.1 Materials and reagents

2.1.1 Reagents

LC-MS grade H2O, acetonitrile (ACN), methanol (MeOH) and isopropanol were purchased from J.T. Baker® (Deventer, Netherlands). LC-MS grade formic acid (HCOOH) and NH4HCOOH was purchased from Fisher Chemical (Madrid, Spain).

2.1.2 Stimulants

Methylephedrine, amphetamine, methedrone and phendimetrazine were purchased from Cerriliant, Ritalinic_acid, benzoylecgonine, isometheptene, methylphenidate, cocaine, norfenfluramine and benfluorex from Sigma (Bornem, Belgium). Ethylphenidate, morazone and fenfluramine from TRC (North York, ON, Canada), 6-hydroxybromantane, benzylpiperazine, carphedone, cyclazodone and famprofazone from NMI (Pymble, Australia), furfenorex from Roussel Uclaf (Romainville, France), oxilofrine and pOH-mesocarb metabolite from the National Measurement Institute (Pymble, Australia), etilefrine from Boehringer-Ingelheim (Brussels, Belgium), adrafinil from Cephalon (Maisons-Alfort, France), heptaminol from Ets. A De Bournonville (Braine L'Alleud, Belgium), pholedrine from Knoll AG (Ludwigshaven, Germany), amfepramone from Lab. Pharm. R.H. Trenker (Brussels, Belgium), pentetrazol from Bios et Coutelier (Brussels), fenproporex from Bottu (Antony, France), fencamine from Laboratoires Miquel S.A, methoxyphenamine from Upjohn (Kalamazoo, USA), nikethamide from Ciba-Geigy (Groot-Bijgaarden, Belgium), propylhexedrine from LGC, mefenorex from Produits Roche (Brussels). Prolintane was a gift from Boehringer & Sohn (Ingelheim am Rhein, Germany), mephedrone from the Moscow anti-doping, ethamivan from Sinclair Pharmaceuticals, pemoline from Boehringer-Ingelheim, fenethylline from Chemiwerk Hamburg. Strychnine and fencamfamine were donated by Merck.

2.1.3 Narcotics

The narcotics are obtained by Cerriliant, apart from hydromorphone that was purchased from Sigma (Bornem, Belgium) and pentazocine that was a gift from Whintrop Laboratories (Newcastle, United Kingdom).

2.1.4 Glucocorticoids

The glucocorticoids were obtained from Sigma (Bornem, Belgium), apart from fluticasone propionate-17b-carboxylic acid, mometasone furoate and mometasone which were purchased

from TRC (North York, ON, Canada), budesonide from Glaxo-Wellcome (Brussels, Belgium), methylprednisolone from Pharmacia (Diegem, Belgium) and 6bOH-TriamAcetonide was a gift from Labaz (Brussels).

2.1.5 HIF (Hypoxia-Inducible Factors)

Enarodustat, IOX2 and FG4592 (raxadustat) were obtained from Med Chem Express, desidustat, vadadustat and GSK1278863 (daprodustat) from TRC and JNJ420 from Calbiochem.

2.1.6 Anabolic Agents

Andarine, andarine O-dephenyl metabolite, gestrinone, LGD4033, ostarine, RAD140, S23 and TFM4 were purchased from TRC (North York, ON, Canada), stanozolol, THG and dehydrochloromethyltestosterone from (Pymble, Australia), GSK288 from WADA and stanozolol-N-glucuronide from Siebersdorf research laboratories.

2.1.7 Diuretics and Masking Agents

Benzthiazide, brinzolamide, cyclothiazide, eplerenone and ethacrynic acid were obtained from Sigma–Aldrich (Bornem, Belgium), azosemide, buthiazide, conivaptan, eplerenone-OH, lixivaptan, methazolamide, methylclothiazide, mozavaptan, relcovaptan and Tolvaptan from TRC (North York, ON, Canada), bendroflumethiazide, bumetanide and hydroflumethiazide from Leo Pharmaceutical Products Belgium (Brussels, Belgium), canrenone and mebutizide from Sinestra, furosemide and piretanide from Hoechst (Brussels, Belgium), chlortalidone, cyclopenthiazide and hydrochlorothiazide from Ciba-Geigy (Groot-Bijgaarden, Belgium), acetazolamide and quinethazone from Cyanamid Benelux (Brussels, Belgium), althiazide from Continental Pharma (Brussels, Belgium), amiloride from Merck Sharp & Dohme (Brussels, Belgium), chlorothiazide from INRS (Québec, Canada), clopamide from Sandoz (Basle, Switzerland), dichlorphenamide from Alcon-Couvreur (Puurs, Belgium), dorzolamide was obtained from European Pharmacopoeia Reference Standards (Strasbourg, France), epitizide from SMB Technology (Marche-enFamenne, Belgium), polythiazide from Pfizer (Brussels), probenecid from Federa (Brussels, Belgium),triamterene from Smith-Kline (Genval, Belgium), trichlormethiazide from Merck (Overijse, Belgium), torasemide from Boehringer Mannheim (Brussels, Belgium) and xipamide was obtained from Laboratoire CUSI (Brussels).

2.1.8 Beta-2 Agonists

The beta-agonists are purchased from RIVM, apart from vilanterol and salmeterol that were obtained from Sigma–Aldrich (Bornem, Belgium), formoterol from Novartis (Arnhem, The Netherlands), tulobuterol from Supelco and bambuterol was donated by the Instituto Nacional do Desporto.

2.1.9 HMM (Hormone and Metabolic Modulators)

The HMMs are obtained from TRC (North York, ON, Canada), apart from SR9009 metabolite D1067, SR9009 metabolite D1066 and letrozole metabolite that were from NMI (Pymble, Australia), trimetazidine from Euorpean Pharmacopeia, anastrozol from Sigma–Aldrich (Bornem, Belgium), androstatrienedione from Steroloids and exemestane metabolite and exemestane were a gift from Astra Zeneca.

2.1.10 Beta Blockers

Befunolol and nebivolol was obtained from Sigma Aldrich (Massachusetts, United States), atenolol and propranolol from ICI (Kortenberg, Belgium), practorol and xamanterol from TRC (North York, ON, Canada), acebutolol from Rhone-Poulenc (Brussels, Belgium), alprenolol from Astra Chemicals (Holstein, Germany), betaxolol from Synthelabo (Brussels, Belgium), bisoprolol from Merck (Darmstadt, Germany), bupranolol from Schwarz Pharma (Monheim, Germany), carvedilol from Roche (Mannheim, Germany), esmolol metabolite from Synfine Research (Richmond Hill, Canada), labetolol from Glaxo (Brussels, Belgium), mepindolol from Schering (Machelen, Belgium), metoprolol from Ciba-Geigy (Groot-Bijgaarden, Belgium), nadolol from Squibb (Braine l'Alleud, Belgium), oxprenolol from CIBA (Dilbeek, Belgium), penbutolol from Thomson (London, United Kingdom), pindolol from Sandoz (Vilvoorde,

Belgium), sotalol from Pfizer (Brussels, Belgium) and timolol from MSD (Brussels, Belgium). Levobunolol and esmolol were a gift from the South African doping control laboratory. Carteolol was a gift from the doping control laboratory from Portugal and following products were obtained from therapeutical preparations: celiprolol (Selectol®, Pharmacia, Brussels) and metipranolol (Beta-Ophtiole®, Tramedic, Sint-Niklaas, Belgium).

2.1.11 Blood samples

Blood samples with different hematocrits were obtained by healthy volunteers and the study was approved by the local ethics committee (ONZ-2022-0625). The samples were collected in ethylenediaminetetraacetic acid (EDTA) tubes and stored in the fridge at 4°C.

2.2 Methods

2.2.1 Sample preparation

For the preparation of DBS and VAMS specimens, whole blood samples obtained from male and female healthy volunteers were fortified with a working solution of 196 compounds that cover 9 different classes (S1, S2, S3, S4, S5, S6, S7, S9, P1) of the WADA prohibited list. In order to exclude dilution effects or the precipitation of proteins, the mixture of compounds was evaporated first. The samples were extensively mixed prior to spotting 20 µL of spiked blood on a DBS sample collection card or Tasso device by using a calibrated pipette or dipping the tip of the 20 µL VAMS device into the spiked blood. The spots, Tasso and VAMS were allowed to dry for at least two hours at room temperature until further analysis.

The whole spots were cut out of the card, transferred to glass tubes and fortified with 500 µL of the extraction solvent. An ultrasonic bath was used for 40 min at 30°C for the extraction. The organic phase was transferred into a fresh Eppendorf tube and the supernatants were evaporated to dryness. The dry residue was resuspended in 50 µL of a 3:1 mixture of the mobile phases A and B (A: H20+5mM NH4HCOOH+0,02% HCOOH; B: 90% ACN+10% H20+5mM NH4HCOOH+0,02% HCOOH) and vortexed. Subsequently, the samples were centrifuged for 5 min at 7000 RPM at 10°C. Finally, all supernatant was transferred into vials that were injected into the LC-MS system.

2.2.2 Method validation

The entire analytical procedure, once developed and optimized, was validated for the screening analysis of prohibited substances in DBS, Tasso and VAMS, according to the ISO 17025 and the WADA requirements. 11 narcotics, 14 corticosteroids, 7 HIFs, 14 anabolic agents, 40 diuretics, 21 beta-2-agonists, 27 beta-blockers, 21 HMMs and 41 stimulants were included in this research. The following parameters were considered: recovery, carryover, selectivity, limits of detection (LOD) and hematocrit effect.

The selectivity was evaluated by analyzing 10 drug-free whole blood samples with different hematocrit levels from five male and five female subjects on two different days, to verify that the analytes of interest were effectively differentiated from endogenous matrix interferences and from those in the reagents/devices used for sample collection and extraction.

For the determination of the LOD, 10 drug-free whole blood samples from five from female and five from male subjects were spiked with the compounds under investigation at a concentration that is displayed in *Table 2*. Six concentration levels were prepared by serial dilutions using the same matrix, and the LODs were calculated according to the WADA guidelines using a sigmoidal module applied to detection rates (/10) at 6 levels.

Carryover was determined by analyzing drug-free whole blood samples immediately after samples containing the compounds of interest at a concentration at least 4 times the concentration in *Table 2*.

The recovery of all analytes was estimated by preparing 1) DBS, Tasso and VAMS samples (pre-spiked) using drug-free whole blood samples fortified with the target analytes at the concentration displayed in *Table 2*, before spotting on to the card, Tasso device, or VAMS

device, and 2) DBS, Tasso and VAMS samples (post-spiked) using drug-free whole blood samples, spotting on to the card, Tasso device or VAMS device, with the same concentration as the pre-spiked samples, after extraction.

The influence of varying hematocrit levels was assessed by analyzing five drug-free whole blood samples with high, medium, and low hematocrit concentrations (33-53%), each spiked with the target analytes.

Table 2: Compounds divided per class with corresponding concentration

Compound	Class	Concentration (ng/mL)
Buprenorphine	NARCOTICS	3,75
Codeine		37,5
Fentanyl		1,5
Hydromorphone		37,5
Methadone metabolite (EDDP)		37,5
Morphine		37,5
Norbuprenorphine		37,5
Norfentanyl		1,5
Pentazocine		37,5
Racemoramide (dextromoramide)		37,5
Sufentanyl		1,5
Budesonide	CORTICOSTEROIDS	67,5
Budesonide-OH		67,5
Clobetasol		45
Desonide		45
Fludrocortisone		45
Flumethasone		45
Fluticasone propionate metabolite		45
Methylprednisolone		45
Mometasone		45
Mometasone-furoate		45
Prednisolone		150,075
Prednisone		450
6bOH-triamacetonide		45
Triamcinolone		45
Desidustat	PEPTIDE HORMONES	12
Enarodustat		12
FG4592 (raxadustat)		12
GSK1278863 (daprodustat)		12
IOX2		12
JNJ420		12
Vadadustat		12
Andarine	ANABOLIC AGENTS	3
Andarine metabolite		3
Gestrinone		7,5
GSK288		3
LGD4033		3
Ostarine		3

Ostarine metabolite		3
RAD140		3
S23		3
Stanozolol		3
Stanozolol-N-glucuronide		3
Tetrahydrogestrinone		7,5
TFM4- AS1		3
Dehydrochloromethyltestosterone		10
Acetazolamide	**DIURETICS**	30
Althiazide		300
Amiloride		300
Azosemide		300
Bendroflumethiazide		300
Benzthiazide		300
Brinzolamide		300
Bumetanide		300
Buthiazide		30
Canrenone		300
Chlorothiazide		300
Chlortalidone		300
Clopamide		300
Cyclothiazide		300
Cyclopenthiazide		300
Conivaptan		300
Dichlorphenamide		300
Dorzolamide		300
Epitizide		300
Eplerenone		300
Eplerenone-OH		300
Ethacrynic acid		300
Furosemide		30
Hydrochlorothiazide		30
Hydroflumethiazide		300
Lixivaptan		300
Mebutizide		300
Methazolamide		300
Methylclothiazide		300
Mozavaptan		300
Piretanide		300
Polythiazide		300
Probenecid		300
Quinethazone		300
Relcovaptan		300
Triamterene		30
Trichlormethiazide		300
Torasemide		30

Tolvaptan		300
Xipamide		300
Bambuterol	BETA2-AGONISTS	30
Brombuterol		30
Cimaterol		30
Cimbuterol		30
Clenbuterol		0,3
Clenpeterol		30
Clenproperol		30
Clorprenaline		30
Fenoterol		30
Formoterol		30
Indacaterol		30
Isosuprine		30
Mabuterol		30
Mapenterol		30
Pirbuterol		30
Procaterol		30
Reproterol		30
Salmeterol		15
Terbutaline		30
Tulobuterol		15
Vilanterol		30
Acebutolol	BETA-BLOCKERS	75
Alprenolol		75
Atenolol		75
Befunolol		75
Betaxolol		75
Bisopropolol		75
Bupranolol		75
Carteolol		75
Carvedilol		75
Celiprolol		75
Esmolol		75
Esmolol acid		75
Labetalol		75
Levobunolol		75
Mepindolol		75
Metipranolol		75
Metoprolol		75
Nadolol		75
Nebivolol		75
Oxprenolol		75
Penbutolol		75
Pindolol		75
Practorol		75

Propranolol		75
Sotalol		75
Timolol		75
Xamanterol		75
Androstatrienedione	HORMONE & METABOLIC MODULATORS	30
Androstatrienedione metabolite		30
Anastrozol		30
Bazedoxifene		30
Clomiphene		30
Exemestane		30
Exemestane metabolite		30
Fulvestrant		30
GW0742 sulfone		3
GW0742 sulfoxide		3
GW1516		3
GW1516 sulfoxide		3
GW1516 sulfone		3
Letrozole metabolite		30
Raloxifene		30
SR9009		15
SR9009 metabolite D1066		15
SR9009 metabolite D1067		15
Tamoxifene metabolite		30
Toremifene		30
Trimetazidine		15
6-Hydroxybromantane	STIMULANTS	75
Adrafinil		75
Amfepramone		75
Amphetamine		75
Benzfluorex		75
Benzylpiperazine		75
Benzoylecgonine		75
Carphedone		75
Cocaine		15
Cyclazodone		75
Ethamivan		75
Ethylphenidate		75
Etilefrine		75
Famprofazone		75
Fencamine		75
Fencanfamine		75
Fenethylline		75
Fenfluramine		75
Fenproporex		75
Furfenorex		75
Heptaminol		75

Isometheptene		75
Mefenorex		75
Mephedrone		75
Methedrone		75
Methoxyphenamine		75
Methylephedrine		75
Methylphenidate		75
pOH-mesocarb metabolite		75
Morazone		75
Nikethamide		75
Norfenfluramine		75
Oxilofrine		75
Pemoline		75
Pentetrazol		75
Propylhexedrine		75
Phendimetrazine		75
Pholedrine		75
Prolintane		75
Ritalinic acid		75
Strychnine		75

2.3 Instrumentation

2.3.1 LC

A Dionex UltiMate 3000 UHPLC system (Thermo Scientific, Bremen, Germany) was used to carry out the chromatographic separation. An Agilent ZORBAX eclipse Plus C18 (2.1x100mm) column was chosen as analytical column. The flow rate was set at 0,200 mL/min and the injection volume at 10µl. The gradient for the mobile phases in the analytical column is displayed in *Figure 6*.

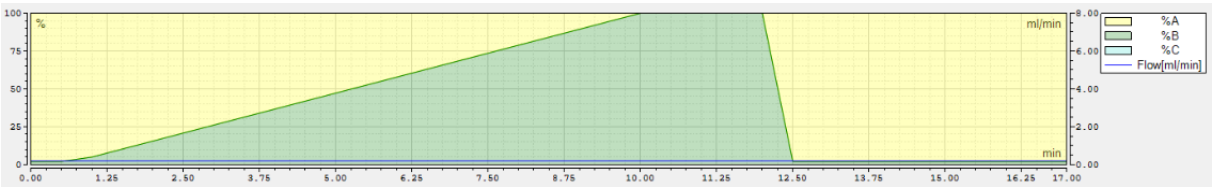


Figure 6: Gradient for mobile phases in the analytical column (A: H20+5mM NH4HCOOH+0,02% FA; B: 90% ACN+10%.

2.3.2 MS

A Thermo Scientific QExactive benchtop Orbitrap-based mass spectrometer, was used for this study. The instrument was operated in the positive-negative polarity switching mode and was equipped with a heated electrospray ionization source. The flow rates of the sheath gas and auxiliary gas, both consisting of nitrogen, were set at 40 and 10 arbitrary units, respectively. The sweep gas flow rate was set to 0 arbitrary units. The temperature of the ion transfer tube was set to 320 °C, while the vaporizer temperature was maintained at 0 °C. The spray voltage was fixed at 4000 V for the positive polarity mode and -3500 V for the negative polarity mode. The instrument was operated in the full scan mode, scanning a mass-to-charge ratio (m/z) range of 100 to 1000, with an Orbitrap resolution of 35000. To ensure accurate mass measurements, the Orbitrap instrument's mass calibration were performed daily using the calibration reagents provided by the manufacturer, both in the positive and negative modes.

2.3.3 Vacuum evaporation
An Eppendorf concentrator plus was used to evaporate in vacuum at room temperature.

2.3.4 Evaporation with N_2
Evaporation with N_2 was done using a TurboVap from Biotage at a temperature of 40°C.

2.3.5 Centrifuge
The centrifuge used was the universal 32 R centrifuge from Hettich Zentrifugen.

3 Results and discussion
The first test with DBS and VAMS were done under the standard conditions for the routine testing of urine samples. No experiments with Tasso were done at this first stage for cost efficiency reasons. These experiments yielded unexpectedly good results where a concentration equivalent to the urinary MRPL was feasible for most compounds.

In the subsequent phases of the study, various combinations of sample preparation techniques were explored, assessing their effectiveness in terms of recovery and consistency of results. While the optimal method is expected to demonstrate superior performance overall, it may not necessarily excel in every individual aspect.

Sample preparation encompasses several key steps: spiking of blood, preparation of DBS, VAMS devices, or Tasso devices, and extraction. The spiking of blood involves a thorough process: initially evaporating the compounds under examination, followed by the addition of blood to the evaporated tube containing the compounds, and thorough mixing. Subsequently, the dried blood spots, VAMS, or Tasso's are created using the spiked blood. The subsequent extraction step is pivotal, where the compounds are transferred from the blood matrix into a blank matrix suitable for injection into the LC/MS system. Various extraction solvents were tested, including methanol, a combination of methanol and acetonitrile, and a combination of methanol and isopropanol. Post-extraction, the solution necessitates evaporation, and two methods were investigated, including vacuum and nitrogen gas. Finally, the samples were reconstituted with a mix of the mobile phases (A: H20+5mM NH4HCOOH+0,02% FA; B: 90% ACN+10% H20+5mM NH4HCOOH+0,02% FA).

For the validation, following parameters were investigated: recovery, LOD, selectivity, carry over and hematocrit effect. Stability testing is still ongoing and thus not integrated in this thesis.

3.1 Optimization of the extraction protocol
To determine the optimal extraction solvent for DBS, Tasso and VAMS, three different solvents were evaluated across samples containing the compounds of interest. The concentrations of these compounds are detailed in *Table 2*. The followed protocol is previously stated (cf. 2.1.1). Each sample comprised six DBS, six Tasso and six VAMS derived from drug-free whole blood obtained from a single individual that was fortified with the mixture of the analytes of interest. For each DBS, Tasso and VAMS, 500 µl of MeOH, a mixture of 250 µl MeOH and 250 µl acetonitrile, or 250 µl MeOH and 250 µl isopropanol was added as extraction solvent. These three combinations of extraction solvents were chosen due to literature and company suggestions of the DBS, VAMS and Tasso.

Additionally, the most effective evaporation technique was investigated. Two DBS, two Tasso and two VAMS per solvent were evaporated using vacuum at room temperature, while another two of each were evaporated using nitrogen gas. These techniques were chosen because they were the available options in the laboratory.

Recovery samples were made following the same protocol, with the only variation being the addition of the standard mixture after the extraction. To ascertain the optimal extraction solvent and evaporation technique, the recoveries were calculated by dividing the area under the curve (AUC) of a recovery sample by that of a normal sample. The results are displayed in *Table 3*.

Recoveries higher than 210% were eliminated. Due to time limitations, these values could not be further investigated.

3.1.1 DBS

Overall, for DBS the best results were obtained with the mixture of methanol/acetonitrile and methanol/isopropanol. From our results, it was clear that evaporation with vacuum was the best technique for both extraction solvents, where there were the highest recoveries. In particular, the beta blockers class exhibits distinct patterns in these findings. Broadly, the outcomes concerning beta blockers were promising, with exceptions noted for select compounds such as mepindolol, practorol, and xamaterol, where recoveries were below 50%. Notably, the recoveries achieved with both methanol/acetonitrile and methanol/isopropanol, utilizing vacuum as the evaporation technique, ranged from 69.3% to 98.3% (excluding the three lowest performing compounds), representing favorable results. Similar findings were observed in the stimulants class, with the exception of five compounds exhibiting recoveries below 50% overall (6-hydroxybromantane, fencamine, adrafinil, pOH-mesocarb metabolite, and sibutramine). The other recoveries ranged from 50% to 208.2%, with the majority falling between 80% and 110% for these two conditions. Also, for the glucocorticoids, beta-agonists and HMM, the same trend is visible where the recoveries in these 2 conditions vary respectively between 61,6% - 121,9%, 56,1% - 146,6% and 56,7% - 153,2% with the majority between 80% and 100% apart from clobetasol, procaterol, reproterol, indacaterol, raloxifene, bazedoxifene and fulvestrant that had recoveries of less than 50% for all or most of the conditions.

Overall, the anabolic agents exhibit higher recovery rates compared to other classes; however, a consistent trend is visible. Remarkably, many recoveries fall within the range of 100 to 120%. Stanozolol-N-glucuronide and dehydrochloromethyltestosterone exhibited recoveries less than 50% for all the conditions. The recoveries of the diuretics were slightly lower compared to other classes, with the majority falling between 60% and 80% under conditions utilizing methanol/acetonitrile or methanol/isopropanol as extraction solvents and vacuum as the evaporation technique. However, a consistent trend persisted within this class as well. Compounds such as amiloride, azosemide, bumetanide, ethacrynic acid, furosemide, piretanide, triamterene, and xipamide exhibited recoveries of less than 50% under these conditions.

For both the narcotics and the HIFs there were more recoveries that were less than 50% than in the other classes but here also the same conclusions could be made.

As an illustrative example of trends, *Figure 7* displays the recoveries per compound, where the blue line represents the condition of methanol as extraction solvent, noticeably lower than the other two conditions. *Figure 8* represents the trends for the different evaporation techniques where the vacuum technique is clearly better in both methanol/acetonitrile and methanol/isopropanol.

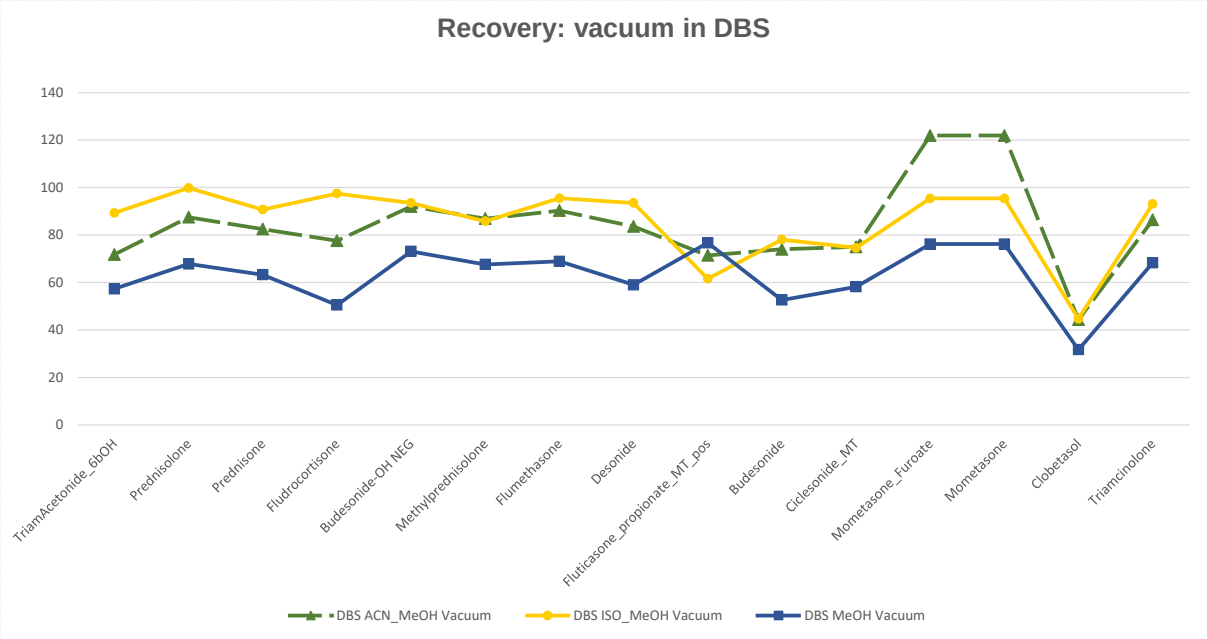


Figure 7: Recovery Plot for Glucocorticoid Compounds in DBS with Vacuum Evaporation Technique. Green line: DBS in methanol/acetonitrile as extraction solvent and vacuum as evaporation technique. Yellow line: DBS in methanol/isopropanol as extraction solvent and vacuum. Blue line: DBS in methanol as extraction solvent and vacuum as evaporation technique.

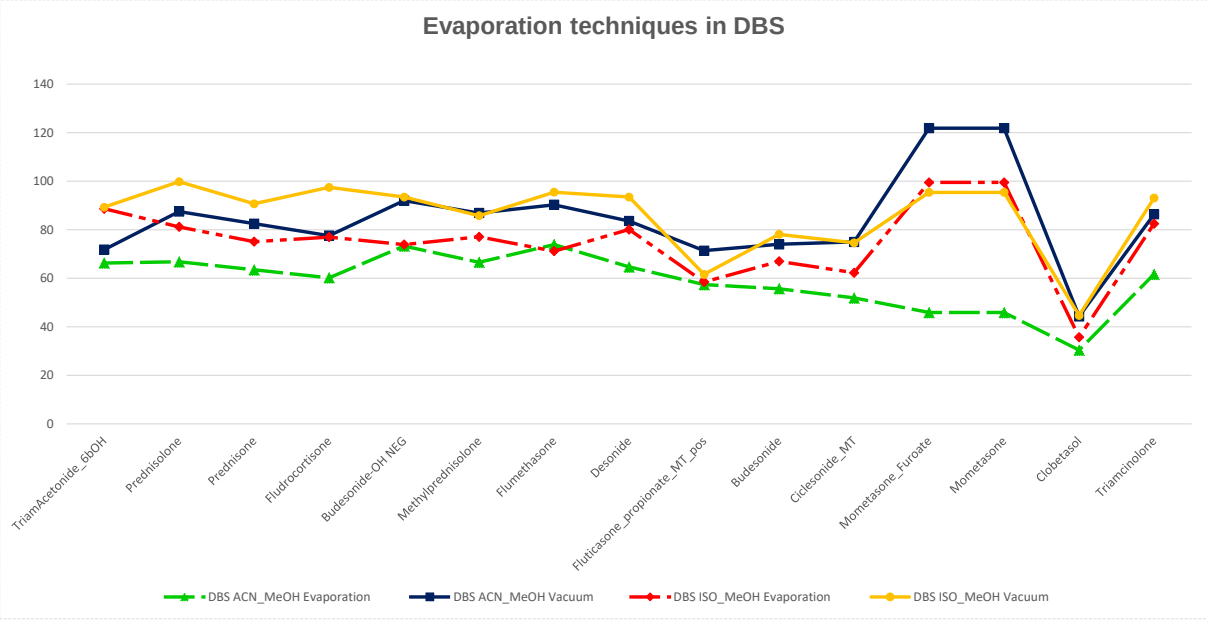


Figure 8: Recovery Plot for corticosteroids compounds in DBS with methanol/acetonitrile and methanol/isopropanol in both vacuum as nitrogen gas evaporation technique. Green line: DBS in methanol/acetonitrile as extraction solvent and nitrogen gas as evaporation technique. Blue line: DBS in methanol/acetonitrile as extraction solvent and vacuum as evaporation technique. Red line: DBS in methanol/isopropanol as extraction solvent and nitrogen gas as evaporation technique. Yellow line: DBS in methanol/isopropanol as extraction solvent and vacuum as evaporation technique.

3.1.2 TASSO

In Tasso samples, methanol and the mixture of methanol/acetonitrile emerged as the most effective extraction solvents, with no clear difference observed in evaporation techniques. However, trends in Tasso samples were less distinct compared to DBS in some classes.

Anabolic agents displayed mostly recoveries exceeding 100%, with no clear trend apparent. Stanozolol, stanozolol-N-glucuronide, and dehydrochloromethyltestosterone exhibited again recoveries below 50%, consistent with DBS results. Similarly, beta-blockers and beta-agonists did not exhibit clear trends, although overall recoveries mostly surpassed 80% across all tested conditions. Stimulants showed optimal results with methanol alone, with most recoveries exceeding 80%. However, there was not a clear difference between the evaporation techniques, vacuum showed more compounds with recoveries less than 50%.

In contrast, diuretics, narcotics, glucocorticoids, and HIFs demonstrated distinct differences in recoveries between methanol/isopropanol and the other extraction solvents, with methanol/isopropanol yielding lower recoveries. For glucocorticoids, recoveries were predominantly above 90%, with approximately half exceeding 100%, except for clobetasol, which displayed recoveries below 50%. Narcotics exhibited only one compound with recoveries below 50% (methadone metabolite EDDP) using methanol/acetonitrile or methanol as the extraction solvent, which was consistent with DBS results. HIFs displayed lower recovery rates compared to other classes, ranging mostly between 60% and 80%, albeit with a consistent trend. Diuretics exhibited a clear trend, with most recoveries exceeding 80% using methanol and methanol/isopropanol as extraction solvents.

For HMMs, trends were less distinct, with recoveries varying more between different conditions tested, although most were above 80%.

As an illustrative example of trends, *Figure 9* displays the recoveries per compound, where the yellow line represents the condition of methanol/isopropanol as extraction solvent, noticeably lower than the other two conditions. *Figure 10* represents the trends for the different evaporation techniques where there could be no clear difference seen.

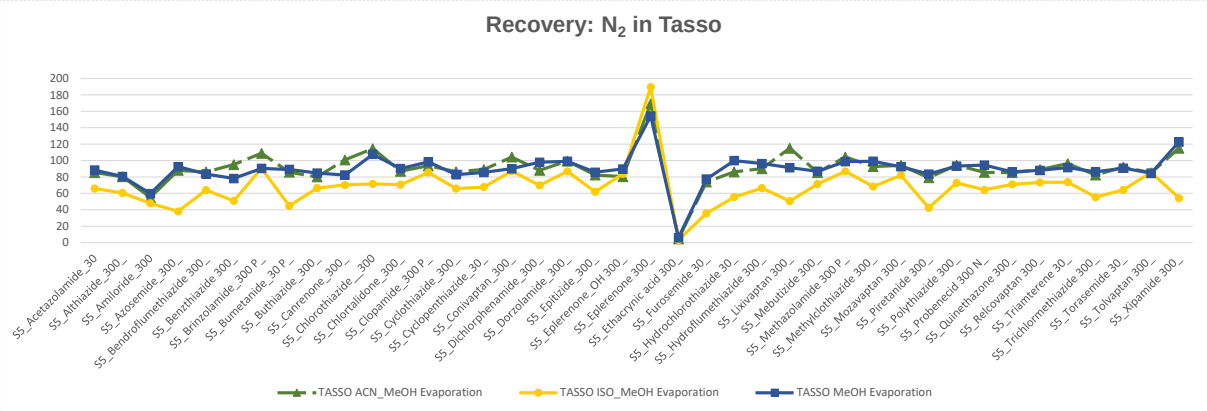


Figure 9: Recovery plot for diuretic compounds in Tasso with nitrogen Evaporation Technique. Green line: Tasso in methanol/acetonitrile as extraction solvent and nitrogen gas as evaporation technique. Yellow line: Tasso in methanol/isopropanol as extraction solvent and nitrogen gas as evaporation technique. Blue line: Tasso in methanol as extraction solvent and nitrogen gas as evaporation technique.

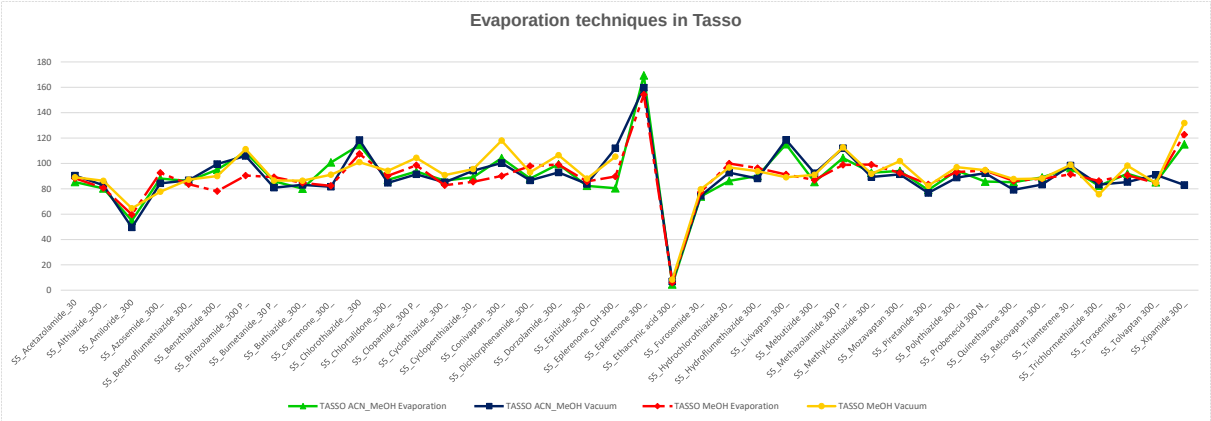


Figure 10: Recovery Plot for diuretic compounds in DBS with methanol and methanol/acetonitrile in both vacuum as nitrogen gas evaporation technique. Green line: Tasso in methanol/acetonitrile as extraction solvent and nitrogen gas as evaporation technique. Blue line: Tasso in methanol/acetonitrile as extraction solvent and vacuum as evaporation technique. Red line: Tasso in methanol/isopropanol as extraction solvent and nitrogen gas as evaporation technique. Yellow line: DBS in methanol/isopropanol as extraction solvent and vacuum as evaporation technique.

3.1.3 VAMS

In the analysis of VAMS samples, discerning a clear trend in the results was challenging, except for one condition that notably yielded inferior results compared to the others. Specifically, this condition involved the use of a mixture of methanol/isopropanol as the extraction solvent and vacuum as the evaporation technique.

In the analysis of the results a notable occurrence of recoveries exceeding 100% was found. This was more prevalent compared to DBS or Tasso device analyses. This observation suggests a potential matrix enhancement effect, although the exact cause remains unclear. The unusually high recoveries pose a challenge as we lack a definitive explanation for their occurrence. Notably, the samples of the DBS, Tasso and VAMS were done in the same batch, so this suggest that instrumentation is not the primary factor influencing these outcomes. Even after incorporating internal standards post-extraction, similar results persisted, indicating that the phenomenon may stem from interference within the sample matrix. However, further investigation is needed to elucidate the underlying mechanisms driving this matrix enhancement effect.

As an illustrative example of trends, *Figure 11* displays the recoveries per compound, where the yellow line represents the condition of methanol/isopropanol as extraction solvent and vacuum as evaporation technique which noticeably lower than the other conditions (in this figure, only the vacuum evaporation technique is displayed).

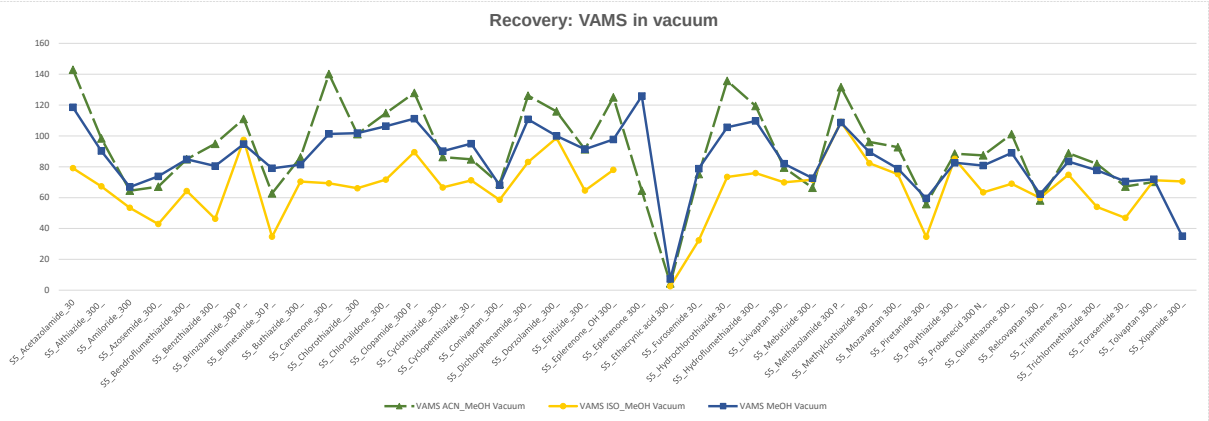


Figure 11: Recovery plot for diuretic compounds in VAMS with vacuum evaporation technique. Green line: VAMS in methanol/acetonitrile as extraction solvent and vacuum as evaporation technique. Yellow line: VAMS in methanol/isopropanol as extraction solvent and vacuum as evaporation technique. Blue line: VAMS in methanol as extraction solvent and vacuum as evaporation technique.

In conclusion, our analysis identifies the mixture of methanol/acetonitrile as the best extraction solvent overall. While this choice yields favorable results for most compounds, it may not be the most suitable for all substances. Additionally, our findings reveal similarities in the preferred evaporation technique between Tasso and VAMS samples. However, in the case of DBS samples, the use of vacuum evaporation was better than using nitrogen gas in terms of recovery

Table 3: Results of the recovery (%) for every device (DBS, Tasso and VAMS), every extraction solvent (methanol/acetonitrile, methanol/isopropanol and methanol) and the different evaporation techniques (N_2 or vacuum). Results for RT (CV%) for DBS) and results for LOD (ng/mL) for every device (DBS, Tasso and VAMS). (indicate a value higher than 200. These values are unexpectedly high and need to be further investigated.) (/ indicate an LOD that could not be calculated)*

	Recovery (%)																		RT (CV%)	LOD (ng/mL)		
	DBS						TASSO						VAMS						DBS	DBS	TASSO	VAMS
	Methanol/acetonitrile		Methanol/isopropanol		Methanol		Methanol/acetonitrile		Methanol/isopropanol		Methanol		Methanol/acetonitrile		Methanol/isopropanol		Methanol					
Compound	N_2	Vacuum	N_2	Vacuum	N_2	Vacuum	N_2	Vacuum	N_2	Vacuum	N_2	Vacuum	N_2	Vacuum	N_2	Vacuum	N_2	Vacuum				
NARCOTICS																						
Buprenorphine	35.2	45.2	35.4	44.2	36.6	34.1	56.5	57.0	44.9	49.2	50.6	51.2	74.3	77.3	63.0	45.3	57.7	56.9	0.13	<0.12	0.12	<0.12
Codeine	64.8	82.1	67.9	82.0	63.8	65.4	86.6	90.2	69.6	70.8	81.1	84.6	105.9	113.9	95.5	68.4	91.0	86.7	0.22	<1.17	<1.17	<1.17
Fentanyl	58.3	76.7	68.7	94.6	63.0	58.2	81.6	80.9	79.4	75.6	89.7	92.1	123.3	119.8	114.3	81.5	101.6	97.9	0.27	0.11	0.14	0.12
Hydromorphone	57.5	71.8	49.3	56.6	44.0	47.3	67.8	66.6	44.1	43.7	50.9	50.0	77.6	83.5	69.6	42.9	55.8	52.2	0.19	<1.17	1.49	<1.17
Methadone met (EDDP)	18.4	40.5	13.2	21.1	26.8	26.3	35.3	38.7	28.3	20.6	60.4	50.5	55.3	86.6	108.9	16.5	50.7	61.3	0.25	1.31	1.31	<1.17
Morphine	57.5	71.8	49.3	56.6	44.0	47.3	67.8	66.6	44.1	43.7	50.9	50.0	77.6	83.5	69.6	42.9	55.8	52.2	0.19	<1.17	1.31	<1.17
Norbuprenorphine	19.7	27.8	23.2	27.8	13.6	21.4	71.8	72.3	56.7	54.0	65.1	68.1	61.3	56.5	65.1	42.8	53.0	51.4	0.27	1.49	19.65	6.1
Norfentanyl	63.3	82.1	60.0	83.8	47.5	61.5	96.9	85.3	73.6	74.4	95.3	94.2	128.0	119.4	126.3	73.1	106.8	117.8	0.18	0.08	0.1	0.07
Pentazocine	49.2	63.0	58.5	71.2	55.5	53.2	88.3	85.9	76.1	79.8	88.5	95.1	107.1	117.1	109.5	77.3	97.9	98.0	0.23	<1.17	<1.17	<1.17
Racemoramide (dextromoramide)	67.4	79.0	74.5	91.5	69.6	66.6	99.9	98.8	90.4	92.6	89.5	105.6	118.1	135.9	117.7	78.3	101.2	104.9	0.31	<1.17	4.49	<1.17
Sufentanyl	32.4	34.8	27.4	25.3	24.3	26.3	87.1	54.1	68.3	75.6	94.0	97.6	59.9	57.2	77.5	62.5	51.8	102.5	0.33	0.2	0.44	0.13
CORTICOSTEROIDS																						
Budesonide	55.7	74.0	67.0	78.1	48.6	52.6	94.4	98.7	80.9	93.4	109.0	112.2	120.1	115.7	99.0	82.7	107.0	106.5	0.13	5.26	8.81	9.33
Budesonide-OH	73.3	92.0	73.9	93.5	66.9	73.1	108.4	105.4	94.0	100.6	112.9	116.8	133.8	127.2	112.1	91.9	108.1	108.8	0.16	2.36	2.92	<2.11
Clobetasol	30.4	44.3	35.7	44.7	28.8	31.7	44.8	47.8	45.6	48.8	46.7	46.5	22.1	20.1	16.8	13.8	17.6	18.0	0.10	4.49	24.39	6.88
Desonide	64.7	83.6	80.1	93.5	56.8	59.0	109.7	102.7	98.1	111.6	121.2	122.1	137.7	135.4	110.0	102.5	120.2	113.8	0.00	<1.41	2.94	<1.41
Fludrocortisone	60.2	77.6	77.0	97.5	45.1	50.5	107.1	114.5	86.0	115.0	107.9	120.3	124.7	141.4	107.5	107.7	111.0	111.0	0.13	2.12	3.25	<1.41
Flumethasone	73.9	90.3	71.2	95.5	70.0	68.9	107.9	111.4	91.0	*	111.9	110.7	130.0	122.9	105.9	90.9	107.7	102.0	0.14	<1.41	2.12	<1.41
Fluticasone propionate Metabolite	57.4	71.4	58.5	61.6	95.7	76.7	85.9	94.0	73.2	69.8	97.3	95.3	67.9	61.0	60.6	53.2	68.8	77.4	0.14	3.51	12.44	9.59
Methylprednisolone	66.6	86.9	77.1	85.8	62.2	67.6	107.0	108.1	96.7	102.2	119.2	116.6	133.4	133.1	114.4	104.4	109.7	102.5	0.16	3.51	3.18	6.96
Mometasone	45.9	121.9	99.5	95.4	63.5	76.2	123.6	209.8	99.2	112.5	125.8	*	95.5	84.7	104.8	67.6	111.5	98.8	0.07	6.22	26.01	11.78
Mometasone-Furoate	45.9	121.9	99.5	95.4	63.5	76.2	123.6	209.8	99.2	112.5	125.8	*	95.5	84.7	104.8	67.6	111.5	98.8	0.10	/	44.73	26.01
Prednisolone	66.8	87.5	81.2	99.8	68.4	67.8	110.5	113.0	98.0	103.5	109.9	116.9	130.7	135.7	130.4	99.9	115.1	118.6	0.12	<4.69	7.88	14.5
Prednisone	63.5	82.5	75.1	90.7	69.7	63.3	111.8	107.6	88.4	97.2	111.5	109.8	130.1	129.9	105.9	97.4	110.7	113.6	0.13	<14.06	<14.06	<14.06
6bOH-TriamAcetonide	66.3	71.8	88.7	89.3	62.0	57.3	109.6	130.9	93.5	91.2	115.5	123.7	121.5	130.2	134.1	96.5	115.0	114.0	0.11	4.49	6.22	5.87

Triamcinolone	61.7	86.4	82.5	93.1	63.8	68.3	112.2	103.2	92.1	94.5	118.4	120.1	122.8	117.3	116.7	91.8	113.6	99.8	0.09	1.94	2.07	1.94
HIF																						
Desidustat	40.8	60.1	27.1	25.4	41.5	49.6	77.2	68.9	37.8	40.1	63.6	58.5	80.9	65.6	56.0	29.5	68.7	62.4	0.14	3.32	5.51	1.95
Enarodustat	77.7	59.6	9.9	65.6	61.0	32.4	57.9	59.0	26.5	32.8	67.3	49.7	80.8	64.1	92.2	26.4	65.2	83.8	0.16	7.16	3.72	5.51
FG4592 (Raxadustat)	28.6	66.0	17.1	17.1	29.2	26.1	62.8	73.5	29.4	36.4	66.7	52.7	58.7	46.1	38.7	24.8	52.6	63.9	0.14	3.49	6.72	1.99
GSK1278863 (Daprodustat)	29.9	62.7	27.4	28.5	24.5	35.2	64.8	67.1	25.7	30.0	55.4	71.1	46.5	35.5	27.3	23.5	51.0	38.1	0.32	6.29	3.32	3.95
IOX2	28.6	66.1	17.1	17.2	29.2	26.1	62.9	73.6	29.5	36.4	66.7	52.7	58.8	46.1	38.6	24.7	52.8	63.8	0.13	3.49	6.72	1.99
JNJ420	63.2	100.5	61.6	59.1	65.7	88.7	88.8	85.7	54.3	55.1	94.7	128.2	71.7	57.4	53.6	43.3	101.7	77.2	0.14	5.51	11.93	5.51
Vadadustat	43.7	67.1	22.5	32.3	42.1	48.6	75.3	69.5	32.9	46.7	74.2	83.1	82.0	61.0	43.6	34.6	78.4	70.9	0.12	2.56	6.94	1.57
Anabolic agents																						
Andarine	49.1	63.4	60.3	69.6	38.1	42.3	121.6	117.8	105.2	118.9	139.0	125.5	111.9	117.8	100.9	126.5	101.3	105.7	0.09	0.23	1.68	0.22
Andarine metabolite	32.0	95.0	88.4	60.5	49.2	62.2	105.6	91.4	34.5	23.4	122.8	110.5	122.5	130.4	202.5	79.7	146.4	142.2	0.14	<0.09	0.11	<0.09
Gestrinone	10.2	19.1	9.6	9.0	30.3	28.2	30.7	22.3	11.6	12.1	42.2	36.4	15.1	10.6	4.3	6.4	18.2	17.0	0.12	0.53	1.09	<0.23
GSK288	48.5	66.1	62.9	79.0	37.0	45.5	124.4	130.1	112.0	117.6	154.7	126.2	103.7	97.1	116.5	116.3	100.2	123.4	0.07	0.14	0.2	<0.09
LGD4033	31.3	49.6	46.5	54.5	15.4	24.0	57.5	45.6	44.8	51.9	52.8	51.2	84.4	86.1	80.7	50.6	78.0	77.9	0.08	0.2	0.79	<0.09
Ostarine	75.4	101.5	89.4	121.1	57.7	69.5	202.8	198.1	134.2	174.7	162.2	185.0	149.7	145.8	142.5	157.9	132.5	139.1	0.08	0.11	0.44	<0.09
Ostarine metabolite	93.5	120.3	89.0	137.5	83.6	87.1	159.2	154.3	129.3	141.6	153.2	172.5	187.3	178.9	155.8	145.2	150.3	157.4	0.00	<0.09	<0.09	<0.09
RAD140	93.7	116.6	105.7	143.2	68.7	86.4	185.4	203.4	173.2	179.0	180.3	177.3	176.2	145.0	177.5	180.9	131.8	141.0	0.09	0.84	/	0.41
S23	86.4	133.7	100.0	127.1	85.6	97.4	148.0	148.5	118.1	138.2	161.9	157.0	180.2	158.1	173.4	169.0	158.4	143.6	0.09	0.79	1.79	0.21
Stanozolol	87.0	133.8	101.8	114.9	97.8	107.1	166.7	168.5	135.6	159.0	161.4	164.9	157.4	131.4	142.9	142.2	145.5	143.4	0.16	/	/	/
Stanozolol-N-glucuronide	99.8	113.6	104.2	124.5	85.5	88.5	148.9	146.9	134.8	146.1	163.1	166.8	195.7	190.5	165.7	156.4	153.9	158.7	0.06	0.25	0.79	0.46
Tetrahydrogestrinone	66.9	110.8	95.5	122.6	61.3	75.5	0.0	41.9	178.2	201.2	*	111.2	142.3	114.0	125.7	152.7	117.7	124.1	0.09	<0.23	4.2	1.15
TFM4- AS1	87.1	185.3	136.6	139.6	146.3	151.3	192.4	*	107.6	174.4	155.4	197.5	194.6	169.9	171.2	158.1	190.8	127.3	0.14	/	1.96	1.57
Dehydrochloromethyltestosterone	59.2	112.4	94.3	121.1	37.5	62.0	*	*	209.2	191.4	161.7	*	159.9	130.5	157.3	151.4	168.3	151.8	0.10	1.66	3.10	1.31
DIURETICS																						
Acetazolamide	67.9	95.1	67.2	83.6	64.0	69.3	85.3	90.4	65.9	60.9	88.3	89.1	127.4	142.9	113.0	79.1	120.3	118.5	0.10	<0.94	<0.94	<0.94
Althiazide	58.5	72.9	56.3	72.4	49.7	55.3	80.3	82.8	60.5	66.3	80.6	86.3	111.8	98.4	93.7	67.3	92.4	90.2	0.16	<9.38	<9.38	<9.38
Amiloride	35.5	47.8	42.9	46.1	41.9	44.3	55.0	49.5	47.9	49.6	59.7	64.6	65.8	64.5	66.5	53.4	76.3	66.9	0.12	<9.38	<9.38	<9.38
Azosemide	48.0	68.5	34.2	38.3	49.9	57.3	88.1	84.3	38.0	42.0	92.6	77.7	72.6	67.1	55.4	42.9	71.5	73.8	0.14	<9.38	15.75	<9.38
Bendroflumethiazide	57.8	74.4	63.1	80.1	49.0	57.1	86.1	86.7	64.0	78.1	83.6	87.2	99.3	84.9	88.5	64.3	82.3	84.8	0.11	<9.38	<9.38	<9.38
Benzthiazide	72.8	112.2	47.5	62.7	77.8	78.6	95.1	99.5	50.8	63.0	78.1	90.0	108.0	94.9	69.5	46.3	89.9	80.5	0.12	<9.38	<9.38	<9.38
Brinzolamide	63.1	72.8	66.8	67.1	56.5	57.1	108.9	105.9	90.5	94.1	90.4	111.2	110.1	111.0	106.8	97.4	92.4	94.7	0.27	<9.38	<9.38	<9.38
Bumetanide	53.5	66.1	42.2	41.8	58.4	61.9	85.9	80.9	44.8	51.4	89.2	86.5	67.5	62.8	52.8	34.7	69.1	79.0	0.13	<9.38	22.23	<9.38
Buthiazide	53.1	62.7	54.3	67.4	44.6	51.0	80.1	83.1	66.4	73.4	84.7	86.4	97.5	86.1	86.3	70.4	77.9	81.4	0.10	<0.94	<0.94	<0.94

Canrenone	51.9	67.1	56.8	75.6	35.2	44.6	100.8	81.6	70.3	72.0	82.3	91.1	122.5	140.1	115.8	69.3	108.5	101.3	0.09	<9.38	19.59	<9.38
Chlorothiazide	71.7	100.4	56.3	61.1	71.7	76.3	114.5	118.5	71.5	70.7	107.5	101.0	115.2	101.2	86.7	66.1	106.5	101.8	0.14	<9.38	<9.38	<9.38
Chlortalidone	68.1	85.2	65.7	80.0	59.7	66.4	87.0	84.7	70.6	78.9	90.2	94.2	123.2	114.8	100.4	71.7	110.1	106.3	0.12	<9.38	<9.38	<9.38
Clopamide	64.6	84.9	80.4	97.0	72.8	64.8	93.6	91.4	85.4	89.9	98.5	104.4	127.5	127.9	125.9	89.5	115.4	111.2	0.15	<9.38	<9.38	<9.38
Cyclothiazide	57.6	76.6	59.8	72.7	47.7	55.5	86.4	85.1	65.8	71.8	82.8	90.8	108.8	86.3	92.5	66.6	89.9	90.1	0.27	<9.38	10.5	<9.38
Cyclopenthiazide	58.7	78.3	61.3	78.8	55.7	61.7	89.1	94.3	67.7	78.1	85.5	95.5	111.3	84.8	99.3	71.2	94.2	95.0	0.14	<9.38	<9.38	<9.38
Conivaptan	48.5	64.2	44.2	53.3	52.4	62.3	104.2	100.3	87.2	88.9	90.1	118.0	72.0	68.9	71.4	58.6	75.5	68.1	0.33	<9.38	/	<9.38
Dichlorphenamide	64.2	86.3	67.8	86.3	68.9	63.3	88.1	86.6	69.7	76.2	97.9	93.0	133.7	126.1	119.6	83.1	107.2	110.7	0.14	<9.38	<9.38	<9.38
Dorzolamide	59.7	72.5	60.3	70.2	58.6	63.8	99.4	92.9	86.8	86.7	99.1	106.5	117.0	115.9	108.7	98.7	100.0	100.0	0.23	<9.38	<9.38	<9.38
Epitizide	59.9	77.1	61.2	76.8	50.8	57.8	82.3	83.8	61.9	68.1	85.8	88.0	107.0	91.3	90.1	64.6	90.3	91.4	0.10	<9.38	<9.38	<9.38
Eplerenone	44.2	82.9	83.6	79.9	78.3	48.9	80.4	111.9	83.8	87.8	89.7	105.3	112.1	125.0	124.9	78.0	107.7	97.7	0.09	<9.38	/	/
Eplerenone-OH	53.3	104.3	66.5	53.9	83.2	75.5	169.4	159.7	189.6	191.2	154.2	*	152.2	64.5	176.3	*	109.5	125.8	0.14	14.13	46.41	14.13
Ethacrynic acid	15.4	22.6	11.8	15.4	13.7	15.0	4.6	6.9	3.1	3.3	6.0	8.4	5.8	4.4	4.1	2.6	6.4	7.3	0.11	15.75	78.54	12.96
Furosemide	52.8	72.3	28.9	32.2	51.3	58.8	73.9	74.5	35.7	40.6	77.4	79.6	89.9	75.3	48.6	32.3	80.9	78.8	0.08	1.19	<0.94	<0.94
Hydrochlorothiazide	64.8	83.6	64.2	79.2	60.9	63.9	86.2	92.7	55.5	59.5	99.9	96.8	118.5	135.7	93.3	73.4	105.0	105.6	0.11	0.94	1.38	<0.94
Hydroflumethiazide	63.7	83.2	69.8	83.9	63.8	62.3	90.1	88.1	66.4	71.4	96.3	93.9	127.6	119.4	104.9	75.9	100.4	109.7	0.07	<9.38	9.38	<9.38
Lixivaptan	48.5	90.3	75.6	69.3	72.9	79.8	115.1	118.6	50.5	44.8	91.3	89.1	83.0	79.5	103.7	69.9	101.0	82.0	0.13	/	/	168.09
Mebutizide	48.5	61.5	52.3	66.5	40.4	48.0	85.3	92.3	71.4	77.6	86.9	91.0	85.9	66.4	88.6	71.6	70.4	72.6	0.09	<9.38	<9.38	<9.38
Methazolamide	60.6	79.8	65.9	76.3	58.5	64.7	104.3	112.0	86.8	80.4	98.8	112.7	133.5	131.6	119.8	109.1	110.1	108.7	0.09	<9.38	<9.38	<9.38
Methylclothiazide	52.9	67.7	59.7	69.4	45.8	50.7	92.7	89.3	68.3	75.3	99.1	91.8	98.7	96.1	94.9	82.3	89.0	89.5	0.11	<9.38	<9.38	<9.38
Mozavaptan	44.9	59.9	56.8	68.8	58.1	46.8	94.1	91.4	81.9	87.1	92.5	101.8	90.7	92.7	82.1	75.2	80.2	78.8	0.29	<9.38	<9.38	<9.38
Piretanide	41.8	58.1	31.4	36.7	44.5	48.9	79.0	76.7	42.4	49.6	83.5	82.3	61.0	55.8	41.2	34.6	60.4	59.5	0.11	<9.38	<9.38	<9.38
Polythiazide	49.7	65.8	55.8	69.2	40.2	48.2	94.4	88.8	72.7	84.5	93.2	97.0	94.3	88.5	97.2	85.1	79.9	82.6	0.14	<9.38	<9.38	<9.38
Probenecid	55.0	67.6	54.8	69.7	51.4	52.9	85.7	92.3	64.3	74.6	94.4	94.7	94.0	87.4	69.9	63.4	83.1	80.8	0.12	<9.38	<9.38	<9.38
Quinethazone	46.6	63.8	54.3	63.6	42.4	50.7	85.2	79.2	71.0	78.4	86.2	87.7	99.7	101.2	72.9	69.0	83.0	89.0	0.11	<9.38	<9.38	<9.38
Relcovaptan	45.4	58.1	47.0	57.9	39.2	46.8	89.0	83.3	73.4	81.7	88.0	88.1	67.8	58.2	62.1	59.8	63.5	62.3	0.10	11.94	12.96	10.5
Triamterene	46.6	55.2	38.6	43.7	46.1	48.2	96.3	98.3	73.7	72.8	91.4	98.8	101.0	88.8	88.7	74.8	97.9	83.4	0.21	0.94	<0.94	<0.94
Trichlormethiazide	51.1	63.1	50.3	59.9	41.5	48.2	82.3	83.3	55.4	60.0	86.2	75.6	86.7	81.9	66.4	54.0	77.4	77.7	0.10	<9.38	<9.38	<9.38
Torasemide	52.8	66.1	49.2	55.5	62.7	57.8	91.9	85.3	64.3	73.9	90.7	98.2	75.7	67.2	53.2	46.9	72.5	70.5	0.11	1.05	<0.94	<0.94
Tolvaptan	45.0	62.8	57.4	67.8	45.5	48.9	85.1	91.0	85.7	99.2	84.6	84.6	80.9	70.2	81.1	71.3	73.1	71.9	0.09	<9.38	11.94	<9.38
Xipamide	51.9	89.9	44.1	48.0	68.0	78.4	115.0	83.0	54.2	60.9	122.7	131.9	59.0	*	36.9	70.5	86.1	35.0	0.27	49.5	98.85	41.46
BETA2-AGONISTS																						
Bambuterol	57.2	78.3	58.5	84.1	53.7	54.1	70.4	78.7	60.8	77.2	75.5	78.7	84.7	85.6	81.5	56.7	77.4	72.0	0.18	1.05	1.96	4.64
Brombuterol	67.2	91.6	83.1	100.6	64.9	69.9	98.1	102.2	92.1	91.5	107.4	108.7	118.3	122.4	134.3	88.8	112.6	111.9	0.27	<0.94	1.3	<0.94

Cimaterol	76.3	101.2	88.4	97.9	77.2	83.1	109.4	99.9	85.6	99.1	107.9	108.9	132.7	135.9	125.1	92.3	118.8	119.7	0.18	<0.94	<0.94	<0.94
Cimbuterol	78.2	100.7	87.6	98.1	76.0	84.2	102.2	101.3	86.6	92.0	106.6	105.3	132.9	145.4	131.8	92.9	124.8	116.7	0.16	<0.94	<0.94	<0.94
Clenbuterol	72.4	122.4	84.6	114.2	106.5	82.9	121.3	38.5	98.4	99.4	106.0	0.0	163.0	132.6	121.2	98.5	105.2	56.1	0.25	0.02	0.05	0.04
Clenpeterol	69.3	89.9	82.1	102.9	75.4	73.9	106.4	101.2	93.6	88.0	110.9	112.0	123.1	135.5	131.3	93.0	112.1	115.1	0.25	1.05	<0.94	<0.94
Clenproperol	75.7	95.0	84.0	105.4	71.6	78.6	109.9	105.1	93.7	97.2	107.8	114.5	128.6	133.9	122.6	96.3	122.6	120.0	0.20	<0.94	<0.94	<0.94
Clorprenaline	80.8	105.7	81.9	118.2	77.7	86.8	90.0	98.0	68.2	94.5	129.6	108.6	94.9	139.0	*	86.0	115.5	117.6	0.21	<0.94	<0.94	<0.94
Fenoterol	64.4	78.6	68.0	85.2	58.4	62.2	89.9	79.0	69.3	71.9	77.3	77.8	107.4	113.9	95.7	67.7	86.0	80.6	0.16	3.05	/	11.56
Formoterol	37.8	57.6	51.4	56.1	38.7	39.6	84.6	81.9	78.2	72.8	78.3	82.3	81.6	62.4	90.9	62.8	80.8	72.0	0.20	<0.94	<0.94	<0.94
Indacaterol	18.6	40.4	24.0	27.2	9.8	13.8	30.2	28.5	47.3	40.8	12.5	0.0	45.2	32.2	75.7	35.4	51.5	35.4	0.40	/	/	16.81
Isosuprine	51.5	65.2	66.4	77.2	51.7	50.4	101.9	103.5	91.2	97.1	*	109.2	131.8	120.5	135.3	96.3	115.4	104.8	0.20	<0.94	<0.94	<0.94
Mabuterol	76.6	89.9	84.5	105.5	68.0	72.8	102.9	102.1	94.8	95.3	108.3	110.2	118.6	137.6	141.6	97.7	112.3	114.9	0.20	<0.94	<0.94	<0.94
Mapenterol	81.7	101.1	68.2	146.6	0.5	1.0	100.8	106.0	0.5	*	*	113.2	117.2	145.7	87.3	116.7	92.5	115.1	0.30	<0.94	<0.94	<0.94
Pirbuterol	61.9	82.2	74.7	91.6	64.8	73.1	81.8	75.3	76.9	84.8	98.9	95.8	97.9	100.5	105.3	75.1	106.6	102.9	0.29	<0.94	<0.94	<0.94
Procaterol	4.9	0.0	6.7	4.4	1.3	4.7	14.0	10.5	15.9	16.9	21.5	20.9	17.4	18.5	30.6	13.9	23.8	23.3	0.21	16.81	19.8	15.72
Reproterol	34.6	47.5	32.5	45.2	40.6	38.7	68.7	66.9	47.2	54.6	69.6	68.9	84.0	80.6	73.5	44.8	76.2	75.9	0.20	4.15	1.3	4.64
Salmeterol	36.7	73.5	58.7	68.7	20.2	32.4	54.2	81.4	122.0	70.7	78.2	117.6	81.2	63.1	117.3	58.6	87.8	89.7	0.36	/	/	7.37
Terbutaline	47.3	65.4	58.1	70.5	53.8	62.9	73.8	72.5	69.0	81.2	89.1	85.1	84.4	83.8	91.5	64.3	92.5	91.5	0.14	1.41	<0.94	<0.94
Tulobuterol	80.2	104.6	71.4	114.1	74.1	84.3	82.5	96.2	70.8	87.8	146.5	106.2	82.9	140.2	*	74.7	110.5	108.6	0.20	<0.94	<0.94	<0.94
Vilanterol	42.2	68.5	57.4	68.1	37.4	38.8	0.0	31.7	94.7	81.1	87.6	0.0	79.4	66.9	97.0	57.3	88.1	76.4	0.37	1.08	/	0.98
BETA-BLOCKERS																						
Acebutolol	83.9	85.4	99.1	94.4	63.2	80.2	119.7	106.3	95.9	101.1	101.2	100.0	125.0	116.8	89.4	90.2	110.0	103.7	0.52	<2.34	<2.34	<2.34
Alprenolol	64.0	88.3	75.1	96.0	71.4	67.7	86.4	103.5	85.8	90.3	104.9	107.6	101.4	121.3	132.9	83.6	99.4	102.4	0.30	<2.34	<2.34	<2.34
Atenolol	76.4	92.7	113.1	93.8	38.3	72.3	99.1	123.8	84.5	125.0	102.9	141.2	176.0	128.7	196.5	155.6	106.0	187.8	0.17	<2.34	<2.34	<2.34
Befunolol	75.4	88.4	79.9	88.1	65.3	69.8	96.6	94.6	82.7	85.0	91.8	102.5	116.6	110.2	108.1	81.0	103.6	92.1	0.21	<2.34	<2.34	<2.34
Betaxolol	58.6	89.7	79.1	98.3	81.3	65.3	93.5	104.9	95.5	92.4	107.0	111.1	117.1	115.9	125.0	83.7	107.0	106.0	0.28	<2.34	<2.34	<2.34
Bisopropolol	66.3	85.3	78.5	94.4	65.7	68.4	99.4	95.9	92.6	89.9	108.9	105.1	125.4	133.4	121.9	89.0	108.0	106.6	0.20	<2.34	3.24	<2.34
Bupranolol	56.6	88.2	72.8	97.2	79.3	67.1	81.9	100.3	90.3	88.9	105.2	113.3	103.0	111.7	131.4	82.2	102.3	103.9	0.32	<2.34	<2.34	<2.34
Carteolol	69.2	84.4	75.5	86.6	69.6	70.3	89.2	90.0	82.2	86.2	96.2	99.7	102.3	105.3	98.2	75.3	99.9	97.8	0.33	<2.34	<2.34	<2.34
Carvedilol	69.2	84.4	75.5	86.6	69.6	70.3	14.5	103.7	82.2	78.4	85.0	0.0	102.7	89.2	113.8	74.2	104.3	95.5	0.52	2.99	/	<2.34
Celiprolol	69.9	96.7	71.9	85.9	65.2	67.0	94.9	92.1	81.0	88.9	99.6	100.6	113.7	106.2	101.9	72.3	100.4	93.4	0.18	<2.34	<2.34	<2.34
Esmolol	52.4	70.3	56.5	71.9	47.2	50.6	59.5	60.3	56.4	57.0	58.6	63.7	76.1	75.3	72.2	47.4	61.2	62.6	0.22	3.53	<2.34	2.99
Esmolol acid	52.4	70.3	56.5	71.9	47.2	50.6	59.5	60.3	56.4	57.0	58.6	63.7	76.1	75.3	72.2	47.4	61.2	62.6	0.22	3.46	<2.34	2.99
Labetalol	55.1	74.1	62.8	73.9	52.2	50.1	103.0	96.9	93.0	87.7	89.2	105.0	94.1	75.8	100.4	69.4	87.9	80.7	0.24	2.63	2.99	<2.34
Levobunolol	66.1	83.9	75.7	90.6	60.2	67.8	93.8	89.6	84.4	83.7	96.7	98.1	108.3	103.0	105.6	79.7	98.3	90.3	0.21	<2.34	<2.34	<2.34

Mepindolol	34.0	38.4	27.8	26.7	26.5	31.9	48.7	54.5	37.8	29.8	69.1	38.0	55.7	61.6	90.9	47.7	56.2	61.6	0.12	23.28	/	10.92
Metipranolol	77.6	95.9	86.0	96.1	70.7	78.1	104.6	101.4	81.8	85.5	95.2	100.4	101.7	106.5	92.7	73.1	99.6	91.2	0.15	<2.34	<2.34	<2.34
Metoprolol	79.9	89.2	78.9	96.7	68.7	74.5	99.1	95.4	87.9	90.6	99.4	110.3	119.1	125.0	128.6	90.0	111.5	103.5	0.19	<2.34	<2.34	<2.34
Nadolol	71.3	85.9	77.9	88.6	67.8	75.4	88.6	84.9	76.9	84.9	90.4	96.8	101.7	106.5	92.7	73.1	99.6	91.2	0.15	<2.34	<2.34	<2.34
Nebivolol	48.6	89.2	68.3	78.0	46.4	48.9	38.3	98.0	80.0	75.4	78.3	108.4	102.9	90.2	121.7	71.8	97.5	84.1	0.49	2.99	/	<2.34
Oxprenolol	67.8	86.0	74.7	95.5	73.3	72.4	96.8	98.3	83.1	92.0	107.4	107.7	121.6	132.8	131.2	89.5	110.8	108.4	0.25	<2.34	<2.34	<2.34
Penbutolol	58.6	85.1	68.8	85.2	57.4	65.5	90.4	100.1	86.1	87.3	94.2	107.3	96.2	95.6	106.6	70.6	90.4	92.8	0.48	<2.34	/	<2.34
Pindolol	53.2	69.3	55.8	69.7	53.3	53.7	87.5	85.2	63.2	62.5	92.2	89.8	103.4	100.1	101.6	74.2	93.0	86.4	0.15	<2.34	<2.34	<2.34
Practorol	30.5	39.0	30.7	37.4	30.2	31.5	61.9	56.5	46.8	49.2	58.0	61.5	72.5	77.1	60.9	49.1	67.8	60.4	0.17	<2.34	<2.34	<2.34
Propranolol	68.0	91.2	76.9	89.9	68.8	69.8	86.6	104.8	94.3	92.9	100.9	114.9	114.8	116.0	127.7	83.2	100.9	100.1	0.32	<2.34	<2.34	<2.34
Sotalol	77.6	95.9	86.0	96.1	70.7	78.1	104.6	101.4	81.8	85.5	95.2	100.4	123.8	125.8	102.0	86.9	107.6	100.0	0.19	<2.34	<2.34	<2.34
Timolol	72.8	84.4	86.3	90.9	54.7	72.9	116.2	103.9	97.7	89.2	97.2	100.4	114.4	121.9	89.6	92.3	104.9	108.4	0.32	<2.34	<2.34	<2.34
Xamanterol	30.5	39.0	30.7	37.4	30.2	31.5	61.9	56.5	46.8	49.2	58.0	61.5	72.5	77.1	60.9	49.1	67.8	60.4	0.30	<2.34	<2.34	<2.34
HORMONE & METABOLIC MODULATORS																						
Androstatrienedione	54.3	66.1	67.5	77.8	40.3	44.6	74.8	65.0	64.2	65.0	74.5	74.6	100.0	103.9	113.9	84.2	79.3	89.4	0.07	<0.94	1.19	1.58
Androstatrienedione metabolite	55.9	85.6	71.8	86.5	46.9	58.7	152.3	122.7	106.5	0.0	147.3	131.6	117.6	107.4	124.3	138.5	136.7	117.5	0.09	1.41	<0.94	2.9
Anastrozol	82.0	102.9	96.2	107.1	69.4	75.5	114.8	106.6	105.2	104.9	119.7	124.6	164.1	181.8	162.3	121.5	131.3	138.7	0.12	<0.94	1.41	<0.94
Bazedoxifene	0.3	8.9	7.1	10.1	0.3	3.3	55.8	59.7	21.4	43.4	46.8	55.3	38.4	0.2	73.8	0.3	24.5	50.5	0.37	/	/	2.17
Clomiphene PC	48.8	135.4	82.3	100.6	74.8	71.6	75.2	84.3	70.6	66.6	65.9	91.9	135.0	126.8	122.7	63.3	111.1	74.3	0.47	2.17	/	1.96
Exemestane	84.5	76.6	73.3	97.3	48.2	46.7	83.3	62.5	72.3	73.9	79.9	137.1	102.7	102.4	115.1	85.8	107.3	93.2	0.12	15.72	/	4.37
Exemestane Metabolite	56.8	82.1	75.1	88.7	45.5	51.5	88.7	88.4	84.6	85.7	104.4	100.3	122.0	122.9	148.3	90.9	113.7	116.2	0.11	1.58	1.3	<0.94
Fulvestrant	0.0	0.0	63.5	112.5	0.0	0.0	100.1	101.5	93.7	95.6	67.2	73.9	99.3	97.1	83.8	62.9	103.3	29.4	0.07	/	17.34	17.9
GW0742 sulfone	81.1	122.5	71.6	79.4	77.1	87.8	99.8	96.6	69.7	80.9	97.6	91.9	104.8	74.3	90.1	57.5	96.4	89.7	0.16	0.15	0.2	0.2
GW0742 sulfoxide	81.3	107.8	71.1	80.6	75.1	86.3	101.5	103.0	70.8	83.9	113.7	123.3	110.6	88.3	87.0	58.5	99.9	94.4	0.14	1.22	/	0.83
GW1516	64.3	153.2	107.3	100.6	94.4	94.4	95.5	108.5	60.7	82.4	77.1	100.7	137.1	110.0	104.8	66.3	121.2	67.9	0.29	1.22	1.57	0.41
GW1516 sulfoxide	72.6	98.4	62.6	76.3	72.6	77.2	102.2	94.3	68.6	79.9	98.3	109.8	108.5	86.6	82.9	52.5	96.3	97.8	0.11	0.11	0.25	<0.09
GW1516 sulfone	77.5	112.6	66.9	79.9	78.7	85.7	108.6	101.4	74.7	85.2	106.7	112.2	102.3	75.7	87.2	59.6	95.9	94.4	0.14	0.14	0.25	<0.09
Letrozole metabolite	76.7	90.2	81.9	101.7	63.5	74.3	102.9	107.9	100.6	116.9	121.2	123.0	158.7	148.3	162.1	120.8	126.3	144.0	0.11	1.05	1.3	<0.94
Raloxifene	28.0	44.6	35.4	43.6	26.8	41.3	87.7	92.3	73.1	74.3	77.9	95.2	75.0	71.9	91.5	59.4	95.6	67.3	0.28	2.17	/	<0.94
SR9009	46.8	137.6	93.7	99.5	100.2	69.0	113.7	127.4	101.0	105.1	84.6	109.7	132.2	104.6	116.3	86.9	116.7	74.2	0.16	/	3.93	3.38
SR9009 metabolite D1066	41.7	56.7	47.6	60.3	34.6	42.2	28.0	44.4	40.4	50.9	43.7	56.7	94.2	96.5	128.3	74.5	91.8	103.5	0.68	4.15	4.66	2.3
SR9009 metabolite D1067	53.8	68.3	61.4	74.0	48.2	52.9	92.9	88.5	81.7	83.6	88.1	100.6	117.7	108.3	109.8	99.1	105.7	97.3	0.27	<0.47	<0.47	<0.47
Toremifen/Tamoxifene metabolite	59.4	76.5	60.3	71.3	66.3	71.7	108.3	109.3	81.1	81.6	110.2	123.2	87.1	77.4	76.2	55.1	92.3	83.7	0.26	<0.94	/	<0.94
Trimetazidine	76.5	91.6	75.2	94.2	70.9	73.7	56.7	73.5	93.3	85.8	98.0	77.6	119.2	0.3	130.3	81.0	114.6	70.5	0.00	<0.47	0.79	<0.47

STIMULANTS																					
6-Hydroxybromantane	31.5	47.4	41.4	56.4	12.3	38.3	58.7	36.5	38.1	43.4	65.6	44.6	102.5	73.8	115.9	66.6	95.3	99.5	0.11	34.46	49.51	10.37
Adrafinil	30.5	48.2	29.7	38.7	28.5	35.8	63.3	63.0	40.4	43.6	68.2	63.7	84.2	81.9	70.1	40.4	80.2	83.8	0.13	<2.34	<2.34	<2.34
Amfepramone	14.6	208.2	25.1	86.8	138.4	54.5	2.4	7.2	6.5	6.2	6.7	7.1	28.0	8.7	*	11.3	27.9	27.7	0.21	24.71	/	15.21
Amphetamine	53.8	93.6	48.1	95.0	68.9	67.3	52.6	55.3	62.0	72.5	78.4	67.6	50.2	64.9	156.3	42.4	69.8	63.1	0.21	<2.34	5.42	<2.34
Benzfluorex	46.0	66.6	53.2	71.6	42.0	51.9	*	*	*	*	50.1	*	89.3	99.1	109.9	70.6	81.4	84.3	0.40	3.53	/	2.63
Benzylpiperazine	49.8	79.2	46.0	80.1	59.7	67.0	62.6	65.3	30.5	74.9	112.2	82.9	*	99.8	167.3	51.0	85.9	89.9	0.51	<2.34	<2.34	<2.34
Benzoylecgonine	73.2	91.4	74.0	90.7	66.3	70.7	94.6	91.9	76.9	80.9	95.1	102.9	134.0	126.7	115.8	78.7	121.9	108.7	0.12	<2.34	<2.34	<2.34
Carphedone	78.2	86.7	89.2	98.8	69.2	72.9	93.6	93.2	92.5	90.1	93.1	107.9	138.3	127.8	130.3	106.1	120.0	113.4	0.10	<2.34	<2.34	<2.34
Cocaine	64.3	89.5	68.3	96.0	63.3	66.4	77.7	80.8	72.5	77.2	85.0	93.7	121.9	140.7	131.4	86.8	103.3	108.5	0.20	<0.47	<0.47	<0.47
Cyclazodone	74.7	91.1	87.5	101.6	75.1	74.9	94.5	93.0	87.0	91.4	99.7	98.3	134.8	155.4	133.1	99.8	122.8	122.9	0.09	<2.34	<2.34	<2.34
Ethamivan	48.5	62.2	58.5	71.9	50.5	49.1	84.7	82.4	77.9	82.4	86.5	87.4	129.7	133.9	123.3	94.0	114.8	112.6	0.09	<2.34	<2.34	<2.34
Ethylphenidate	62.6	95.0	50.7	97.8	75.7	71.7	65.2	73.4	68.5	76.2	156.1	91.2	85.3	117.5	*	62.8	91.2	104.0	0.25	<2.34	<2.34	<2.34
Etilefrine	63.6	93.4	48.3	96.6	77.2	75.7	68.2	91.7	74.6	79.3	140.9	101.2	111.9	169.1	¨*	83.2	123.2	132.8	0.14	<2.34	<2.34	<2.34
Famprofazone	54.9	66.2	56.6	75.5	45.1	52.2	82.7	79.9	66.5	73.1	82.2	78.5	106.3	107.9	95.7	68.9	87.0	95.3	0.06	<2.34	2.99	2.34
Fencamine	27.6	34.0	29.3	34.2	27.7	28.5	66.3	61.8	58.7	58.3	65.7	71.6	77.9	78.6	68.1	52.6	67.3	68.3	0.21	<2.34	<2.34	<2.34
Fencanfamine	54.4	88.8	51.6	93.0	77.8	69.6	44.7	62.3	56.7	57.7	189.1	74.1	48.7	83.8	*	33.9	65.7	73.1	0.28	<2.34	<2.34	<2.34
Fenethylline	70.5	82.9	79.2	91.2	62.4	67.0	91.8	86.1	84.5	82.1	88.3	95.9	122.0	117.1	115.5	93.0	106.4	103.6	0.20	<2.34	<2.34	<2.34
Fenfluramine	32.7	109.9	79.1	127.7	89.1	59.9	26.3	38.0	27.2	53.5	*	73.7	42.2	23.7	205.7	32.1	64.6	60.2	0.27	<2.34	2.99	<2.34
Fenproporex	54.6	92.6	34.5	91.8	75.8	69.7	51.2	77.0	53.5	66.3	152.5	92.6	75.7	105.0	*	56.9	90.1	94.7	0.20	<2.34	<2.34	<2.34
Furfenorex	26.0	81.4	20.5	63.6	65.2	46.0	28.2	16.5	24.5	18.4	55.5	23.7	69.4	39.2	*	31.5	46.7	55.1	0.18	<2.34	4.9	2.63
Heptaminol	68.7	94.1	*	101.8	64.5	78.7	84.8	80.0	68.2	*	103.5	96.7	95.2	121.5	177.4	83.5	107.5	117.7	0.19	<2.34	<2.34	<2.34
Isometheptene	37.6	106.3	96.4	149.0	86.9	60.4	36.9	37.1	34.9	59.4	*	74.3	45.9	33.9	*	28.8	77.3	61.1	0.17	<2.34	3.46	<2.34
Mefenorex	46.2	88.6	39.8	99.7	82.0	62.7	30.3	48.6	38.5	50.3	191.7	69.5	40.8	55.9	*	26.0	53.6	64.4	0.26	<2.34	<2.34	<2.34
Mephedrone	27.9	93.8	35.6	76.2	76.2	47.7	32.3	38.5	26.8	35.0	112.9	43.6	48.1	50.7	128.0	24.3	49.2	57.8	0.22	<2.34	<2.34	2.99
Methedrone	57.1	94.1	57.7	94.6	71.9	68.8	66.9	70.8	46.8	55.1	134.0	86.4	68.7	106.3	*	43.9	81.0	88.6	0.22	3.24	3.24	3.46
Methoxyphenamine	61.1	85.3	60.5	120.1	65.7	64.8	67.5	76.7	42.0	131.2	*	100.8	70.4	116.3	*	35.7	101.4	98.5	0.16	<2.34	<2.34	<2.34
Methylephedrine	45.0	74.4	43.3	111.6	62.1	58.5	47.0	67.4	30.4	59.8	*	80.5	65.9	100.8	*	27.6	84.5	91.8	0.16	<2.34	<2.34	<2.34
Methylphenidate	60.9	89.0	52.0	90.2	69.4	67.7	61.2	79.2	66.0	74.2	145.9	95.6	76.6	119.2	*	64.6	89.5	101.3	0.24	<2.34	<2.34	<2.34
pOH-mesocarb metabolite	14.6	23.2	21.6	27.8	7.3	11.3	66.2	60.7	53.7	54.7	71.8	71.2	78.4	84.6	81.4	58.1	73.8	76.4	0.13	60.75	<2.34	2.99
Morazone	85.7	105.8	96.2	118.4	89.6	84.3	152.5	152.7	132.3	143.7	160.6	171.4	179.9	178.5	157.6	154.2	148.8	144.5	0.00	<2.34	<2.34	<2.34
Nikethamide	77.9	102.7	45.9	101.5	93.3	91.1	73.2	92.3	67.6	89.3	129.0	101.7	107.9	130.6	*	72.3	114.1	113.0	0.12	<2.34	<2.34	<2.34
Norfenfluramine	52.5	90.5	51.9	120.1	57.6	59.5	71.4	69.4	80.3	133.3	113.9	89.9	51.9	55.0	*	48.1	81.8	75.1	1.12	<2.34	<2.34	<2.34
Oxilofrine	43.8	52.7	42.4	50.0	37.4	43.6	57.9	66.1	53.0	63.0	69.2	58.2	75.9	86.1	80.2	55.5	75.5	72.2	0.57	2.99	<2.34	<2.34

Pemoline	73.7	91.3	79.5	96.8	69.7	72.4	97.3	97.5	83.4	90.2	99.2	103.5	138.5	140.8	127.4	104.5	122.0	117.3	0.10	<2.34	<2.34	<2.34
Pentetrazol	71.8	99.8	39.6	103.0	80.6	80.4	80.4	99.2	73.4	85.3	150.3	122.7	122.1	165.8	*	85.7	130.3	121.6	0.14	<2.34	<2.34	<2.34
Propylhexedrine	45.9	96.0	69.3	189.1	70.8	61.2	43.9	51.5	31.4	69.9	*	82.5	41.1	58.0	*	21.6	82.8	76.7	0.25	<2.34	2.99	<2.34
Phendimetrazine	31.1	183.7	58.5	117.6	171.7	86.4	37.0	32.6	46.8	39.6	173.7	37.1	110.3	28.4	*	39.8	87.5	84.8	0.21	<2.34	7.2	7.07
Pholedrine	53.5	91.9	55.5	79.5	73.0	76.8	72.5	79.1	58.0	56.9	90.3	80.0	85.2	128.4	164.2	71.1	89.6	97.0	0.24	<2.34	<2.34	<2.34
Prolintane	23.3	62.0	24.2	56.0	67.0	45.3	42.0	34.6	68.9	39.9	172.2	49.6	55.7	39.9	*	24.8	42.1	49.6	0.24	<2.34	<2.34	<2.34
Ritalinic acid	83.0	85.8	70.9	80.7	72.9	76.8	89.8	87.8	72.7	75.1	96.9	102.2	108.1	104.9	87.2	70.1	105.3	102.9	0.11	<2.34	<2.34	<2.34
Strychnine	59.9	72.3	64.6	79.7	57.5	59.6	87.0	89.1	74.6	71.9	87.6	92.6	112.4	107.8	104.7	78.5	97.2	90.7	0.19	<2.34	<2.34	<2.34

3.2 Validation

The initial testing procedure developed in this study was tested and validated in DBS, VAMS and Tasso in view of the potential application for the determination of the prohibited compounds in the context of doping screening in athletes. The extraction solvent selected was methanol/acetonitrile and following parameters were investigated: LOD, carry over, selectivity and hematocrit effect.

3.2.1 LOD

The LODs for all the compounds are displayed in *Table 3*. The LODs were calculated according to the WADA guidelines using a sigmoidal module applied to detection rates (/10) at 6 levels. For simplicity, the values in *Table 3* will be rounded. To analyze these results, they will be compared to two other studies [34,35], where Mazzarino et al. reported LODs in VAMS and Garzinski et al in DBS. Nevertheless, here also the Tasso results will be compared to these papers, even though there is a clear influence of the sampling device in some cases. Additionally, the panel of compounds was not the same across studies although ours as well as those from Mazzarino et al. and Garzinksi et al. each show a very broad range of substances. Direct comparisons can however only be made between compounds that were present in each study. Overall comparison between classes will nevertheless be attempted as many substances from the same class often have similar LODs, although as shown from our results this is not universal.

For the anabolic agents, our results in DBS range from 0,1 to 1,5 ng/mL. Garzinski et al. had comparable results for ostarine and S-23, but for andarine our results were ten times better. In our study, the LOD of TFM4-4AS-1 could not be calculated in DBS, and stanozolol was not detectable at the spiked concentrations in any of the devices. The andarine metabolite, ostarine metabolite, and tetrahydrogestrinone had LODs below 0.1 ng/mL, 0.1 ng/mL, and 0.2 ng/mL, respectively, as these were still detectable at the lowest tested concentration. The exact LODs for these compounds would require further investigation with even lower concentration levels. For Tasso, the LODs ranged between 0.1 ng/mL and 2 ng/mL, with the exception of tetrahydrogestrinone and dehydrochloromethyltestosterone, which have an LOD of 4 ng/mL and 3 ng/mL, respectively. RAD 140 and stanozolol were not detectable at the spiked concentrations. In VAMS, Mazzarino et al. reported LODs between 0,1 ng/mL and 1 ng/mL. These results are close to our findings. Andarine metabolite, GSK288, LGD4033, ostarine, ostarine metabolite, and RAD 140 had LODs of less than 0.1 ng/mL. Gestrinone had an LOD of less than 0.20 ng/mL, which all requires further investigation with lower concentrations. The remaining compounds had LODs between 0,2 and 2 ng/mL. The differences in LODs for some substances between the devices, clearly indicates that the choice of microsampling device matters and that it always needs to be validated separately. As a general conclusion however, under the current analytical conditions, it seems that the Tasso device is having slightly higher LODs than the other two devices tested.

For the peptide hormones, growth hormones, related substances, and mimetics, Mazzarino et al. her results ranged from 1 to 1,5 ng/mL while Garzinksi' et al. his results varied more widely, from 5 to 100 ng/mL. Our findings for DBS ranged from 2.5 to 7.5 ng/mL, for Tasso from 3.3 to 12 ng/mL, and for VAMS from 1.5 to 5.5 ng/mL, with VAMS demonstrating the lowest detection limits. Thus, our LODs were generally lower than those from Garzinski et al. but slightly higher than those from Mazzarino et al. In general, however, it can be concluded that the overall performance is quite similar.

For the beta-agonists, most of the LODs were less than 1 ng/mL; indeed for 11, 12, and 13 compounds out of 21 for DBS, Tasso, and VAMS, respectively, the lowest level at which these substances were spiked was still detected in every single sample. This indicates the need for further investigations with lower concentrations if the true LOD would need to be established. The current LOD estimations show however the potential of the methodology as very low concentrations (vs the urinary MRL values). In DBS and Tasso, the other compounds had LODs lower than 2 ng/mL, except for procaterol, which had LODs of 17 ng/mL and 20 ng/mL

in DBS and Tasso, respectively. Indacaterol and salmeterol were not detectable in DBS and Tasso, so no LOD could be determined for these compounds. Fenoterol and vilanterol were also not detectable in Tasso, preventing LOD determination. In VAMS, clenbuterol and vilanterol had LODs less than 1 ng/mL, while bambuterol and reproterol had LODs under 5 ng/mL. Salmeterol had an LOD of 7.5 ng/mL, and the other compounds had LODs between 11.5 ng/mL and 17 ng/mL. Mazzarino et al. reported LODs for beta-agonists between 0.5 and 2 ng/mL, which mostly align with our results. Garzinski et al. reported LODs between 2.5 and 50 ng/mL, where our results were generally better or within the same range, except for indacaterol and salmeterol, which were not detectable and thus had no determined LODs.

For the hormone and metabolic modulators in DBS, most LODs were below 2 ng/mL. Which also include androstatrienedione, anastrozole, SR9009 metabolite 1067, toremifene, tamoxifen metabolite, and trimetazidine, where further investigation with lower concentrations is needed to determine precise LODs. Exemestane and SR9009 metabolite D1066 had LODs of 15.5 ng/mL and 4 ng/mL, respectively. Bazedoxifene, fulvestrant, and SR9009 were not detectable, hence no LODs could be determined for these compounds. Comparing these results to other studies, Garzinski et al. reported mostly higher LODs (1,25-25) than our results. For Tasso devices, LODs were generally in the same range as for DBS. However, LODs for bazedoxifene, clomiphene, exemestane, GW0742 sulfoxide, raloxifene, toremifene and tamoxifen, could not be determined, and the LOD for SR9009 was 4 ng/mL. In VAMS, six compounds had LODs below 1 ng/mL, two had LODs below 0.01 ng/mL, and two had an LOD below 0.5 ng/mL, all of which require further investigation with lower concentrations. Ten compounds had LODs below 5 ng/mL, and fulvestrant had an LOD of 18 ng/mL, which is comparable to the LOD in Tasso. Mazzarino's LODs were between 0.5 and 2 ng/mL, making them comparable to our findings.

Most diuretics exhibited an LOD of less than 10 ng/mL (the lowest level tested for most diuretics), acetazolamide, buthiazide, and hydrochlorothiazide displayed an LOD of less than 1 ng/mL. Investigations with experiments involving lower concentrations are needed to precisely determine the exact detection limit for these compounds. Furosemide, hydrochlorothiazide, triamterene, and torasemide demonstrated an LOD of 1 ng/mL in DBS, indicating their high detectability in this medium. Conversely, OH-eplerenone, ethacrynic acid, and relcovaptan exhibited higher LODs of 14, 16, and 12 ng/mL, respectively. Garzinski et al. reported a much higher LOD for relcovaptan, which was 50ng/mL. Xipamide presented an LOD of 50 ng/mL, contrasting with the lower LOD reported by Garzinski et al. which was 2,5 ng/mL. The LOD of lixivaptan in DBS could not be determined. In comparing LODs between DBS and VAMS, most compounds exhibited comparable LODs, except for eplerenone and lixivaptan, where the LODs could not be determined and reached 170 ng/mL, respectively. Mazzarino et al. reported LODs ranging between 0.5 and 3 ng/mL, posing challenges in direct comparison because most compounds need further investigation. However, given the upper limit of our LODs, the performance can be estimated as similar (or better). Notably, discrepancies were observed between our findings and those of Mazzarino et al. for lixivaptan and xipamide where they reported LODs of 3 and 0,5 ng/mL, respectively, for these compounds.

Additionally, when considering Tasso, LODs were comparable to those observed in other devices. However, notable deviations were observed for azosemide, bumetanide, canrenone, cyclothiazide, relcovaptan, and tolvaptan, with LODs ranging from 10 to 20 ng/mL. Furthermore, for conivaptan, eplerenone, and lixivaptan, the LODs could not be calculated, while hydrochlorothiazide exhibited an LOD of 1 ng/mL. Notably, eplerenone displayed a higher LOD of 50 ng/mL, and ethacrynic acid exhibited the highest LOD among the diuretic compounds at 80 ng/mL.

The analysis of stimulants revealed that most compounds exhibited a limit of detection of less than the lowest tested level of 2,5 ng/mL. However, further investigations with experiments at lower concentrations are necessary to refine these detection limits. In DBS, 6-

hydroxybromantane, amfepramone, and the pOH-mesocarb metabolite presented higher LODs of 35, 25, and 60 ng/mL, respectively. Garzinski et al. reported a wide range of LOD values between 0.1 and 125 ng/mL, with our results generally showing better sensitivity. The obtained result also provide evidence that, as observed in the field of toxicology and forensics [36], stimulants and opioids show very low detection limits in DBS.

In the Tasso sampling device, most compounds had an LOD of less than 5 ng/mL, except for 6-hydroxybromantane and phendimetrazine, which had LODs of 50 and 7 ng/mL, respectively. The LODs for amfepramone and benfluorex could not be determined in this device. For VAMS, the majority of LODs were below 5 ng/mL, with the exceptions of 6-hydroxybromantane, amfepramone, and phendimetrazine, which had LODs of 10, 15, and 7 ng/mL, respectively. This is mostly consistent with Mazzarino et al.'s findings, who reported LODs between 0.1 and 3 ng/mL.

The analysis of narcotics revealed that most compounds exhibited a LOD of less than 2 ng/mL across all devices tested. Exceptions were norbuprenorphine, with an LOD of 20 ng/mL in Tasso and 6 ng/mL in VAMS, and racemoramide, with an LOD of 4 ng/mL in Tasso. For approximately half of the narcotics, further investigations are necessary using experiments with lower concentrations to refine these LOD values.

Comparing these results with existing literature, Mazzarino et al. reported LODs between 0.5 and 2 ng/mL and Garzinski et al. from 0,625 to 5 ng/mL, which align closely with our findings. The data from the field of toxicology by Ambach et al. are also similar [37]. These comparisons indicate that our LOD values are consistent with previously reported data, though specific compounds like norbuprenorphine and racemoramide highlight areas where detection sensitivity could be further improved.

In DBS, the LODs for most glucocorticoids ranged between 2 and 6 ng/mL. Notable exceptions include desonide and flumethasone, with LODs of less than 1 ng/mL, prednisolone with an LOD of less than 5 ng/mL, and prednisone with an LOD of less than 14 ng/mL. These compounds require further investigations at lower concentrations to precisely determine their LODs. The LOD for mometasone furoate could not be determined in DBS. Compared to the literature Garzinski et al. reported LODs between 10 and 25 ng/mL. Our results are generally better to these reported values.

In the Tasso device, six compounds had LODs of less than 5 ng/mL, five compounds had LODs of less than or equal to 10 ng/mL, two compounds had LODs of 25 ng/mL, and mometasone furoate had the highest LOD of 45 ng/mL. This variation suggests that while Tasso performs well for most compounds, certain glucocorticoids, particularly mometasone furoate, exhibit significantly higher detection limits.

For the VAMS, desonide, fludrocortisone, and flumethasone demonstrated LODs of less than 1 ng/mL. 6OH-Budesonide had an LOD of less than 2 ng/mL, and prednisone had an LOD of less than 14 ng/mL. These compounds also require further testing at lower concentrations to accurately define their LODs. The majority of other LODs in VAMS fell between 5 and 10 ng/mL. Notably, triamcinolone showed a lower LOD of 2 ng/mL, whereas mometasone furoate and prednisolone exhibited higher LODs of 25 ng/mL and 15 ng/mL, respectively. Mazzarino et al. reported LODs ranging from 0.5 to 3 ng/mL, indicating that our results generally exhibit higher LODs.

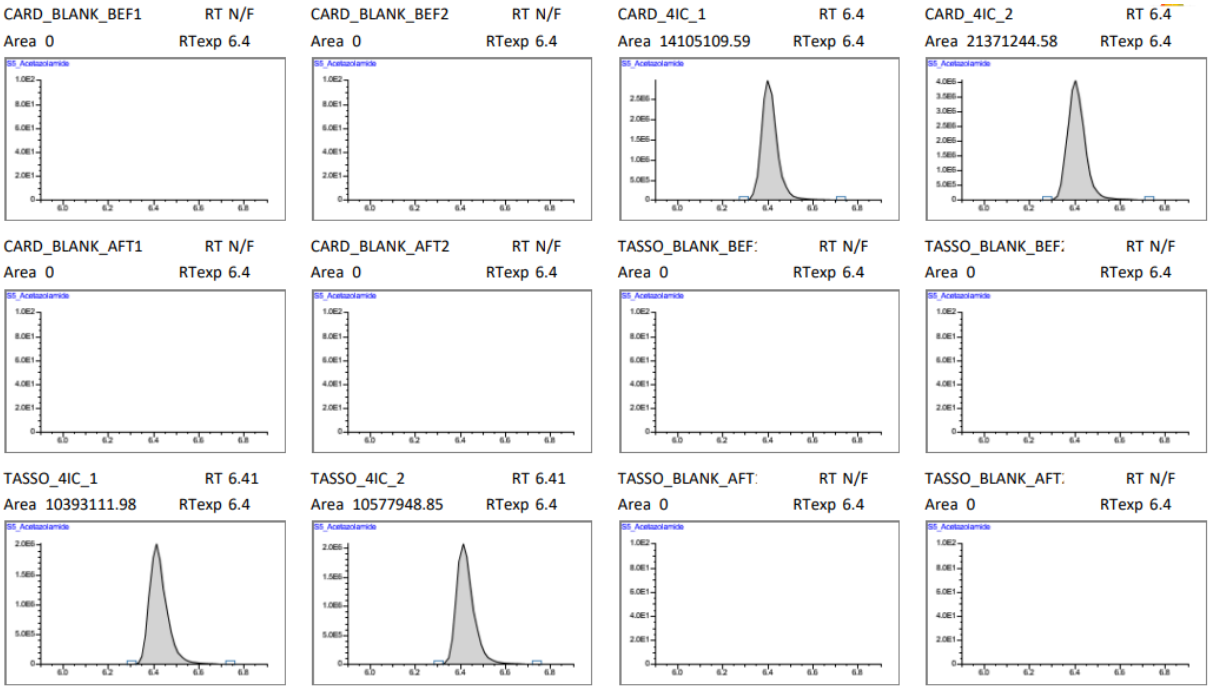
Lastly, he analysis of beta blockers across the three sampling devices showed promising results, with almost all samples exhibiting LODs below 2.34 ng/mL. This indicates a high sensitivity in detecting beta blockers, but further investigations with lower concentrations are necessary to determine the precise LODs for these compounds. Most of the LODs for the remaining beta blockers were around 3 ng/mL, except for mepindolol, which presented significantly higher LODs of 23 ng/mL in DBS and 10 ng/mL in VAMS. In the Tasso device, the LODs for four compounds—carvedilol, mepindolol, nebivolol, and penbutolol—could not be

determined, suggesting potential limitations in sensitivity or technical issues with this particular sampling method.

When comparing our findings with the literature, Mazzarino et al. reported LODs ranging from 0.3 to 1.5 ng/mL, while Garzinski et al. reported even lower LODs between 0.25 and 1.25 ng/mL. Our study results could be in the same range but further research need to be conducted to determine the precise values of the LODs.

3.2.2 Carry over

An experiment was done to determine if there was carry over between the samples. The protocol followed as previous stated. For DBS, Tasso and VAMS, two blank samples were injected first, followed by two samples, spiked with the compound of interest at a concentration four times the concentration in *Table 2*, lastly, another two blank samples were injected. The chromatograms were evaluated and for the compounds integrated in this study, no or less than 1% carry over was observed. In compounds exhibiting <1% carryover, the carryover was mostly observed in the TASSO_BLANK_BEF sample *(Figure 13).* This detection in the before sample of the Tasso may be attributed to the sequential injection of samples from the three different devices. In *Figure 12*, an example of a compound with no carry over is displayed.



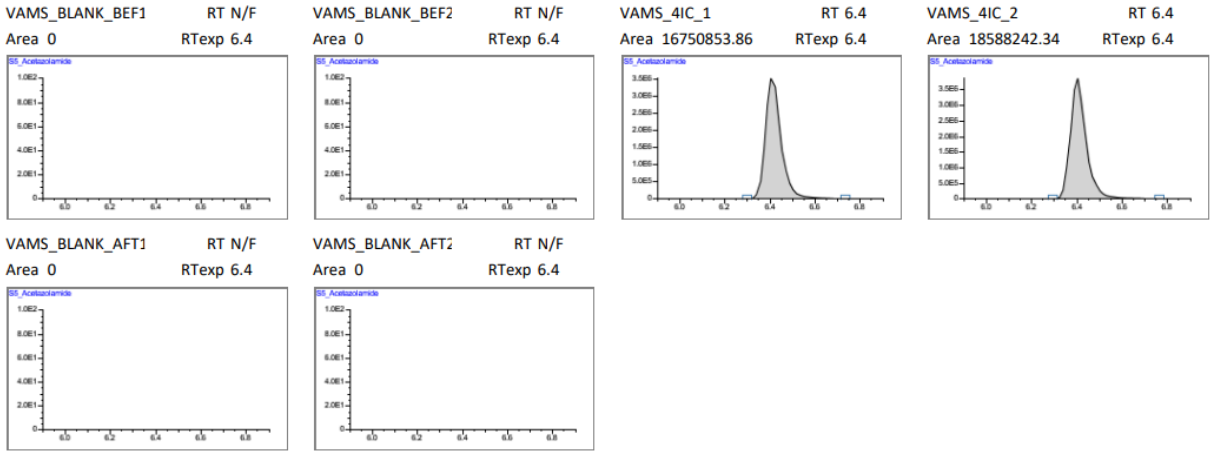


Figure 12: Example of compound (acetolamide) that demonstrated no carry over, where there are no peaks in the blank samples and clear peaks in the spiked samples.

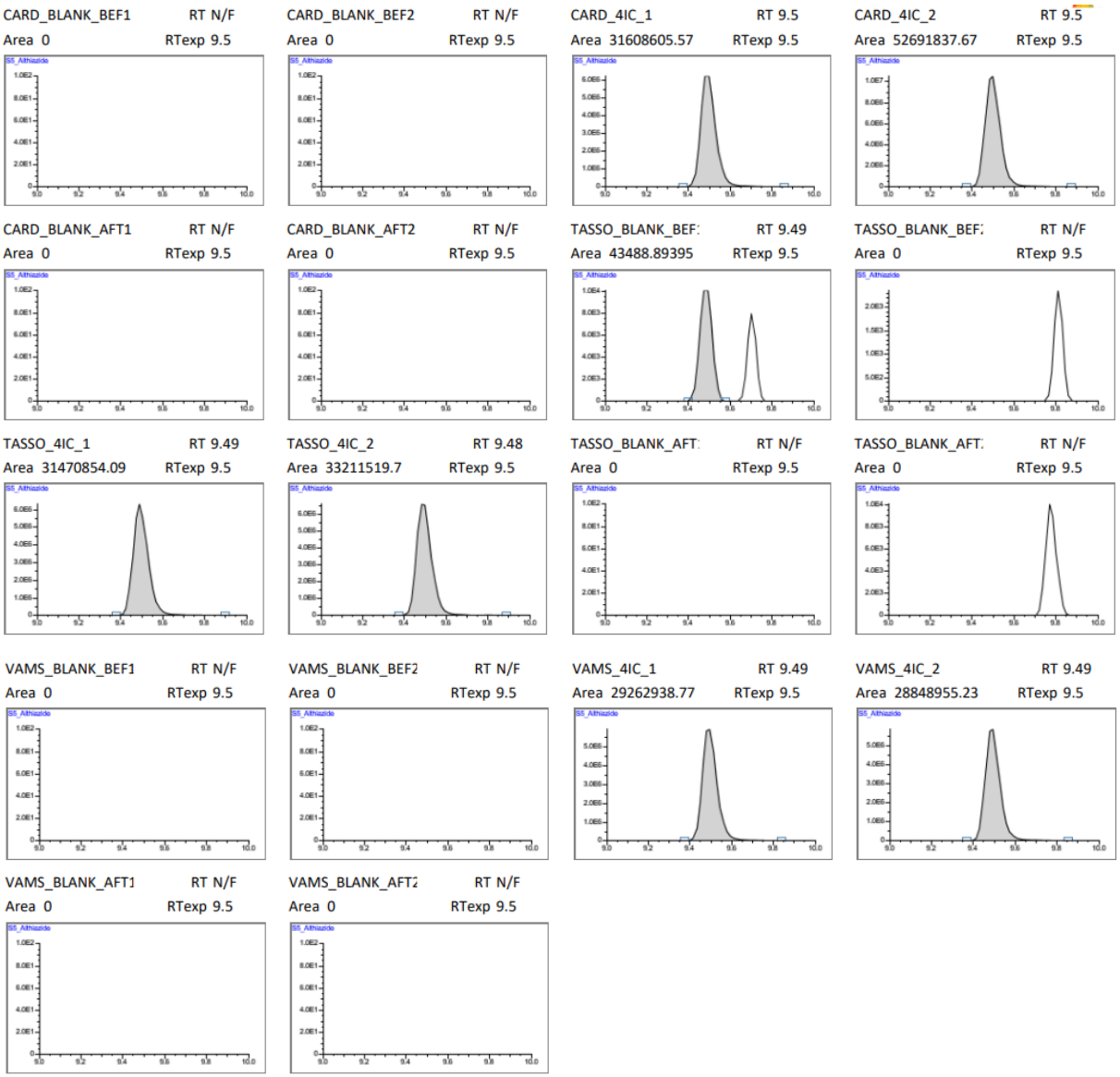


Figure 13: Example of compound (althiazide) that demonstrated <1% carry over in the TASSO_BLANK_BEF sample.

3.2.3 Selectivity

The selectivity was evaluated by analyzing at least 10 drug-free whole blood samples with different hematocrit, from five male and five female subjects, in two different days to verify that the analytes of interest were effectively differentiated from endogenous matrix interferences or in the reagents/devices used for sample collection and extraction. In *Figure 14*, two model compounds per class are displayed. No interferences were observed in the analysis of narcotics. However, for the other classes, approximately half of the compounds exhibited some level of interference or background signal in the 10 blank samples. The intensity of these signals ranged between 1.2E+03 and 8E+05, with the exception of one compound (niketamide), which displayed a background signal of 2.5E+07. Despite the relatively high background signal, the compound itself exhibited a signal of approximately 8E+08, representing less than 1% interference. Despite some background, all the analytes were clearly distinguishable in whole blood.

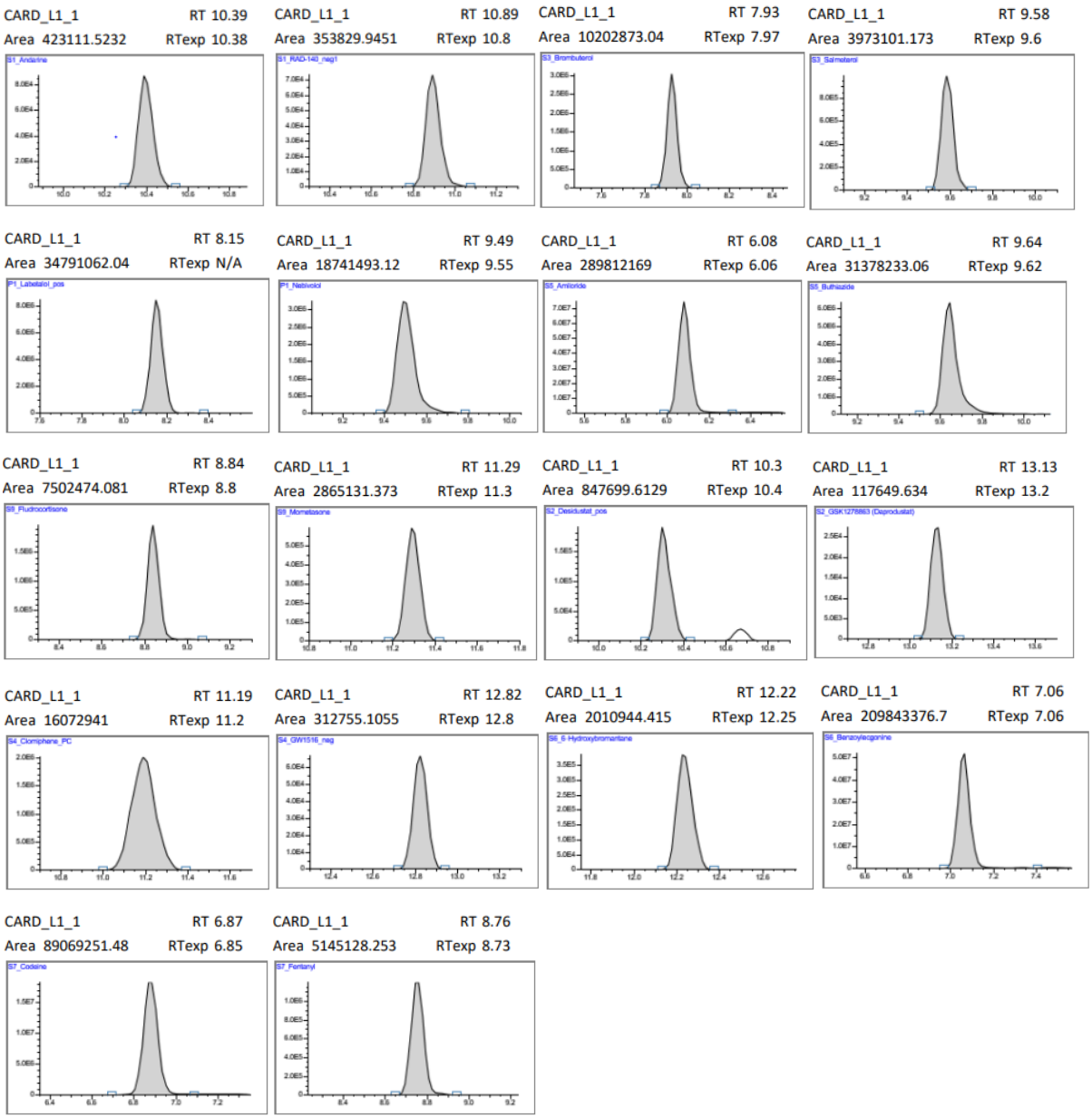


Figure 14: Extracted chromatograms at the RTs of two model compounds for the included classes in a negative sample spiked with the compounds under investigation at the highest concentration in Table 2. The samples were in DBS and the extraction solvent was methanol/acetonitrile.

3.2.4 Hematocrit effect

To assess the impact of varying hematocrit levels on result efficacy, five whole blood samples with different hematocrit values ranging from 33 to 55% were analyzed. There were no clear differences observed in the AUC between the high and low hematocrits.

4 General conclusion

Dried blood spots (DBS), Tasso devices and VAMS provide a promising and minimally invasive method for detecting doping agents. The use of liquid chromatography-mass spectrometry (LC-MS) for analyzing DBS allows for effective and sensitive detection of various substances, including anabolic steroids and stimulants, commonly abused in sports.

The method developed shows high sensitivity and specificity, with satisfactory limits of detection for multiple doping substances. The minimal carry over and minimal impact of hematocrit levels on the analysis underscore the robustness and reliability of this approach. The study highlights the potential of DBS, Tasso and VAMS for routine sports drug testing, offering advantages such as cost-effectiveness, ease of sample collection, and better storage and transport conditions compared to traditional blood or urine samples.

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6 Poster

FACULTY OF MEDICINE AND HEALTH SCIENCES

DoCoLab

Detection of doping agents in dried blood spots via liquid chromatography-mass spectrometry

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Introduction

In the domain of competitive sports, the persistent battle against doping has become integral to upholding the principles of fairness and integrity. The World Anti-Doping Agency (WADA) plays an important role in defining and enforcing the Prohibited List, categorizing prohibited methods and substances. Detection methods have primarily involved urine, serum, and whole blood analyses. However, the evolution of technology has paved the way for innovative microsampling techniques, such as Tasso, Volumetric Absorptive Microsampling (VAMS), and Dried Blood Spot (DBS) testing, revolutionizing the landscape of anti-doping strategies.

Figure 1: WADA prohibited list

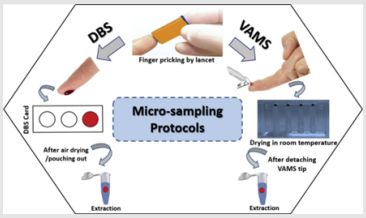

Figure 2: micro-sampling workflow for DBS and VAMS

Method

- Development and validation of a qualitative LC/MS method using online solid phase extraction
- Analysis of blood microsamples

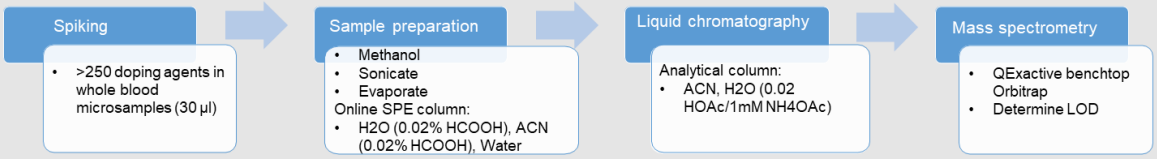

Figure 3: Flowchart of the method for qualitative analysis of spiked whole blood with >250 compounds

Table 1 provides a comprehensive overview of the eleven classes included in the WADA prohibited list. The chosen target compounds encompass all prohibited substance classes (S0, S1, S2, S3, S4, S5, S6, S7, S8, S9, P1).

Table 1: WADA prohibited substance classes

S0 (non-approved substances)	S6 (stimulants)
S1 (anabolic agents)	S7 (narcotics)
S2 (peptide hormones, growth factors, related substances, and mimetics)	S8 (cannabinoids)
S3 (beta-2 agonists)	S9 (glucocorticoids)
S4 (hormone and metabolic modulators)	P1 (beta-blockers)
S5 (diuretics and masking agents)	

Preliminary results

Figure 1 illustrates the chromatographic profiles of Mapenterol, a beta-2-agonist, showcasing examples of negative, borderline, and positive chromatograms. Based on such evaluation, the calculated limit of detection (LOD) for Mapenterol in VAMS is 1,755 ng/ml.

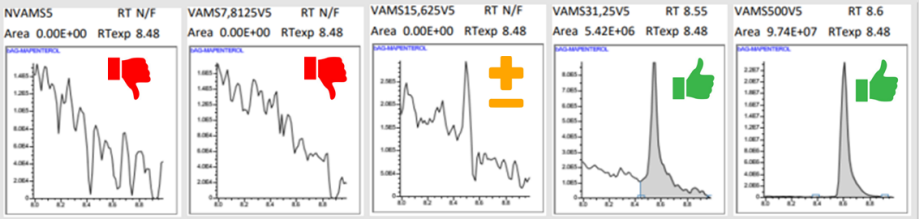

Figure 4: Chromatograms illustrating the elution profiles of the beta antagonist Mapenterol at various concentrations, from left to right: blanco, 0.78125 ng/ml, 1.5625 ng/ml, 3.125 ng/ml, and 50 ng/ml.

Further experiments will explore various sample preparations and analytical chromatographic settings. The LOD of all target analytes will then be estimated.

Conclusion

The first (preliminary) results indicate detection of a wide-range of doping agents is possible using LC-HRMS in DBS and VAMS microsamples at concentration levels in the low ng/ml range.

Further experiments to optimize the method will be performed to expand the list of substances and attempt to lower LOD's and hence extend the detection window of doping use.

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