University of São Paulo

School of Pharmaceutical Sciences of Ribeirão Preto

and

University of Technology Sydney

Centre for Forensic Science

Sweat as an alternative sample in doping control

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2019

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A Thesis presented to the Post Graduate Program in toxicology and Forensic Sciences in fulfilment of the requirements for the award of the Doctor of Sciences and Doctor of Philosophy

Area: Toxicology and Forensic Sciences

Supervisors: Prof. Dr. Bruno Spinosa De Martinis and Dr. Philip Doble

Co- supervisors: Prof. Dr. Claude Roux

2019

ACKNOWLEDGEMENT

First and foremost, I would like to express my deepest appreciation to my supervisors, Professor Bruno Spinosa De Martinis and Dr. Lucas Blanes and my co-supervisors Professor Maurício Yonamine and Professor Claude Roux. Thank you for giving me the opportunity to undertake this project, supporting and encouraging me throughout this journey. Thank you for giving me the freedom to try and follow my ideas and the opportunity to grow as a researcher.

I would like to thank Professor Philip Doble and Dr David Bishop for their constant laboratory support and invaluable contributions during the thesis preparation. I cannot put in words how grateful I am for everything you have done for me.

I also would like to show my gratitude to everyone involved in the preparation of this thesis. Your constructive feedback greatly improved the written work related to this PhD and I am extremely grateful for your time and efforts.

A sincere thank you to all the volunteers who participated in this study. During gathering this material, interviewing the participants, and collecting the data and samples, I had invaluable assistance from all of you. Without these, the completion of this study would not have been possible. I would like to thank Dr Claudio Sodré for the support with the laboratorial exams.

And to all my colleagues at the University of Sao Paulo (USP) and University of Technology Sydney (UTS) I cannot even begin to thank you all for everything you have done for me throughout my lifetime, but especially these past 4 years dealing with all my ups and downs. You are my “second family” you guys were always there for me made a world of difference.

I want to thank my partner Kevin for his support throughout the whole time. He has been by my side throughout the hardest year, to whom I can share my true emotions especially in the down times. His unconditional love, support, trust, and confidence in me and has helped and will continue to help me overcome many tough waves in life. I know the last few months have not been easy, and I greatly appreciate all the sacrifices you made to make my life easier even though they made your life harder. You have been there for me, always provided constructive feedback and advices.

Words cannot express how grateful I am and how much I love my mum Marcia, my dad Dorival, my sister Aryanne. It is because of them that today I have the chance to follow my dreams. I could not be where I am today without their love, hard work, support, and sacrifice.

I would like to thank all my family and friends back home in Brazil. Despite all these kilometres including an ocean separating us, you have been there for me every step of the way. You have always believed in me, even when I doubted myself.

"There are only two ways to live your life. One is as though nothing is a miracle. The other is as though everything is a miracle".

Albert Einstein

ABSTRACT

Bordin, Dayanne Cristiane Mozaner. **Sweat as an alternative sample in doping control.** 2019. 149p. Thesis Doctor Degree. School of Pharmaceutical Sciences- USP and Centre of Forensic Sciences-UTS. 2019.

Sport has become a major industry of invaluable awards and major investments. To overcome opponents, many athletes resort to illegal means to improve their performance, as the World Antidoping Agency (WADA) prohibits drug use in sport. In addition to the ethical aspects, drug use can cause serious damage to an athletes' health by directly influencing physiological capacity and removing physical and psychological barriers, and their health damage may be irreversible. Urine is the standard matrix used in doping control; however, these analyses can also be performed on alternative biological samples; such as sweat. Toxicological analysis in sweat samples present some advantages over urine, such as less chance of sample tampering, greater detection windows, non-invasive collection, and the possibility of finding the parent drugs. The use of sweat as an alternative sample also allows obtaining a history of drug abuse.

The identification of doping cases has been an important area of research and development in recent years due to the continued increase in the number of prohibited substances. For the detection of prohibited substances, sophisticated analytical instruments such as liquid (LC), gas (GC) chromatography coupled to mass spectrometry and capillary electrophoresis (CE) are generally used. This thesis focused on the development of methods for evaluation of sweat as an alternative biological matrix for doping control, using different methods for sample preparation and detection to overcome its limitations. In addition, an outline of the profile of drugs used for sports practice and the acute effects caused in the organism of abusers were investigated.

A survey was applied to the volunteers to evaluate the adverse effects of androgenic anabolic steroids (AAS), dietary supplements (DS) and multiple drug use. In parallel, the volunteers hematological and biochemical parameters were measured. Through toxicological urine analysis, self-reporting and the parameters measured within the study revealed that the use of doping agents and drugs are subject to different factors, which are normally guided by the type of physical activity or aesthetic appearance. This abuse can increase the chances of health problems causing synergistic side effects, increasing the risk to develop diseases. We developed a method to determine the presence of 13 amphetamines and cocaine related

substances and their metabolites in sweat and urine using disposable pipette extraction (DPX) tips and GC-MS. The validated method was used to analyze 40 urine and sweat samples whose athletes self-reported the use of drugs and/or stimulants. It was verified that all consumed drugs and metabolites detected in urine were also present in sweat samples indicating that sweat was a viable matrix to perform doping tests.

We also developed a screening alternative method for stimulants detection in sweat samples using a microchip capillary electrophoresis instrument (Agilent Bionalyzer). Although functioning, the method was not sensitive enough to detect the low concentrations of drugs and metabolites present in sweat samples.

A one step fully automatized derivatisation and headspace (HS) SPME extraction method followed by GC-MS was developed for the analysis for amphetamine-type drugs and cocaine. The HS-SPME/GC-MS method was used to detect concentrations between 0.1 to 1 ng/mL of the target analytes without any additional sample preparation, suitable for routine analysis of drug traces in biological samples, such as urine and sweat.

Keywords: sport, doping, alternative biological matrix, sweat stimulants, urine, and analytical methods.

RESUMO

Bordin, Dayanne Cristiane Mozaner. **Suor como matriz alternativa no controle do doping.** 2019. 149p. Tese (Doutorado). Faculdade de Ciências Farmacêuticas de Ribeirão Preto – Universidade de São Paulo, Ribeirão Preto, 2019.

O esporte tornou-se uma indústria de valiosos prêmios e grandes investimentos. Na tentativa de vencer adversários, muitos atletas recorrem a meios ilegais para melhorar seu desempenho. A Agência Mundial Antidoping (WADA) proíbe o uso de drogas no esporte. Além dos aspectos éticos, o uso de drogas pode causar sérios danos à saúde de um atleta, influenciando diretamente a capacidade fisiológica e removendo barreiras físicas e psicológicas, e seus danos à saúde podem ser irreversíveis. A urina é a matriz padrão utilizada no controle de doping. No entanto, essas análises também podem ser realizadas em amostras biológicas alternativas; como o suor. A análise toxicológica em amostras de suor apresenta algumas vantagens em relação à urina, como menor chance de adulteração de amostras, maior janela de detecção, coleta não invasiva, além da possibilidade de encontrar principalmente as drogas consumidas. O uso de suor como amostra alternativa também permite obter um histórico de exposição ao abuso de drogas. A identificação de casos de doping tem sido uma área importante de pesquisa e desenvolvimento nos últimos anos devido ao aumento contínuo do número de substâncias proibidas. Para a detecção de substâncias proibidas, geralmente são utilizados instrumentos analíticos sofisticados, tais como cromatografia líquida (LC), cromatografia gasosa (GC) acoplados à espectrometria de massas e eletroforese capilar (CE). Esta tese centrou-se no desenvolvimento de métodos para avaliação do suor como uma matriz biológica alternativa para o controle de doping, utilizando diferentes métodos de preparação e detecção de amostras para superar suas limitações. Paralelamente, foi realizado um esboço do perfil das drogas utilizadas para prática esportiva e os efeitos agudos causados no organismo dos usuários. Foi aplicada uma pesquisa aos voluntários para avaliar os efeitos adversos dos esteroides anabolizantes androgênicos (AAS), suplementos alimentares (DS) e uso múltiplo de drogas. Em paralelo, foram realizadas medidas de parâmetros hematológicos e bioquímicos dos voluntários. Através de análises toxicológicas em urina, o auto-relato e a medição de parâmetros, o estudo revelou que o uso de agentes dopantes e drogas recreativas são submetidos a diferentes fatores, que são normalmente guiados pelo tipo de atividade física ou aparência estética. Seu uso pode aumentar as chances de problemas de saúde causando efeitos colaterais sinérgicos, aumentando o risco de desenvolver doenças. Desenvolvemos um

método para determinar a presença de 13 anfetaminas e substâncias relacionadas à cocaína e seus metabolitos em suor e urina usando Pipetas de Extração descartáveis (DPX) e GC-MS. O método validado foi utilizado para analisar 40 amostras de urina e suor cujos atletas auto-relataram o uso de drogas. Verificou-se que todas as drogas consumidas e seus metabólitos encontradas na urina também estavam presentes em amostras de suor indicando que o suor é uma matriz viável para realizar testes de doping. Também desenvolvemos um método alternativo de triagem para detecção de estimulantes em amostras de suor usando um instrumento de eletroforese capilar de microchip (Bionalyzer). Embora funcionasse, o método não era suficientemente sensível para detectar baixas concentrações de drogas e metabólitos presentes em amostras de suor. Foi desenvolvido um método de extração por headspace (HS) e derivatização totalmente automatizado de apenas um passo seguido por analises em GC-MS para determinação de drogas tipo anfetaminas e cocaína. O método HS-SPME/GC-MS foi utilizado para detectar concentrações entre 0,1 a 1 ng/mL dos analitos sem qualquer preparação adicional de amostra, apresentando potencial aplicação para análise de rotina de traços de drogas em amostras biológicas, como urina e suor.

Palavras-chave: esporte, doping, matriz biológica alternativa, estimulantes, agentes anabolicos esteroides, suor, urina e métodos analíticos.

CERTIFICATE OF AUTHORSHIP AND ORIGINALITY

I, Dayanne Cristiane Mozaner Bordin, declare that this thesis, is submitted in fulfilment of the requirements for the award of Doctor of Philosophy, in the Faculty of Science at the University of Technology Sydney.

This thesis has also been submitted for qualification at University of São Paulo as it is the result of a research candidature conducted as part of a collaborative Doctoral degree.

This document has not been submitted for qualifications at any other academic institution. This thesis is wholly my own work unless otherwise reference or acknowledged. In addition, I certify that all information sources and literature used are indicated in the thesis.

This research is supported by an Australian Government Research Training Program, Brazilian Federal Agency for Coordination of Improvement of Higher Education Personnel (CAPES) and São Paulo Research Foundation (FAPESP).

Production Note:
Signature removed
prior to publication.

Dayanne Cristiane Mozaner Bordin

03.04.2019

LIST OF PUBLICATIONS

The presented work in this thesis have been published, accepted for publication or prepared for submission to journals as follows:

Published:

D.C.M. Bordin, F. Monedeiro, L.H.Bueno, M.N.R.Alves E.G. De Campos, B.S. De Martinis. Novas perspectivas em preparo de amostras biológicas com interesse forense, Scientia Chromatographica, 2015, DOI: **/10.4322/sc.2015.022**

D.C.M. Bordin, M.N.R.Alves, E.G. De Campos, B.S. De Martinis. Review Article: Disposable pipette tips extraction: Fundamentals, applications and state of the art, ***Journal of Separation Science,*** 2016, *39*, 1168–1172, DOI: 10.1002/jssc.201500932

D.C.M. Bordin, B.B. Bettim, G.C. Perdona, E.G. De Campos, B.S. De Martinis. Understanding alterations on blood and biochemical parameters in athletes that use dietary supplements, steroids and illicit drugs**,** ***Toxicology*****,** 2017**, 376,** 75-82, DOI:org/10.1016/j.tox.2016.05.019

A.A. Ishikawa, D.C.M. Bordin, E.G. De Campos, L. Blanes, P. Doble, B.S. De Martinis. Analysis of methylenedioxy derivatives in vitreous humor using liquid-liquid extraction and GC/MS. ***Journal of Analytical Toxicology***, 2018, 42 (9), 661-666. Doi.org/10.1093/jat/bky044

Submitted and final preparation:

1. Dayanne Mozaner Bordin, Eduardo Geraldo de Campos, Lucas Blanes, Philip Doble, Claude Roux and Bruno Spinosa De Martinis. A new approach for determination of doping used-stimulants drugs in sweat and urine using pipette tips extraction (DPX) and GC/MS. (article) Drug Testing Analysis **(final preparation)**

2. Dayanne Mozaner Bordin, Eduardo Geraldo de Campos, Fernanda Monedeiro, Lucas Blanes, Philip Doble, Claude Roux and Bruno Spinosa De Martinis. Sweat as a tool for drug testing, metabolomics and diseases detection (**final preparation**)

3. Lidiane Alves, Dayanne Mozaner Bordin, Fernanda Monedeiro, and Bruno Spinosa De Martinis. Investigation of Ayahuasca β-carboline alkaloids and Trytamines present in sweat samples after a religious ritual. Drug Testing Analysis **(final preparation)**

LIST OF CONFERENCES

The research conducted during this project was presented at several international conferences listed below.

Year	Conference	Presentation
2014	52rd The International Association of Forensic Toxicologists (TIAFT), Buenos Aires, Argentina	A rapid assay for determination of MDMA, MDA, MDEA, Methamphetamine and Amphetamine in sweat using Disposable Pipette Extraction (DPX) and GC-MS
2015	53rd The International Association of Forensic Toxicologists (TIAFT), Firenze, Italy	Side effects and trends in the consumption of anabolic-androgenic steroids (AASs) and psychotropic drugs in bodybuilders
2015	9th Congress of Toxicology in Developing Countries XIX Congresso Brasileiro de Toxicologia, Natal, Brazil	Side effects in the consumption of Anabolic Androgenic Steroids And Dietary Supplements
2016	54rd The International Association of Forensic Toxicologists (TIAFT), Brisbane, Australia	A new approach for the analysis of stimulants in sweat using a disposable pipette extraction (DPX) and GC-MS
2016	International Symposium on Advances in Separation Science (ASASS), Hobart, Tasmania	Application of screening method for stimulants in sweat samples and urine using a modified technique DPX and GC-MS
2017	Forensic and Clinical Association Meeting (FACTA), Melbourne, Australia	One step automatized analysis of drug stimulants using HS-SPME/GC-MS

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SUMMARY

ABBREVIATIONS

WADA	World Antidoping Agency
LC	Liquid Chromatography
GC	Gas Chromatography
MS	Mass Spectrometry
CE	Capillary Electrophoresis
DPX	Disposable Pipette Extraction Tips
AAS	Anabolic Androgenic Steroids
DS	Dietary Supplements
EI	Electro Ionization
HS	Headspace
SPME	Solid Phase Micro Extraction
IOC	International Olympic Committee
S	Substances
M	Methods
HIF	Hypoxia-inducible factor stabilizers
CG	Chorionic Gonadotrophin
GH	Growth Hormone
SERM	Selective estrogen receptor modulators
mL	Millilitre
CNS	Central Nervous System
DAT	Dopamine
NOR	Noradrenaline
SER	Serotonin
MA	Methamphetamine
MDA	3,4-methylenedioxyamphetamine

MDMA	3,4-methylenedioxymethamphetamine
MDEA	3,4-methylenedioxyethylamphetamine
BE	Benzoylecgonine
EME	Methylecgonine ester
DHEA	Dehydroepiandrosterone
cAMP	Cyclic adenosine monophosphate
RNA	Ribonucleic
LH	Luteinizing Hormone
FSH	Follicle-stimulating Hormone
T4	Thyroxine
T3	Triiodothyronine
IRMS	Isotope Ratio Mass Spectrometry
LLE	Liquid–Liquid Extraction
SPE	Solid-Phase Extraction
RP	Reversed-Phase sorbent
CX	Cation Exchanger sorbent
WAX	Anion Exchanger sorbent
PDMS	Polydimethylsiloxane
PA	Polyacrylate
CAR	Carbowax
CI	Chemical Ionisation
QMS	Single Quadrupole Mass Spectrometer
S/N	Signal-to-noise
QQQ	Triple Quadrupole
ME	Microchip electrophoresis
EOF	Electroosmotic flow

MEKC	Micellar Electrokinetic Capillary Chromatography
UV	ultra-violet
CID	Collision-Induced Dissociation
PES	Performance Enhancing Substances
THCCOOH	11-nor-9-carboxy-9-tetrahydrocannabinol
d	Deuterium
mg	Milligram
C	Celsius
°	Degrees
MSTFA	N-methyl-N-(trimethylsilyl) trifluoroacetamide
µg	Microgram
IS	Internal Standard
M	Molar
rpm	Rotation per minute
v	Volume
mm	Millimetre
m	Metre
µm	Micrometre
SIM	Selected Ion-Monitoring
m/z	Mass-to-charge ratio
min	Minutes
LOD	Limit of Detection
LOQ	Limit of Quantification
PC	Positive Control
THC	Cannabis
BCAA	Amino acids

MCV	Mean corpuscular volume
MCH	Mean corpuscular haemoglobin
MCHC	Mean corpuscular haemoglobin concentration
GFR	Glomerular Filtration Rate
dL	Decilitre
L	Litre
CKD	Chronic kidney disease
GGT	Gama-Glutamyl Transferase
GOT	Glutamico-Oxalacetic Transaminase
GPT	Glutamic-Pyruvic Transaminase
HDL	High-density lipoprotein
LDL	Low-density lipoprotein
VLDL	Very Low-density lipoprotein
MPT	Mephentermine
AMP	Amphetamine
AMP D11	Amphetamine D11
METAMP	Metamphetamine
AMPHEP	Amphepramone
MDMA	3,4-Methylenedioxymethamphetamine
MDMA D5	3,4-Methylenedioxymethamphetamine D5
MDA	3,4-Methylendioxyamphetamine
MDEA	3,4-Methylenedioxyethylamphetamine
MPH	Methylphenidate
FENP	Fenproporex
COC	Cocaine
COC D3	Cocaine D3

BE	Benzoylecgonine
COE	Cocaethylene
MEPHE	Mephedrone
SWGTOX	Scientific Working Group Toxicology guidelines
R^2	Coefficient of determination
QC	Quality control
ng	Nanogram
LOC	Lab-on-a-chip
mmol	Millimol
LED	Fluorescence detection
FITC	Fluoresceine isothiocyanate
mM	Millimol
kV	Kilovolts
ppt	Parts per trillion
cEF	ethyl chloroformate

Chapter 1: **Literature Review**

Chapter 1: Literature Review

Historical records indicate that the use of drugs by athletes to improve physical performance was occurring as far back as the Classical Era. Roman athletes to obtain advantage in competitions empirically abused stimulants and hallucinogens substances. During time this practice evolved in the same proportion of the importance given to the sport (YONAMINE, PAULA, 2008; WADA, 2010).

The sports environment became a market, in which high performance is required over a short period of time inducing athletes to use all available resources, legal or not (YONAMINE, et al, 2005). Besides the ethical aspects, these substances remove physical and psychological barriers, which are aspects of particular concern as they may lead to increased chances for injury, dependence and in extremes cases death (YONAMINE, PAULA, 2008; SEGURA, et al, 1998; CAMPOS, et al, 2005).

Misuse of drugs during competitive games has been recognized by sports organizations as an important problem since the International Olympic Committee (IOC) Medical Commission was established in 1967 (WADA, 2010). The death of a Danish cyclist caused by amphetamine intoxication during the Olympic Games in Rome and the indiscriminate consumption of stimulants, such as amphetamines, by athletes were the facts that forced the authorities to create regulations and doping control (WADA, 2010).

In 1967, the International Olympic Committee (IOC) published a list of banned substances that included stimulant drugs and narcotic analgesics, making official the doping control. The IOC introduced the first doping tests in Mexico at the Summer Olympic Games in 1968. In 2000, the World Anti-doping Agency (WADA) was formed in Canada by Olympic Movement members' and government's representatives from five continents and became the responsible organization for coordinating the prevention and doping control. Since then, the list of prohibited substances and methods is reviewed annually and used for sports regulations (YONAMINE, PAULA, 2008; SEGURA, et al, 1998; WADA, 2010).

According to WADA statistics, drug abuse by athletes for performance improvement continues to occupy a prominent place in sport (YONAMINE, PAULA, 2008; WADA, 2012). According to the report published in the year 2014, anabolic agents and stimulants now constitute more than 90% of positive cases reported

(WADA, 2015). In 2014, 267,000 urine samples were analysed; of those 1.7% tested positive. Of those positive results, anabolic steroids were the most common, detected in 2279 cases (50.6%); followed by the stimulants with 679 cases (15.5%) (WADA, 2015).

These substances are responsible for serious consequences to the athlete's health and are related to many cases of deaths. They mask the signs of fatigue and pain; and increase significantly an athlete's ability to tolerate high levels of anaerobic metabolism (CHASIN et al, 2008; BERGO, 2007). The disturbances in temperature body regulation, blood pressure variations, and altered external factors such as high temperature in the environment, can contribute death. Some of the drugs have positive reinforcement produced by their pleasant pharmacological effects that can lead to the continuous self-administration, leading to dependence (SEGURA et al, 1998; BOLLMANN et al, 2006).

The development of analytical techniques capable to detect the prohibited substances has a significant inhibitory effect on such abuse by athletes. The effective way to minimize doping in sport has been this systemic control by performing toxicological analyses on biological material provided by the athlete. This control has been performed during the competition period or during training. The analytical aspects of doping testing remain challenging. These approaches are improving in parallel with the requirements of increasing sensitivity and selectivity for detection of prohibited substances in biological samples (SEGURA et al, 1998; LAURE, 2003; BERGO, 2007).

Urine is the official body fluid used in doping analysis. The main reasons of this choice are that collection is easy and non-invasive, and large amounts can often be collected. However, this matrix can easily be adulterated, subjected to various masking agents, and requires invasive constraints for collection. Additionally, drugs are usually found in their metabolite form and most of them are only present in urine for 2-3 days after consumption (CAPLAN, GOLDBERGER, 2001; KARCH, 2007; GALLARDO, QUEIROZ, 2008; RIVIER, 2000).

According to WADA (in paragraph 5.2.4.4 of the International Standard for Laboratories), the use of alternative matrices is permitted, however the respective result shall not be used to counter adverse analytical or atypical findings from urine

(GALLARDO, QUEIROZ, 2008). In this sense, the use of alternative biological specimens can provide significant benefits in toxicological analysis.

Sweat is an alternative biological matrix that has gained prominence in recent years in toxicological analysis. Several studies have been successfully conducted for analysis of drugs of abuse in sweat (DE MARTINIS et al, 2007, CONE et al, 1994; HUESTIS et al, 1999; KACINKO et al, 2005; COLE et al, 1994; HUESTIS et al, 2000; KINTZ et al, 1997). Sweat testing has several advantages in relation to the conventional biological matrices, blood and urine (DE MARTINIS et al, 2008) including the sample collection is non-invasive and does not cause embarrassment (HUESTIS et al, 1999; DE MARTINIS et al, 2007; TAYLOR et al 1998). "Sweat patches" are used for the collection; those can be worn continuously for longer periods of time which does not disturb the ability to carry out normal activities including swimming and showering without removing the patch (HUESTIS et al, 2000; TAYLOR et al, 1998). The detection of parent compounds as predominant species, rather than metabolites (CAPLAN, GOLDBERGER, 2001) in a longer period post consumption (from several days up to several weeks) is a significant advantage (DE MARTINIS et al, 2008), and in addition, the possibility of sample adulteration is reduced since it is possible to identify if a patch has been removed and reapplied (DE MARTINIS et al, 2007).

1.1. Prohibited substances in the sport

Doping in sports may be defined as the use of prohibited substances or methods. The WADA Prohibited List has defined more than two hundred substances forbidden both in- and out-of-competition. These drugs are classified into nine categories; two groups of analytes prohibited in particular sports and three forbidden methods. According to WADA code, all the prohibited substances shall be considered as "specified substances" except substances in classes S1, S2, S4, S5, S6 and prohibited methods M1, M2 and M3 (BADOUD et al 2011; WADA, 2016). The classification of the prohibited substances and methods are presented in the Table 1.1 below.

Table 1.1: List of prohibited substances and methods published by WADA in 2016 (WADA, 2016).

WADA classification	Type	Brief description and/or examples
* S0	Non-approved substances	Any pharmacological with no current approval by any governmental regulatory health authority for human therapeutic use
* S1	Anabolic Agents	1) Anabolic Androgenic Steroids (AAS): exogenous and endogenous when administered exogenously 2) Other anabolic agents
* S2	Peptide Hormones, Growth Factors, Related substances and mimetics	1) Erythropoietin - Receptor agonists 2) Hypoxia-inducible factor (HIF) stabilizers 3) Chorionic Gonadotrophin (CG) and Luteinizing Hormone (LH) 4) Corticotrophins and their releasing factors 5) Growth Hormone (GH) and its releasing factors
* S3	Beta-2 Agonists	Beta-2 agonists, including all optical isomers
* S4	Hormone and metabolic modulators	1) Aromatase inhibitors 2) Selective estrogen receptor modulators (SERMs) 3) Other anti-estrogenic substances 4) Agents modifying myostatin function 5) Metabolic modulators
* S5	Diuretics and Masking Agents	Including all optical isomers

Table 1.1 continued: List of prohibited substances and methods published by WADA in 2016 (WADA, 2016).

WADA classification	Type	Brief description and/or examples
* M1	Manipulation of Blood and Blood Components	1) The Administration or reintroduction of any quantity of autologous, allogenic (homologous) or heterologous blood, or red blood cell products of any origin into the circulatory system 2) Artificially enhancing the uptake, transport or delivery of oxygen 3) Any form of intravascular manipulation of the blood or blood components by physical or chemical means
* M2	Chemical and Physical Manipulation	1) Tampering, or Attempting to Tamper, to alter the integrity and validity of Samples collected during Doping Control 2) Intravenous infusions and/or injections of more than 50 mL per 6 hour period except for those legitimately received in the course of hospital admissions, surgical procedures or clinical investigations
* M3	Gene Doping	1) The transfer of polymers of nucleic acids or nucleic acid analogues; 2) The use of normal or genetically modified cells
**S6	Stimulants	Including all optical isomers
**S7	Narcotics	
**S8	Cannabinoids	Natural and cannabimimetics
**S9	Glucocorticoids	All glucocorticoids are prohibited when administered by oral, intravenous, intramuscular or rectal routes

Table 1.1 continued: List of prohibited substances and methods published by WADA in 2016 (WADA, 2016).

WADA classification	Type	Brief description and/or examples
**P1	Alcohol	Prohibited in: Air sports, archery, automobile, powerboating
**P2	Beta-Blockers	Prohibited in: Archery, automobile, darts, golf, skiing

*Substances and methods prohibited at all times (in- and out-of-competition)

**Substances and methods prohibited in-competition

- ✓ S: means substance
- ✓ M: means method
- ✓ P: means substances prohibited in particular sports

Depending on the intended biological effects, substances may be prohibited in- and out of competition or at all times. The stimulants are short-acting drugs prohibited in competition while the activity of anabolic agents outlasts its circulation and potential detection in blood and urine. Both compounds are prohibited and controlled in- and out-of-competition (BADOUD et al, 2011; WADA 2016). The Figure 1.1 bellow summarizes the WADA findings of prohibited substances in urine in their last report, 2014.

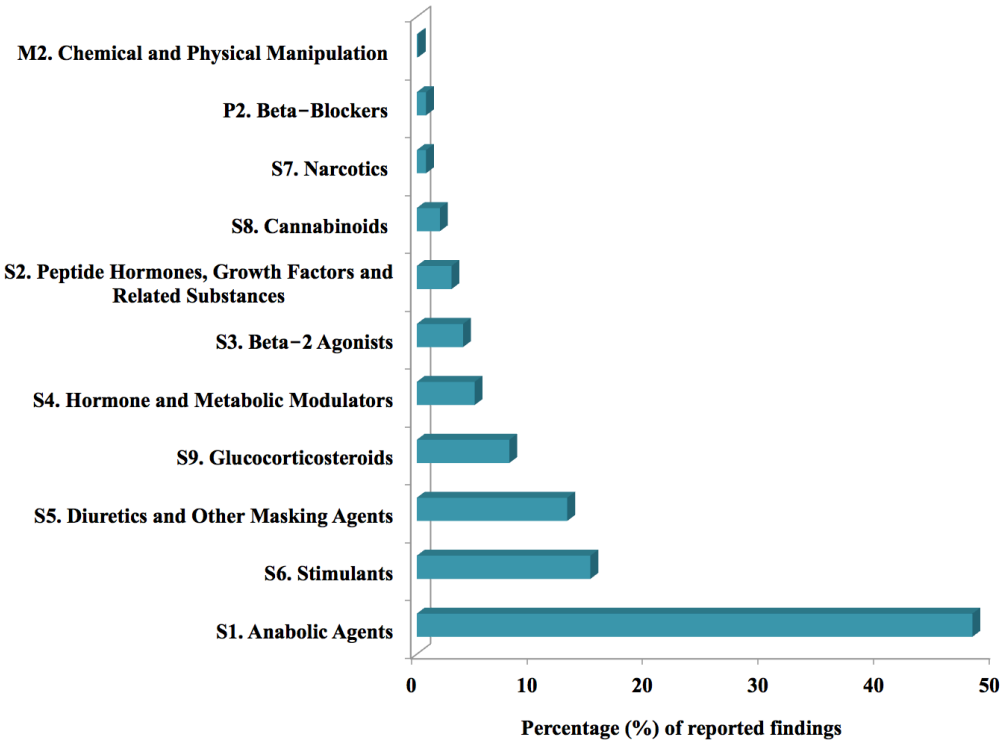


Figure 1. 1: Percentage of substances identified by WADA in each drug class during the year 2014 (WADA, 2015).

1.2. Stimulants abuse in the sport

The stimulants represent one of the oldest classes of doping agents; they were the first groups of substances to be prohibited by most of the international sports federations. This group is included within drug class S.6 under the in-competition testing section of the List of Prohibited Substances and Methods (WADA, 2012).

The use of stimulants in doping presents a major problem in elite sports. About 20% of positive reported cases by IOC accredited laboratories are related to those substances, being the second class most used among athletes. The desired effects include a sense of wellbeing, a decrease in the perception of fatigue and appetite, and an increase in self-confidence and motor function (WADA, 2015; LAURE, 2006; BERGO, 2007).

In this class are included psychomotor, sympathomimetic, and various central nervous system (CNS) stimulants, which act primarily by influencing the mechanism of action of specific neurotransmitters by increasing, blocking or directly stimulating their release. Their mechanisms are influenced by 3 major modes of

neurotransmission at the nerve terminal; including an elevated release of neurotransmitters dopamine (DAT), noradrenaline (NOR), and/or serotonin (SER) into the synaptic cleft, the direct stimulation of postsynaptic receptors, and the inhibition of neurotransmitter reuptake (TREVIS et al, 2010; KAHLIG et al, 2005). The main examples of abused drugs of this class are caffeine, amphetamines, ephedrines and cocaine (CHASIN et al, 2008, BERGO, 2007, SEGURA et al, 1998, BOLLMANN, et al, 2006).

Amphetamines correspond to a group of substances chemically related to β-phenethylamine and composed of amphetamine and its derivatives, such as methamphetamine, 3,4-methylenedioxyamphetamine (MDA), 3,4-methylenedioxymethamphetamine (MDMA), 3,4-methylenedioxyethylamphetamine (MDEA), among others. They act as sympathomimetic amines of the α and β adrenergic receptors, stimulating the noradrenergic, serotonergic and dopaminergic systems. In the dopaminergic system acts increasing the DAT secretion; manipulating the Na^+/Cl^- dependent DAT transporter. The presence of extracellular amphetamines reverses the DAT physiological function and bursts of dopamine are released into the synaptic cleft in a channel-like mode, intensifying the dopaminergic neurotransmission significantly (OGA, CAMARGO, BATISTUZZO, 2008; TREVIS et al, 2010; KAHLIG et al, 2005).

Cocaine is an alkaloid with stimulating properties presenting in its structure based on tropane and indole nuclei. It is an illicit drug with a high potential for addiction. It has local anaesthetic properties. Cocaine acts binding and blocking DAT resulting in the elevation of concentrations of dopaminergic and noradrenergic transmitter at the neuronal synapse; presenting in this way a sympathomimetic action (THEVIS et al, 2010; KAHLIG et al, 2005). Its main metabolites are benzoylecgonine (BE) and methylecgonine ester (EME) (OGA; CAMARGO; BATISTUZZO, 2008).

The stimulants main toxic effects are increased blood pressure, temperature body regulation disorders, tachycardia, arrhythmia, vasoconstriction, mydriasis, nervous excitement, anxiety, and seizures. Some stimulants have a marked positive reinforcement for their use, since their action in the reward centres of the brain produces pleasant pharmacological effects that can lead to their continuous self-administration, leading to dependence (CHASIN et al, 2008; BERGO, 2007;

SEGURA, et al, 1998; BOLLMANN, et al, 2006).

In addition to being responsible for serious health consequences to the athlete, many cases of sports deaths are related to the abuse of these substances. By significantly increasing athletes' ability to tolerate high levels of anaerobic metabolism they mask the signs of fatigue and pain. Disturbances in the body regulation temperature add to the external factors, such as the elevated temperature of the environment, may contribute to the occurrence of these deaths. In the case of lethal intoxication, death comes after convulsion, coma, and cerebral haemorrhage (CHASIN et al, 2008; BERGO, 2007; SEGURA, et al, 1998; BOLLMANN, et al, 2006).

A survey carried out in France in 2001 with 402 physicians showed that only 34.5% of them followed the French anti-doping legislation and 11% said they had been paid to prescribe doping agents for athletes, mainly stimulants (LAURE, 2003). According to the IOC statistics the most abused CNS stimulants were amphetamines and cocaine. But low-profile CNS stimulants, such as ephedrine derivatives, anorexic and miscellaneous stimulant drugs are the main substances found in doping control. Figure 1.2 bellow summarizes the WADA stimulants findings in urine in their last report, 2014.

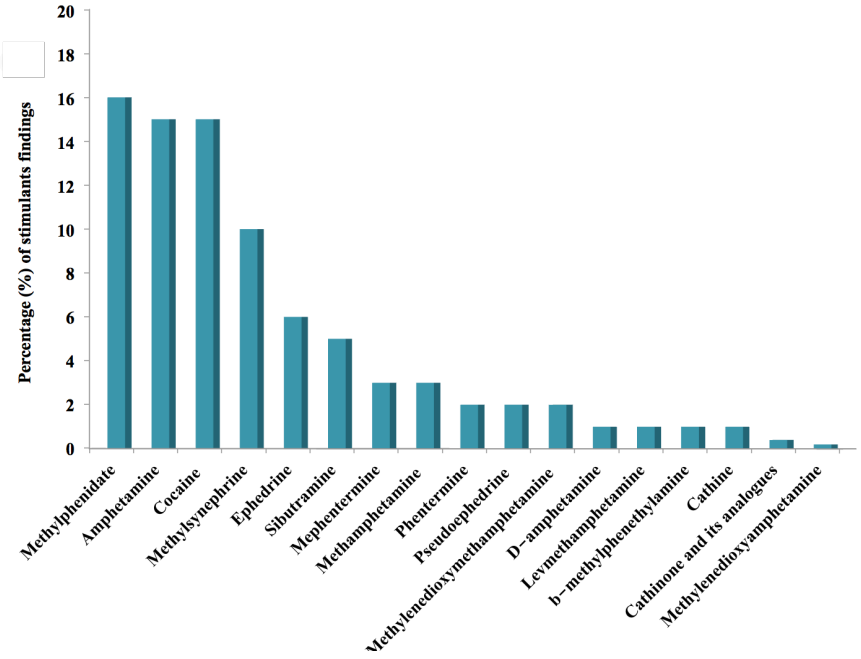


Figure 1.2: Percentage of stimulants identified by WADA during the year 2014 (WADA, 2015).

Stimulants have different activities on different levels, mediated by specific receptors in the organism. They are usually consumed prior to competition to increase the concentration and awareness, to become more aggressive and develop endurance, to deploy more instant energy and speed (WADA, 2015; SEGURA, *et al*, 1998; LAURE, 2003; BERGO, 2007).

1.3. Anabolic androgenic steroids (AAS)

Anabolic androgenic steroids (AAS) are synthetic derivatives of the male hormone testosterone. They are the most common adverse finding declared by WADA accredited laboratories; being around half of the positive tested samples. The AAS abuse in sports starts as agents supporting recovery after massive stress, exhaustion and to enhance athletic performance (DUTRA, PAGANI, RAGNINI, 2012; FORTUNATO, ROSENTHAL, DE CARVALHO, 2007).

Testosterone is the principal androgen in males produced by the testes; its biosynthesis occurs in the Leydig cells. Around 95% of the circulating testosterone is secreted by testicular origin; and the remaining amount derives from peripheral metabolism of a weaker androgen, androstenedione (ROMMERTS 2004; LUKE and COFFEY 1994; O'MALLEY and STROTT 1999). The blood rate of total testosterone is approximately 3-7 mg/day. Dihydrotestosterone (DHT) is the main intracellular androgen in reproductive tissue, it's more potent than testosterone and arises either from testosterone by 5α reduction or, to a much lesser extent, from androstenedione. Dehydroepiandrosterone (DHEA), DHEA sulphate, and androstenedione are weak androgens produced in the adrenal gland. The androgenic effects are responsible for the males' sexual maintenance; for the development of the male reproductive tract and the secondary sexual characteristics, as well as the maintenance of the reproductive function. The anabolic effects refer to the stimulation of muscle growth, which occur through nitrogen fixation, causing a positive nitrogen balance, by increasing the protein in several tissues (YONAMINE, PAULA, 2008; KICMAN, 2008).

Most of the AAS can be administered either orally or intramuscularly. Once absorbed and distributed they act on a wide variety of tissues in the body. In their mechanism of action, after diffusion through the plasma membrane of target cells, they bind to specific intracellular protein receptors. The process of entry into the cell

generates greater production of cyclic adenosine monophosphate (cAMP), thus increasing cell metabolism. The receptor-hormone complex translocates to chromatin in the cell nucleus, thereby promoting transcription of the gene and subsequent synthesis of messenger RNA, and consequent protein transduction, which is modulated by androgens. These androgen receptors have already been characterized in some tissues including: reproductive organs, brain, kidney, liver, skin, skeletal muscle, heart muscle, bone, larynx, thymus, hematopoietic and adipose tissue (CELOTTI, CESI, 1992; SHAHIDI, 2001; SEGURA et al, 1998). Figure 1.3 illustrates the general mechanism of action of steroids in cells.

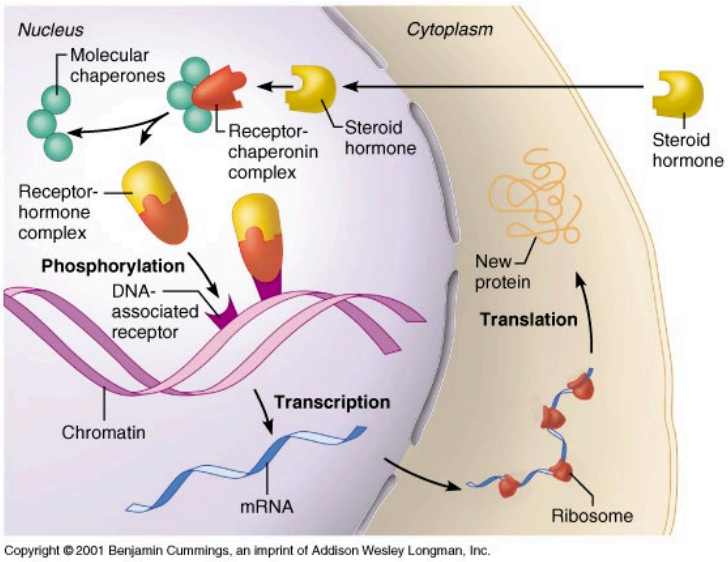


Figure 1.3: Illustration of the general mechanism of action of AAS (Figure adapted from CUMMINGS, 2001)

Despite the existence of data that shows the toxic effects and health damages, AAS are intensely used by the sports community to increase muscle mass, strength, aggressiveness and endurance. The actual number of users is larger than the reported statistics. The steroid abusers intend to improve their physical performance, aesthetic appeal, stress compensation, and in some cases, well-being. The most common toxic effects occur on the cardiovascular, hepatic and endocrine systems, changes in growth patterns and masculinization in women are also common (BOLLMANN, et al, 2006; SEGURA, et al, 1998).

As anabolic agents, they have direct effects on cardiac growth, myocyte metabolism and platelet function. Cardiovascular diseases, such as myocardial infarction, sudden death due to arrhythmia and stroke have been cited as causes of athlete deaths related to the abuse of these substances. Studies show significant changes in the lipid profile. These unfavourable changes include an increase in LDL concentration and a decrease in HDL concentration of 30 to 50%. Metabolic dysfunctions are related to atherosclerotic cardiovascular diseases and hypertension. (LARSSON et al, 2005).

Liver diseases such as hepatic peliosis, liver tumours, cholestasis, and elevated levels of hepatic transaminases have been reported after steroid abuse. Hepatic dysfunction is most commonly associated with alkylated 17-alpha steroids. Occasionally, cholestasis jaundice occurs, reflecting production disturbances, metabolism and/or excretion of bilirubin, but this effect may disappear within 3 months after drug discontinuation. There are reports in the literature of hepatocellular carcinoma and hepatic tumour rupture deaths in athletes caused by extensive steroid abuse. The hepatic events are also associated with hepatic impairment and dysfunction causing hepatic cirrhosis (HEINZ et al, 2001).

Male AAS users present a decrease in serum testosterone levels, due to suppression of the hypothalamic-pituitary axis. A decrease in luteinizing hormone (LH) and follicle-stimulating hormone (FSH) levels is also present. This decay is responsible for testicular atrophy, infertility and loss of libido. Testicular atrophy and oligospermia or azoospermia usually disappear after discontinuation of drug use, but the sperm count and morphology may remain abnormal for up to 6 months. Another consequence of continued use is the development of the breast tissue, a condition called gynecomastia, which results from the peripheral conversion of androgens to estradiol. This side effect may be exacerbated in people with liver disease by reducing hepatic clearance. In women the use of AAS is associated with menstrual cycle abnormalities and masculinizing effects, which include excessive hair growth, acne, thickening of the voice, clitoris hypertrophy, and male pattern baldness. The literature reports that AAS abuse can increase the basal metabolism; as a biochemical response the plasma levels of thyroxine (T4); triiodothyronine (T3) and insulin decrease. These decreasing hormones are responsible for glucose tolerance and adverse effects in the

metabolism (BAXTER et al, 2005).

Controlled studies correlate the association between the AAS abuse, neurological disorders, and serious psychiatric side effects. Altered mental status and behavioural changes, including irritability, aggressiveness, euphoria, depression, mood swings, libido, and psychosis are reported. In addition, symptoms of withdrawal, dependence and pathological concern with the image itself are also described. It is observed that abrupt withdrawal can produce central adrenergic symptoms, such as hyperactivity, anxiety, irritability, insomnia, hot flashes, sweating, chills, anorexia, myalgia, nausea, vomiting, tachycardia and hypertension (VAN AMSTERDAM et al, 2010; MARAVELIAS et al, 2005).

1.4. Biological matrices analysed for doping control

The choice of the biological matrix should consider the context and the main purpose of the analysis. The applicability and usefulness can vary depending on the results required. The detection window; type of investigation; biology of drug incorporation; sample collection; complexity and integrity of the sample under analysis; cost, validity and reliability of their results are significant aspects to be considered and differ the matrices. Blood and urine are the most prevalent conventional matrices used to perform toxicological analyses (CHASIN et al, 2008; GALLARDO e QUEIROZ, 2008; KARCH, 2007).

1.4.1. Urine

Urine is a metabolic product obtained from kidneys ultrafiltration that contains little endogenous interference. The urine is mainly composed of water (95%); organic (such as urea, uric acid and creatinine) and inorganic substances (chloride and sodium ions). It presents high concentrations of metabolites and is the matrix of choice for drug screening and identification. A healthy individual excretes about 1.2 litters per day; however, ingestion factors and fluid intake may alter this volume. This matrix has many advantages including easy collection, large volumes available for analysis and reduced number of interferences when compared to other matrices (MENG *et al*,

2015).

As previously mentioned, the standard biological material mandatory to control doping is urine. The analyses performed in this matrix provide information on recent drug use (1 to 7 days depending on the type of drug used and the frequency), thus making test scheduling an issue for many applications. In addition, this matrix is easily susceptible to tampering and adulteration, this fact has generated major problems for the doping control organizations (JICKELLS, NEGRUSZ, 2008; GALLARDO, QUEIROZ, 2008; KARCH, 2007).

The easy manipulation of urine during the collection procedure remains for this process an important practical issue, increasing the probability of false-negative results. *In vivo* adulteration, *in vitro* adulteration and substitution are common actions that may result in false-negative results in urine analyses. *In vivo* adulteration attempts to reduce the drug concentration below cut-off levels in urine using large volumes of water ingestion; herbal teas or the intake of any other substance aimed to interfere the drugs tests. To detect this adulteration the quantification of urinary creatinine excretion rate is recommended. *In vitro* urine adulteration can be diluted or modified after collection using an oxidizing (bleach) agent such as vinegar and/or lemon juice for example. In the substitution practice a drug-free specimen is substituted for the positive urine specimen. An adequate chain-of-custody and the supervised sample collection are essential to avoid invalidation of the results. Even though in some cases; such as Russia during Socchi Olympics this chain of custody can be bypassed (ROUEN et al, 2001).

1.4.2. Blood

Blood is a complex biological fluid; 80% of its composition is soluble proteins, fats, salts and suspended cells. It is a matrix conventionally used to provide correlation between the drug concentration and the potential pharmacological and toxicological effects. The drug/ metabolite ratio is useful to predict the period since administration; it can also be used to differentiate between acute and chronic drug use. However, this sample presents an invasive collection method involving a high contamination risk and presents a narrow detection window for drugs (JICKELLS & NEGRUSZ, 2008; SAMANIDOU *et al,* 2011).

Blood is required for doping control analyses to establish the Athlete Biological Passport (ABP) to monitor variables (biomarkers of doping), which over time reveal the effect of doping. The ABP provides valuable information to be used as a complement to analytical methods and target testing or investigations more effectively consequently to refine and strengthen the anti-doping strategies. These monitoring include haematological (manipulation of blood and blood components) and steroidal module (markers of steroid doping) (WADA, 2017).

1.5. Alternative Biological Matrices

Recently, the interest in the use of alternative biological matrices to identify and quantify drugs has grown and gained a significant relevance in toxicology. The main reasons for this are the advantages presented by these matrices when compared to the traditional samples. Among the alternative matrices are included: sweat, oral fluid/saliva, hair, and nail. These alternative specimens offer different detection times and significantly different metabolic profiles when compared to urine. Alternative samples collection is less invasive and can be assisted (which makes tampering difficult); unchanged drugs are incorporate rather than biotransformation products to these matrices; in addition, the detection window is larger for some drugs. However, they present some disadvantages, such as low concentration of analytes, a lack in number of studies that show complementary quantitative data, and limited sample size requiring techniques with greater sensitivity (GALLARDO; QUEIROZ, 2008). Table 1.2 bellow shows the comparison of the main characteristics related to the different biological matrices discussed.

Table 1.2: Summary of main characteristics related to the different biological matrices (Table adapted from De MARTINIS, 2008).

	Urine	Blood	Sweat	Saliva	Hair
Detection period	1-3 days	1-48 hours	1-14 days	1-36 hours	7-100+days
Level of invasiveness	High	Very high	Low	Low	Low
Drug disposition	Incremental	Incremental	Cumulative	Incremental	Cumulative
Risk of false-positives	Low	Low	Moderate	Low	High
Risk of false-negatives	High	High	Low	High	Low
Risk of adulteration	Very high	Low	Moderate	Moderate	Moderate
Matrix interferences	Low	High	Low	Low	High
Sample availability	High	Moderate	Moderate	Moderate	Moderate
Predominant species in the sample	Metabolized drugs	Unchanged drugs and metabolized	Unchanged drugs	Unchanged drugs and metabolized	Unchanged drugs and metabolized
Technological development	Established	Established	Needed	Needed	Needed

The analytical techniques and sample preparation advances were crucial for the development of toxicological analyses. They enabled the detection of drugs and their metabolites in low concentrations in extremely complex samples. The most commonly used techniques are gas chromatography coupled to mass spectrometry (GC-MS) and liquid chromatography coupled to mass spectrometry or in tandem mass spectrometry (LC-MS, LC-MS/MS). These techniques present high sensitivity, and they permitted the introduction of multi-targeted screening methods to simultaneously test many drugs of abuse in alternative biological matrices

(FARRELL; KERRIGAN; LOGAN, 2007; JONES, HOLMGREN e KUGELBERG, 2007; PAWLISZYN, 2002; SMERAGLIA, BALDREY; WATSON, 2002).

1.5.1. Sweat as an alternative Biological Matrix

Sweat is an alternative biological sample that has gained prominence in recent years in toxicological analysis. Several studies have been conducted for the analysis of drugs of abuse in sweat, including amphetamines (DE MARTINIS et al, 2007), cocaine (CONE et al, 1994; HUESTIS et al, 1999; KACINKO et al, 2005), opiates (COLE et al, 1994; HUESTIS et al, 2000; KINTZ et al, 1997) nicotine and alcohol (HUESTIS et al, 1999). Also, studies comparing the efficacy of monitoring drug use using sweat and urine have shown that sweat provides satisfactory results and may be used as an alternative biological matrix. Sweat present several advantages compared to the conventional biological matrices (blood and urine) (DE MARTINIS et al, 2008), the sample collection is non-invasive and does not cause embarrassment (HUESTIS et al, 1999; DE MARTINIS et al, 2007; TAYLOR et al 1998).

1.5.1.1. The Physiology of Sweat

The epidermis and the dermis are the two major layers, which form the skin composition. The epidermis layer of the skin is composed by stratified epithelium and the stratum corneum. This layer acts as a barrier to restrain the passage of water and solutes in either direction across the skin. Beneath the epidermis lies the dermis, the second major layer, and it is comprised of dense fibroelastic connective tissue. The dermis supports extensive vascular and nerve networks in addition to specialized excretory and secretory glands (POTTTS & GUY, 1993; ODLAND, 1991; FORTNER, 2007).

Sweat secretion is a homeostatic mechanism whose main function is the maintenance of a constant body temperature. It's secreted to the surface of the skin causing body heat loss after evaporation. In addition, it plays an important role in the immunological protection and hydration of the skin. It is composed of 99% hypertonic aqueous solution, as well as lactate, urea, ammonium ions, some enzymes

and other organic compounds, such as excreted exogenous and exogenous substances, and may also contain significant amounts of proteins depending on the type of sweat glands (KINTZ, SAMYN, 2000; DE MARTINIS, 2008).

The amount of sweat secreted is highly variable depending on the daily activity, emotional state and environmental temperature. The trunk is responsible for 50% of the total volume of sweat produced; followed by, 25% from the legs and the 25% from the head and upper extremities (KINTZ, SAMYN, 2000; DE MARTINIS, 2008; HUESTIS, CONE, 1998). Between 300 and 700 ml of sweat is produced per day, throughout the body, however during physical exercise this volume can increase to 2 to 4 litters per hour in short periods of time. The mean pH of sweat in resting individuals is 5.8; however, during physical activities in general the flow increases and the pH value may increase to 6.8 (HUESTIS, OYLER, CONE, 1999; LEVISKY, 2000).

The human body has 3-4 x 10^6 sweat glands. The glands are classified into two types: eccrine and apocrine (Figure 1.4). The eccrine glands are the most numerous; they are distributed throughout the body and are particularly abundant in the palms of the hands, soles of the feet, the face and the chest. The apocrine glands are larger and are located primarily in the axillae, pubis and mammary gland (SAGA, 2002). Both types of glands are derivatives of the epidermis located at different body sites, the secretory portion of the gland is in the dermis, and an excretory duct discharges the secretion at the surface of the skin that occur in almost every part of the skin. The developments of sweat glands are in close association with hairs and their ducts sometimes open into hair follicles (DE MARTINIS, 2008).

Besides aqueous secretions, the skin also receives sebaceous secretions, especially on the face, scalp and back regions. These secretions are sterile and odourless and are mainly composed of lipids, the major lipids being in cholesterol (75%), triglycerides and fatty acids (20%). Blood capillaries nourish the sweat glands, in a similar way to the hair follicles (KIDWELL et al, 1998; KINTZ, SAMYN, 2000). The high lipid content of sebaceous secretions may transport and absorb many drugs. Sweat and sebum are mixed on the skin and are normally collected together; most studies related to sweat sampling do not make difference between both specimens and refer to this mixed as sweat (DE GIOVANNI, FUCCI, 2013).

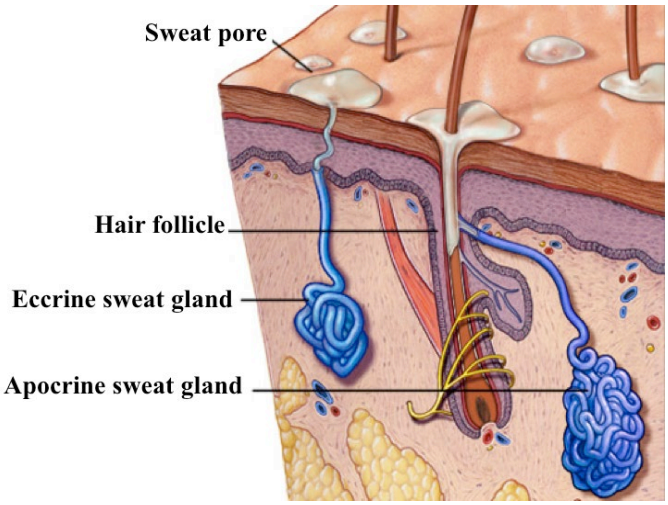


Figure 1.4: Illustration of structures found on the skin; sweat glands: eccrine and apocrine; follicle and pores (Figure adapted from Mayo Foundation, 2007).

1.5.1.2. Incorporation of Drugs into Sweat

The mechanism of drug incorporation into sweat is not fully understood, and there are several potential mechanisms by which drugs may be secreted in sweat including passive diffusion from blood into sweat glands and transdermal migration of drugs across the skin. Delivery of high concentrations of the drug to the skin surface by sebum and sweat could produce deposition on the stratum corneum and allow the skin to serve as a shallow drug depot. Many illicit drugs may diffuse through the dermal and epidermal layers of the skin. Passive diffusion of drugs from capillaries in the skin into perspiration seems to be the main drug pathway but excretion of substances via sebum and intercellular diffusion also may contribute (DE MARTINIS, 2008; HUESTIS et al, 1999; KACINKO et al, 2005; KINTZ, SAMYN, 2000). An alternate mechanism could involve drug diffusion through the stratum corneum to the skin surface where the drug dissolves in the sweat. Drug excretion via the sebum occurs by transcellular diffusion and keratinocytes. The keratinocytes transport can be delayed for many days (LEVISKY et al, 2000).

Excretion into sweat depends upon a drug's physic-chemical properties such as molecular mass, pH and pKa, protein binding and lipophilicity. Sweat is slightly acidic; the pH is generally in the range of 4 to 6.8, with the average sweat pH of

resting individuals 5.8. With the increased flow rate (following exercise or temperatures above 31°C), sweat pH may increase to 6.8. Non-ionized, basic drugs and low protein binding drugs, present higher transfer rate from blood to other fluids and/or cells. Therefore, parent drugs that more easily cross the membranes are expected to accumulate in sweat in greater concentrations than polar hydrophilic metabolites (CONE et al, 1994; UEMURA et al, 2004). The main analyte found on the skin surface is predominantly the parent drug. The time interval between drug consumption and detection on the skin surface depends on the nature of the particular drug and on the sensitivity of the analytical method used. In chronic abusers drug molecules are permanently present on the skin due to temporary reservoir of the stratum corneum (LEVISKY, 2000; KINTZ, SAMYN, 2000). Table 1.3 summarizes some important characteristics, such as the type of collection device, application site of the patch on human body, time of wearing.

Table 1.3: Summarizes important characteristics of sweat analysis, between them the type of collection device, application site of the patch on human body, time of wearing.

Author	Drugs and Metabolites	Area and Period of Collection	Analytical Method	Application
Kintz P. 1996	Opiates, Cocaine, Cannabinoids, Buprenorphine, Metadone, Nordiazepam	Back / 1 Week	GC-MS	Clinical setting
Kintz P. et al 1997	Opiates	Back /24 Hours	GC-MS	Clinical setting
Kidwell DA. et al 2001	Cocaine, Metamphetamine Heroin	Arms	GC-MS	Environmental contamination
Chawarski MC et al 2007	Opiates	Not Specify	GC-MS	Clinical setting

Table 1.3 continued: Summarizes important characteristics of sweat analysis, between them the type of collection device, application site of the patch on human body, time of wearing.

Author	Drugs and Metabolites	Area and Period of Collection	Analytical Method	Application
Huestis M. et al 2000	Opiates	Abdomen, Back/ 1 Week	ELISA- GC-MS	Clinical setting
Saito T. et al 2004	Cannabinoids	Skin /12 Hours	GC-MS-NICI	Method validation, clinical setting
Follador MJ. et al 2004	Cocaine, Cocaetilene	Sweating Part Of The Body / 3-7 Days	GC-MS	Method validation
Brunet BR. et al 2008	Methadone, Heroin, Cocaine	Not Specified / 7 Days	GC-MS	Method validation, pregnancy
Barnes AJ. et al 2009	MDMA	Back, Abdomen / 2 Hours - 7 Days	GC-MS	Controlled administration
Levisky JA. et al 2001	Cocaine, Metamphetamine	10 -14 Days	GC-MS	Drug users
Samyn N. et al 2002	Ecstasy	Wiping With Cotton Over Forehead	GC-MS	Controlled administration
Marchei E. et al 2010	Methylphenidate	Back/ 24 Hours	LC-MS	Pilot study
Brunet BR et al 2010	Cocaine, Opiates	Back. Arm /1 Week	GC-MS	Pregnancy
Kacinko S.L. et al 2005	Cocaine	Back, Abdomen / 4-15 Hours	GC-MS	Controlled administration
Uemura N. et al 2004	Cocaine	Back, Shoulder / 1-72 Hours	GC-MS	Controlled administration
Schwilke E.et al 2006	Opiates	Abdomen, Back / 1 Week, 1-15 Hours	GC-MS	Controlled administration
Liberty HJ. et al 2004	Cocaine	Biceps/ Various Time	GC-MS	Controlled administration

Table 1.3 continued: Summarizes important characteristics of sweat analysis, between them the type of collection device, application site of the patch on human body, time of wearing.

Author	Drugs and Metabolites	Area and Period of Collection	Analytical Method	Application
Spiehler V. et al 1996	Cocaine	Skin / 7 Days	Immunoassay GC-MS	Drugs users, controlled administration
Burns M. et al 1995	Cocaine	Up To 7 Days	RIA GC-MS	Controlled administration
Liberty H. et al 2003	Crack	One Per Hand /15-30 Minutes	GC-MS	Controlled administration
Fogerson R. et al 1997	Opiates	Skin /1-10 Day	EIA GC-MS	Controlled administration, adulteration study
Pichini S. et al 2003	MDMA	24 Hours (Back)	Immunoassay GC-MS	Controlled administration

1.5.1.3. Sweat Collection

Various methods have been developed to collect sweat samples from human skin. Significant advances have been made in recent years to develop sweat patch technology for the routine collection of sweat samples over an extended period. The collected material is always a mixture of sebum and the two different types of sweat according to the area of the skin, since not all types of glands are evenly distributed throughout the body (KIDWELL et al, 1998).

The use of this matrix was possible due to the development of sweat collection devices, patches, available commercially as PharmChek® patches (Figure 1.5). The "sweat patch" device consists of an adhesive layer on a thin transparent film of surgical dressing to which a rectangular absorbent pad is attached. The drugs are

deposited into the absorbent bandage of cellulose that is adhered to the skin by an adjoining adhesive. A polyurethane layer protects the device from external contamination, while allowing the gas exchange between skin and environment, not harming the skin (KIDWELL, SMITH, 2000; KACINKO et al, 2005, UEMURA et al, 2004).

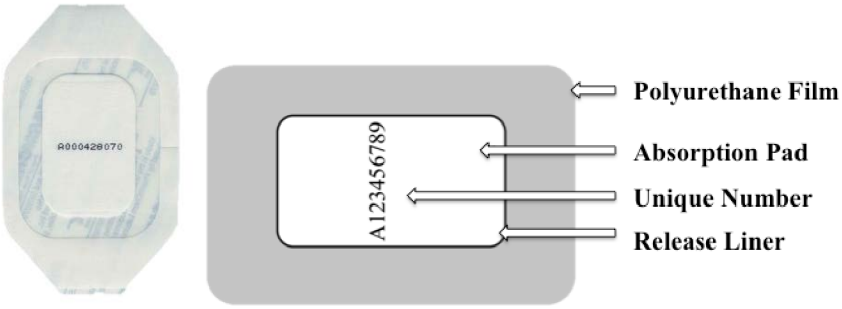


Figure 1.5: Photo of a PharmChek® adhesive used for the sweat collection.

The sweat patch acts as a specimen container for non-volatile and liquid components of sweat, including doping agents. Non-volatile substances from the environment cannot penetrate the transparent film, which is a semi-permeable membrane over the pad that allows oxygen, water and carbon dioxide to pass through the patch, leaving the skin underneath healthy. Over a period of several days, sweat saturates the pad and slowly concentrates it, while drugs present in sweat are retained (HUESTIS, OYLER, CONE, 1999; KINTZ, SAMYN, 2000).

Prior to the application of the adhesive the skin is cleaned with isopropyl alcohol to remove existing contaminants, as shown in Figure 1.6 (KIDWELL, SMITH, 2000; KACINKO et al, 2005, UEMURA et al, 2004).

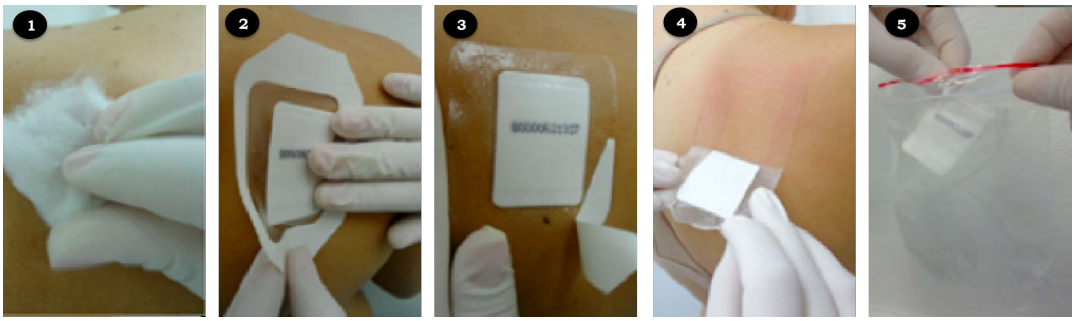


Figure 1.6: Sweat collection procedure. (1) Clean the skin with isopropyl alcohol; (2 and 3) adhesive fastening; (4) adhesive removal; (5) adhesive storage (Figure modified from TAVARES, 2014).

1.5.1.4. Advantages and disadvantages of sweat as an alternative matrix

Sweat testing has several advantages over blood and urine such as: the non-invasiveness, a cleaner matrix, the ease and simple collection that allow carrying out normal activities including swimming and showering without removing the patch, and a larger detection window (up to 14 days); it has low risk of biological contamination, being harmless for the analyst when compared to blood collection for example; presents less chance of tampering because each device has unique serial number printed thereby maintaining the chain of custody; and the predominant species found in sweat are unchanged compounds, not the drug metabolites. The absence of metabolites reduces steps during sample preparation, to avoid conjugated metabolites). The effects of vigorous or prolonged exercise can increase the transfer of drugs in sweat, which indicates that the collection should be peformed after the competition. Depending upon the period of collection, the sweat testing can provide a cumulative estimate of drug exposure (DE MARTINIS, 2008; HUESTIS et al, 1999; KINTZ, SAMYN, 2000). The disadvantages of sweat as an alternative specimen include lack of information about dose-response relationships, lower analyte concentrations; environmental contamination possibility and a shortage of laboratories performing sweat analysis (KIDWELL et al, 1998; DE GIOVANNI, FUCCI, 2013; SMERAGLIA, BALDREY E WATSON, 2002).

1.6. Techniques for analytical drug testing in biological matrices

The analytical aspects for detection of doping agents in biological samples remain a challenge. Different techniques can be applied during the preparation of biological samples. To deal with the necessities of the growing number of doping agents, fast and accurate analysis methods are mandatory. The existing procedures used for detecting the wide range of WADA prohibited substances rely on a combination of extraction methods and analysis techniques. The expansion of the Prohibited List coupled with developments in analytical technology has meant that laboratories constantly must develop new methods to cope with increased demand. The most used analytical techniques for drug detection are immunoassays, gas (GC) or liquid chromatography (LC) and capillary electrophoresis (CE). The LC and GC coupled with mass spectrometry are the state of art instrumentation used in those investigations (RIVIER, 2000; WADA, 2017).

1.6.1. Analytical workflows in doping control

High sensitivity and selectivity are essential for the analytical strategy in doping control. Doping analyses mostly involve complex matrices; compounds with different physicochemical properties and molecular weight; limited sample volumes and very fast delivery timing requirements (results within 24–48 h for important sport events). Besides that, to confirm the extension of detection window the characteristic drug metabolites are also included to the analyses; increasing the number of target compounds (BADOUD et al, 2011).

Basically, to fulfil the WADA requirements the anti-doping laboratories require two steps. The first one is performing sample screening. The principal aim of this step is to detect the highest number of relevant analytes. It must be fast, selective, sensitive and detect the presence or absence of a doping agent. Once a doping agent is detected, the confirmatory step must be performed to assess the suspected substance and quantify it. This requires the employment of precise and accurate methods (BADOUD et al, 2011).

Appropriate workflows are needed to differentiate between particular

compounds such as endogenous substances from exogenous intake as neither GC– nor LC–MS/MS techniques are able to provide this information. Prevention of testosterone abuse, for example, is particularly difficult because it is identical to testosterone produced by the body. The urinary norandrosterone and testosterone, and isotope ratio mass spectrometry (IRMS), based on enrichment of 13C, are used to distinguish between endogenous and synthetic steroid analogues (BADOUD et al, 2011; VAN DER KORK et al, 2000; CAMONO et al, 2005; YONAMINE, PAULA, 2008). Figure 1.7 below shows the anti-doping workflow for sample analysis.

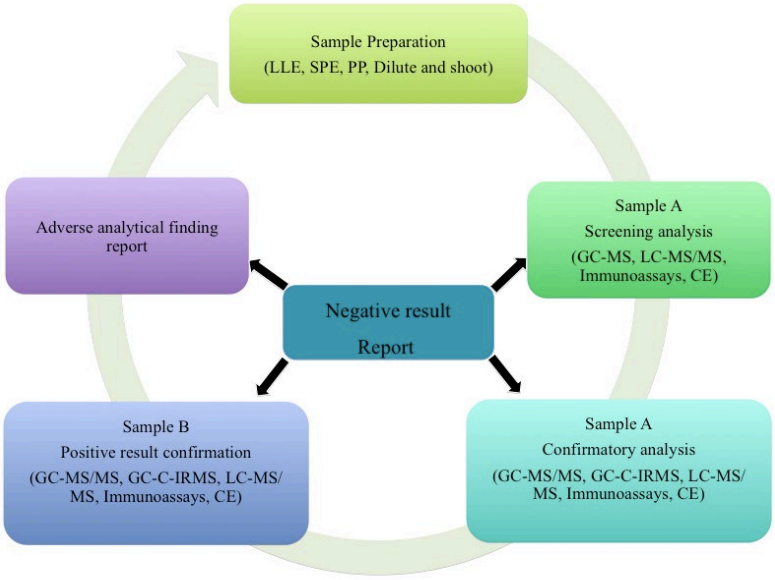


Figure 1.7: The anti-doping workflow for sample analysis. The urine sample is divided in two aliquots (A and B). Sample A is used for the screening testing and the confirmatory analysis; while the sample B is kept for cross-examination in positive cases (WADA, 2015).

Analytical workflows for doping control rely on chromatographic separation techniques. Adequate sample preparation is a fundamental process to obtain successful chromatographic analyses of biological samples. Furthermore, the sample preparation has an impact on the method selectivity and may have limitations that compromise the analysis (FU et al, 2005). Sample preparation methods have been constantly developed and optimized, with the aim of designing cheaper, faster, "clean" and effective approaches than those currently available. Liquid–liquid

extraction (LLE) and solid-phase extraction (SPE) are highly used off-line extraction procedures which may reduce the risk of cross-contamination and carryover. The advantages of LLE are that it is simple, efficient and low cost. However, it presents disadvantages such as a requirement of high volumes of solvents and sample, lower recovery and the lack of automation possibilities.

SPE methods are commonly used in doping analyses. They combine sample clean-up, analyte pre-concentration and high selectivity. In addition, they present compatibility with several analytical techniques and are constantly evolving to increase the speed of analysis. One of the recently developed promising SPE methods is disposable pipette tip extraction (DPX) (BREWER, 2003), with solid-phase micro-extraction (SPME) also providing rapid sample preparation/analysis. Both techniques can improve the selectivity, sensitivity and reduced matrix effects over liquid extraction.

1.6.2. Sample Preparation Methods

As mentioned above, sample preparation is a crucial part of the analytical procedure. It reduces the sample interferents assisting the method selectivity and sensitivity. The improvement of traditional extraction methods is extremely important for optimization of the analysis. This process is most often intensive, accounting for up to 80% of the total analysis time and increases the likelihood of analyst errors. The main characteristics required for an extraction technique are minimum sample loss with effective clean-up, high recovery, low time consuming and low cost. Currently a wide variety of sample preparation methods are available and many of these methods can be and have been employed in doping analyses (SMERAGLIA et al, 2002; FU et al, 2005; RITCHER, 1999; PAVLOVIC, 2007).

1.6.2.1. Liquid-liquid extraction (LLE)

LLE is a sample preparation method based on the partitioning between two immiscible phases (organic and aqueous). The extraction efficiency depends on the interaction ratio between the investigated analyte and the organic solvent. The process involves the addition of the extractive solvent followed by pH adjustment, mechanical stirring to promote maximum contact between the phases and centrifugation.

Currently, there are a wide variety of extraction methods; however, the LLE is still the technique of choice for being fast, simple and cheaper when compared to SPE. The major disadvantage of LLE is the generation of solvent residues, which are often high toxic to the analyst and environment (WU et al, 2005; MYASEIN et al, 2009).

1.6.2.2. Solid Phase Extraction (SPE)

SPE is based on the same principle of separation as liquid phase chromatography consisting of liquid-solid separation. The steps of this technique include retention of the analytes from the biological matrix, removal of interferents, concentration of the sample, and elution. Traditionally SPE is available in normal phase, reverse phase and ion exchange modes, with reverse phase being the most used. Due to the variation of physicochemical properties of analytes the traditional formats are not always adequate, and the availability of different stationary phases and different approaches are required. In this technique, the analytes contained in a matrix are extracted after passing through a cartridge containing a sorbent solid. A selective organic solvent is generally used to remove the interferents and then another solvent is used to remove the analytes of interest from the solid phase. Conventional SPE offers several advantages over LLE such as lower volumes of solvents, shorter operating time, high recovery, and the possibility of automation. However, it also presents some limitations, such as the desorption step that requires the use of toxic solvents and the occurrence of matrix effects (BERRUETA et al, 1995; KRISHNAN et al, 1994; FU et al, 2005).

1.6.2.3. Disposable pipette tip devices

SPE is an extremely versatile and well-established technique to extract drugs of abuse, which has been successfully applied to analysis of bio samples (BRUNET et al, 2008; DE MARTINIS et al, 2007; KACINKO et al, 2005). However, recently miniaturized techniques based on SPE have been proposed as alternatives for processing biological samples in general, including sweat. One of these techniques is Disposable Pipette Tip Extraction (DPX), which it's a pipette tip with a small amount of dispersed sorbent inside. This type of tip allow better contact of the solid phase with the sample and minimizes the amount of solvent and sorbent needed for extraction (BORDIN et al, 2016; KOLE et al, 2009).

DPX is a modification of conventional SPE developed by Dr. William Brewer (University of South Carolina, USA) to significantly reduce the extraction time and the amount of solvents. DPX extraction consists of a standard pipette tip with a capacity of 1 or 5 mL, filled with a sorbent SPE in two ways. In the original version, the powdered sorbent is freely dispersed inside the pipette tip. In the second version, the sorbent is poorly dispersed between two disks one located at the lower portion (which allows the bidirectional flow of fluids) of the pipette tip and the other at the top (which prevents contamination of the syringe with sample) (Figure 1.8) (BREWER, 2003; BORDIN et al, 2016).

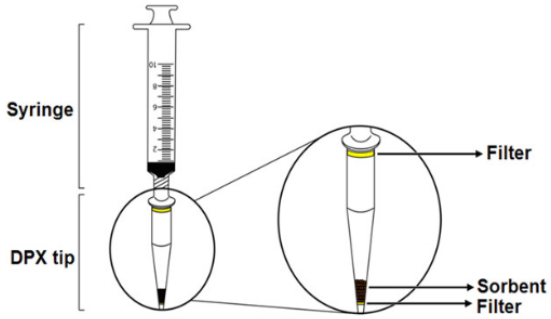


Figure 1. 8: Schematic diagram of the manual DPX extraction (BORDIN et al, 2016).

As in other SPE devices, different solid phases are commercially available for DPX tips, which can be used to extract a large amount of analytes in different samples. Tips containing reversed-phase sorbent (DPX-RP) can be used for non-polar to medium polar compounds. Strong cation exchanger sorbent (DPX-CX) is used for cations and non-polar compounds and weak anion exchanger sorbent (DPX-WAX) is used for anions and non-polar compounds. Graphitized carbon is another solid phase used in DPX extraction.

DPX extraction is based on the same principles and uses similar steps as that of traditional SPE. First, an appropriate solvent or a combination of solvents is used in the conditioning step for activation the sorbent sites. The activation of the sorbent sites allows the appropriate molecular interaction between this solid phase and the analyte in the sample. The sample is drawn up into the tip and is then subjected to suction with air to promote the mixing and held in contact with the sorbent (BORDIN

et al, 2014; GUA, STEWART, 2014). An organic solvent or a mixture of solvents is used to remove the interferents and finally a solvent is used to elute the analytes.

The mode of distribution of the sorbent inside the pipette tip allows dynamic mixing with the solvent, which leads to a rapid adsorption equilibrium between the solid phase and the analyte, selectively retaining the analytes of interest. The mixing/contact time must be controlled to allow an adequate chemical interaction (by van der Waals forces in reversed-phase mode or by electrostatic interactions in ion exchange modes), reaching the equilibrium time for analyte adsorption in sorbent particles and consequently provide an efficient extraction and good repeatability. After the required time to establish the dynamic equilibrium between analytes and sorbent, the sample is discarded from the tip. The next step consists in washing step in which one or more solvents are used to remove interferents. The selection of solvent requires the consideration of the chemical interaction between sorbent and analyte and the chemical structure of probable matrix interferents. The process is similar to the sample aspiration step: the washing solvent is aspirated with air and discarded out one or more times. The last step is the elution of analytes. The elution solvent is aspirated several times with air through the tip to rupture the sorbent–analyte interaction. The analyte desorbs from the sorbent and migrates to the liquid phase (elution solvent). The elution solvent must be chosen according to the type and chemical nature and the sorbent as well as the washing solvent. After this process, the extract can be subjected to quantitative or qualitative chromatographic analysis (Figure 1.9) (BORDIN et al, 2014; BORDIN et al, 2016; GUAN, STEWART, 2014).

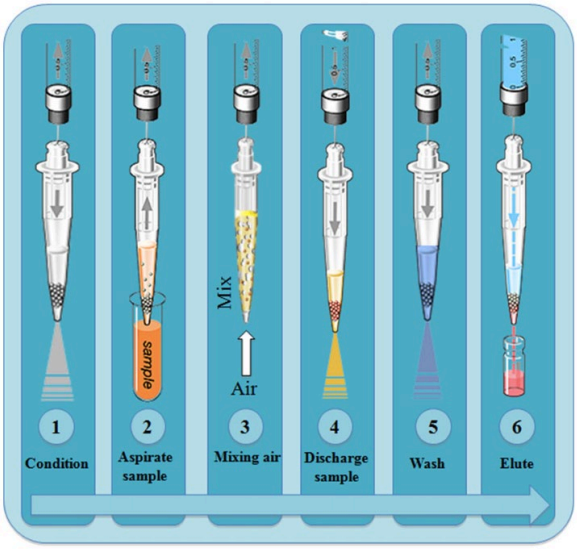


Figure 1.9: Schematic and modified diagram of the process of DPX extraction (BORDIN et al, 2016).

DPX extraction is a promising sample preparation method with several advantages over conventional SPE (Table 1). DPX is a simple and fast sample preparation method, which requires low volumes of sample and organic solvents. In addition, with DPX the extraction time is reduced and less sample manipulation is required. DPX also provides high recovery and efficiency. The processes can be automated including the injection into a chromatographic system. However, as DPX is a relatively new technique, the amount of extracting phases commercially available and the high cost compared to the traditional solid phase cartridges represent a limitation to its applicability in routine analysis (BRUNET et al, 2008; BORDIN et al, 2016).

1.6.2.4. Solid Phase Micro Extraction (SPME)

SPME is an excellent technique for sample preparation in toxicological analysis. The method is simpler and faster than traditional SPE and can be easily automated; allows multiple sampling, guarantees the preservation of the sample and present minimal risk of contamination. SPME shows good recoveries and an increase in the limits of detection compared with conventional SPE; fundamental for sweat analysis. An additional advantage is a reduction in solvents use, reducing the cost of analysis

and avoiding the exposure of analysts to these potentially toxic substances. It has been used for the analysis of volatile compounds of the human skin, in the evaluation of the characteristics of odours and drugs. For analytes that need to be derivatised, this step can be performed simultaneously during the extraction with the fibre, significantly reducing the analysis time (KOLE, 2010; VALENTE, AUGUSTO, 1999).

SPME consists of a chemically modified fused silica capillary fibre coated with a polymer film that is the active site for the extraction process. Different polymer film phases are available such as polydimethylsiloxane (PDMS), polyacrylate (PA) or Carbowax (CAR). SPME functions similarly to a syringe: by pushing its plunger, the fibre (extractive phase) is exposed and placed in contact with the sample, which can be liquid, solid, or with its headspace, for sufficient time until equilibration (adsorption, or partition) between the phases is achieved. In this process, the organic analytes migrate to the adsorbent surface by affinity, so the fibre can be repositioned into the needle (DUEIRA, GAUJAC, NAVICKIENE, 2008; VALENTE, AUGUSTO, 1999). Finally, the sampler is taken and introduced into the GC where the analytes are thermally desorbed from the adsorbent fibre under the flow of the entrainment gas (VALENTE, AUGUSTO, 1999). Figure 1.10 below shows the headspace extraction mode using SPME fibre.

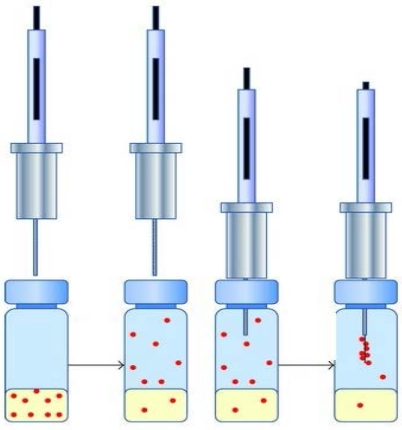


Figure 1.10: SPME extraction using the headspace mode (Figure adapted from, INDELICATO et al 2014).

1.6.3. Analytical methods applied in doping control

Technological advances of analytical instrumentation have improved the selectivity and sensitivity of the techniques and permitted the introduction of multi

target screening methods to simultaneously test a large number of drugs of abuse in biological matrices. These techniques include gas chromatography-mass spectrometry (GC-MS), liquid chromatography or tandem mass spectrometry (LC-MS, LC-MS/MS) and microchip capillary electrophoresis (SMERAGLIA, BALDREY; WATSON, 2002).

1.6.3.1. Gas Chromatography-mass spectrometry (GC-MS)

GC-MS is a fundamental part of the analytical arsenal in toxicological analysis laboratories; it is considered standard technique for the detection of drugs of abuse. GC techniques generally require the analytes to be chemically extracted from the biological samples, and in most cases derivatised to make them volatile prior to the injection. GC is responsible for the separation of compounds based on differences in volatility and solubility in the liquid, solid and gaseous phases (SMITH et al, 2007).

After separation in the chromatograph the molecules are ionized as they sequentially enter the ion source. The ionisation techniques used are electron ionisation (EI) and chemical ionisation (IC), both with the purpose of producing charged species that are later analysed according to their mass-charge-ratio (m/z) (CODY, 2003).

Electron ionisation is a widely used technique characterized by the bombardment of the target molecule with an electron, which removes electrons from the molecule, producing unstable positive ions (molecular ions), which are then fragmented into more stable ions. The energy used is usually 70eV, but can be adjusted. As a result of this energy interaction the m/z and the abundance of characteristic fragments are obtained for the compound being analyzed. The charged fragments are transferred to the mass detector, thereby obtaining the analyte identification via a unique fingerprint of ion fragments (PETERS, MAURER, 2002).

Chemical ionisation (CI) is typically used to improve the yield of the pseudo molecular ion or increase sensitivity, especially with halogenated compounds. The technique uses a charged reactive gas (ammonium NH_4 or methane CH_4) to transfer the charge to the compound. These charged species are more stable than the ions formed in EI, and, negatively and positively charged molecule formations may occur.

GC coupled to a single quadrupole mass spectrometer (QMS) is routinely used to identify and quantify drugs of abuse in biological fluids and tissues (KACINKO et al 2005). To perform a valid quantification in a lower concentration range, the signal-to-noise ratio (S/N) must be reduced. One approach to noise reduction is MS-MS, which have LODs of less than 1 part per trillions (ppt). The GC-MS-MS offers high sensitivity, high mass accuracy and more importantly, structural information due these combinations it became a common technique in toxicology laboratories (SMITH et al, 2007).

1.6.3.2. Capillary Electrophoresis (CE) and Microchip Electrophoresis (ME)

During the past decade, capillary electrophoresis (CE) emerged as a promising, effective and economic separation technique for a large variety of substances in forensic toxicology. CE separates compounds on based on differences in electrophoretic mobility, phase partitioning, isoelectric point, molecular size or a combination of one or several of these properties. Advantages of CE include automation, small sample size, minimal sample preparation, use of very small amounts of organic solvents and the low cost of capillary columns in comparison with GC or LC columns (RIVIER, 200).

Microchip electrophoresis (ME) is a miniaturized version of a capillary electrophoresis (CE) instrument, in which a device containing micro-channels replaces the conventional capillary. The separation occurs under the influence of an electric field formed through the application of a difference of potential. The analytes/compounds/ions migration and separation occur inside the micro channels filled with the running electrolyte after the voltage application. The capillary wall in general has a negative electric charge allowing the formation of a double layer between the wall of the capillary and the positive ions of the electrolyte. After the high voltage application by the electrodes (positioned at the ends of the capillary), the ions and the electrolyte migration towards the cathode takes place. This movement of the electrolyte is called electroosmotic flow (EOF). The ionic or ionisable compounds injected into the capillary migrate differently within the electrolyte depending on their

mass/charge ratio; cations tend to migrate towards the cathode (negative electrode), while the anions tend to migrate towards the anode (positive electrode). Neutral species generally migrate with the same velocity of the electro osmotic flow. To separate neutral or lightly charged species, it is necessary to use micellar electrokinetic capillary chromatography (MEKC). In MEKC, a surfactant is added to the electrolyte above its critical micelle concentration. Generally, negatively charged micelles act as a "pseudo-stationary phase" allowing the separation of neutral compounds. In CE or ME any type of substance in general can be separated and detected, depending only on the choice of a specific electrolyte considering the detector used (HANCU et al 2012; XU et al 2009; LLOYD, et al, 2014).

1.6.3.2.1. Bioanalyzer Microchip Electrophoresis (ME)

The Agilent Bioanalyzer is a commercial lab-on-a-chip device designed for the analysis of biological molecules; such as DNA, RNA and proteins. The microchips (Figure 1.11), fabricated from soda lime glass are etched with an array of micro-channels that inter-connect 12 sample wells to the separation channel.

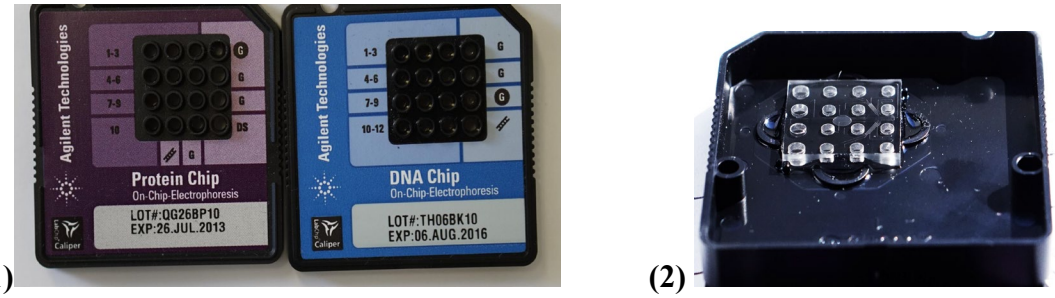


Figure 1.11: Photos showing the upper part of the microchip used in the Bioanalysis equipment containing the reservoirs (1) and glass chip and their micro channels (2).

The micro-channels are interconnected as shown in figure 1.12. There are 12 sample wells (A1-A3, B1-B3, C1-C3 and D1-D3). The wells located in column 4 (A4-D4) are reserved for the electrolyte only. The separation of the sample is obtained by the application of voltages between the micro channels (Agilent, 2008).

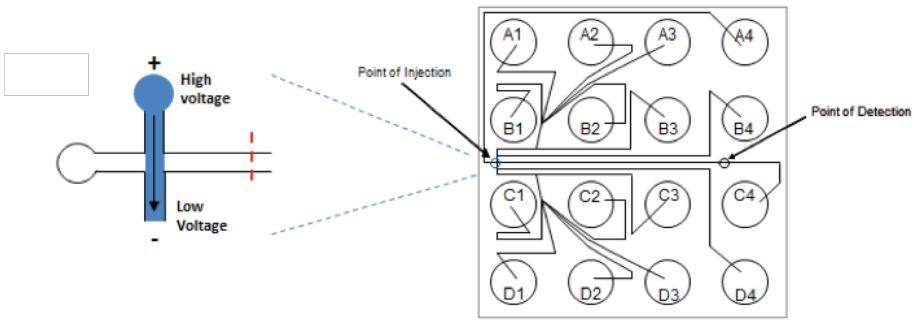


Figure 1.12: Schematic illustrating the micro-channels interconnected with the wells, injection and detection points (Figure taken from and adapted from the Agilent Technologies website, 2008).

The microchip fits into a portable platform fitted with individual electrodes that controls the sequential movement of each sample into the separation channel, through the application of a high voltage (Figure 1.13).

Figure 1.13: Bioanalyzer Agilent ME. The electrodes are centred in the reservoirs of the microchip when closing the lid.

Chapter 2: Definition and justification of aims

Research Aims

As outlined in Chapter 1, sport has become a major industry of valuable awards, resulting in athletes resorting to illegal means to overcome their opponents causing serious damage to the athletes' health. The identification of doping cases has been an important area of research and development in recent years due to the continued increase in the number of prohibited substances. Urine is the standard matrix used in doping control; however, sweat can be used as an alternative biological matrix providing additional information, complementary to results obtained from urine.

Therefore, the ***overall aim*** of this thesis was the development of methods for evaluation of sweat as an alternative biological matrix for the doping control using different methods of sample preparation and detection to overcome it current limitations. In parallel an outline of the profile of drugs used for sports practice and the acute effects of abusers were drawn.

This aim was fulfilled via consideration of the following five objectives:

1. Evaluation of the adverse effects of AAS, dietary supplements and multiple drug abuse using a self-questionnaire and toxicological urine analysis.
2. Verification of alterations caused by multiple drug use by the measurement of haematological and biochemical parameters from volunteers.
3. Development and validation of an analytical method for several stimulant psychoactive substances in urine and sweat samples from athletes using disposable pipette (DPX) tips extraction and GC-MS-EI.
4. Investigation of the Agilent Bioanalyzer platform for the analysis of amphetamine-type stimulants in sweat samples.
5. Development and validation of a solid phase micro extraction (SPME) fully automated method for cocaine and amphetamines-type drugs analyses by GC-MS.

Ethical Aspects

Human research is committed to safeguarding the integrity of all concerned including preserving privacy, minimizing risks and discomforts, seeking benefits, non-discrimination and protecting vulnerable groups of people. Among the strategies used to protect the individuals studied here are free informed consent terms and the evaluation by Research Ethics Committees. The preliminary evaluation of the projects made by a Research Ethics Committee aimed to ensure that the ethical and methodological aspects were adequate. The process of free and informed consent aimed to allow the person who is invited to participate in a research project to understand the procedures, risks, discomforts, benefits and rights involved, in order to allow an autonomous decision (GOLDIM et al, 2003).

In compliance with Resolution 466/12 of the National Health Council, the Research Ethics Committee of the School of Pharmaceutical Sciences of Ribeirão Preto (USP) approved this project; in October 3, 2014, protocol number CEP/FCFRP number 333 (Appendix I).

Chapter 3: Evaluation of the adverse effects of multiple drug abuse using a self-questionnaire and toxicological urine analysis

Chapter 3: Introduction

In recent years it was verified that the number of teenagers and young adults using a combination of DS, AAS and drugs of abuse were growing at an alarming rate. With AAS being one of the most frequently abused performance enhancing substances. Serious side effects can occur as a result of this practice (DODGE & JACCARD, 2006).

The abuse of steroids is not only intended to improve physical performance, but it is also related to stress compensation. In some cases, steroid abuse is purely aimed for an aesthetic appeal to reach a feeling of wellness in some users (BAHRKE et al, 2000; DODGE AND JACCARD, 2006). Bodybuilders are a group of elite athletes that have been using steroids during the last five decades to increase muscle mass and to intensify training regimens (YESALIS, BAHRKE, 1995). This trending consumption of AAS among adolescents and young adults can be considered a public health problem since the actual number of users is higher than indicated by the statistics (DAHER et al, 2009). In a recent study review the use of AAS in adolescents and young male adults were ranging from 4% to 6% while for females the estimative ranged from 0.2 to 2.9% (DODGE, JACCARD, 2006; HOFFMAN et al, 2007; YESALIS, BAHRKE, 1995; NILSSON, 1995). On the other hand the number of users that practice power sports or weight lifting were generally higher with prevalence ranging from 20% to more than 50% (BEEL et al, 1998; KANAYAMA et al, 2009).

In addition to AAS abuse, some commercially available DS claim to improve athletic/physical performance. The concern is that the consumption of these supplements has become as common as the consumption of AAS. According to the Brazilian Association of Nutritional Products Companies (Abenutri) it is estimated that 2% of the population (about 4 million people) are supplement consumers. These products cover a wide range of products including vitamins, minerals, herbs, meal supplements, sports nutrition products, natural food supplements, and other related

products used to boost the nutritional content of the diet. Some of these consist of high-protein products, such as amino acid supplements, while other products contain nutrients that support metabolism, energy, and athletic performance and recovery. They are found in pill, tablet, capsule, powder and liquid form. Although some of these supplements are used for replenishment of nutrients lost during physical activity, others mimic the effects of AAS and may have side effects similar to those associated with AAS use. Moreover, prohibited substances can be added deliberately during the supplement manufacturing process, or included inadvertently through contamination. They are not administered as prescribed medication, but as fitness shops products; bought by people unaware of their contents and their toxicological effects (WADA, 2017; DODGE, JACCARD, 2006).

The misuse of AAS and DS can cause serious side effects, inducing several health problems. These agents' effects can occur in different body systems such as central nervous, cardiac, reproductive, liver and kidney (BÜTTNER, THIEME, 2010). They are modulated according to the type of steroid used, the amount consumed, the duration of use and the type of administration (BOLDING et al, 2002; BÜTTNER AND THIEME, 2010; NIEMINEN et al, 1996; SALAS-RAMIREZ et al, 2010; SANTORA et al, 2006; SULLIVAN et al, 1999).

In conjunction with AAS and DS, users have also been reported the use of other licit and illicit drugs. The studies have suggested that similar physiological mechanisms may be responsible for AAS abuse and the abuse of specific types of substances (KANAYAMA et al, 2009). Kanayama and collaborators have found that AAS abuse and opioid abuse tend to co-occur, but AAS abuse and the abuse of other drugs (e.g., alcohol) do not.

The aim of this chapter was to evaluate the adverse effects of AAS, DS and multiple drug use using a self-questionnaire. A second purpose was to identify and characterize the relationship between AAS abuse and the use of other drugs through toxicological urine analysis.

3.1. Material and Methods

3.1.1. Chemicals and Reagents

The following standards of illicit drugs and metabolites were investigated: amphetamine (AMP); methamphetamine (MA); N-methyl-3,4-methylenedioxymethamphetamine (MDMA); benzoylecgonine (BE); 11-nor-9-carboxy-9-tetrahydrocannabinol (THCCOOH). The deuterium internal standards used were AMP-d11, MDMA-d5, BE-d3 and THCCOOH-d3. All standards were purchased from Cerilliant (Round Rock, TX,USA) as 1 mg/mL or 100 mg/mL solutions in acetonitrile or methanol. Standard stock solutions and internal standards were prepared by appropriate dilution with methanol and stored at 2°C, protected from light.

Ethyl acetate, n-hexane, methanol and acetic acid were obtained from J. T. Baker (USA). All solvents and reagents were of analytical grade. Sodium hydroxide was purchased from Synth (Diadema, Brazil). The silylating reagent used for derivatisation was the N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) was purchased from Sigma-Aldrich (Milwaukee,WI, USA).

3.1.2. Calibrators and quality controls

All investigated drug standards were mixed and diluted with methanol in order to obtain three calibration solutions at 1.0; 2.0 and 10 µg/mL. Internal standards solutions were prepared in methanol at 10 µg/mL. Daily quality controls were prepared adding by spiking the blank urine with the standards solutions and IS.

3.2. Individuals

Forty male volunteers, (20 soccer, and 20 bodybuilder's professional athletes) from Ribeirão Preto city, state of São Paulo, Brazil, aged between 21 to 27 years participated of this study. Individuals provided informed consent consistent with the National Council of Health from Brazilian Ministry of Health and received a copy of the signed form upon completion. The study was approved by the Human Research Committee from the Faculty of Pharmaceutical Sciences of Ribeirão Preto of the University of São Paulo process number 048/2014.

3.2.1. Questionnaire and data collection

The volunteers were subjected to a 22 questions self-completion questionnaire (Appendix II), which was constructed specifically for this study. The interviews took between 10 and 15 minutes to be completed. The questionnaire contained information about the use of doping agents, patterns of AAS, dietary supplements and recreational drug use. Target questions based on the socio-demographics, sports or physical activity, motivations for substances used and adverse or physical effects may be related to the use of doping agents within the volunteers were also recorded. The questionnaire was not subject to psychometric testing.

3.2.2. Urine samples collection

The collection of urine samples was carried out in universal collectors, without the addition of stabilizers, with a minimum volume of 100 mL per individual. The urine samples were stored in a freezer at -20˚C until GC-MS analysis.

3.3. Development of a method for toxicological urine analysis

3.3.1. Urine Sample Preparation

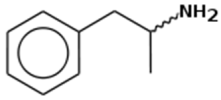
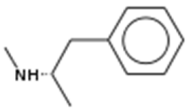
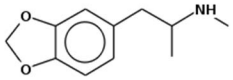
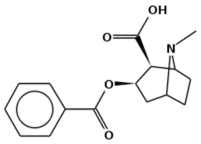
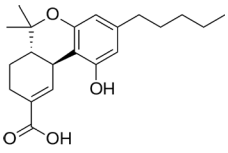
The urine samples were processed by LLE followed by two steps in basic and acid conditions. An aliquot of 0.5 mL of urine samples was spiked with the internal standards (AMP-d11, MDMA-d5, BE-d3 and THCCOOH-d3) to obtain a final concentration of 100 ng/mL. Subsequently, 125 µL of 6 M sodium hydroxide was

added for removing glucuronide and heated for 15 minutes at 40 °C. Two milliliters of ethyl acetate was added to the hydrolyzed urine sample to perform the first step of the LLE. The mixture was vortex-mixed and then centrifuged for 7 minutes at 2800 rpm. The supernatant was transferred into a clean tube. 100 µL of acetic acid was added to the remaining sample to adjust to pH 4.5. Two milliliters of n-hexane: ethyl acetate mixed solution (9:1, v/v) was added to the aforementioned sample to perform the second step of the LLE. This sample was vortex-mixed and centrifuged for 3 minutes at 2800 rpm. 1.5 mL of supernatant of the acidic extraction was mixed to the supernatant of that basic extraction. The solvents were dried using an evaporator under a nitrogen stream (KIM et al, 2013). The extracts were derivatised with MSTFA. The residue was reconstituted with 80 µL of ethyl acetate and transferred to an insert containing 30 µL of MSTFA and heated at 90˚C for 20 min.

3.4. Instrumentation

GC–MS analyses were carried out using a 7890A GC instrument coupled with a 5975C mass spectrometer (Agilent Technologies, Palo Alto, CA, USA). A 1 µL of samples were injected in a splitless mode at 280˚C. The capillary column used was HP-5MS (30 m x 0.25 mm x 0.25 µm film thickness) from J&W Scientific (Agilent Technologies, USA). The carrier gas used was helium at flow rate of 1.0 mL/min. The initial column temperature of 90˚C was held for 2 min, ramped to 220˚C at 10˚C/min and increased to a final temperature of 290˚C at 20˚C/min and held for 4 min (run time 20 minutes). The temperature of the MS interface, source and quadrupole were 280, 230 and 150˚C, respectively. The mass spectrometer was operated in an electron ionization (EI) mode 70eV. During urine analysis, three characteristic or two for the same stable isotopes mass fragments were monitored using the selected ion-monitoring (SIM) mode with respect to significant ions for each compound and internal standard. Table 3.1 summarizes the ions (m/z) selected for identification to each compound and their respective retention times.

Table 3.1: m/z ions selected to identify the target substances present in urine samples and the respective retention times.

Analytes		SIM ions (m/z)	Retention time (min)
AMP D11		98, ***120***	7.79
AMP		91, ***116***, 192	7.80
MA		91, ***130***, 206	8.99
MDMA D5		***135***, 255	13.08
MDMA		100, ***130***, 250	13.13
BE D3		85, ***243***, 364	16.76
BE		82, ***240***, 361	16.78
THCCOOH D3		***374***, 476	17.41
THCCOOH		371, ***473***, 488	17.43

*The underline ions were selected for the quantification measurement (quantifier ions).

3.5. Data analysis

Compounds were identified by comparing retention times (±2%) based on the IS and all combined ions of each analyte (±20%) to the corresponding average values of calibrators assayed. Peak area ratios of the target analytes and their respective IS were calculated using the software supplied by the manufacturer (Agilent Chemstation).

3.6. Method validation

The screening method was validated according to the parameters for validation of qualitative methods that includes: selectivity/specificity, limit of detection (LOD), extraction recovery and repeatability (intra-assay precision). The analysis was performed in a one-day assay protocol, based on the analysis of five drug-free urine samples and four replicates of positive control (PC) samples at two different concentrations (PC low and PC high) (Jimenez et al, 2002).

Sensitivity and selectivity parameters were evaluated using five drug-free urine samples obtained from different healthy volunteers. The presence of the target substances was confirmed when the analyte peaks present a signal-to-noise ratio 3:1 considering the three ions monitored.

The LODs were determined through the analysis of four extracted urine specimens fortified with the lowest concentrations recommended (PC low). It was established at a signal-to-noise ratio of at least 3:1 for all the ions.

Extraction recoveries were evaluated in four replicates for each analyte of PC high that consists of a blank urine sample spiked with the analyte at a concentration 10 times higher than PC low. The recovery was evaluated using the post extraction addition method, with the analytes added after the extraction of the blank matrix.

PC low and PC high samples were used to perform the repeatability measurements (intra-assay precision) with values accepted at 25 and 15% of the standard deviation, respectively.

3.7. Results and Discussion

3.7.1. Questionnaire and data collection

3.7.1.1. Prevalence of substances use

From the universe of 40 male volunteers, 20 reported consumption of AAS and DS; while 20 reported to be DS consumers only (Table 2.2). The mean age of the volunteers was 23 years (range 21-27). All the AAS abusers affirmed the use of illicit drugs at least once time during their lifetime and seventeen reported to be frequent users. None of them reported to be current cigarette smokers. Table 3.2 below shows the obtained results from the report.

Table 3.2: Self-report of 40 volunteers about lifetime consumption and current use of AAS, DS, pharmaceutical and recreational drugs.

Substance	Lifetime use (%)	Current users (%)
AAS	50	50
DS	100	100
Pharmaceutical drugs	100	97.5
Cigarette	15	0
Alcohol	100	15
Cannabis (THC)	25	15
Amphetamine	20	15
Ecstasy	15	5
Cocaine	10	7.5
LSD	5	0

Through the self-report of 40 questionnaires collected it was verified that the consumption of DS is predominant in all athletes. The anabolic steroid users confirmed their awareness of the adverse effects of these substances and the related increased health risk.

Table 3.3 shows the main dietary supplements used by the volunteers' that include proteins, amino acids, creatine, stimulants (caffeine, ephedrine, pseudoephedrine, mephentermine), vitamins, antioxidants, minerals, carbohydrate and various extracts from plant sources.

Table 3.3: Self-report of 40 volunteers about daily use of dietary supplements (DS).

Dietary supplements (DS)	Percentage of Users (%)
Whey Protein	100
Amino acids (BCAA)	100
Creatine	55
Carbohydrate	75
Stimulants	
Caffeine	25
Ephedrine	10
Pseudoephedrine	5
Mephentermine	5
Vitamins	
Vitamin C	75
Vitamin D	15
Multivitamin	65
Herbals extracts	
Tribulus terrestris	25
Green tea	20
Other (s)	10

3.7.1.2. Motivation for use of AAS

Figure 3.1 shows the volunteers' motivation for AAS use. The most frequently reported reasons for using AAS were to attain a more attractive body, have larger muscles, enhance performance, gain strength, decrease body fat, and to perform better

in sports. Additional other reported motives included curiosity and to become brave and self-confident.

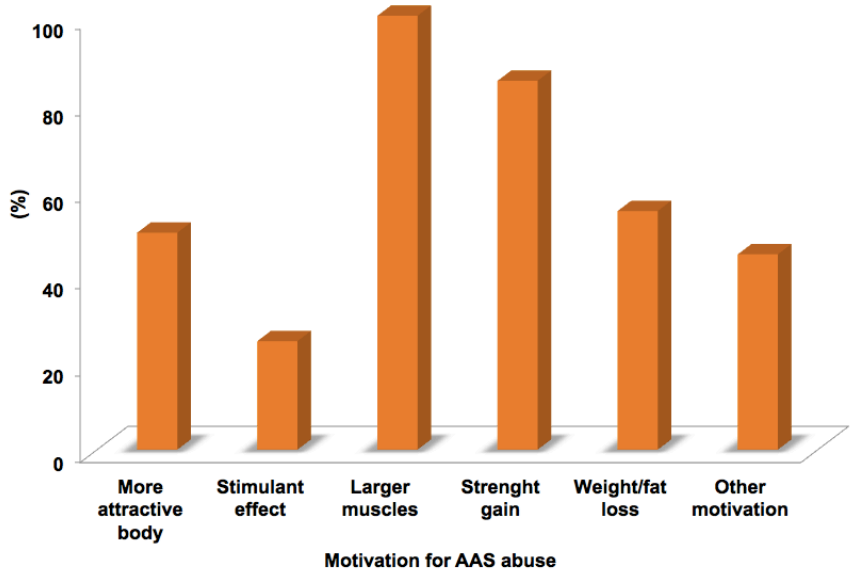


Figure 3.1: Self-reported motivations for use of AAS (n=20); several volunteers reported multiple motivations and "other motivation(s)".

The motivation for AAS use was consistent with previous studies on AAS users. These studies also describe that some of the reasons for AAS abuse appeared to reflect a direct effect on the CNS, because the AAS reduced anxiety and perhaps reduced feelings of empathy. For this reason, some abusers may perceive AAS as a means to enhance the quality of life in aspects unrelated to physical performance only. It suggests that the susceptibility of the drug users to the pharmacological and physiological effects of AAS was similar to that of AAS users that did not have a substance abuse habit. These studies also correlated the AAS use to certain psychiatric symptoms, such as depression or aggressiveness (PARKINSON and EVANS, 2006; KANAYAMA et al, 2008; PETERSSON et al, 2010).

3.7.1.3. Volunteer's AAS abuse pattern

Twenty athletes also confirm the self-administration of AAS for longer and/or short periods (several weeks or months) in quantities as high as 1 g per week during the entire year. They reported the oral and/or injectable administration of the AAS.

Only one volunteer reported the use of AAS prescribed by a general practitioner. Table 3.4 shows the list of the AAS substances that this group of athletes have been used daily or occasionally.

Table 3.4: Volunteers' self-reports of 20 athletes that consumed AAS.

AAS Type	Volunteer's (%)	Amount used / week (mg)
Boldenone	10	250-1000
Drostanolone	5	350-800
Methenolone	5	750
Nandrolone	10	400-1000
Stanozolol	25	500-5000
Esters of testosterone	20	500-1000
Trenbolone	10	350-1000
Fluoxymesterone	5	600
Mesterolone	5	550
Methandienone	5	500-1000
Oxandrolone	15	200-1000
Oxymetholone	5	450

The potential harm from using such high doses of these substances is recognized by health organizations. In supraphysiologic doses, AAS can affect multiple organ systems causing an increased morbidity and mortality in abusers. Exogenous testosterone shuts down the hypothalamic–pituitary–gonadal axis subsequently inhibiting testicular function and causing infertility in males. The reported amounts considerably exceed the highest limit usually prescribed for clinical purposes (BÜTTNER, THIEME, 2010). A 12 years follow-up study showed the mortality in 62 power lifters, strongly suspected of having used megadoses of AAS over several years, was 12.9% (mean age at death 43 year) compared with 3.1% in the control group of 1094 subjects (mean age not documented) (PARSSINEN et al, 2000).

3.7.1.4. Effects and adverse effects of multi-drug use

The adverse effects perceived by the AAS users are shown in Table 3.5, between them are included nausea, headaches, anxiety, depression, altered libido, site reaction, insomnia and vomiting.

Table 3.5: Self-report of 20 volunteers who suffer adverse effects caused when using the combination of AAS and DS.

Adverse Effects	Volunteer's (%)
Dry mouth	60
Headache	55
Insomnia	50
Stomach pain	50
Tachycardia	40
Altered libido	35
Reduced appetite	35
Irritability	30
Increased libido	25
Site reaction	25
Oily skin	25
Testicular atrophy	25
Acne	20
Diarrhoea	20
Gynecomastia	20
Vomiting	20
Anxiety	15
Depression	15
Loss of hair	15
Aggressiveness	10
Nausea	10
Increased appetite	5
Other (s)	20

The AAS abuse disrupts the normal hormones production in the body and may result in reversible or irreversible effects. The mentioned adverse effects such as: headaches, gastrointestinal irritation, diarrhoea, stomach pains, and an oily skin are considered acute toxic responses. The effects can be reversible with cessation of AAS use. The local reaction can cause serious infections at the injection site, causing pain and abscess. Acne development is a common result of AAS abuse due to the growth of sebaceous glands and the increase of secretion of natural oil sebum (VAN AMSTERDAM et al, 2010).

The gynecomastia reported is because of the abuse of high doses of AAS. Aromatization is the process by which steroid hormones are interconverted. Testosterone and other aromatizable AAS are metabolized in part to estradiol and other estrogen agonists. As a result, of this process the circulating estrogen levels are typical of women during a normal menstrual cycle. This can lead to breast pain in men and, the often irreversible, gynecomastia (VAN AMSTERDAM et al, 2010; MARAVELIAS et al, 2005).

Chronic adverse effects associated with the reported AAS abuse include testicular atrophy, urogenital problems, aggressive acne, impotence and difficulty or pain in urinating. With continued abuse these effects can become irreversible (BÜTTNER, THIEME, 2010; MARAVELIAS et al, 2005).

AAS users also reported irritability, anxiety and depression. Studies associated neuropsychiatric effects are with abuse of AAS. Behavioural and psychiatric effects, which vary from still socially acceptable mild irritation, anxiety and body training drive to uncontrolled aggression, hostility and depression. The frequency of these effects is generally dependent on the dose used. In a controlled study AAS produced clear psychiatric effects in individuals using doses of 1000 mg/week. High doses of AAS can also increase the risk to develop dependency to AAS. As a result of such withdrawal symptoms they may develop depressive symptoms, anhedonia, fatigue (PERRY et al, 1990; PERRY et al, 2003; CLARK and HENDERSON, 2003; POPE et al, 2000).

3.7.2. Method Validation results

Table 3.6 summarizes the validated method via GC-MS to detect AMP, MAMP, MDMA, BE, THCCOOH in urine samples.

Table 3.6: GC-MS validation method for the analysis of 5 different illicit drugs in urine.

Analytes	LOD (ng/mL)	Repeatability tests		Extraction recovery (%)
		PC low (ng/mL) and RSD (%)	PC high (ng/mL) and RSD (%)	
AMP	10	50 (22.0)	100 (14.5)	70.0
MAMP	10	50 (24.7)	100 (12.0)	68.0
MDMA	5	25 (21.0)	50 (12.8)	83.0
BE	15	75 (17.5)	150 (10.3)	75.0
COOHTHC	10	50 (17.0)	100 (14.0)	77.0

The selectivity, specificity, LODs and repeatability tests are consistent with other LLE and GC-MS methods used for amphetamines, cannabinoids and cocaine in urine (Kim et al, 2013; Marais and Laurens, 2009; Saito et al, 2007).

3.7.2.1. Toxicological urine analysis results using GC-MS

Using the proposed GC-MS method it was observed that approximately 27.5% of the urine samples (n = 11) contained amphetamine, MDMA and benzoylecgonine (a cocaine metabolite). Fifteen percent of the urine samples (n = 6) were positive for Δ^9-THC metabolite (COOH-THC) that indicates the use of Marijuana. Figure 3.2 shows the chromatogram obtained after the LLE of the drug-free urine sample spiked with all investigated analytes.

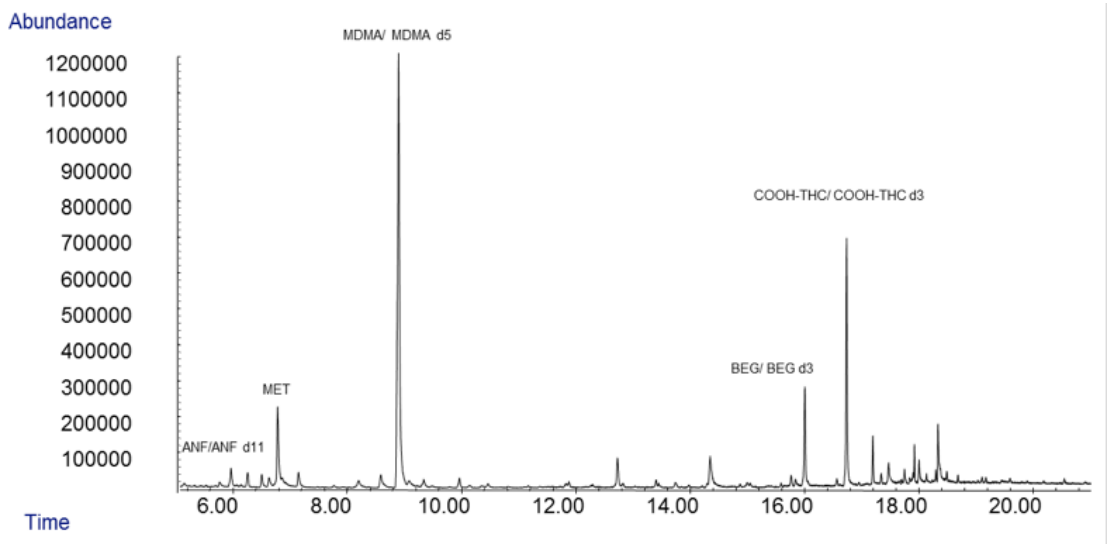


Figure 3.2: Total ion current chromatogram obtained after LLE extraction of a drug-free urine sample spiked with 100 ng/mL of: AMP; MAMP; MDMA; BE; THCCOOH and the deuterium internal standards AMP-d11, MDMA-d5, BE-d3 and THCCOOH-d3 (100ng/mL) for toxicological urine analysis.

Toxicological urine analysis confirmed that individuals in this studied group used multiple drugs. 27.5% of the AAS abusers also consumed amphetamine, MDMA and cocaine, while 15% were cannabis users. These results corroborate with other studies indicating that AAS abusers may be more inclined to use other drugs. Evidence found in the literature has shown that AAS and psychotropic substances affect the same reward regions of the brain, suggesting that the mechanisms are similar for both types of misuse (CALFEE, 2006; SKARBERG, 2009). Studies focused on AAS effects in male rats suggested that androgens might impact the sensitivity of the brain reward systems. Treatment with amphetamine-type stimulants prior to and after the androgen administration shows that the AAS may enhance the sensitivity of the brain due to an amphetamine-induced reward effect (TARTER, 1998). Studies conducted with rats showed that the use of cocaine can result in systemic toxicity. The combination of cocaine and AAS administrated barely or in combination resulted in increased aggression. It was also noted that these animals had an increased risk of coronary heart disease. In rodents testosterone induces a conditioned place preference, and is voluntarily consumed through oral, intravenous and intracerebral self-administration in hamsters. Male rats develop a conditioned place preference to testosterone injections into the nucleus accumbens, an effect

blocked by dopaminergic antagonists. Nonetheless, androgen reinforcement is not comparable to that of cocaine or heroin. Instead, testosterone resembles other mild reinforces, such as caffeine or benzodiazepines. Currently, the effects of cannabinoids and androgens interaction are unknown. However, it should be said that further investigations are needed to fully elucidate the complex interactions of drugs of abuse and AAS (MOZAYANI & RAMON, 2004; PENDERGRAFT III et al, 2014; SPEALMAN, 1995).

3.8. Conclusion

This study revealed that the use of doping agents among the volunteers was subject to different factors, which are normally guided by the type of physical activity or aesthetic appearance. It was verified that 50 % of the volunteers were users of AAS, DS and illicit drugs in different degrees. The use of these substances for long periods and/ or in high amounts can increase the chances of health problems causing synergistic side effects, increasing the risk to develop diseases. The importance of prevention is obvious and the regulation in the market place of the products should be intensified, alongside people seeking professional guidance prior to usage. The side effects of AAS studies are particularly important to improve health education and to reach more effective prevention strategies.

Chapter 4: Verification of the alterations caused by multiple drug use measuring haematological and biochemical parameters

Chapter 4: Introduction

Multiple drug use has been linked to many pathological conditions. Long-term steroids abuse is associated with cardiovascular disease, hypertension, heart attack and stroke. A study has suggested the growing number of reports of death related to apparent cardiac problem among AAS users in their early 20s and 30s (KANAYAMA et al, 2008). The mechanisms of AAS cardiovascular toxicity are not fully understood, although various studies have suggested direct toxicity in the cardiac tissue, resulting in a cardiomyopathy. Lipid abnormalities are described as a major risk factor for coronary heart disease. Several studies suggest that AAS cause profound changes in lipid metabolism, including the increase of triglyceride levels; reductions in high-density lipoprotein (HDL) levels and elevations in low-density lipoprotein (LDL) (BÜTTNER, THIEME, 2010).

Cellular structure and liver function can also be altered by drug abuse. Cholestasis hepatitis, hepatic peliosis, hyperplasia, and hepatocellular adenomas may occur with prolonged use. Orally active AAS can cause hepatotoxicity, including hepatic neoplasms (PERTUSI et al, 2001).

On the male reproductive system, AAS induced hypogonadism reduces the release of gonadotropins and decreases the production of endogenous testosterone, resulting the testicular atrophy and deficiency in spermatogenesis (oligoospermia and azoospermia). In addition, exogenous testosterone can shut down the hypothalamic–pituitary–gonadal axis subsequently inhibiting testicular function and causing infertility in males (MARAVELIAS et al, 2005).

Acute kidney injuries have been reported due to the combined or individual consumption of AAS, vitamins and supplements (DAHER et al, 2009; ROCHA et al, 2011). The abuse of these products is commonly associated with cases of acute renal failure, renal calcification, poisoning and hypervitaminosis (HAGELOCH et al, 1988). In addition, the use of AAS can increase the levels of serum creatinine, urine nitrogen and uric acid levels (MARAVELIAS et al, 2005; MOCHIZUKI, RICHTER, 1988). Some studies also suggest that AAS can induce the formation and growth of tumours in the presence of other carcinogens (MARAVELIAS et al, 2005; LAMB, 1984; WATANABE & KOBAYASHI, 1993).

The aim of this chapter was to verify the alterations caused by multiple drug use by measuring haematological and biochemical parameters in 40 athletes.

4.1. Material and Methods

4.1.1. Individuals

Forty male volunteers, (20 soccer, and 20 bodybuilder's professional athletes) from Ribeirão Preto city, state of São Paulo, Brazil, aged between 21 to 27 years participated in this study. Individuals provided informed consent consistent with the National Council of Health from Brazilian Ministry of Health and received a copy of the signed form upon completion. The study was approved by the Human Research Committee from the Faculty of Pharmaceutical Sciences of Ribeirão Preto of the University of São Paulo process number 048/2014.

4.1.2. Questionnaire and data collection

The volunteers were subjected to a 22 question self-completion questionnaire (Appendix II), which was constructed specifically for this study. The questionnaire took between 10 and 15 minutes to be completed. The questionnaire contained information about the use of doping agents, patterns of AAS, dietary supplements and recreational drug use. Target questions based on the socio-demographics, sports or physical activity, motivations for substances used and adverse or physical effects may be related to the use of doping agents within the volunteers were also recorded. The questionnaire was not subject to psychometric testing. The reference values for each analysed parameter follow international and European guidelines of clinical recommendations.

4.1.3. Sample analysis

Blood and urine samples were analyzed in the same day of collection in the Sodré Laboratory, Bauru city- São Paulo/Brazil. Blood cells were measured using a hematology analyzer ABX Micros 60 (Horiba medical). Serum urea, uric acid and creatinine were measured using a BS 200 (Mindray) chemistry analyzer with enzymatic reagents (Dialab). For sodium and potassium analysis an ion selective electrode was used (Bayer Diagnostics). The hormones levels were performed by immunoassay using the Tecman Colter unicel DXi 800 (Beckman Coulter). Urine samples were stored in a freezer at -20˚C until GC-MS analysis.

4.1.4. Statistics

The data was analyzed in two groups (abusers and non-users of substances). The Mann–Whitney test was used to compare the groups since some variables were not normally distributed. To display the comparison of measurements of the groups, box plots graphics were used.

4.2. Results and Discussion

4.2.1. Assessment of blood parameters and hormones levels

The results of blood parameters and hormones levels obtained from the athletes' volunteers are summarized in the Table 4.1.

Table 4.1: Blood parameters measured in users and non-users in comparison with reference levels.

Blood measures (unit)	Reference values	Volunteer's	Mean / SD	Median (5%, 95%)	p-value
Erythrocytes (Millions/mm³)	3.8 – 5.8	**Non-users**	5.05 (1.36)	5.05 (4.4; 5.7)	<0.0001
		Abusers	5.72 (2.62)	5.6 (4.9; 6.4)	
Haemoglobin (g/dL)	11 – 16.5	**Non-users**	14.29 (0.65)	14.25 (13.2; 15.3)	<0.0001
		Abusers	16.07 (0.65)	15.95 (14.4; 17.6)	
Leukocytes (cells/mm³)	4,000 to 11,000	**Non-users**	6265 (1250)	6,300 (4,246; 8,353)	0.776
		Abusers	6583 (1446)	6,000 (3620; 8962)	
Platelets (cells/mm³)	150,000 to 400,000	**Non-users**	183,750 (2253)	18,500 (148092; 22,1907)	<0.0001
		Abusers	287,350 (6192)	27,600 (174,136; 389,113)	
Haematocrit (%)	36 - 48	**Non-users**	43.2 (2.2)	43.1 (39.3; 47.1)	<0.0001
		Abusers	48.9 (3.2)	48.7 (45.0; 53.9)	
MCV (%)	80-92	**Non-users**	85.8 (4.1)	85.5 (80.1; 94.4)	0.4170
		Abusers	86.3 (3.9)	86.5 (77,1; 92.9)	
MCH (%)	30-35	**Non-users**	33.1 (0.7)	33 (32.1; 34.6)	<0.0001
		Abusers	28.6 (1.1)	28 (27.0; 31.3)	
MCHC (%)	27-29	**Non-users**	28.4 (1.8)	28.3 (26.1; 32.0)	<0.0001
		Abusers	33.1 (0.7)	33 (31.5; 34.0)	

Table 4.1 continued: Blood parameters measured in users and non-users in comparison with reference levels.

Blood measures (unit)	Reference values	Volunteer's	Mean / SD	Median (5%, 95%)	p-value
Total Testosterone (ng/dl)	175 – 781	**Non-users**	545.2 (156.0)	552.2 (283.4; 744.4)	0.2560
		Abusers	649.45 (340.9)	594 (133.3;1607.5)	
LH (mUI/mL)	1.5 – 9.3	**Non-users**	4.73 (1.49)	4.8 (2.3; 7.2)	<0.0001
		Abusers	0.81 (1.21)	0.38 (2.7; 0.05)	
FSH (mUI/mL)	1.6 – 8.0	**Non-users**	3.43 (1.47)	3.15 (0.7; 5.6)	<0.0001
		Abusers	0.97 (1.18)	0.31 (2.9; 0.1)	

MCV: Mean corpuscular volume
MCH: Mean corpuscular haemoglobin
MCHC: Mean corpuscular haemoglobin concentration
FSH: Follicle-stimulating hormone
LH: Luteinizing hormone

Erythrocytes, haemoglobin, platelets, haematocrit (volume percentage of erythrocytes in the blood) and mean corpuscular haemoglobin concentration (MCHC) levels were statistically higher in abusers than in non-users, at 5% of significance ($p < 0.0001$). The MCV values were higher for the abusers' group but with a statistically significance variation in relation to the non-abusers group ($p > 0.005$). The increases observed in these parameters are related to increased risk of thrombosis, transient ischemic attacks, stroke and myocardial infarction (MARAVELIAS et al, 2005).

The higher values regarding to the erythrocytes and leucocytes blood count in

abusers correspond to the stimulation of the erythropoiesis and granulopoiesis by androgenes with a reversible increase in the level of erythropoietin in the organism. The same applies to the higher values obtained in thrombocytes of the abusers' group. An increase in hematocrit and thrombocytes is critical since the hematocrit values were correlated with a cardiovascular risk and the tendency of thrombocytes to aggregation increases in connection with testosterone (KANAYAMA et al, 2008).

The increased numbers of blood platelets have a pivotal role in the pathogenesis of arterial thrombosis. Androgen-mediated enhancement of platelet aggregability and augmenting of thrombosis have been shown in experimental animal models of thrombosis (EMMS AND LEWIS, 1985). Deep venous thrombosis, pulmonary embolism, ventricular thrombosis cerebrovascular events, myocardial infarctions and peripheral arterial thromboses in this population have been described (LILJEQVIST et al, 2008). A possible prothrombotic effect through the aromatization of testosterone to estradiol has been suggested, as estrogen-based therapies have been shown to increase the risk of venous thromboembolism (LILJEQVIST et al 2008, LOWE 2002). The possible mechanism contributing to elevated levels in AAS abusers of haemoglobin is the AAS-induced increase of erythropoietin levels, a common finding in AAS users (TERUEL et al, 1995). According to the Framingham data, increased hematocrit values were correlated with an increase in the cardiovascular risk and total mortality (GAGNON et al 1994). Combined with an increase in platelet aggregability, it is likely that the elevated hematocrit in AAS users will contribute to the increased risk of thrombosis (MARAVELIAS et al, 2005).

Abusers presented higher testosterone levels in relation to non-users with distinct inter-individual variations in abusers. The average levels of total testosterone of the users showed an increase of 20 to 60% compared to reference values. Figure 4.1 shows a comparison between the levels of testosterone on non-users and abusers (AAS and DS) groups.

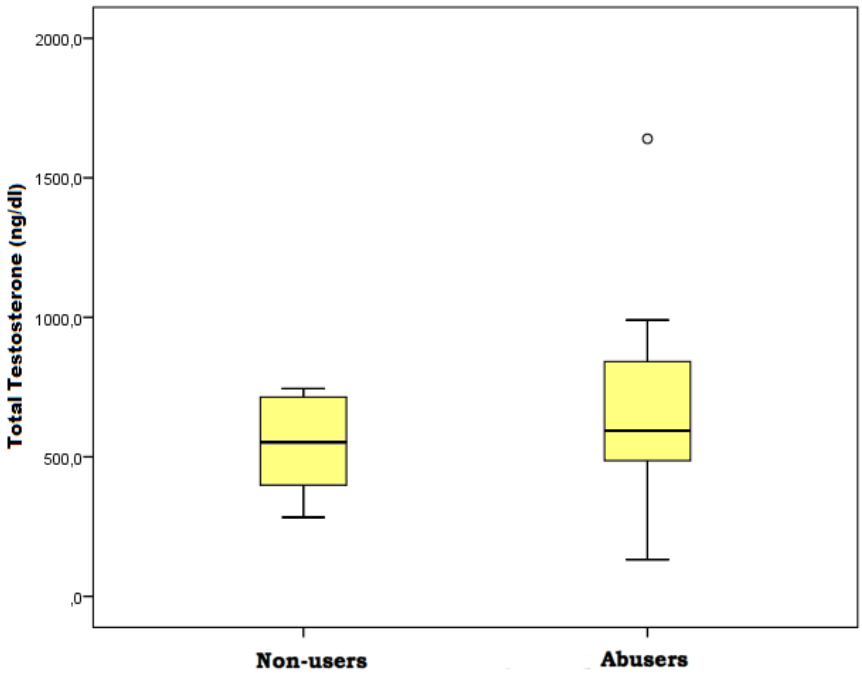


Figure 4.1: Boxplot of total testosterone measures from non-users and abusers (AAS and DS) groups.

The LH and FSH levels were statistically lower in abusers than in non-users, at 5% of significance ($p < 0.0001$). The observed reduction in the concentrations of these gonadotropins of the AAS abusers is related to the negative feedback under the hypothalamic-pituitary axis, its suppression. Since AAS are testosterone derivatives they act on the sex hormones and the reproductive system. Consequently, AAS abuse disturbs the endogenous production of testosterone and the gonadotrophins LH and FSH. In males, this suppression induces testicular atrophy and reduces semen production and quality (KANAYAMA et al, 2008). The reduction of FSH levels is associated with morphological changes and a decrease in sperm count, while the reduction of LH is related to the interference of signalling synthesis and release of testosterone. Long-term administration of supratherapeutic doses of AAS in male athletes may lead to infertility within months. The gonadotrophins levels full recovery after poly drug administration may take at least 4-5 months, in some cases up to one year (MARAVELIAS et al 2005). Some of the athletes' volunteers reported that they try to prevent this disturbance of the reproductive system by using human chorionic gonadotrophin (hCG) or clomifene together with the AAS or immediately after the

end of the AAS course.

4.2.2. Biomarkers of renal function

The results of renal function biomarkers and glomerular filtration rate (GFR) are presented in Table 4.2. The GFR was calculated using the Cockcroft-Gault method (COCKCROFT, GAULT, 1976). According to The International Renal Association, a normal GRF level is observed above 90 mL/min.

Table 4.2: Analysis of uric acid, urea, creatinine and the glomerular filtration rate (GFR) in the blood of athletes that are users or non-users of AAS, DS and drugs of abuse.

Biochemical Parameter	Reference Values	Volunteers	Mean / SD	Median (5%,95%)	p-value
Uric acid (mg/dL)	4.5 - 8.1	**Non-users**	5.1 (0.8)	4.9 (4.0; 6.4)	<0.007
		Abusers	5.9 (0.8)	5.8 (4.9; 7.5)	
Urea (mg/dL)	17 - 42	**Non-users**	34.6 (4.7)	33,0 (29.0; 44.9)	<0.0001
		Abusers	42.8 (6.4)	41,0 (36.1; 60.6)	
Creatinine (mg/dL)	0.7 – 1.3	**Non-users**	1.1 (0.1)	1,1 (1.0; 1.3)	<0.0001
		Abusers	1.7 (0.2)	1.65 (1.4; 1.9)	
GRF (mL/min)	> 90	**Non-users**	106.3 (4.5)	105 (103.0; 106.0)	<0.0005
		Abusers	89 (11.3)	89 (79; 91)	
Sodium (mEq/L)	130 – 155	**Non-users**	141 (2.3)	141 (138; 145)	0.0134
		Abusers	141 (11.7)	139 (119; 160)	
Potassium (mEq/L)	3.5 – 5	**Non-users**	4.5 (0.14)	4.6 (138; 145)	<0.0005
		Abusers	4.1 (0.19)	4 (3.7; 4.4)	

The mean levels of uric acid, urea and creatinine were statistically higher in AAS abusers' group than in non-users, at 5% of significance. Uric acid level was the

only biomarker that followed a normal distribution. The abusers group showed lower values in relation to normal GRF value, that is considered a GRF reduced rate. The levels of sodium and potassium in serum did not show significant differences between both groups. The relationships between creatinine and hematocrit measurements were calculated by Pearson/Spearman correlation. P-value <0.05 shows statistical significance. In Figure 4.2, the graph shows a positively correlation between creatinine and hematocrit values (r = 0.710) indicating that creatinine levels increased linearly with hematocrit levels.

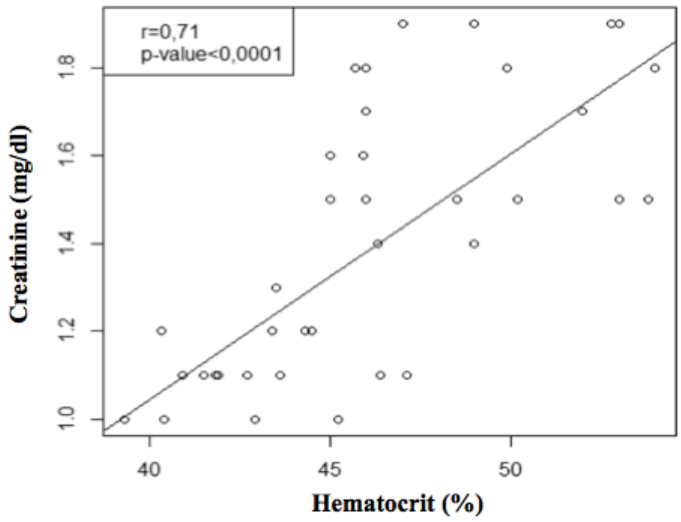


Figure 4.2: Correlation graph of creatinine and hematocrit variables.

It was observed in AAS abusers the retention of urea, creatinine and uric acid in blood, reaching values of up to 60.6; 1.9; and 7.5 mg/dL, respectively, which are typical values found in individuals with renal dysfunctions. The clinical relevance of these elevated renal biomarkers remains unclear. Urea production is increased when a greater number of amino acids are metabolized in the liver (ALMUKHTAR et al, 2015; DAHER et al, 2009). The high levels of amino acids found in these individuals are associated with the high consumption of proteins and / or dietary supplements.

The creatinine levels in combination with weight and age parameters are used to estimate the glomerular filtration rate (GRF). According to The International Renal Association, the GRF level is considered normal above 90 mL/min. The

determination of GFR must be assessed to detect and to evaluate a chronic kidney disease (CKD) (LEVEY et al, 2007). In this study, the mean GRF value observed in the AAS abusers' group was 89 ml/min with values as low as 79 mL/min for some individuals. If this value is maintained lower than the 90 mL/min for a period equal or higher than three months this number can be indicative of CKD (SILVA, BRUNE, 2011). As in this study preliminary data about GRF of the individuals were not collected, we could not associate their GRF values with a CDK. However as high protein diet increases the creatinine levels (and consequently the GFR levels) (ALMUKHTAR et al, 2015), these individuals should further investigate the possibility of having some renal malfunction.

Uric acid level was the only biomarker that follows a normal distribution with a mean of 5.8 and a maximum of 7.5 mg/dL that are still below the maximum expected of 8.1 mg/dL.

Several adverse effects caused by AAS abuse are well known, however few studies establishes the correlation with renal injury in humans. Some studies suggest that androgens may cause a direct toxic effect on glomerular cells, leading to mesangial matrix accumulation and podocyte depletion (DAHER et al, 2009; ROCHA et al, 2011). The susceptibility to these effects is primarily due to the high degree of filtration of potentially toxic products. For example, recent reports have shown that long-term abuse of AAS is related with severe forms of focal segmental glomerulosclerosis (FSGS) (HERLITZ et al, 2010). The FSGS is associated with hyperfiltration induced by; high protein diets; increased body mass; and/or reduced renal mass (ALMUKHTAR et al, 2015; HARRINGTON, 2011; HERLITZ et al, 2010; WINNETT, 2011). In addition to the harmfully effects caused by the concomitant abuse of AAS, DS and illicit drugs is another factor that can contribute to disrupt the renal functions. Several illicit drugs are linked with nephrotoxic effects (PENDERGRAFT et al, 2014).

4.2.3. Biomarkers of liver function

The results of liver enzymes obtained from the athletes' volunteers are summarized in the Table 4.3.

Table 4.3: Measurement results of liver enzymes alkaline phosphatase, gama-glutamyl transferase, glutamico-oxalacetic transaminase and glutamic-pyruvic transaminase through the blood of athletes that are users or non-users of AAS, DS and drugs of abuse.

Biomarker of liver function	Reference values	Volunteers	Mean / SD	Median (5%,95%)	p-value
Alkaline Phosphatase (U/L)	53 - 128	**Non-users**	108 (21.9)	111 (75; 147)	<0.0001
		Abusers	73.4 (18.1)	70.5 (41; 103)	
GGT (U/L)	< 55	**Non-users**	31.7 (19.2)	27 (27; 58)	0.06541
		Abusers	22.1 (7.87)	20 (7.1; 35)	
GOT (U/L)	7 - 34	**Non-users**	33 (12.1)	30 (9.9; 50)	<0.0001
		Abusers	55.6 (24.5)	46.5 (6.1; 96)	
GPT (U/L)	7 – 36	**Non-users**	24.8 (11.1)	24.5 (6.2; 43)	<0.0001
		Abusers	49.9 (12.9)	50 (28; 71)	

GGT: Gama-Glutamyl Transferase

GOT: Glutamico-Oxalacetic Transaminase

GPT: Glutamic-Pyruvic Transaminase

The mean levels of alkaline phosphatase GOT and GPT were statistically higher in AAS abusers group than in non-users, at 5% of significance. The measure GGT in serum presented identical distribution between both groups showing correlation ($p > 0.005$). In the literature, some authors associate increased liver enzyme activity with 17-α-alkylated steroids. Some authors correlate these prolonged changes with episodes of hepatocellular carcinoma, cholestasis and hepatitis. However, it is described that with the interruption of the use of anabolic steroids, these levels tend to normal after a period up to 5 months (MARAVELIAS et al, 2005).

High intensity and strength exercises cause increases in transaminases. Usually, the ratio of GOT/ GPT is higher than 1.0, which was observed in three cases

of abusers in this study. Literature shows that the GPT -values are on average higher than GOT values in strength athletes with or without anabolic steroid consumption, which was in contrast to the obtained results showing higher GOT concentrations. In our investigation, the correlation between the GPT activity of abusers leads to the assumption that the higher transaminase values of abusers might primarily be the manifestation of an impairment of the liver function induced by AAS (DICKERMAN et al, 1999; URHAUSEN et al; 2003).

4.2.4. Blood lipid levels

The results of blood lipid levels obtained from the athletes' volunteers are presented in the Table 4.4.

Table 4.4: Measurement results of lipid levels in the blood of athletes that are users or non-users of AAS, DS and drugs of abuse.

Blood lipid Parameter	Reference Values	Volunteers	Mean / SD	Median (5%,95%)	p-value
Triglyceride (mg/dL)	≤150	**Non-users**	51.4 (15.1)	48.5 (23.7; 73.3)	0.0037
		Abusers	71.35 (22.4)	65 (28; 108)	
Total Cholesterol (mg/dL)	200 - 230	**Non-users**	152.7 (23.4)	148 (109; 186)	0.2791
		Abusers	187.9 (81.0)	158 (24; 320)	
HDL Cholesterol (mg/dL)	Low < 40 High > 60	**Non-users**	50.75 (9.9)	50 (33; 66)	0.01785
		Abusers	39.85 (19.7)	41 (8.6; 72)	
LDL Cholesterol (mg/dL)	100 – 129	**Non-users**	92.1 (17.2)	91.5 (63; 119)	0.8075
		Abusers	123.3 (82.6)	89 (46; 259)	
VLDL Cholesterol (mg/dL)	< 30	**Non-users**	10.55 (3.1)	10 (4.9; 15.1)	0.0036
		Abusers	14.6 (4.21)	14.5 (7.6; 21.5)	

HDL: High-density lipoprotein

LDL: Low-density lipoprotein

VLDL: Very Low-density lipoprotein

The measure trygliceride and VLDL in serum did not show any significant differences between both groups. The total cholesterol, LDL and HDL values were higher for abusers' group but without a statistically significance variation in relation to non-abusers group ($p > 0.005$). The alterations observed in these parameters present a potential risk related to cardiovascular problems. Epidemiological studies have shown that changes in cholesterol fractions lead to an increase in the risk of cardiovascular events due to atherosclerosis, such as ventricular thrombosis and acute myocardial infarction (MARAVELIAS et al, 2005; DE LUIS et al, 2001).

The decrease in HDL-cholesterol and an increase in LDL-cholesterol are the most frequently documented side effects of an AAS; these effects increase the risk of coronary artery disease. Notably the alkylated and orally used AAS such as stanozolol lower the levels of HDL-cholesterol. High LDL and low HDL levels increase the risk of atherosclerosis. However, in general, serum levels return to baseline levels within several weeks to months after drug cessation (VAN AMSTERDAN et al, 2010).

4.3. Conclusion

The statistically higher results obtained during the blood parameters assessment associated with serum lipoprotein profiles are related with increase the risk of cardiovascular diseases; including risk of thrombosis, transient ischemic attacks, stroke and myocardial infarction. The increased measurements of testosterone associated to the lower measurements of LH and FSH in abusers may cause negative effects on the reproductive system leading to infertility associated with morphological changes, decrease in sperm count and interference of synthesis and release of testosterone. The retention of urea, creatinine and uric acid in blood, reached values of 60.6; 1.9; and 7.5 mg/dL, respectively, which are typical values found in individuals with renal malfunctions. In addition to the harmfully effects several illicit drugs are linked with nephrotoxic effects. Our results showed that the supratherapeutic AAS use in combination with the multidrug abuse for long periods could increase the risk of health problems. A variety of pathophysiologic mechanisms suggest an increased risk of kidney diseases and cardiovascular damages.

Chapter 5: Determination of doping used-stimulants in sweat and urine using disposable pipette extraction (DPX) tips and GC-MS

Chapter 5: Introduction

Stimulants are a major problem in elite sports and were the first group of substances prohibited by most of the international sports federations (WADA, 2012). About 25% of positive reported cases by IOC accredited laboratories are related to those substances, being the second class most used among athletes (WADA, 2012; LAURE, 2006; BERGO, 2007).

Sweat samples have some advantages over urine in doping tests such as less chance of sample adulteration, longer time-detection window, non-invasiveness and possibility of finding both parent drug and metabolites. According to the WADA code, in paragraph 5.2.4.4 of the International Standard for Laboratories, the use of alternative samples are permitted, however the respective result shall not be used to counter adverse analytical or atypical findings from urine. (GALLARDO, QUEIROZ, 2008). The Disposable Pipette Extraction Tips (DPX) consists of a solid-phase extraction in which the sorbent is loosely dispersed in a pipette tip, which allows a quick and dynamic contact between the aspirated analyte from the sample and the solid phase. It is a technique used particularly in forensic analysis, since it requires a small amount of sample and solvent (BRUNET et al, 2008; DE MARTINIS et al, 2007; KACINKO et al, 2005).

This study shows the full development of a method to determine the presence of 13 amphetamines-type drugs, cocaine and the new substance psychoactive mephedorne and their metabolites in sweat and urine samples using DPX and GC-MS.

5.1. Material and Methods

5.1.1. Chemicals and Reagents

The following illicit drugs and metabolites were investigated: amphetamine (AMP); methamphetamine (MAMP); N-methyl-3,4-methylenedioxymethamphetamine (MDMA); 3,4-Methylenedioxyamphetamine (MDA); 3,4-Methylenedioxyethylamphetamine (MDEA); mephentermine (MPT); methylphenidate (MPH); amphepramone (AMPH); mephedrone (MEPHE);

phemproporex (PHEMP); cocaine (COC), benzoylecgonine (BE) and cocaethylene (COE). Deuterium internal standards (IS) of AMP-d11, MDMA-d5, COC-d3 were used. All standards were purchased from Cerilliant (Round Rock, TX,USA) as 1 mg/mL or 100 mg/mL solutions in acetonitrile or methanol. Standard stock solutions and IS were prepared by appropriate dilution with methanol and stored at 2°C, protected from light.

Methanol, ethyl acetate, dichloromethane, 2-propanol, heptane, acetic acid, ammonium hydroxide, potassium phosphate monobasic, potassium phosphate dibasic, sodium chloride, urea, ammonium hydroxide and concentred hydrochloric acid were obtained from J. T. Baker (USA). All solvents and reagents were of analytical grade. Sodium hydroxide, ammonium chloride, lactic acid was purchased from Synth (Diadema, Brazil). The silylating reagent used for derivatisation was N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) purchased from Sigma-Aldrich (Milwaukee,WI, USA).

The DPX tips (DPX-CX1) were acquired from Gerstel (USA). PharmChek™ sweat patches were supplied by PharmChem Inc (Fort Worth, TX, USA).

Artificial sweat was used to perform the validation of the method. The artificial sweat solution contained 327 mmol/L ammonium chloride, 166 mmol/L lactic acid, 83 mmol/L urea, 42 mmol/L acetic acid, 34 mmol/L sodium chloride in deionized water and pH was adjusted to 4.7 with 2 mol/L sodium hydroxide.

5.1.2. Calibrators and quality controls

All investigated drug standards were mixed and diluted with methanol to obtain three calibration solutions at 1.0, 2.0 and 10 µg/mL. Internal standards solutions were prepared in methanol at 10 µg/mL. Daily calibration curves from 10 to 500 ng/patch; quality controls were constructed adding the calibrators' solutions to blank urine and sweat patches. 500 µL of artificial sweat were added to unused drug-free sweat patches evenly distributed onto the pad and allowed to dry for 30 minutes at room temperature. For preparation of calibration curves, these patches were spiked with standard solutions final concentrations of 10, 25, 50, 100, 250 and 500 ng/patch. Internal standards were added to each patch at concentrations of 100 ng/patch. QC samples also were prepared daily by spiking these blank patches with control

solutions to concentrations of 15, 200 and 350 ng/patch for all analytes and 100 ng/patch of IS. After fortification, all patches were allowed to air dry for 10minutes at room temperature.

5.2. Individuals

Forty male volunteers, (20 bodybuilders and 20 soccer professional soccer athletes) from Ribeirão Preto city, state of São Paulo, Brazil, aged between 21 to 27 years participated of this study. Individuals provided informed consent consistent with the National Council of Health from the Brazilian Ministry of Health and received a copy of the signed form upon completion. The study was approved by the Human Research Committee from the Faculty of Pharmaceutical Sciences of Ribeirão Preto of the University of São Paulo process number 048/2014 (Appendix). The volunteers were subjected to a self-completion questionnaire, which was constructed specifically for this study in order to personal data and information discussed in Chapter's 2 and 3 (Appendix). The questionnaires took between 10 and 15 minutes to be completed. The questionnaire contained information about the use of doping agents, patterns of AAS, dietary supplements and recreational drug use. Target questions based on the socio-demographics, sports or physical activity, motivations for substances used and adverse or physical effects may be related to the use of doping agents within the volunteers were also recorded. The questionnaire is in the Appendix. The questionnaire was not subject to psychometric testing.

5.3. Sweat and Urine samples collection

The collection of urine samples was carried out in universal collector, without the addition of stabilizers, with a minimum volume of 100 mL per individual. For collection of the sweat samples, two PharmCheck® patches were used with one of these applied on the back region and the second was applied on the abdominal area. Before sampling, the skin was wiped with 2-propanol. The patches were removed after the individuals carried out any physical activity (up to 24 hours of use) and individually stored in sealed plastic bags. Sweat and urine samples were stored in a freezer at -20˚C until GC-MS analysis.

5.4. Development of a method for toxicological analysis in sweat and urine samples

5.4.1. Sample Preparation

5.4.1.1. Urine

For the DPX extraction of urine samples, a volume of 500 µL of each sample was transferred to a conical flask and an aliquot of IS solutions corresponding to 100 ng of each compound (AMP-d11, MDMA-d5 and COC-d3) and 200µL of 0.1M hydrochloric acid (HCl) solution was added. The DPX pipette tips were conditioned with a 40% (v/v) aqueous solution of acetonitrile. Then, the sample solution was aspirated and held in contact with the solid phase for 60 seconds with continuous air aspiration. The sample solution was discarded, and the tip was then washed with 1 mL of deionized water. Finally, the elution step was conducted two times with a dichloromethane/isopropanol/ammonium hydroxide solution (78:20:2, v/v/v) and the eluate was dried at 40°C under flow of nitrogen. The residue was reconstituted with 40 µL of ethyl acetate and transferred to a vial containing 30 µL of MSTFA. The derivatisation reaction was carried out by the thermal incubation method (heating at 90˚C for 20 min). Then, 1 µL of the extract was injected into the GC-MS.

5.4.1.2. Sweat

The patches fortified with the calibrators, QC samples and collected samples were folded (twice) and placed into a conical flask. 2 mL of methanol was added to perform a liquid-liquid extraction before the DPX clean-up. After the solvent addition the samples were transferred to a horizontal shaker (250 RPM) for 10 minutes. The extracted sample was transferred to a clean conical flask and 200µL of 0.1M HCl solution was added. The DPX conditioning, washing and elution steps were identical to those described above for urine analysis. The eluate was dried at 40°C under a nitrogen flow and the residue was reconstituted with 40 µL of ethyl acetate and mixed with 30 µL of the MSTFA at 90˚C for 20 min for derivatisation. One microliter of the extract was injected into the GC-MS.

5.5. Instrumentation

GC–MS analyses were carried out using an Agilent Technologies 7890A GC coupled with a 5975C mass spectrometer (Agilent Technologies, Palo alto, CA, USA). 1 µL of the extracted sample was injected in splitless mode at 280˚C at flow of 1 mL/min. A capillary column HP-5MS (30 m x 0.25 mm x 0.25 µm film thicknesses) from J&W Scientific (Agilent Technologies, USA) was used. The carrier gas used was helium at flow rate of 1.0 mL/min. The initial column temperature of 90˚C was held for 2 min, ramped to 220˚C at 10˚C/min and increased to a final temperature of 290˚C at 20˚C/min and held for 4 min (run time of 20 minutes). The temperature of the MS interface, source and quadrupole were 280, 230 and 150˚C, respectively. The mass spectrometer was operated in electron ionization (EI) mode 70eV. During the analysis, three characteristic mass fragments were monitored using the selected ion-monitoring (SIM) mode with respect to significant ions for each compound and IS. Table 5.1 presents the ions (m/z) selected for identification to each compound and their respective retention times.

Table 5.1: m/z of ions used to identify the analytes in urine and sweat samples and the respective retention times.

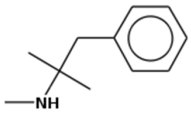
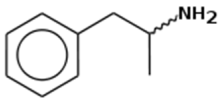
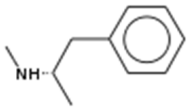
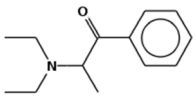
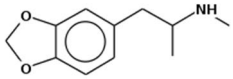
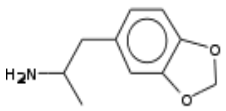
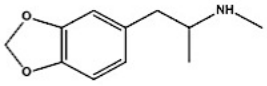
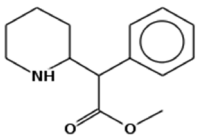
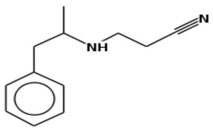
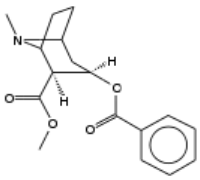
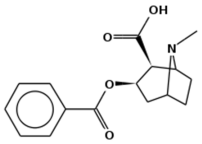
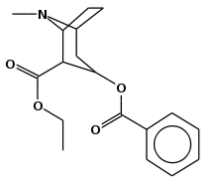
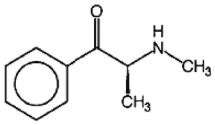
Compound	Structure	Quantifier (m/z)	Qualifier (m/z)	Retention times (Minutes)
Mephentermine (MPT)		58	91,105	6.80
Amphetamine (AMP)		116	91, 134,192	8.20
Amphetamine D11 (AMP D11)		120	98	8.22
Metamphetamine (MA)		130	91, 206	8.99
Amphepramone (AMPHEP)		100	77,105, 91	11.05
3,4-Methylenedioxymethamphetamine (MDMA)		130	100, 250	11.73
3,4-Methylenedioxymethamphetamine D5 (MDMA D5)		135	255	11.78
3,4-Methylendioxyamphetamine (MDA)		116	135, 236	11.95
3,4-Methylenedioxyethylamphetamine (MDEA)		144	72, 264,135	12.05
Methylphenidate (MPH)		84	91,115,172	12.89
Fenproporex (FENP)		97	91,115,135	13.77
Cocaine (COC)		182	477	17.81
Cocaine D3 (COC D3)		185	85, 306	17.83

Table 5.1 continued: m/z of ions used to identify the analytes in urine and sweat samples and the respective retention times.

Compound	Structure	Quantifier (m/z)	Qualifier (m/z)	Retention times (Minutes)
Benzoylecgonine (BE)		240	82, 303	18.02
Cocaethylene (COE)		196	371, 488	18.43
Mephedrone (MEPHE)		59	119, 177	19.85

5.6. Data analysis

Compounds were identified by comparing retention times (±2%) based on the IS and qualifier ion ratios (±20%) to the corresponding average values of calibrators assayed. Peak area ratios of the target analytes and their respective IS were calculated using the software supplied by the manufacturer (Agilent Chemstation). Calibration was conducted by linear regression analysis.

5.7. Method validation

Method validation was conducted according to the Scientific Working Group Toxicology guidelines (SWGTOX, 2013). The parameters evaluated were selectivity, sensitivity, linearity, recovery, precision (intra- and inter-assay) and accuracy, carry over and stability.

5.7.1. Selectivity

Selectivity was defined as the ability to identify and quantify analytes with or without in the presence or absence of endogenous or exogenous compounds. To verify potential endogenous interferents or adverse matrix effects blank urine and sweat patches provided by drug-free volunteers were analysed (N=6). In addition, the assay was performed using the fortified urine and sweat samples with the analyte concentration at the limit of quantification (LOQ) and possible interferences that may be present in actual analyses at 1000 ng/mL. The concentration RSD (%) should not vary by more than 20% in the presence of any interference investigated. Retention times had to be within ±0.2 min of the mean calibrator retention time. The interferents were alprazolam, caffeine, clomipramine, clonazepam, THC, diazepam, dipyrone, lorazepam and nicotine.

5.7.2. Limits of detection (LOD) and limits of quantification (LOQ)

The LODs and LOQs were determined based on the signal-to-noise ratio of 3:1 and 10:1 respectively. The experiments were performed by adding aliquots of the target standards to the samples in decreasing concentrations in triplicate.

5.7.3. Linearity

Linearity was provided using six calibration points with a concentration range from 10 to 500 ng/mL. Internal standards were added to each patch at concentrations of 100 ng/patch. The samples were inserted into a conical flask and spiked with the IS and the standard solution of each analyte in those concentrations selected for the calibration curve. Linearity was evaluated by the method of least squares and expressed as coefficient of determination (R^2). A 1/x-weighting factor was utilized. Calibrators were required to satisfy all identification criteria and quantify within 20% of target concentration.

5.7.4. Precision and accuracy

Precision and accuracy assay was determined using three QCs, covering the range adopted in the calibration curve (n=5). The concentrations of QC were 15 ng/mL (QC1), 200 ng/mL (QC2) and 350 ng/mL (QC3) for all the analytes. The intra-assay precision was evaluated according to the analysis of five replicates between the results obtained in the same day. The inter-assay precision was assessed according to the results of analysis over five different days. Precision was expressed as a percent of the relative standard deviation (RSD%). Accuracy was determined by comparing measured concentrations with target values over runs and expressed as a percent of the target concentration.

5.7.5. Recovery

The recovery was determined in samples of the three QCs for each analyte through the comparison between the quantification results of the fortified samples after extraction and the results of the fortified samples before extraction. The IS was added prior to the processing of the samples in the two recovery test conditions.

5.7.6. Carry over

To assess the carry over effect, extracts of blank samples of sweat and urine were analysed immediately after the analysis of the sample extract fortified which highest point in the calibration curve (500 ng/mL).

5.8. Analysis of real samples

The method was applied to 40 urine and sweat samples collected from professional and non-professional athletes. These volunteers stated that they had used at least one of the investigated substances in their lifetime.

5.9. Results and Discussion

5.9.1. Method Validation

Figure 5.1 shows the chromatographic separation of a sweat sample fortified with 100 ng/mL of all the analytes. Tables 5.2 and 5.3 show the results of the analytical validation of the DPX-GC-MS method presented. All the parameters presented acceptable values according to the SWGTOX guidelines.

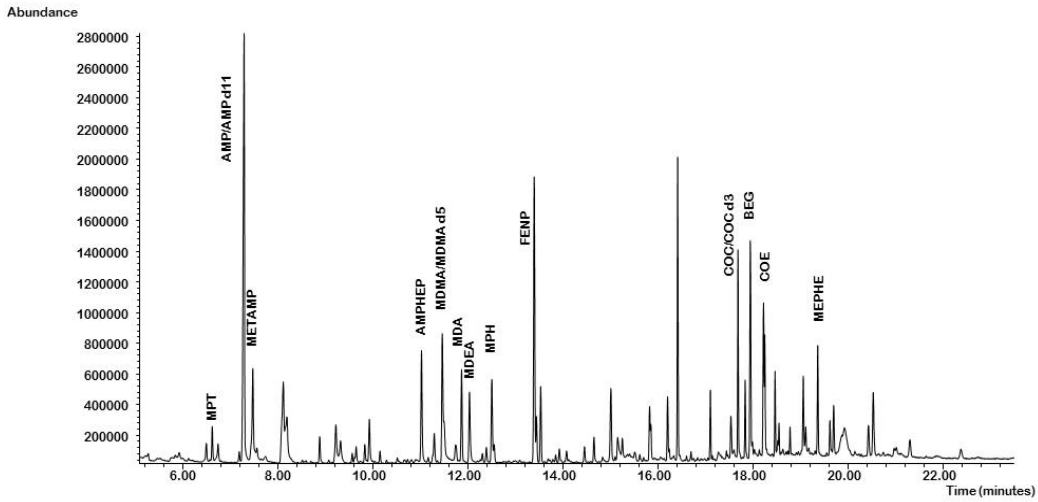


Figure 5.1: Total ion current chromatogram obtained after DPX extraction of a sweat sample fortified with 100 ng/patch of all the analytes.

Table 5.2: Analytical validation results of the DPX-GC-MS method for the analysis of urine fortified with 13 stimulants.

Analyte	R^2	LOD (ng/mL)	LOQ (ng/mL)	*Quality controls(QC)*	*Intra-assay RSD (%)*	*Inter-assay RSD (%)*	*Accuracy (%)*	*Recovery (%)*
MPT	0.99736	3.0	10.0	15	12.0	7.50	93.2	80.0
				200	13.50	12.10	101.5	75.0
				350	14.55	7.20	95.6	70.0
AMP	0.99901	2.0	10.0	15	7.50	8.30	99.3	93.0
				200	3.70	11.70	91.7	82.9
				350	2.05	7.85	102.0	93.0
MDA	0.99712	2.5	10.0	15	14.08	14.10	99.5	81.8
				200	7.30	7.75	95.4	92.3
				350	9.10	11.35	96.4	77.0
MDEA	0.95321	3.5	10.0	15	12.40	9.05	97.4	91.7
				200	13.28	13.35	90.2	90.5
				350	13.45	9.43	103.4	90.8
MPH	0.99204	3.0	10.0	15	8.40	4.70	99.0	96.0
				200	8.70	15.30	97.1	89.0
				350	7.20	9.30	99.2	85.0
AMPH	0.98973	5.0	10.0	15	12.10	6.40	100.1	92.5
				200	12.08	7.70	99.7	90.4
				350	9.50	12.60	95.6	65.8
FENP	0.99606	5.0	10.0	15	7.15	5.05	98.2	93.0
				200	12.30	13.10	93.4	77.0
				350	7.32	11.90	99.1	69.0
MET	0.99870	1.5	10.0	15	5.20	6.40	98.6	85.1
				200	6.20	7.70	93.4	79.0
				350	5.50	6.55	98.8	76.1
MDMA	0.99460	2.5	10.0	15	4.67	10.10	90.0	91.7
				200	12.40	7.30	90.7	90.5
				350	8.23	8.60	94.2	88.8
BE	0.99059	5.0	10.0	15	12.75	5.10	103.4	95.0
				200	6.25	10.15	101.1	86.0
				350	15.60	14.30	100.5	94.2
COC	0.99834	3.5	10.0	15	10.35	12.5	100.7	94.0
				200	9.12	11.20	99.2	88.4
				350	7.24	12.90	101.6	81.9
COE	0.99723	2.5	10.0	15	15.0	13.20	94.1	76.7
				200	8.32	11.70	97.6	74.0
				350	7.56	15.15	98.5	69.0
MEPHE	0.99887	2.0	10.0	15	5.60	13.05	97.7	75.0
				200	7.30	12.70	104.6	79.0
				350	12.05	11.10	100.2	80.7

Table 5.3: Analytical validation of the DPX-GC-MS method for the analysis of artificial sweat fortified with 13 stimulants.

Analyte	**R^2**	**LOD (ng/mL)**	**LOQ (ng/mL)**	***Quality controls(QC)***	***Intra-assay RSD (%)***	***Inter-assay RSD (%)***	***Accuracy (%)***	***Recovery (%)***
MPT	0.99534	3.0	10.0	15	8.85	9.80	95.0	90.0
				200	5.79	13.45	91.0	88.0
				350	4.87	9.45	95.5	77.0
AMP	0.99807	2.0	10.0	15	5.83	9.70	99.0	94.0
				200	7.56	13.5	98.0	89.0
				350	3.40	11.50	97.0	87.0
MDA	0.99734	2.5	10.0	15	3.39	8.90	97.5	90.5
				200	10.60	8.85	98.0	92.8
				350	12.85	11.91	90.0	89.0
MDEA	0.99453	3.5	10.0	15	5.98	9.70	105.5	91.7
				200	6.29	9.95	102.0	81.5
				350	15.66	13.05	93.0	82.9
MPH	0.99204	3.0	10.0	15	7.64	8.60	96.0	86.4
				200	3.70	8.85	89.0	85.0
				350	17.80	11.91	97.5	87.8
AMPH	0.99687	5.0	10.0	15	11.01	8.75	103.0	85.5
				200	10.12	6.40	90.7	80.2
				350	9.15	6.30	96.0	93.8
FENP	0.99761	5.0	10.0	15	10.10	6.90	98.0	86.0
				200	7.54	4.90	97.7	95.0
				350	12.28	5.90	95.0	78.0
MET	0.99170	1.5	10.0	15	8.75	10.40	99.0	85.0
				200	9.70	14.30	98.0	78.0
				350	5.98	13.40	104.0	70.5
MDMA	0.99343	2.5	10.0	15	13.10	4.25	96.0	91.7
				200	9.90	4.05	94.1	90.5
				350	12.50	11.60	99.0	81.8
BE	0.99627	5.0	10.0	15	13.05	15.35	98.0	83.0
				200	11.24	12.50	98.8	84.9
				350	5.90	12.70	104.0	84.0
COC	0.99777	3.5	10.0	15	9.65	6.05	96.0	97.0
				200	4.20	14.85	99.0	91.0
				350	11.60	13.0	99.5	101.3
COE	0.99349	2.5	10.0	15	9.85	8.25	98.8	90.6
				200	5.30	10.42	97.1	74.0
				350	9.40	11.60	98.0	70.0
MEPHE	0.99299	2.0	10.0	15	4.72	14.08	99.0	87.5
				200	9.20	11.50	98.0	82.5
				350	12.50	7.70	99.7	68.0

The method showed acceptable selectivity and sensitivity for all analytes examined in both the urine and sweat samples. LOD and LOQ values were considered appropriate for the analysis of the 13 studied substances. Acceptable accuracies values for all the lower controls were obtained, with values less than 20% to the low control in the presence of interferents. It is important to emphasize that the selected interferents were those commonly found in real samples and were not related to the consumption of performance drugs, and none of interferents presented the same retention time as that of the target analytes.

The precision was expressed as the relative standard deviation (%RSD) and the values are considered acceptable within the range of up to 15%, and accuracy between 80 and 120%. The assays of precision results are within the acceptable range up to 15% for QC1 and up to 20% for QC2 and QC3.

The developed method also presented acceptable linearity, supported by the correlation coefficient (R^2) values greater than 0.99 in the analysis of all target compounds over the range of 10 to 500 ng/mL. The extraction technique using DPX pipette tips were efficient, with recovery mean values ranging from 65 to 96%. Although recoveries close to 100% are expected, lower values are acceptable once the method presents high precision and accuracy.

5.9.2. Real-case samples results

The validated method was applied to the analysis of urine and sweat samples of 40 professional and non-professional athletes. 27 individual samples (urine and sweat) were negative to all tested analytes, and in two cases, drugs detected in sweat were not observed in urine samples. Table 5.4 presents the detected drugs in urine and sweat samples and Figure 5.2 shows the samples concentrations correlation. The DPX-GC-MS analysis showed that 13 out of 40 samples presented one or more measurable concentrations of at least one of the analysed drugs.

Table 5.4: Analytical and reported results obtained from the volunteers.

Sample number	Urine (ng/mL)	Sweat (ng/patch)	Report drug use
1	120 (AMP)	300 (AMP)	Amphetamine
2	640 (BE)	200 (COC)	Cocaine
3	116 (BE)	150 (COC)	Cocaine
4	260 (AMP)	100 (AMP)	Amphetamine
5	244 (AMP)	180 (AMP)	Amphetamine
6	550 (MDMA)	200 (MDMA)	MDMA
7	100 (MDMA)	115 (MDMA)	MDMA
8	397 (AMP)	270 (AMP)	Amphetamine
9	N.D	115 (AMP)	Amphetamine
10	130 (MPT)	150 (MPT)	Mephentermine
11	250(MDMA)	155 (MDMA)	MDMA
12	580 (BE)	300 (COC)	Cocaine
13	N.D	135 (MPH)	Methylphenidate

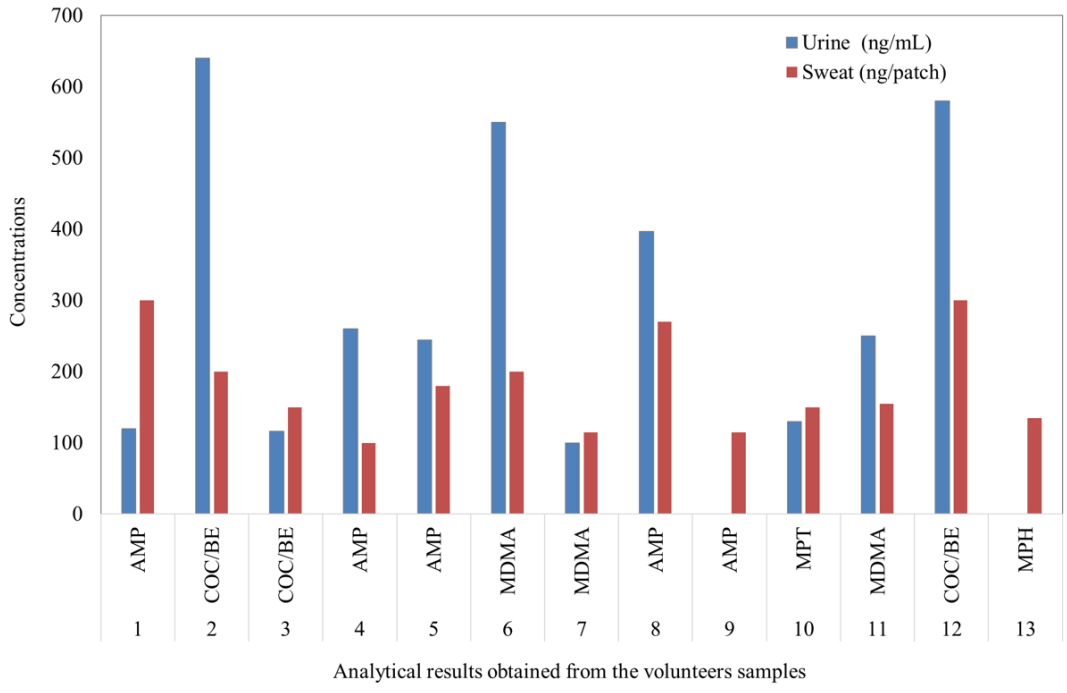


Figure 5.2: Sweat and urine samples concentrations correlation.

In samples which amphetamine was detected, the concentration ranged between 120 and 397 ng/mL in urine and between 100 and 300 ng/patch in sweat.

Cocaine levels were in the range of 150 to 300 ng/patch in sweat and the concentration of cocaine metabolite, benzoylecgonine, was between 116 and 640 ng/mL in urine. In sweat samples, MDMA concentrations were in the range of 115 to 200 ng/patch and, in urine samples, in the range of 100 to 550 ng/mL. The concentration of mephentermine in urine sample was equal to 130 ng/mL and in sweat equal to 150 ng/patch. Methylphenidate concentration in sweat sample was equal to 135 ng/patch.

According to the WADA 2014 Anti-Doping Testing Figures, the results of anti-doping testing analyses conducted by accredited laboratories showed that, in the class of stimulants, methylhexaneamine, methylphenidate, amphetamine and cocaine were the most commonly found substances in the samples (WADA, 2014). Less occurrences of MDMA in the samples were recorded. In our analysis of real sweat samples from professional and non-professional athletes, similar results to that described in the WADA report were observed. In 38.4% of sweat samples (n=5) and 30.8% of urine samples (n=4), amphetamine was found. 23.1% of the sweat samples (n=3) presented cocaine and in 23.1% of the urine samples, benzoylecgonine was detected. MDMA was detected in 23.1% of urine and sweat samples (n=3). Mephentermine was found in one sample of urine and sweat, corresponding to 7.7%. Methylphenidate was detected in one sweat sample only.

Comparing the toxicological analyses results with the information about drugs use provided by volunteers, the results are consistent. All drugs found in the sweat and urine samples are those previously stated by the users. In two samples (9 and 13, respectively), amphetamine and methylphenidate detected in sweat was not detected in urine. These variations are possible related to toxickinetic aspects. The time interval between drug consumption and detection on the skin surface depends on the chemical characteristics of the drugs and on the sensitivity of the analytical method used. As the pH of sweat is lower in relation to the pH of plasma, alkaline drugs tend to redistribute and to accumulate in sweat (DE LA TORRE, 2004; DE GIOVANNI, FUCCI, 2013). In addition, the renal excretion of amphetamine and an analogue compound may depend on the pH of urine, increasing the elimination at lower pH values (acidification of urine) (DE LA TORRE, 2004). The physical-chemical

properties of amphetamine and methylphenidate, two weak bases, may explain the presence of those compounds only in sweat samples.

Additionally, drug binding to skin fractions and reabsorption of drugs from the skin can explain the obtained results. Literature data also shows a continued presence of drugs on the skin surface results in the time period when blood or urine levels are already undetectable (DE GIOVANNI, FUCCI, 2013).

5.10. Conclusions

The proposed DPX-GC-MS method was successful applied to the detection of all 13 target stimulant drugs in sweat and urine samples with acceptable recovery, accuracy and precision. This method was successfully applied in real case samples. Our results suggest that sweat can be useful as a complementary matrix to perform urine-doping tests. It was verified that all consumed drugs detected in urine were also detected in sweat samples after physical activities.

Chapter 6: Lab-on-chip analysis of sweat samples for prohibited stimulants in sports

Chapter 6: Introduction

Lab-on-a-chip (LOC) devices are an emerging low-cost technology that has been used in numerous applications in forensic analyses such as in-field amphetamine drug testing, explosives detection in soil (LLOYD et al, 2011). The identification of cases of doping during competitive games has become an important area of research and development as rapid analyses are required to deal with the growing number of doping control samples and the short response time required during sport events (WADA, 2012; LAURE, 2006; BERGO, 2007).

LC- and GC- MS are sensitive techniques and can detect prohibited substances, however they are expensive and require extensive processing of samples before analysis and are not available at the site of competitive games. To achieve faster identification of doping at the site of competitive games, analysis via LOC is showing potential (GALLARDO, QUEIROZ, 2008).

Commercial LOC devices such as the Agilent Bioanalyzer microchip capillary electrophoresis system offers good sensitivity, inherent miniaturization of the detection/separation system, low cost, portability, can be easily operated, requires little maintenance, is portable for onsite analysis and only requires small sample volumes with detection limits in the ng/mL. In addition, this device can analyze prohibited substances in several matrices. Recent growth in the area of microchip technologies has improved the 'functionality' and robustness of portable systems, enhancing their capability for high-throughput analyses. Previous studies have demonstrated the use of the Bioanalyser for the analysis of stimulants in various matrices including (LLOYD, et al, 2011; LLOYD, et al, 2013; LLOYD, et al, 2014). The aim of this study was to investigate the suitability of the Bioanalyzer for stimulants screening of sweat samples.

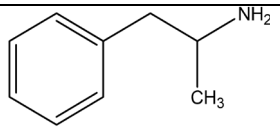
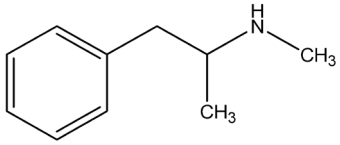
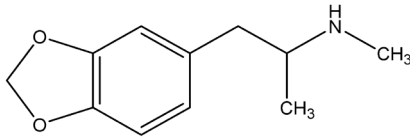
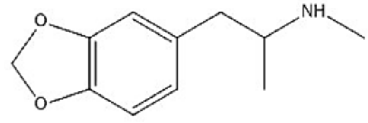
6.1. Materials and Methods

6.1.1. Chemicals and reagents

The following illicit drugs and metabolites were investigated: amphetamine (AMP); methamphetamine (MAMP); N-methyl-3,4-methylenedioxymethamphetamine (MDMA); **3,4-Methylenedioxyethylamphetamine** (MDEA). All standards were purchased from the National Measurement Institute (Sydney, Australia) as 1 mg/mL or 100 mg/mL solutions in acetonitrile or methanol. Table 6.1 lists the chemical structures of the target compounds.

Methanol, acetonitrile, hydrochloric acid, acetone sodium hydroxide, sodium dodecyl sulphate, nile blue chloride, sodium tetraborate, ammonium chloride, lactic acid and Fluorescein isotiocyanate I (FITC) (>90%) were purchased from Sigma Aldrich (Sydney, Australia). All solvents and reagents were of analytical grade.

Table 6.1: Chemical structures of the target compounds

Analyte	Structure
Amphetamine (AMP)	
Mephentermine (MPT)	
3,4-Methylenedioxymethamphetamine (MDMA)	
3,4-Methylenedioxyethylamphetamine (MDEA)	

6.2. Preparation of solutions and reagents

6.2.1. Standard solutions

Standard stock solutions of amphetamine (AMP), methamphetamine (MA), N-methyl-3,4-methylenedioxymethamphetamine (MDMA), 3,4-, and Methylenedioxyethylamphetamine (MDEA) were prepared by appropriate dilution with ultra-pure water in order to obtain the final concentrations of 1, 2 and 10 µg/mL. The solutions were stored at 2°C, protected from light.

6.2.2. Nile blue solution

A concentration of 1 mM nile blue dye was diluted in the running buffer and primed through the micro channels prior to analysis for laser focusing.

6.2.3. Electrolyte preparation

The separation electrolyte consisted of 50 mM sodium tetraborate buffer at a pH of 9.66 with the addition of 50 mM SDS. The electrolyte was mixed, sonicated for 5 minutes and filtered through a 0.2 µm syringe filter prior to injection (Millipore, Billerica, MA, USA).

6.2.4. Fluorescein isotiocyanate I (FITC) stock solution

A 10 mM stock solution of FITC was prepared in analytical reagent grade acetone and stored in a plastic bottle wrapped in aluminium foil at -6 °C.

6.2.5. Artificial sweat solution

Artificial sweat was used to perform the method tests. The artificial sweat solution contained 327 mmol/L ammonium chloride, 166 mmol/L lactic acid, 83

mmol/L urea, 42 mmol/L acetic acid, 34 mmol/L sodium chloride in ultra-pure water and pH was adjusted to 4.7 with 2 mol/L sodium hydroxide.

6.3. Instrumentation

All experiments were performed on an Agilent 2100 Bioanalyzer using the Agilent 2100 Expert software (Agilent Technologies, Waldbronn, Germany) with a fluorescence detection system (LED Blue Emitting Diode (LED-IF), wavelength of excitation (λ_{ex}) 470 nm and emission (λ_{em}) 525 nm. All separations were performed using standard DNA 1000 microchips obtained from Agilent Technologies (Forest Hill, Australia). The chips were fabricated from soda lime glass and have a separation channel length of 15mm, micro-channel depth of 10 µm and width 50 µm. Injection was performed at 1.5 kV for 2 seconds with a separation voltage of 1.5 kV. Table 6.2 shows the retention time of each analyte investigated.

Table 6.2: Retention time of each analyte investigated.

Analyte	Retention time (seconds)
AMP	30
MA	35
MDMA	45
MDEA	40

6.4. Development of a lab-on-chip method for stimulants analysis in sweat samples

The procedure developed for extraction of the analytes in sweat and application of the samples in the microchips is shown in Figure 6.1:

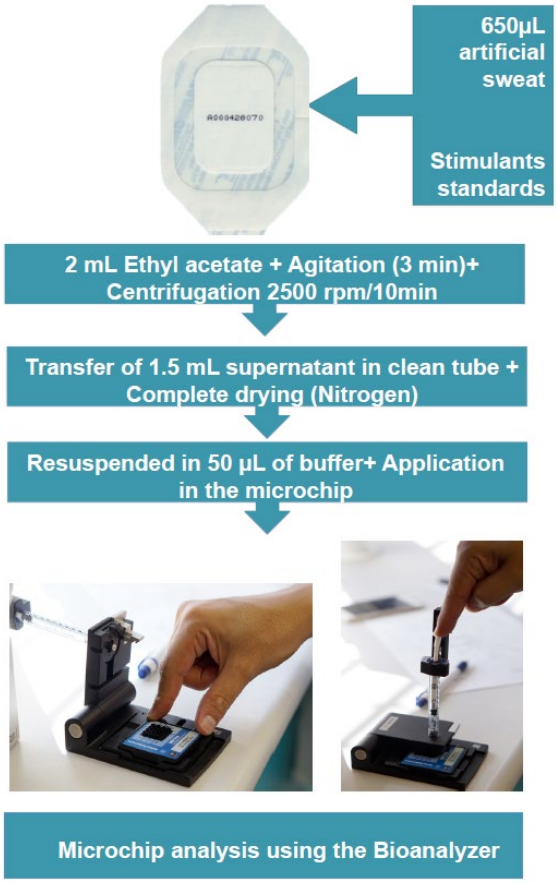


Figure 6.1: Diagram of the analytical procedure for the extraction of stimulants in sweat samples and the application in the Bioanalyzer microchip.

Extraction procedure: patches containing 650 µL of artificial sweat were fortified with standards (AMP, MA, MDMA and MDEA). Once completely dry (15 minutes at room temperature), they were folded and placed in clean conical flasks and subjected to the liquid extraction with 2 mL of ethyl acetate, and then vigorously mixed for 3 minutes using a vortex. 1.5mL of the extract (supernatant) was transferred to a clean conical flask and evaporation to dryness at 40ºC using a nitrogen flow (N2) sample concentrator. After complete evaporation of the solvent, the extract was resuspended with 500µL of run buffer.

Derivatisation procedure with FITC: Derivatisation was performed with FITC (10mM) in which 250 µL of the solution was added to 500 µL of the extracted samples. The mixture was transferred to a suitably sealed 2 mL amber vial and heated for 3 minutes at 90ºC in a dry block. After reaching room temperature, the extract was

diluted in buffer (1:1) and applied to the microchip for analysis.

6.5. Results and Discussion

For the development of the method, the Bioanalyzer separation conditions and the amphetamines derivatisation procedure were optimized to obtain a satisfactory separation and adequate resolution of the peaks. The run time for the analysis was 150 seconds.

6.5.1. Electrolyte solution analysis

To verify the electrophoretic profile of the running electrolyte and possible changes in its composition, two injections were made, one at the beginning (before the analytes) and the other at the end (after analysis). Figure 6.2 below shows the electropherogram obtained when the running electrolyte is injected. No peaks were observed.

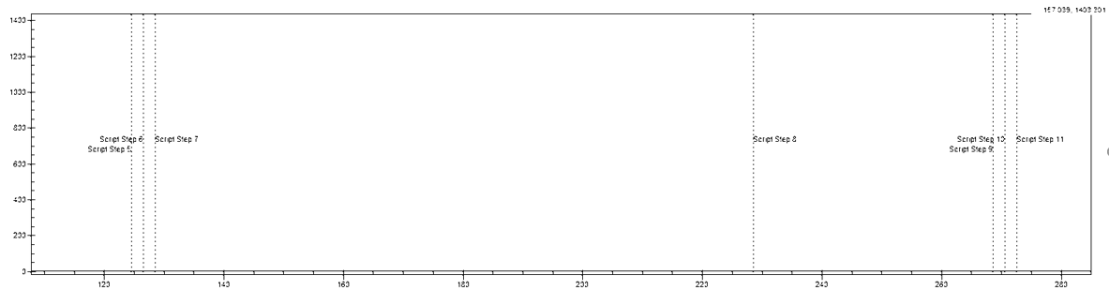


Figure 6.2: Electropherogram of running electrolyte solution using LED-IF (λem 470, λem 525). Conditions: 25□C; injection time 2 seconds; injection voltage 1.5 kV; separation voltage 1.5 kV.

6.5.2. Derivatisation

The derivatisation reaction follows the methods described by Lloyd et al, 2014 and Moser & Hage 2009. In the procedure, fluorescein isothiocyanate (FITC) was used as derivatising agent. FITC is a fluorescein derivative modified with an isocyanate (-N = C = S) reactive group, substituting a hydrogen atom for the lower ring of the structure, figure 6.3. FITC reacts with amine groups of organic molecules

resulting in stable fluorophores. This compound has excitation and emission wavelengths of approximately 495 nm at 521 nm and is therefore compatible with the fluorescence detection system used in Bioanalyzer: the IF-λem = 510-540 nm, λex = 470 nm).

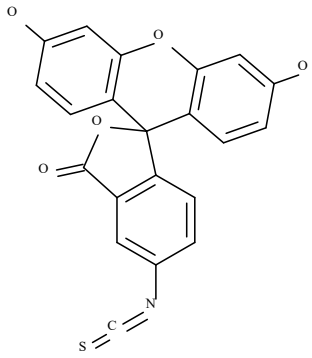


Figure 6.3: Fluoresceine Isothiocyanate (FITC) chemical structure.

In the reaction, the amphetamine amine nucleophile present in the molecule attacks the central carbon of the isocyanate group, resulting in the bond between FITC and the primary amine.

FITC was individually injected in order to determine its migration time. Figure 6.4 below shows the electropherogram of the FITC analysis only.

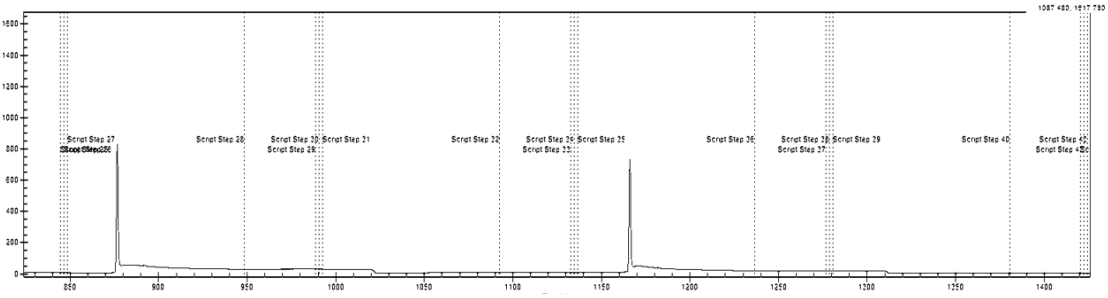


Figure 6.4: Electropherogram of FITC solution using LED-IF (λem 470, λem 525), in two different runs. Conditions: running electrolyte (50 mM SDS plus 50 mM sodium tetraborate, pH 9.66); 25°C; injection time 2 seconds; injection voltage 1.5 kV; separation voltage 1.5kV.

Figure 6.5 below show the electropherogram of the stimulants (AMP, MA,

MDEA and MDMA) derivatised with FITC. All compounds were detected in less than 1 minute, and the peaks showed excellent baseline resolution.

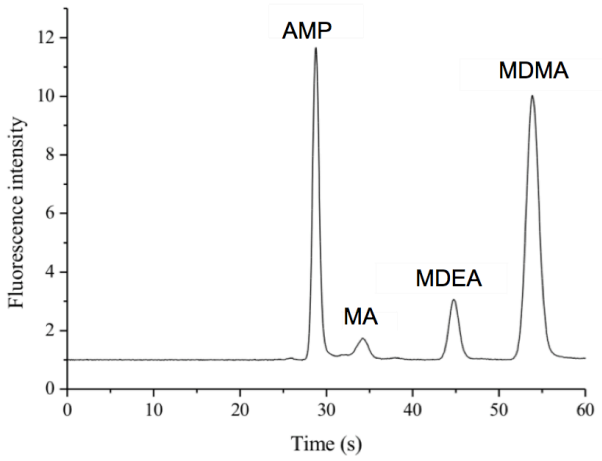


Figure 6.5: Electropherogram of 50µg of derivatised AMP, MA, MDEA and MDMA using LED-IF (λex 470, λem 525). Conditions: running electrolyte (50 mM SDS plus 50 mM sodium tetraborate, pH 9.66); 25°C; injection time 2 seconds; injection voltage 1.5 kV; separation voltage 1.5 kV.

6.5.3. Solvent extractor selection

Although sweat is considered a relatively clean biological matrix, it presents some organic compounds that may interfere the analysis. To reduce the interferences of the matrix and concentrate the analytes; liquid extraction procedures were tested. For the extraction procedure of the analytes on the adhesive, the following organic solvents were tested: ethyl acetate, acetonitrile, dichloromethane and methanol. Each of these solvents was added in separate tubes containing the artificial sweat-containing adhesives and the analytes in question, followed by vortexing, centrifugation, and evaporation of the organic phase. Samples were subjected to the analysis described above. The solvent that presented the best extraction efficiency was ethyl acetate.

6.5.4. Limits of Detection (LOD) and Limit of Quantification (LOQ)

The limit of detection (LOD) is the lowest concentration of an analyte present in the sample that can be detected and identified and is determined by the lowest concentration at which the analytes result in peaks with magnitudes greater than or equal to 3 times that of the noise (signal to noise ratio ≥ 3: 1). The detection limit was determined by analysing duplicate adhesives containing 1, 2, 4, 5, 10 and 15 µg of each of the drugs studied and was the lowest concentration that obtained signal to noise ratio greater than or equal to 3 in both analyses.

The limit of quantification (LOQ) is the lowest concentration at which analytes can be detected and quantified with a signal-to-noise ratio greater than or equal to 10. This parameter was obtained through the analysis of fortified artificial sweat samples with decreasing concentrations of the analytes to the lowest quantifiable level (signal to noise ratio ≥10: 1), with acceptable accuracy and accuracy. The values found for the LOD and LOQ are shown in Table 6.3.

Table 6.3: LODs and LOQs obtained with the Bioanalyzer.

Analyte	LOD (µg/patch)	LOQ (µg/patch)	LOQ Precision (% RSD)
AMP	1.0	1.5	17.5
MA	9.0	10	16.2
MDMA	3.0	5.0	12.7
MDEA	3.0	5.0	13.8

Data present in the literature and international guidelines for drugs of abuse analysis in alternative biological samples indicate that the concentrations of drugs found in sweat of individuals in recreational use situations are in the range of 25 to 1000 ng/patch, that is, 0.0025 to 1 µg/patch; the latter in cases of heavy use of drugs. Table 6.4 below shows cut-off values for drugs in sweat samples recommended by the

Substance Abuse and Mental Health Services Administration in the year 2014 (SAMHSA).

Table 6.4: Drugs of abuse cut-off values proposed by SAMHSA.

Drug/ Class	Drugs in sweat	Cut off in ng/patch
Amphetamines/ metabolities	Amphetamine	25.0
	Methamphetamine	25.0
Cannabinoids	THC	4.0
Cocaine/ metabolities	Cocaine	25.0
	Benzoylecgonine (BE)	25.0
Opiates	Morphine	25.0
	Codeine	25.0

The limits of detection obtained in the Bioanalyzer do not meet the requirements for the analysis of this type of biological sample. In addition, the composition of sweat has some organic components, such as lactate, urea, ammonium ions, amino acids, some enzymes and other organic compounds, which showed interferences with the proposed derivatisation process (KINTZ, SAMYN, 2000; DE MARTINIS, 2008).

6.6. Conclusion

Despite pre-concentrating samples and the advantages presented regarding the use of the Bioanalyzer, the method did not present the necessary sensitivity to detect the very low concentrations of the proposed drugs in sweat samples. Microfluidic devices need improved sensitivity before they can be accepted as routine screening devices for substances of abuse in sweat or an alternative technique should be applied for this type of sample, such as capillary electrophoresis coupled to mass spectrometry.

Chapter 7: One step automated Solid Phase Micro Extraction sample preparation for stimulant drugs analysis

Chapter 7: Introduction

Technological advances of the analytical instruments have improved the selectivity and sensitivity of drug analysis. These techniques include GC-MS that is considered a reference technique for drugs analysis in biological samples. Sample preparation remains a vital step that impacts in the method selectivity and may have limitations that compromise the analysis (KOLE, 2010).

Sample preparation in toxicological analysis is a time-consuming step which generally involves extraction with organic solvents, extract concentration and derivatisation. New instrumental methods are constantly being developed to simplify and improve drug analysis as well as test unconventional biological samples that are becoming more common. Solid-phase micro extraction (SPME) is an effective sampling preparation technique that has enabled miniaturisation, automation and cost reduction. The attraction of SPME is that the extraction is simple, fast, can be done usually without solvents, and detection limits can reach parts per trillion (ppt) levels for certain compounds. SPME is a technique that is considered green, as it is organic solvents free which simplifies the preparation of the sample when compared to conventional approaches. The use of SPME with GC-MS is increasing for drug analysis as the extraction and concentration are included in a single step and the extracts do not require additional procedures of cleaning (DUEIRA, GAUJAC, NAVICKIENE, 2008).

The aim of this study is the analysis of amphetamine-type drugs and cocaine using a fully automatized derivatisation and headspace (HS) SPME extraction methods followed by GC-MS analysis.

7.1. Materials and Methods

7.1.1. Chemicals and Reagents

Ethyl chloroformate (eCF) and acetonitrile used were supplied from Aldrich Chemical Company (Milwaukee, WI). 1 mg of amphetamine (AMP), methamphetamine (MA), N-methyl-3,4-methylenedioxymethamphetamine (MDMA) and cocaine (COC) standards were acquired from National Measurement Institute (Sydney, Australia) as a powder. Standard stock solutions were prepared in acetonitrile at a concentration of 1 mg/mL, then the working solutions were daily prepared by appropriate dilution with acetonitrile. SPME fibre were purchased from Sulpelco (Bellefonte, PA, USA). The fibre examined were poly(dimethylsiloxane) (PDMS, 100µm film thickness), and Polydimethylsiloxane-carboxene-divinylbenzene (DVB/CAR/PDMS) 50/30µm film thickness. The fibres were thermally conditioned as recommended by the supplier.

7.1.2. Solutions

All investigated drug standards were mixed and diluted with acetonitrile to obtain the calibrations solutions at 1.0 and 10 µg/mL. Daily calibration curves from 1 to 1000 ng and quality controls were prepared. The pure derivatisation agent, eCF, was diluted in acetonitrile to obtain a final concentration of 500 µg/mL.

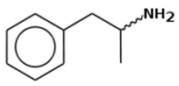
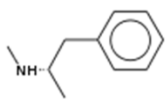
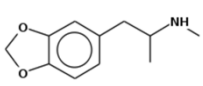
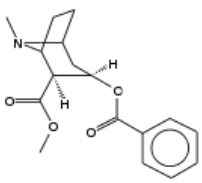
7.2. Solid-Phase Micro Extraction procedure

In 100 µL of sample analytes were derivatised with the addition of 20 µL ethyl chloroformate (500 µg/mL) under basic conditions using 50 µL of borate buffer pH 9 while being simultaneously extracted using Headspace (HS) mode and a SPME fibre. The incubation time was 10 minutes at 65˚C under constant agitation. The fibre conditioning was performed into the injector at 250˚C for 20 minutes daily before the extraction. Total extraction and desorbed time were 20 and 10 minutes, respectively. The SPME extracted sample was desorbed in the injector for 3 minutes in a splitless mode at 250 ˚C (splitless time 1.5 ml/min).

7.3. Instrumentation

GC–MS analyses were carried out using a Thermo Fisher Scientific Trace 1310 GG-TSQ 8000 MS. The SPME desorption was made in a splitless mode 1.5ml/min at 250˚C. A capillary column HP-5MS (30 m x 0.25 mm x 0.25 µm film thickness) from J&W Scientific (Agilent Technologies, USA) was used. The carrier gas used was helium with a flow rate of 1.0 mL/min. The initial column temperature of 90˚C was held for 3 min, ramped to 240˚C at 20˚C/min for 3 min, and increased to a final temperature of 290˚C at 50˚C/min and held for 3 min (total run time of 17.3 minutes). The temperature of the MS transfer line and ion source were both 280˚C. The mass spectrometer was operated in an electron ionization (EI) mode 70eV, positive mode. The retention times and characteristic mass fragments were determined in *Full scan* mode; mass range m/z 50–310 starting after 7 minutes. During the analysis, three characteristic mass fragments were monitored using the Selected Ion-Monitoring (SIM) mode with respect to significant ions for each compound. The dwell time was 0.30 seconds. Table 7.1 presents the ions (m/z) selected for identification to each compound and their respective retention times.

Table 7.1: m/z of ions used to identify the analytes and the respective retention times.

Compound	Structure	Quantifier (m/z)	Qualifier (m/z)	Retention times (Minutes)
Amphetamine (AMP)		116.1	91.1; 71.1	8.91
Methamphetamine (MA)		130.1	91.1; 102.1	9.09
3,4-Methylenedioxymethamphetamine (MDMA)		130.1	102.1; 135.1	11.07
Cocaine (COC)		182	82; 303	13.45

7.4. Data analysis

Compounds were identified by comparing retention times (±2%) and qualifier ion ratios (±20%) with the corresponding average values of calibrators assayed. Peak areas of the target analytes were calculated using the software supplied by the manufacturer (Chromeleon). Calibration was conducted by linear regression analysis.

7.5. Method validation

The method validation was examined by determining the linearity, recovery, reproducibility; limit of detection and limit of quantification for all target compounds. Table 6.1 shows the method validation results of the target analytes after the HS-SPME extraction.

7.5.1. *Limits of detection (LOD) and limits of quantification (LOQ)*

The LODs and LOQs were determined based on the signal-to-noise ratio of at least 3:1 and 10:1, respectively. The experiments were performed adding aliquots of the target standards before extraction in decreasing concentrations, in triplicate.

7.5.2. *Linearity*

Linearity was determined using five calibration points corresponding to the concentrations of 10 to 1000 ng/ml, using SIM mode. Linearity was evaluated by the method of least squares and expressed as coefficient of determination (R^2). The calibration curves for each analyte obtained using the PDMS fibre are presented below in Figure 7.1.

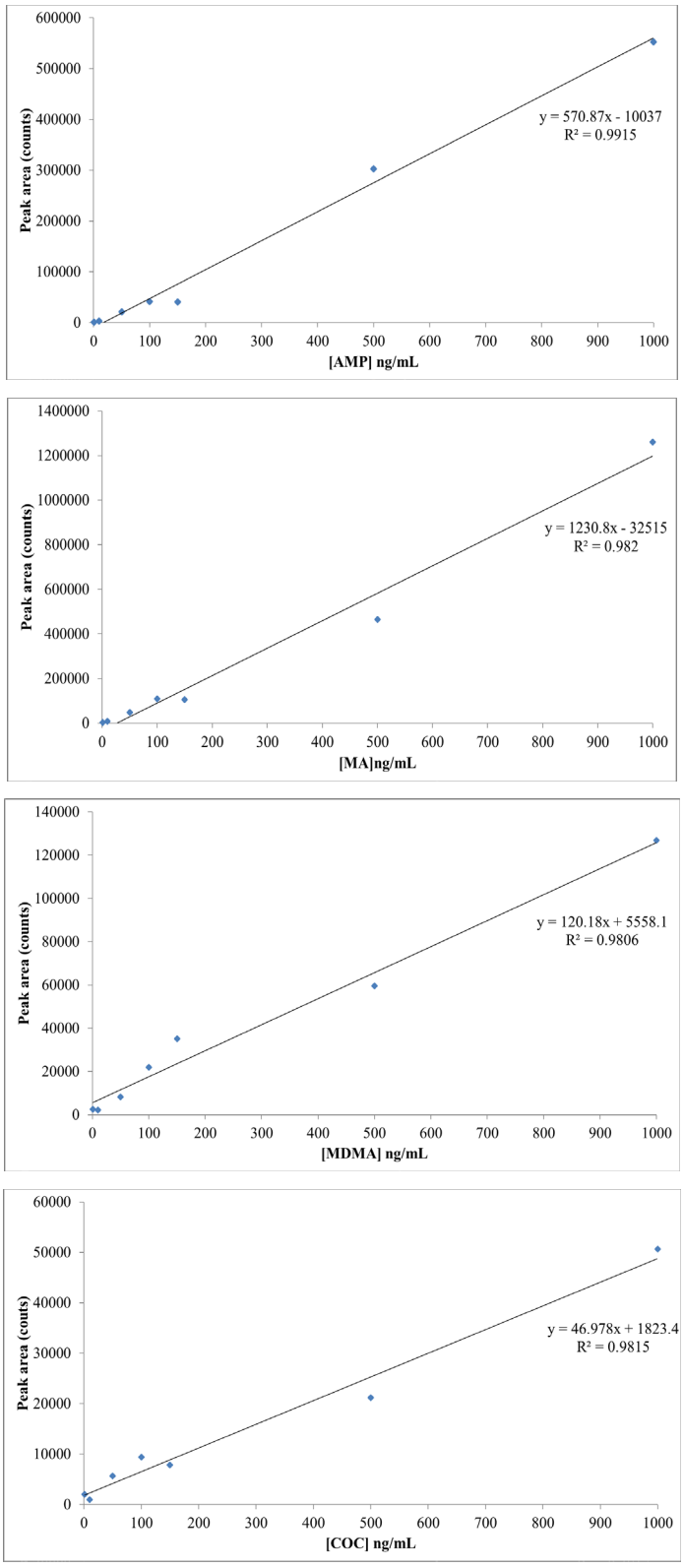


Figure 7.1: Calibration curves obtained for all investigated analytes (AMP, MA, MDMA and COC) after the headspace SPME extraction and GC-MS analysis.

7.5.3. Precision and accuracy

Precision and accuracy were determined using three QCs, covering the range adopted in the calibration curve, in triplicate. The concentrations of QC were 50 ng/mL (QC1), 250 ng/mL (QC2) and 750 ng/mL (QC3) for all the analytes. Accuracy was determined by comparing measured concentrations with target values over runs and expressed as a percent of the target concentration.

7.5.4. Recovery

The recovery was determined for each analyte through the comparison between the liquid injection responses after derivatisation and the responses samples after SPME extraction.

7.5.5. Carry over

To assess the carry over effect, extracts of blank samples were analysed immediately after the analysis of the sample extract fortified with highest point in the calibration curve.

7.6. Results and Discussion

The SPME parameters that required optimization included extraction pH, ionic strength of the sample, extraction and desorption times. The simultaneous extraction and derivatisation of the analytes showed the improvement of the extractability of amphetamines and permitted their determination by GC–MS. The limits of detection ranged from 0.1ng/mL and 1ng/mL; the limit of quantification was 10 ng/mL. The method was linear resulting in R of 0.99 for all analytes with a dynamic range of 10 to 1000 ng/mL. HS-SPME recoveries ranged from 54–74% with extraction relative standard deviations (RSD) lower than 20%, showing that the results are consistent

with the literature.

7.6.1. Optimization of SPME

The typical fibre coating used in the literature is PDMS. Two SPME fibres of different polarities were investigated, PDMS (non-polar) and PDMS-CAR-DVB (bipolar). All the experiments were performed in the HS-SPME and it was carried out in triplicate for each fibre. Figure 7.2 below shows the comparison of the relative response obtained with the two different SPME coatings (n=3) after the extraction procedure.

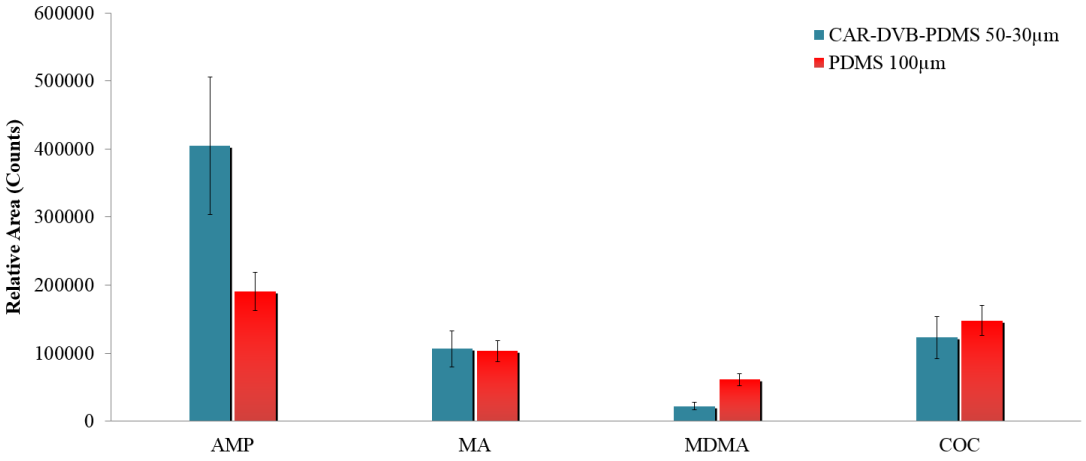


Figure 7.2: Comparison of the relative response obtained with the two different SPME coatings CAR-DVB-PDMS and PDMS (n=3) after the extraction procedure.

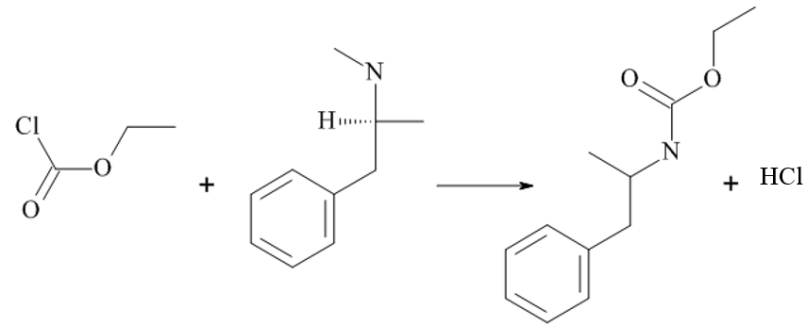
As shown in Figure 7.2, PDMS fibre produced the best results, yielding similar areas for all analytes apart of AMP. For that compound, the CAR-DVB-PDMS fiber produced a 10% higher response than PDMS.

7.6.2. Optimization of SPME parameters

Experiments were carried out to evaluate the effect of derivatisation agent volume, NaCl and buffer.

7.6.2.1. Derivatisation

Most derivatisation reactions use non-polar reagents that require organic solvents and are not directly compatible with aqueous samples. Contrary to this fact, chloroformates reagents such as ethyl chloroformate (eCF) can be used to convert amines into carbamates in a reaction that occurs rapidly, even at room temperature.



Ethyl choroformate (eCF) | Methamphetamine (MA) | MA-EtC

Figure 7.3: Methamphetamine derivatisation reaction.

Three different volumes of eCF (500 µg/mL) 10, 20 and 30µL were examined to determine the optimal derivatisation yield. The derivatisation was performed by adding eCF to 100 µL of the mixed standard, followed by heating the solution for 10 minutes at 65˚C. Initially liquid injections were performed to evaluate the eCF reagent quality and its derivatisation capacity. The GC-MS parameters used were cited above in the item 7.4.

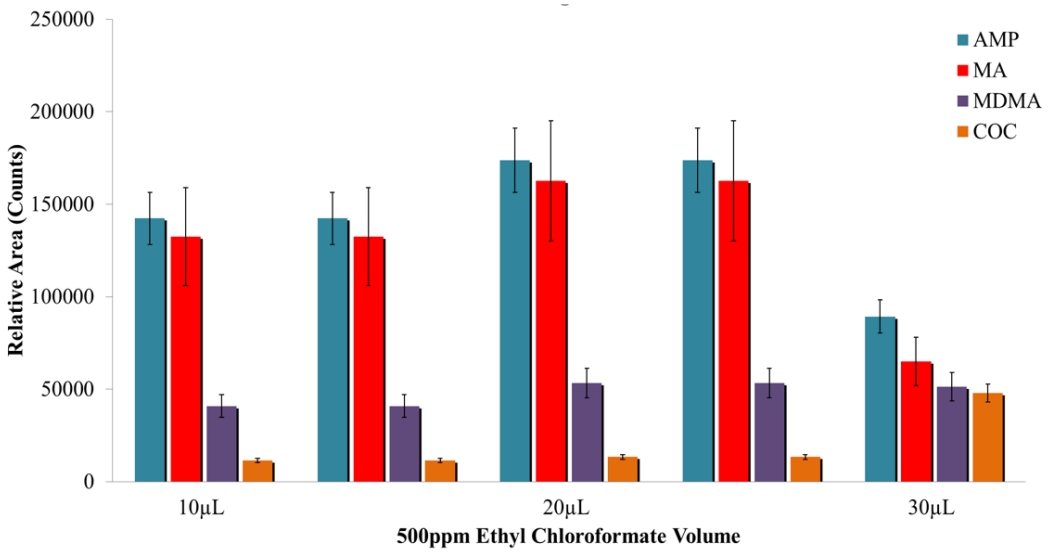


Figure 7.4: Effect of the ethyl chloroformate (500 µg/mL) volume on the analytes response after PDMS-SPME extraction (n=3).

As shown in Figure 7.4, 20 µL of eCF (500 µg/mL) produced the best results, yielding similar areas for all analytes but AMP. For that compound, the CAR-DVB-PDMS fiber produced a 10% higher response than PDMS.

7.6.2.2. Salting out effect

The addition of an insoluble salt into the sample increases the ionic strength of the sample solution. The addition of NaCl in two concentrations was investigated to verify the salting out effect to the tested analytes. The obtained results are shown in the Figure 7.5 bellow.

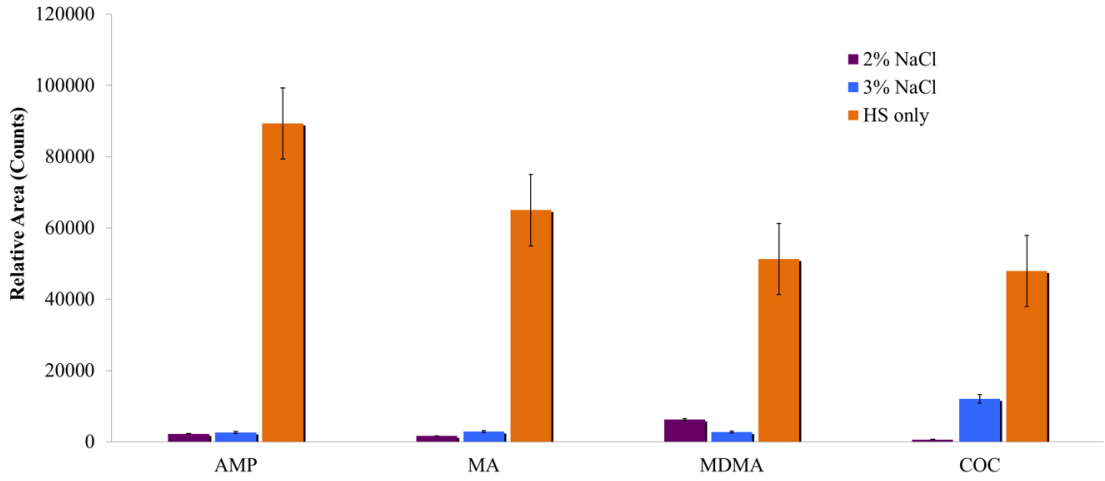


Figure 7.5: Salting out effect in the concentrations of 2 and 3% of NaCl on the analytes response after PDMS-SPME extraction (n=3).

For most of the organic compounds, aqueous solubilities decrease when salt was added to the sample, except for high polar compounds. With the decreased aqueous solubility, the extractability increased and consequently the method sensitivity is improved. However, in some cases, an increase in ionic strength of the sample solution improved the extraction efficiency for both the target and interfering compounds. The addition of salt did not present a positive response on the tested analytes. The ionic strength showed reduction the analytes SPME extraction; this can be attributed to the lower vapour pressure of these analytes. The combination of NaCl and high temperatures in HS extractions resulted into the SPME fibre degradation after some experiments, reducing fibre life and leading to lower reproducibility.

7.6.2.3. pH effect

SPME coatings extract the neutral species of analytes, its extraction efficiency is enhanced by the conversion of analytes into neutral forms. This can be achieved by buffer addition to adjust the sample pH. For basic compounds, the extraction efficiency is improved with high pH values. Two different buffers at 100mM (Borate and Phosphate) were used to perform these tests. The obtained results are shown in the Figure 7.6 bellow.

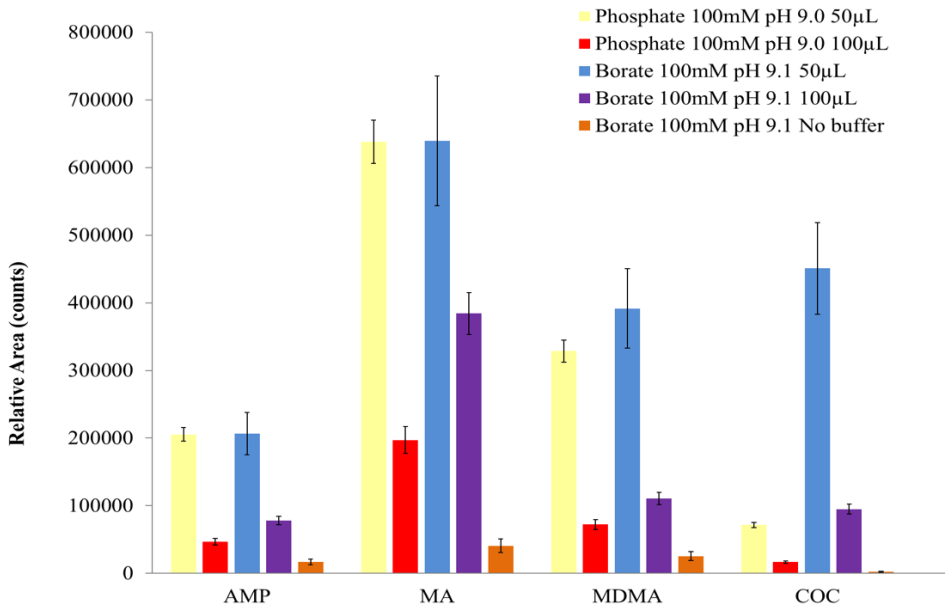


Figure 7.6: Buffer addition effects on the analytes response after SPME extraction (n=3).

The addition of 50 µL of borate 100mM showed positive response for all tested analytes. A basic condition, pH higher than 9, catalyses the derivatisation reaction. The buffer addition showed an increase the basic catalyzer concentration and the salting-in effect, due to the increase of ions in solution.

7.6.3. Method Validation

Figure 7.7 shows the chromatographic separation of a sweat sample fortified with 100 ng/mL of all the analytes. Table 7.2, the results of analytical validation of the HS-SPME/GC-MS method are presented.

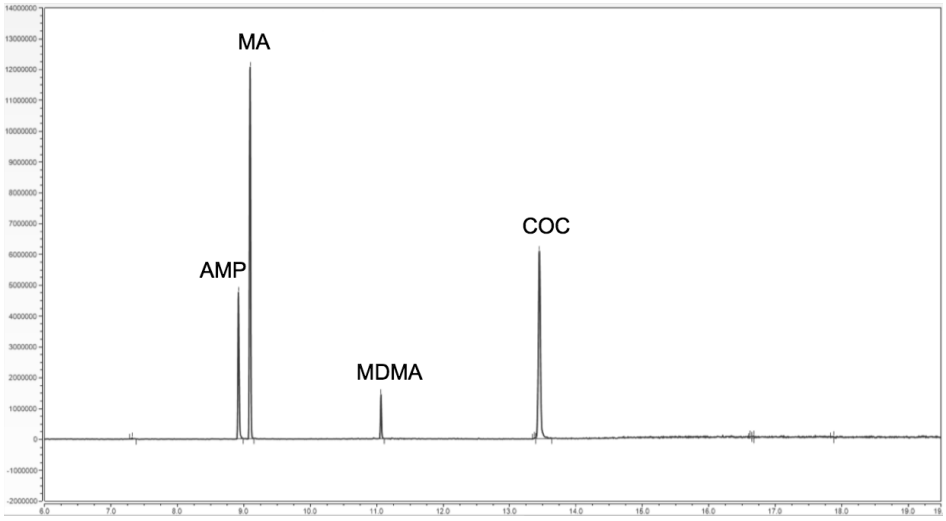


Figure 7.7: Selected ion chromatographic separation obtained from a 100 ng/mL mixed standards injection (AMP, MA, MDMA, COC) after the derivatisation procedure and HS-SPME (PDMS) extraction.

Table 7.2: Analytical validation results of the HS-SPME/GC-MS method for the stimulants analysis.

Analyte	**R^2**	**LOD (ng/mL)**	**LOQ (ng/mL)**	***Quality controls (QC)***	***Intra-assay precision (%)***	***Accuracy (%)***	***Recovery (%)***
AMP	0.99534	1.0	10.0	50	17.35	84.0	56.0
				250	16.78	129.0	68.0
				750	11.12	81.7	64.0
MA	0.99807	0.1	10.0	50	16.95	120.0	71.0
				250	15.70	110.0	60.0
				750	14.70	93.0	69.6
MDMA	0.99734	0.1	10.0	50	13.24	96.3	64.7
				250	6.35	98.0	68.8
				750	12.29	115.0	54.0
COC	0.99453	1.0	10.0	50	18.90	71.0	59.8
				250	9.75	84.0	61.5
				750	11.90	77.0	66.0

The method showed acceptable selectivity and sensitivity for the analytes examined. The simultaneous extraction and derivatisation of the analytes showed the improvement of the extractability of amphetamines and permitted their determination by GC–MS. The limits of detection ranged from 0.1ng/mL to 1ng/mL; the limit of quantification was 10 ng/mL, LOD and LOQ values were considered appropriate for the analysis. The developed method also presented acceptable linearity, supported by the correlation coefficient (R^2) values greater than 0.98 in the analysis of the range of 10 to 1000 ng/mL.

The precision values are considered acceptable within the range of up to 15%, and accuracy between 80 and 120%. The assays of precision results are within the acceptable range up to 15% for QC1 and up to 20% for QC2 and QC3. HS-SPME recoveries ranged from 54–74% with extraction relative standard deviations (RSD) lower than 20%, showing that the results are consistent with the literature.

The carry-over effect was tested by performing a blank analysis after the high concentration (750 ng/mL) analysis; no traces of the analytes were detected in the blank desorption, being evident that 3 minutes are enough for complete desorption of the analytes. However, SPME fibres were desorbed for three additional minutes before the next extraction, in order to assure the elimination of remaining analytes on the fibre.

7.7. Conclusion

The HS-SPME/GC-MS method was able to determine low concentrations of the target analytes, ranging between 0.1 to 1 ng/mL of analytes. The proposed method is a fast and reliable alternative to the standard methods used in toxicology analysis of amphetamine-type related substances and cocaine. This method can be conducted simultaneously with headspace SPME and GC-MS without the need for dissolution and chemical processing, presenting potential application for drug traces in biological samples.

Chapter 8: Conclusions and Future Research

The work presented in this thesis provided an approach to doping investigations and addressed a variety of aspects including health concerns due substance abuse and development of methods for evaluation of sweat as an alternative biological matrix for the doping control.

The identification of doping cases is an increasingly important area of research and development due to the continued rise in the number of prohibited substances. Furthermore, there is a strong need for sensitive, selective and portable screening techniques in this field. Toxicological analysis plays a significant role in the investigation of sport drug-related cases. These analyses provide crucial information for investigations and a broader understanding of drug abuse in sport.

Through toxicological urine analysis, self-reporting and the haematological and biochemical parameters measured within, the study revealed that the use of doping agents and drugs were subject to different factors, which were normally guided by type of physical activity or aesthetic appearance. Furthermore, the results also showed that the supratherapeutic AAS use in combination with multidrug abuse for long periods can potentially harm multiple organs causing synergistic side effects, increasing the risk of disease development. The variety of pathophysiologic mechanisms also suggested an increased risk of kidney diseases and cardiovascular damage.

Our findings corroborate previous findings of AAS and multiple drug abuse. This abuse has potentially serious public health concerns as it has serious implications due the direct influence on physiological capacity by removing endurance and psychological barriers, leading to potentially irreversible health damage. Furthermore, there needs to be ongoing research to investigate trends in AAS abuse and multiple drug use. Future research should focus on the long-term, intermittent and withdrawal aspects, as well as on the long-term health effects of recalcitrant abusers. In addition, investigation of the alterations of the reproductive, cardiovascular, hepatic and renal systems, as well as psychological and behavioural alterations, are required.

A full DPX-GC-MS method determined the presence of 13 amphetamines and cocaine related substances and their metabolites in sweat and urine with acceptable selectivity, sensitivity, LODs and LOQs. The validated method was applied to the analysis of urine and sweat samples of 40 professional and non-professional athletes. It was verified that all consumed drugs and metabolites detected in urine were also

present in sweat samples indicating that sweat was a viable matrix to perform doping tests.

We also developed an alternative screening method for stimulants detection in sweat samples using a microchip capillary electrophoresis instrument (Agilent Bionalyzer). Although functioning, the method was not sensitive enough to detect the low concentrations of drugs and metabolites present in sweat samples. Despite the pre-concentrating step the obtained LOD ranged from 1 to 9 µg/patch.

A one step fully automatized derivatisation and headspace (HS) SPME extraction method followed by GC-MS was developed for the analysis for amphetamine-type drugs and cocaine. The HS-SPME/GC-MS method was used to detect concentrations between 0.1 to 1 ng/mL of the target analytes without any additional sample preparation, suitable for routine analysis of drug traces in biological samples, such as urine and sweat.

Urine is the standard matrix used in doping control; however, the results presented in this thesis has shown that sweat can be a useful alternative matrix to provide additional information in a short period of time, as well as complementary results obtained from urine for stimulants doping analysis. In addition, this matrix has less chance of sample tampering, greater detection windows, non-invasive collection, and the possibility of finding the parent drugs. The use of sweat as an alternative sample also allowed documentation of the history of drug abuse. Sweat analysis can also be an option to identify steroid abuse. There is a requirement for further long-term and additional research in this area such as the diversity of sweat glands, the variation of local metabolisms, threshold determinations, and quantitative data for the potential application of sweat for doping control analyses.

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APPENDICES

School of Pharmaceutical Sciences of Ribeirão Preto Ethical Committee Approval

UNIVERSIDADE DE SÃO PAULO
Faculdade de Ciências Farmacêuticas de Ribeirão Preto
Comitê de Ética em Pesquisa

Of. CEP/FCFRP nº. 048/2014
kmz

Ribeirão Preto, 22 de outubro de 2014.

À pós-graduanda
Dayanne Cristiane Mozaner Bordin
Orientador: Prof. Dr. Bruno Spinosa de Martinis
FCFRP-FFCLRP/USP

Prezada Pós-graduanda,

Informamos que o Comitê de Ética em Pesquisa da FCFRP aprovou, em sua 135ª Reunião Ordinária realizada em 03/10/2014, o projeto de pesquisa intitulado **"AVALIAÇÃO DO SUOR COMO AMOSTRA ALTERNATIVA NO CONTROLE DO DOPING NO ESPORTE"**, apresentado por Vossa Senhoria a este Comitê, Protocolo **CEP/FCFRP nº. 333.**

Lembramos que, de acordo com a Resolução 466/2012, item IV.5, letra d, o TCLE deverá "ser elaborado em duas vias (uma para o pesquisador e outra para o participante da pesquisa), rubricadas em todas as suas páginas e assinadas, ao seu término, pelo convidado a participar da pesquisa, ou por seu representante legal, assim como pelo pesquisador responsável, ou pela(s) pessoa(s) por ele delegada(s), devendo as páginas de assinaturas estar na mesma folha. Em ambas as vias deverão constar o endereço e contato telefônico ou outro, dos responsáveis pela pesquisa e do CEP local".

Informamos que deverá ser encaminhado ao CEP/FCFRP o relatório final da pesquisa em formulário próprio deste Comitê, bem como comunicada qualquer alteração, intercorrência ou interrupção do mesmo, tais como eventos adversos e eventuais modificações no protocolo ou nos membros da equipe, através da interposição de **emenda** na Plataforma Brasil.

Atenciosamente,

Production Note:
Signature removed prior to publication.
PROFª. DRª. CLENI MARA MARZOCCHI MACHADO
Coordenadora do CEP/FCFRP

Avenida do Café S/N: - Monte Alegre – CEP 14040-903 – Ribeirão Preto – SP
Comitê de Ética em Pesquisa – cep@fcfrp.usp.br
Fone: (16) 3602-4213 ou 3602-4216 – Fax: (16) 3602-4892

CONSENT TERM

RESEARCH TITLE: "Evaluation of sweat as an alternative sample in the control of doping in sport."

RESPONSIBLE RESEARCHERS:
Responsible Researcher: PhD Dayanne Mozaner Bordin, e-mail: daybordin@fcfrp.usp.br
Coordinator: Prof. Dr. Bruno Spinosa De Martinis - CRQ N ° 04.100.360 e-mail: martinis@usp.br

RESEARCH PROMOTERS
Department of Clinical Analysis, Toxicology and Food Science, School of Pharmaceutical Sciences of Ribeirão Preto; and Department of Chemistry, Faculty of Philosophy, Sciences and Letters of Ribeirão Preto - University of São Paulo – USP.

You are being invited to volunteer for a survey. You will receive all the information and clarification. If you agree to be a participant of this survey, please sign the two copies of this document. One copy is yours and the other copy is for the responsible researcher. Your participation is not compulsory. You may opt out of the survey at any time. You will not be harmed and have the right to ask whatever you want during the survey. You will not pay anything and neither will you receive any additional money or benefit.

RESEARCH AIMS

In an attempt to overcome opponents and to improve physical ability and/or appearance, professional and non-professional athletes, resort to illegal means to improve their performance. The drug use in sport is prohibited. In addition to the ethical aspects, drug use can cause serious damage to health by directly influencing physiological capacity and removing physical and psychological barriers, and their health damage may be irreversible. The objective of this research is to investigate the possibility of using sweat as an alternative for doping control and to evaluate the alterations in the biochemical and hematological parameters associate with these substances. Three steps are involved: 1—the administration of a self-completion questionnaire; 2—the assessment of hematological and biochemical parameters and; 3—toxicological analysis.

RESEARCH PROCEDURES

If you agree to be a participant of this survey you will be invited to answer a self-completion questionnaire, which was constructed specifically for this study. The interview will take between 10 and 15 minutes to be complete. You will receive a universal collector to collect your urine sample; that must be delivered to the researcher. For collection of the sweat samples, two PharmCheck® patches will be applied on the back region. Before sampling, the skin will be wiped with 2-propanol. The patches will be removed after you carried out any physical activity (up to 24 hours of use). These adhesive devices are 10cm x 5cm in size, serve exclusively to absorb sweat. These samples will be examined for the presence of anabolic and stimulant substances. If you agree, after this procedure you will be invited to perform

biochemical and hematological clinical exams on ___ / ___ / ___, time __: __. You will receive an informational leaflet with all the guidelines to ensure that the procedure is performed safely and effectively.

RISKS

Collecting urine will not bring any predictable discomfort or risk to you. The adhesive for sweat collection is appropriate not to cause allergies, there are no reports of allergic reactions or any damage to the skin. The possible discomfort that may occur is discomfort and/or irritation in the region where the patch is placed. There is no need for epilation of the application site. If you feel any discomfort, you can remove the adhesive at any time, without any penalty.

BENEFITS

The information and samples obtained will be very useful to develop and evaluate a laboratory method that may be proposed for future implantation for the doping analysis. In addition, the participant's assessment of the participant's biochemical and hematological parameters provides important data regarding his / her health and physiological conditions.

CONFIDENTIALITY

Your information is confidential. A number will identify you. The information obtained in the interview, the result of the analysis on the presence or absence of the substances in the urine and sweat samples will be kept secret. You will not be identified in any work that may result from this research. At the end of the research, if you are interested, I will be able to make available the results obtained in the analysis of your samples, and if there are any unexpected results you will be contacted immediately and informed about them.

PROBLEMS OR QUESTIONS

If you have any questions or any doubts about this research, please contact me Dayanne Mozaner Bordin or Prof.Dr. Bruno Spinosa De Martinis by phone: (16) [redacted] (16) [redacted] or address Avenida Bandeirantes, 3900, Monte Alegre, Ribeirão Preto-SP. Or contact the Research Ethics Committee of the School of Pharmaceutical Sciences at: (16) 3602-4213, or address Avenida do Café no number, Monte Alegre, Ribeirão Preto-SP.

THESE ARE YOUR RIGHTS DURING THE RESEARCH PARTICIPATION (NATIONAL COUNCIL RESOLUTION 466/12 OF THE NATIONAL HEALTH COUNCIL / MINISTRY OF HEALTH):

1- At any time (before, during and after the research), the guarantee to receive the answer to any question or clarification of any doubt about the procedures, risks, benefits and others related to the research;

2- The freedom to withdraw their consent at any time and to stop participating in the study without any prejudice;

3- The security of not being identified and the confidential nature of all information related to your privacy;

4- The commitment to receive updated information during the study, even if it affects their willingness to continue participating;

5- The availability of medical treatment and indemnification to which you are legally

entitled, in case of justified damages, caused directly by the research;
6- If there are additional expenses, these will be absorbed by the research budget.

SIGNATURES

If you have read the consent term and you have understood the information and voluntarily agree to participate of this study, please sign below:
Term of Consent:
I, (Mr/Ms)_____,
having received information about the research and my rights above, hereby declare that I am aware and duly informed and that I agree to participate in this study.
Ribeirão Preto, ____ / _____ / _____

_____
Volunteer signature

I've explained the purpose of this study to the volunteer. I am sure he understood the purpose, procedures, risks and benefits of this study.

_____
Responsible Researcher - Dayanne C Mozaner Bordin -RG: 403731653

"This project was approved by the Research Ethics Committee of the School of Pharmaceutical Sciences of Ribeirão Preto - CEP/FCFRP. In case of doubts about your rights as a research volunteer, please contact the Committee: Av Monte Alegre, no number, FCFRP/USP, CEP 14040-903, Ribeirão Preto-SP. Phone +55 (16) 3602-4213; E-mail: cep@fcfrp.usp.br "

STUDY PROTOCOL: SELF-QUESTIONNAIRE

DATE: _____ / _____ / _____ SAMPLE NUMBER: _____
PHARMA CHECK CODE NUMBER: _____

NAME: _____
CONTACT (e-mail / telephone):_____
ADDRESS:_____
DATE OF BIRTH: _____ / _____ / _____
GENDER ▢Male ▢Female
Weight _____
Height _____
Percentage of fat_____

1. CIVILITY STATEMENT

▢Married ▢Single ▢Divorced ▢Widower

SCOLARITY
▢Primary and secondary education
▢ High school
▢Graduate
▢Undergraduate
▢Post graduate

2. TYPE OF PHYSICAL ACTIVITY PERFORMED
▢Run
▢Cycling
▢Bodybuilding
▢Fight
▢Swimming
▢Dance
▢Soccer
▢Other _____

3. DO YOU USE DIETARY SUPPLEMENTS?
▢No (go to question number 9) ▢Yes WHICH / WHAT?

▢Whey Protein
▢BCAA
▢Creatine
▢Albumin
▢Glutamine
▢Multivitamins
▢Pre workout
▢Thermogenic
▢Casein
▢Others_____
_____

4. HOW OFTEN DO YOU USE DIETARY SUPPLEMENTS?
▢Daily
▢Weekly
▢ Up to 3 times per week
▢3 to 6 times a week
▢ Fortnightly
▢Monthly
▢Other _____

5. DO YOU USE DIETARY SUPPLEMENTS FOR WHAT REASON?
▢Muscle mass gain
▢Stimulant
▢Strength gain
▢Weight/fat loss
▢Other _____

6. DID YOU RECEIVE ANY INFORMATION ABOUT THE BENEFITS RELATED TO THE USE OF THESE DIETARY SUPPLEMENTS? WHICH / WHAT?
▢No

▢Yes _____

7. DID YOU RECEIVE ANY INFORMATION ABOUT THE DAMAGES/RISKS OF THE USE OF THESE DIETARY SUPPLEMENTS? WHICH / WHAT?
▢No

▢Yes _____

8. DID YOU FEEL ANY DISCOMFORT DURING THE USE OF SUPPLEMENTS? WHICH / WHAT? (Check all that apply to you)
▢No

▢Yes

▢Nausea

▢Anxiety

▢Depression

▢Altered libido

▢Insomnia

▢Vomiting

▢ Headache
▢Irritability
▢Aggressiveness
▢Increased libido

❑Reduced libido
❑Tachycardia
❑Stomach-ache
❑Increased appetite
❑Reduced appetite
❑Hyperactive Reflections
❑Confusion
❑Hypertension
❑Hypotension
❑Dry mouth
❑Diarrhoea
❑Cramps
❑Skin changes
❑Acne
❑Muscle Weakness
❑Other(s) _____

9. HAVE YOU USED ANDROGENIC ANABOLIC STEROIDS? WHICH / WHAT?
❑No (go to question number 15)

❑Yes
❑Testosterone
❑Molesterolone (Proviron)
❑Metandrostenolone / Metandienone (Dianabol)
❑EnantatoMetenolone (Primobolan)
❑Nandrolone Decanoate (Deca-durabolin)
❑Nandrolone (Durabolin)
❑Genenona (Equipeise / Equifort)
❑Clostebol (Trofodermin)
❑Stanozolol (Winstrol / Wistrol V / Estrombol / Stanzol)
❑Trenbolone (Fina)
❑Oximetolone (Anadrol-50 / Hemogenin)
❑Oxandrolone (Anavar)
❑Fluoxymesterone (Halotestin)
❑Mibolerone (Drops Check)
❑4-Chlorodehydromethyltestosterone (Turinabol)
❑Other(s) _____

10. WHEN WAS THE LAST TIME YOU HAVE USED ANDROGENIC ANABOLIC STEROIDS?
❑1 day ago
❑2 to 6 days ago
❑Last week
❑15 days agos
❑Last month
❑3 months ago
❑6 months ago

▢1 year ago
▢More than one year
▢Other _____

11. HOW OFTEN DO YOU USE ANDROGENIC ANABOLIC STEROIDS?
▢Daily
▢Weekly
▢ Up to 3 times per week
▢3 to 6 times a week
▢ Fortnightly
▢Monthly
▢Other _____

12. WHAT DOSAGE OF ANDROGENIC ANABOLIC STEROIDS IS ADMINISTERED?

_____

_____

13. DID YOU FEEL ANY DISCOMFORT DURING THE USE OF ANDROGENIC ANABOLIC STEROIDS? WHICH / WHAT? (Check all that apply to you)
▢No

▢Yes

▢Nausea

▢Anxiety

▢Depression

▢Altered libido

▢Insomnia

▢Vomiting

▢ Headache
▢Irritability
▢Aggressiveness
▢Increased libido
▢Reduced libido
▢Tachycardia
▢Stomach-ache
▢Increased appetite
▢Reduced appetite
▢Hyperactive Reflections

▢Confusion
▢Hypertension
▢Hypotension
▢Dry mouth
▢Diarrhoea
▢Cramps
▢Skin changes
▢Acne
▢Muscle Weakness
▢Testicular atrophy
▢Menstrual cycle change
▢Loss of hair
▢ Hair growth
▢Other(s) _____

14. WAS THERE DOCTOR PRESCRIPTION FOR THE ANDROGENIC ANABOLIC STEROIDS USE?
▢No ▢Yes

15. DO YOU USE AAS FOR WHAT REASON?
▢Muscle mass gain
▢Stimulant
▢Strength gain
▢Weight/fat loss
▢Other _____

16. HAVE YOU SMOKED CIGARETTE?
▢No (go to question number 17) ▢Yes

17. WHEN WAS THE LAST TIME YOU SMOKED CIGARETTE?
▢1 day ago
▢2 to 6 days ago
▢Last week
▢15 days agos
▢Last month
▢3 months ago
▢6 months ago
▢1 year ago
▢More than one year
▢Other _____

18. HOW MANY CIGARETTS DO YOU SMOKE PER DAY?
▢1-5 cigarettes/day
▢5-10 cigarettes/day
▢10-20 cigarettes/day
▢More than 20 cigarettes/day

19. HAVE YOU USE ANY DRUGS IN YOUR LIFETIME? WHICH ONE HAVE YOU USED?

▢No (go to question number 20) ▢Yes

▢Alcohol
▢Marijuana
▢Cocaine
▢Crack
▢LSD
▢Ecstasy
▢MDMA
▢Solvents
▢Other(s) _____

20. WHEN WAS THE LAST TIME YOU TAKEN THIS DRUG?
▢1 day ago
▢2 to 6 days ago
▢Last week
▢15 days agos
▢Last month
▢3 months ago
▢6 months ago
▢1 year ago
▢More than one year
▢Other _____

21. HOW OFTEN DO YOU USE THIS/THESE DRUG (S)?
▢Daily
▢Weekly
▢ Up to 3 times per week
▢3 to 6 times a week
▢ Fortnightly
▢Monthly
▢Other _____

22. HAVE YOU TAKEN ANY MEDICATION IN THE LAST MONTH? WHICH/WHAT?
▢No ▢Yes

_____

_____

VOLUNTEER INFORMATION FOR BLOOD AND URINE COLLECTION

Responsible Researcher: PhD Dayanne Mozaner Bordin

If you have any questions please do not hesitate to contact us

BLOOD COLLECTION: blood collection procedure is simple and presents low risk. Complications of routine blood collection are rare and usually small. There is a slight discomfort from the puncture that will go away within a few days, a red area and even a bruise (purple), and small blood loss from the vein at the puncture site may occur, and in rare cases infection occur.

- ✓ You must go tho the blood collection in the morning, in FASTING for at least 12 hours;
- ✓ You can drink water;
- ✓ The dinner of the day before of the collection should be light (avoid fried food and other fatty foods);
- ✓ Avoid drinking alcoholic beverages the day before the collection;

URINE COLLECTION

1) Wash your hands with soap and water prior to collection.

2) Hygiene of the genital area with plenty of soap and water (do not use antiseptic).

3) URINE should be collected in a bottle provided by the researcher, on the morning of the day destined to the delivery of the material.

4) Deliver the urine on the day and time marked to remove the sticker.

List of tests to be performed: blood glucose, blood lipids, creatinine, uric acid, hepatic enzymes, complete blood count, total and free testosterone, urea, sodium, potassium, Urine 1.