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NEW ANALYTICAL METHODOLOGIES FOR DOPING CONTROL

DETECTION OF ANABOLIC ANDROGENIC STEROIDS IN HUMAN URINE

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ABSTRACT

The use of anabolic androgenic steroids (AAS) and other banned substances to enhance athletic performance has important health and social implications. The AAS are a major group included in the prohibited list of the world anti-doping agency (WADA) as well as of major sports authorities. This class of drugs, along with other anabolic agents, represent 64,9 % of all adverse analytical findings reported by WADA accredited laboratories, as stated in the WADA statistic report for 2009. The AAS are a class of hormones that include the natural male sex hormone, testosterone, and its many synthetic derivatives. They exert multiple actions affecting both the physiology of the human body and the individual behaviour. Under intensive training, the AAS induce the synthesis of proteins in muscle and bone causing an accelerated growth of these organs. Furthermore, during acute endurance workout, as well as during competition, androgen's action seems to be critical to enhance the performance capacity, since they affect the production of red blood cells and increase neural conduction. In addition, after intense exercises, androgens are thought to prevent muscle catabolism and exhaustion and to speed up the recovery process.

In general, the normal proceeding for AAS determination includes chromatographic separation coupled to mass spectrometry detectors. The use of GC-MS methodologies is the most employed strategy for AAS control. However, over the last years, with the development of suitable LC-MS and LC-MS/MS systems, some AAS presenting poor chromatographic properties for GC-MS analysis, even after derivatisation, are being analysed by LC-MS(/MS) procedures.

The aim of the research programme presented in this thesis was, primarily, the development of a new screening method based on mass spectrometry (MS) using the soft ionisation technique matrix-assisted laser desorption/ionisation (MALDI). The major goals to be achieved were the development of an accurate, sensitive and robust methodology able to improve the screening of AAS for doping control in both analysis time and sample throughput. Additionally, the developed method should be capable to overcome the GC-MS limitation related to thermo-labile and polar AAS, so that the initial screening method could be extended to all AAS included in the prohibited list.

In parallel with the development of a screening procedure based on MALDI-MS(/MS) techniques, and applying the deep expertise of the research group on reaction enhancement by delivery energy based techniques, the improvement of the global sample preparation for the analysis of AAS by anti-doping control laboratories was also included in the research programme.

In **Chapter I**, a brief overview on the doping abuse topic and, particularly, AAS abuse is presented. Emphasis is given to the historical background of doping abuse and control, to the role of AAS in the human body and to the analytical detection of AAS, including the current methodologies and techniques employed by WADA anti-doping laboratories and the methodologies and techniques developed during the present thesis.

Chapter II deals with the analysis of anabolic androgenic steroids by the analytical technique of MALDI-TOF-MS. In this study, the applicability of a variety of matrices for MALDI analysis was tested for 15 compounds. Nine organic and two inorganic matrices were evaluated in order to determine the best matrix for the identification of steroids in terms of ionisation yield and interference by characteristic matrix ions. The best results were achieved for the organic matrices 2-(4-hydroxyphenylazo) benzoic acid (HABA) and trans-3- indoleacrylic acid (IAA). Good signals were obtained for concentrations as low as 0.010 and 0.050 µg / mL on the MALDI sample plate for the HABA and IAA matrices, respectively.

For these two matrices, the sensitivity achieved by the technique of MALDI-TOF-MS is comparable to the sensitivity shown by GC-MS. Additionally, the accuracy and precision of the mass spectra obtained by MALDI-TOF-MS was very good, when compared to the theoretical masses of the molecular ions. The experimental methodology developed was applied to urine samples spiked with AASs. The results obtained present a good indicator concerning the use of MALDI strategies for the rapid screening of anabolic steroids in doping analysis in a near future. These results are particularly relevant to the analysis of exogenous steroids, in which its mere presence is a case of doping.

In Chapter III, a novel database search engine - Mlibrary - to assist the detection and identification of anabolic androgenic steroids, by the analytical technique of MALDI-TOF-TOF-MS, was developed in collaboration with the SING - Next Generation Computer Systems - group. The detection and identification of banned substances is achieved in two steps. In the first step, the developed software identifies the presence of a possible positive in the mass spectrum against a database that contains the theoretical mass of the molecular ions of AAS. In this operation the software includes possible changes that may have occurred to the AAS during the sample treatment. After the identification of possible positives and subsequent analysis of those ions by tandem mass spectrometry, in the second step, the mass spectrum obtained, after fragmentation of the molecular ion, is compared with a library of mass spectra contained in the database. The mass spectra library is easily constructed and introduced into the system by the user. With the help of the MLybrary software application, the use of MALDI techniques for doping control is simplified and the time required for data analysis is reduced. Moreover, by applying simple statistical tests, the software facilitates the interpretation of the results.

Additionally, several tandem mass spectra are presented and the characteristic fragmentation of each compound is discussed.

Chapter IV describes the development and optimization of a rapid sample treatment methodology for the analysis of anabolic androgenic steroids in urine by GC-MS. The new procedure makes use of ultrasonic energy to reduce the reaction times and increase the overall sensitivity of the current methodology used to analyse these compounds in WADA accredited laboratories. Ultrasonic energy was applied to the key steps of the methodology, which include the enzymatic hydrolysis with the enzyme β-glucuronidase from *Escherichia coli K12* of the steroids excreted in urine as conjugated compounds, and subsequent derivatisation of the free compound to improve the analytical characteristics for analysis by GC-MS. The results indicate that, under an ultrasonic field, it was possible to reduce the hydrolysis time for 10 minutes, about six times lower than the reaction time normally required. After the enzymatic hydrolysis, the derivatisation procedure of the compounds with the trimethylsilyl (TMS) reagent, methyl-N-trimethylsilyltrifluoroacetamide (MSTFA)/NH4I/dithioerythritol (DTE) (1000:2:4, v/w/w) was also accelerated with the application of ultrasonic energy. The results demonstrated that after 3 min of ultrasonication, 19 of the 35 compounds studied showed similar reaction yield to those obtained with the classic procedure that last for 30 min; 13 increased to higher silylation yields and for the steroids 1-testosterone, danazol and etiocholanolone-D5, the same results were obtained using a sonication time of 5 min. The overall applicability of the ultrasonic-based sample treatment method was tested by the analysis of five urine samples. The results were similar to those obtained by the routine procedure and are an indication that this methodology provides robustness to be introduced in routine analysis.

In **Chapter V** the use of microwave energy to enhance the key steps mentioned in Chapter IV was evaluated and compared with the ultrasonic energy based methodology. The study covers the current methodology applied in anti-doping laboratories for the analysis of anabolic steroids, as well as other banned substances in urine, by GC-MS. The results indicate that the effect of microwave energy in the enzymatic hydrolysis step, with the enzyme β-glucuronidase from *Escherichia coli K12*, has no advantage over the conventional method. Regarding the reaction of derivatisation, the application of microwave energy enabled us to accelerate the reaction to 3 minutes and, more important, increased the yield of the reaction for 18 of the 55 compounds used in this study. When compared to the ultrasonic method, the microwave method shows some fragility, particularly, at the enzymatic reaction. Regarding the reaction of derivatisation, the results obtained with the two irradiation systems were similar. Nevertheless, the microwave reactor allows higher sample throughput.

In **Chapter VI** the effect of ultrasonic energy on the enzymatic hydrolysis kinetic parameters, as well as on the enzymatic activity of β-glucuronidase from *Escherichia coli K12* was assessed. The study was performed using the compounds 4-nitrophenyl-β-D-glucuronide and 4-nitrophenol. The results showed that the main difference in the reaction kinetics between the conventional methodology (incubation at 55 ° C) and the ultrasonic energy based methodology,

concerns the values of the initial velocity, which is higher when ultrasound waves are employed. All other kinetic parameters have similar values. Moreover, when ultrasound waves are applied to the reaction medium, denaturation of the enzyme occurs shortly after the start of the reaction. These results support the idea that the use of ultrasound waves to accelerate enzymatic reactions has great impact in the starting minutes of the reaction.

In **Chapter VII**, a general discussion and conclusion is presented, showing the most significant results achieved within this thesis.

RESUMO

O programa de investigação abrangido pela presente tese teve como principal objectivo o desenvolvimento de um método de rastreio para a detecção de esteróides androgénicos anabolizantes com base em técnicas de espectrometria de massa, em particular a técnica de *Matrix Assisted Laser Desorption/Ionisation* (MALDI) acoplada a um espectrómetro de massas de tempo de voo (TOF, do inglês time-of-flight). Os principais objectivos a alcançar eram o desenvolvimento de uma metodologia analiticamente sensível, robusta e precisa, capaz de melhorar, ao nível do tempo da análise e capacidade de processamento de amostras, os métodos actuais para o rastreio de esteróides anabolizantes. Os métodos tradicionais usados para o rastreio destes compostos pelos laboratórios de anti-dopagem baseiam-se na técnica de cromatografia gasosa acoplada a espectrometria de massa (GC-MS) e apresentam algumas limitações na análise de compostos termo-lábeis e polares. Consequentemente, o método desenvolvido deveria ser capaz de superar essas limitações de modo a que o rastreio inicial possa ser alargado a todos os esteróides anabolizantes incluídos na lista de compostos proibidos.

Em paralelo com o desenvolvimento de uma nova metodologia de rastreio baseada nas técnicas de MALDI-MS(/MS), parte do programa da tese de doutoramento consistiu no melhoramento dos métodos actuais de tratamento de amostra. Para esse efeito, tivemos como base a larga experiência do nosso grupo de investigação na optimização de reacções por aplicação de técnicas de irradiação com ondas ultra-sónicas e de microondas.

No **Capitulo I** da tese apresenta-se o estado da arte actual sobre a dopagem no desporto, e as principais técnicas analíticas usadas para a sua detecção

O **Capitulo II** da tese aborda a análise de esteróides androgénicos anabolizantes pela técnica analítica de MALDI-TOF-MS. Neste estudo, a aplicabilidade de uma variedade de matrizes para MALDI foi testada para a análise de 15 compostos. Para este estudo foram avaliadas nove matrizes orgânicas e duas matrizes inorgânicas, a fim de determinar a melhor matriz para a identificação de esteróides em termos de rendimento de ionização e menor interferência por iões característicos da matriz. O limite de detecção alcançado pela técnica de MALDI-TOF-MS, com a matriz ácido 2-(4- hidroxi-fenil-azo)-benzóico (HABA, do inglês 2-(4-hydroxyphenylazo)benzoic acid) é comparável com a sensibilidade demonstrada por GC-MS, que é a técnica convencional utilizada para detecção de AAS. Após a optimização das condições experimentais para a análise dos esteróides por MALDI-TOF-MS, aplicou-se o procedimento desenvolvido a amostras reais de urina. Os resultados obtidos constituem uma boa indicação para o uso, num futuro próximo, da técnica de MALDI-TOF-MS para o rastreio rápido de esteróides anabolizantes em análises de dopagem. Estes resultados são particularmente relevantes para a análise de esteróides exógenos, em que a simples presença destes compostos na urina constitui um caso de dopagem.

No **Capitulo III** da tese é apresentado um software desenvolvido em colaboração com o grupo de ciências informáticas SING - Next Generation Computer Systems, para assistir na detecção e identificação de esteróides androgénicos anabolizantes pela técnica analítica de MALDI-TOF-MS. O software desenvolvido permite ao utilizador reduzir o tempo de análise de dados e aplicar testes estatísticos simples que facilitam a interpretação dos resultados.

No **Capitulo IV** da tese é descrito o desenvolvimento e optimização de uma metodologia rápida de tratamento de amostra, para a análise de esteróides anabolizantes em urina por GC-MS. O novo procedimento baseia-se na aplicação de energia ultra-sónica aos passos chave da metodologia usada correntemente para a análise destes compostos, de modo a reduzir os tempos de reacção e aumentar a sensibilidade geral da análise.

O **Capitulo V** da tese é a continuação do quarto capítulo e descreve outra abordagem para melhorar a metodologia padrão aplicada nos laboratórios de anti-dopagem para a análise de esteróides anabolizantes e outras substâncias proibidas em urina por GC-MS. O estudo efectuado engloba o desenvolvimento e optimização de uma metodologia rápida de tratamento de amostra com recurso à energia de microondas e a comparação do método desenvolvido com o método de ultra-sons.

No **Capitulo VI** da tese foi feito um estudo de cinética enzimática para avaliar o efeito dos ultra-sons nos parâmetros cinéticos da reacção enzimática de hidrólise de compostos glucuronidos com o enzima β-glucuronidase. Os parâmetros cinéticos da reacção foram calculados aplicando a equação de Michaelis-Menten aos dados experimentais. Os resultados obtidos mostram que o valor da constante de Michaelis não altera com a aplicação de ultra-sons ao meio reaccional. A principal diferença na cinética da reacção entre o método convencional de incubação a 55ºC e o método de aceleração com os ultra-sons, está relacionada com os valores da velocidade inicial, velocidade máxima e, consequentemente, a constante catalítica, que são superiores quando se aplica ultra-sons.

No **Capitulo VII** da tese é apresentado uma discussão final sobre os resultados obtidos e as principais conclusões.

ABBREVIATIONS

AAS   Anabolic androgenic steroids

ACN   Acetonitrile

ACTH   Adrenocorticotropic hormone

AD   4-androsten-3,17-dione

APCI   Atmospheric pressure chemical ionisation

AR   Androgen receptor

AVP   Vasopressin

BALCO   Bay Area Laboratory Co-operative

Bol   Boldenone

CI   Chemical ionisation

CID   Collision-induced dissociation

CRH   Corticotrophin releasing hormone

CYP   Cytochrome P450

DHB   Dihydroxybenzoic acid

DHEA   Dihydroepiandrosterone

DHT   5α-dihydrotestosterone

DMAPP   Dimethylallyl pyrophosphate

DNA   Deoxyribonucleic acid

DTE   Dithioerythritol

E   Epitestosterone

ECD   Electron capture dissociation

EI   Electron impact

EPO   Erythropoietin

ESI   Electrospray ionisation

ISTD   Internal standard

Etio   Etiocholanolone

FA   Ferulic acid

FAB   Fast atom bombardment

FD   Field desorption

FSH   Follicle-stimulating hormone

G   Glucuronide

GC   Gas Chromatography

GDR   German Democratic Republic

GnRH   Gonadotropin releasing hormone

GT   Girard T

HABA	2-(4-hydroxyphenylazo)benzoic acid
HDL	High density lipoprotein
hGH	Human growth hormone
HPA	Hydroxypicolinic acid
HPLC	High performance liquid chromatography
HSA	Human serum albumin
HSP	Heat shock protein
IAA	Trans-3-indoleacrylic acid
IAAF	International Amateur Athletic Federation
IOC	International Olympic Committee
IPP	Isopentenyl pyrophosphate
IR	Infrared
IRMS	Isotope ratio mass spectrometry
ISO	International Organisation for Standardisation
kcat	Catalytic constant
KM	Michaelis-Menten constant
LC	Liquid chromatography
LDL	Low density lipoprotein
LH	Luteinizing hormone
LLE	Liquid-liquid chromatography
MA	17β-hydroxy-17-methylandrosta-1,4-dien-3-one
MALDI	Matrix assisted laser desorption/ionisation
MeOH	Metanol
MS	Mass Spectrometry
MSTFA	Methyl-N-trimethylsilyltrifluoro acetamide
MRPL	Minimum required performance limit
MT	17α-methyltestosterone
MW	Microwave
NADPH	Nicotinamide adenine dinucleotide phosphate
Nan	Nandrolone
PNP	4-nitrophenol
PNP-G	4-nitrophenyl glucuronide
RFC	Response factor calibration
RIA	Radioimmunoassay
RNA	Ribonucleic acid
RSD	Relative standard deviation
SA	Sinapinic acid
SHBG	Sex-hormone-binding hormone

SID	Surface-induced dissociation
SIM	Select ion monitoring
SIMS	Secondary ion mass spectrometry
SPE	Solid phase extraction
SR	Sonoreactor
StAR	Steroidogenic acute respiratory
SULT	Sulphotransferases
T	Testosterone
TFA	Trifluoroacetic
THAP	2,4,6- trihydroxyacetophenone
TMS	Trimethylsilyl
TMIS	Trimethyliodosilane
TOF	Time of flight
TUE	Therapeutic Use Exemptions
UGT	Uridine diphospho-glucuronosyltransferase
UNESCO	United Nations Educational, Scientific and Cultural Organization
UP	Ultrasonic probe
US	Ultrasound
USADA	United States Antidoping Agency
UV	Ultraviolet
Vmax	Maximum velocity
WADA	World Antidoping Agency
WADC	World Antidoping Code
WADP	World Antidoping Program
α-CHCA	α-cyano-4-hydroxycinnamic acid

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PART ONE

GENERAL ASPECTS

CHAPTER I

I.1 DEFINITION OF DOPING

Ever since international agencies implicated with sports started to worry about the doping issue, the first concern was to find a precise definition for the concept. Over the years the definition of doping has changed with the development of new doping strategies. Until few years ago, doping could be shortly defined as the attempt to enhance the athlete's performance in sport by illegal administration of pharmaceuticals or application of prohibited methods [1, 2]. However, since 2003, with the introduction and acceptance by most sport organizations of the World Anti-Doping Code (WADC), the definition of doping has broadening [2, 3]. Currently, violations of "non-analytical" rules are also considered as doping offenses. In the WADC "*Doping is defined as the occurrence of one or more of the anti-doping rule violations set forth in Articles 2.1 through Article 2.8 of the Code*", which are (i) "*the presence of a prohibited substance or its metabolites or markers in an athlete's sample*"; (ii) the "*use or attempted use by an athlete of a prohibited substance or a prohibited method*"; (iii) "*refusing or failing without compelling justification to submit to sample collection after notification as authorized in applicable anti-doping rules, or otherwise evading sample collection*"; (iv) "*violation of applicable requirements regarding athlete availability for Out-of-Competition Testing, including failure to file required whereabouts information and missed tests which are declared based on rules which comply with the international standard for testing*"; (v) "*tampering or attempting to tamper, with any part of doping control*"; (vi) "*possession of prohibited substances and prohibited methods*"; (vii) "*trafficking or attempted trafficking in any prohibited substance or prohibited method*" and (viii) "*administration or attempted administration to any athlete of any prohibited method or prohibited substance*"[3].

I.2 HISTORICAL BACKGROUND

I.2.1 DOPING: ORIGIN OF THE WORD

Despite the ancient usage of physical enhancing substances, the word "doping" appeared for the first time in an English dictionary in 1889 [1]. At that time the word was used to describe a remedy containing opium commonly used to increase racehorse's performance [1]. The origin of the word is still unclear. Some authors attribute its origin to the African Zulu warrior's beverage called "Dope" or "doop", which was used as a stimulant [1, 4]. Others claim that it comes from the slang word "dope" for opium, used by North Americans [4].

I.2.2 EARLY HISTORY OF DOPING

The use of doping substances to enhance the physical performance is as old as the beginning of civilization [4, 5]. Several reports point out the use by ancient people of substances extracted from plants and animals to increase their physical capacity. Those substances were used mainly to increase strength and to surpass fatigue and possible injuries, which were valuable attributes in war, society and athletic competition [1, 4, 6].

Extracts from Ephedra, one of the oldest medicinal plants know by man, has been used by the Chinese Traditional Medicine for more than 5000 years. The use of the Ma Huang, as it was known, was recommended for its stimulant and antiasthmatic effect by Chinese physicians [7].

South America tribes used to chew coca leaves as well as other plants containing powerful stimulants. Among those tribes, the Incas are an excellent example regarding the effect of those stimulants; their famous ability to run around 1750 Km between Cuzco and Quito in few days was only possible under the effects of cocaine [1, 6]. With the same purpose West Africans tribes made use of *Cola acuminita* and *Cola nitida* to enhance their running ability [6].

Early uses of "testosterone" are dated to the ancient Greeks [8]. The alteration of the animal's behavior after castration provided them the evidence that the testicles were involved in the secondary male sex characteristics [4, 8]. Following a primitive medicine practice known as *similia similibus* that consisted of treating an organ with itself, the consumption of testicular tissue was frequently used to enforce virility [8]. For the same reason, the Huns were known to eat testicles before battles to increase their strength and performance [1]. During the time of the Roman Empire, gladiators as well as soldiers used stimulants to overcome fatigue and injury [4]. One of those stimulants was the extract from the plant Ephedra [7].

Regarding the Ancient Olympic Games, the earliest record of a major sport competition (see Figure I.1), the use of specific diets, opium, hallucinogenic mushrooms, strychnine, alcoholic beverages as well as other substances by the Olympic athletes is continuously documented [1, 4-6, 9].

Figure I.1 – Painting of the athletes in the Ancient Greek Olympic Games (Reproduced from Kristine Toohey and A. J. Veal, *The Olympic Games: a social science perspective*, CAB international, 2007).

Through ancient history, the use of enhancing substances is almost omnipresent in all civilizations. It was not a specific behaviour linked with sports, but to society in general as a way to achieve an advantage over others.

I.2.3 DOPING AND ITS EMINENT PROHIBITION

Despite the use of doping substances for centuries, their prohibition dates back to the beginning of the 20th century [1, 6]. Before that, the use of such substances was not considered cheating at all. For instance, it is remarkable that at the Ancient Olympic Games the widespread of doping among the athletes was not considered cheating, whereas other dishonest behaviours, such as bribing an opponent, was punished severely [1, 4, 6]. Those athletes were banished for life and their name alongside with their family's were inscribed in stones so that everyone could know their offence. It has been also reported that even death penalties were given at that time to those who violate the Game's rules [1]. From the old Olympic Games until the 19th century there is a gap of records concerning the use of enhancing substances or its prohibition, possibly because it was already an intrinsic behaviour in society.

In the second half of the 19th century, the new developments in science and medicine were crucial to identify and purify the active compounds behind the doping extracts [1, 10]. This new knowledge together with the emergence of the first synthetic organic pharmaceuticals met the ideal conditions to the growth of modern doping [1, 10]. In the late 19th century the use of stimulants to

improve the physical capacity was usual amongst athletes and without any kind of prohibition there was no need to cover its use. It was common for trainers to develop their own secret recipes with several enhancing substances [4, 6].

Around the end of the 19th and beginning of the 20th century, the professionalization of amateur sports gave an additional contribution to increase doping attempts. The beginning of the "Six Day" cycling races together with boxing and animal races demanded more physical strength and endurance from the competitors [4]. Caffeine, alcohol, ether, nitro-glycerine, and later on strychnine, cocaine and heroin were usual components of the enhancing mixtures taken by athletes [1, 4]. These levels of doping use and experimentation led to the death of the English cyclist, Arthur Linton, which was the first known death caused by extreme doping [1, 4].

At the beginning of the 20th century, doping abuse was an inevitable phenomenon within all sports [6]. At this point, for the first time, the use of doping substances was considered inappropriate and restrictions to their use were introduced [4]. Few years later, in 1928, the International Amateur Athletic Federation (IAAF) prohibited the use of doping in sports, specifically stimulants, which were the main doping agents at that time [1, 4]. Following the IAAF decision, other sport organizations joined the anti-doping cause, however official testing was not yet performed and the restrictions remained ineffective due to the absence of valuable tests [1, 4].

In 1935, testosterone, the main male sex hormone, was isolated from the testes as a crystallised pure substance by Ernst Lacqueur [4]. In that same year, the chemical synthesis of testosterone was achieved by two distinct studies carried out by Adolf Butenandt and Leopold Ruzicka [11, 12]. This discovery earned to both scientists the joint Nobel Prize for Chemistry for 1939 [1, 13]. The elucidation of the chemical synthesis of testosterone enabled its production worldwide and, given that the effect of these compounds had already been associated with the secondary male sex characteristics, it is unsurprising that their use was quickly introduced in sports, first as agents supporting recovery after massive stress and exhaustion and then as agents enhancing the physical performance of athletes [1].

Russian athletes were putatively the first to use anabolic steroids, in particular, testosterone, to improve athletic performance in international competitions [6, 8]. Nevertheless, its use quickly spread within sports and, soon after, research funded by pharmaceutical companies started in an attempt to find a compound with the anabolic effects of testosterone, but without its androgenic effects [6, 8]. Consequently, few years later, methandrostenolone (Dianabol) was synthesised by the pharmaceutical company Ciba and it became one of the first anabolic steroids marketed and introduced in sports [8]. In parallel, other pharmaceutical companies started to synthesise several testosterone derivatives [11]. The use of these substances has spread in such a way, that at the end of the 1950s this class of drugs became the principal group in the statistics of doping cases [1].

Following the use of anabolic steroids, the use of "new" stimulants in sports also increased around this time. Although synthetic phenylethylamine derivatives were first identified in 1887, their introduction as an ergogenic aid began in the mid 1930s with the identification of its activity as a

central nervous system stimulant [6]. The first systematic use of these compounds occurred within the military services with the aim to improve concentration and vigilance during the Second World War. Its use as strong-acting stimulants quickly spread to sports [1, 4]. Within these substances, amphetamine and methamphetamine were the most used amongst athletes. Their use was particularly established in cycling, reaching its peak between the 1960s and the 1970s [4, 6]. During this period of time, the death of the Danish cyclist Knut Jensen, in 1960, at the Olympic Games in Rome, and the first televised doping death of the English cyclist, Tom Simpson, during the Tour de France, in 1967, highlighted the urgency of an anti-doping policy [4, 6]. Soon after the death of the English cyclist, in that same year the International Olympic Committee (IOC) founded its own Medical Commission with the aim to ban the misuse of doping substances within sports [1, 14, 15]. Following its establishment, the IOC Medical Commission created its first list of prohibited substances [1, 2, 14]. Stimulants, analeptics, some alkaloids and narcotics, such as amphetamines, ephedrine, or cocaine became prohibited as the first classes of doping agents [1, 16]. Although the use of anabolic steroids was thought to be extremely extensive among sports, the first list of prohibited substances did not included that class of compounds because there was no viable analytical test available for them [3, 14]. IOC doping tests were first introduced during the Winter Games in Grenoble and at the Summer Olympic Games in Mexico in 1968 [1, 3, 6, 15, 17]. The doping control carried out in Mexico may be considered as a pilot project to the official and systematic antidoping tests performed in all sports at the Olympic Games in Munich in 1972 [15, 17]. It was at these Olympic Games that mass spectrometry (MS) was introduced coupled with gas chromatography (GC) as a confirmatory method to identify doping substances [17]. Professor Manfred Donike, from the Institute of Biochemistry at the German Sport University in Cologne, was the main initiator for the introduction of mass spectrometry in doping analysis [4, 16].

In 1974, the anabolic steroids were finally banned, first by the IAAF and then by the IOC Medical Commission that included these compounds in the list of banned substances [2]. The screening method to detect the presence of anabolic steroids in the 1976 Montreal Olympics was based in radioimmunoassay (RIA) while GC/MS was used for confirmation purposes [18]. The introduction of reliable tests for the anabolic steroids determination resulted in numerous disqualifications in the late 1970`s, especially among strength related sports [2, 16].

With the emerging of several laboratories performing antidoping analyses, together with the increasing complexity of the analyses and the impact of the analytical result to the athlete, a set of restrictive rules for the analytical procedures used and a system for the accreditation of the doping analyses laboratories were created by the IAAF to insure the quality of the analytical results [1, 16]. Following the implementation of the accreditation program, in 1981, the IAAF in a joint accomplishment with the IOC, accredited the first 6 anti-doping laboratories around the world [1].

I.2.4 HARMONISATION OF ANTIDOPING POLICIES

In the beginning of the 1980s a new challenge concerning the use of anabolic steroids arose within doping control agencies. Despite its capacity to detect anabolic steroids, anti-doping laboratories were not prepared to positively detect the misuse of exogenous testosterone, such as testosterone esters, which have the same excretion metabolites than its endogenous counterpart [17, 18]. It is perhaps unsurprising that until 1983, when Professor Donike, using GC-MS based methodologies, introduced a new screening method for testosterone, numerous testosterone abuses have gone unpunished [1, 17, 18]. The method purposed by Donike, established a testosterone to epitestosterone ratio threshold, above which it was considered a doping offence [1, 17, 18].

Along with the use of endogenous anabolic steroids administered exogenously, the anti-doping laboratories were also confronted with the emergence of new drugs and new methods, such as the human growth hormone (hGH) and blood transfusion [16]. At the time blood doping was already considered unethical but it was not officially prohibited for inexistence of a valuable test [2]. However, right before the opening of the 1984 Los Angeles Games, the authorities uncovered a clandestine operation carried out by some cyclists that were transfused with whole blood [2, 6]. The impact of this behaviour on the media and consequently on society, led the medical commission of the IOC to ban these drugs and methods, although no tests were available for their detection [1, 2, 6]. Along with the introduction of blood doping, beta-blockers in 1985, diuretics in 1987 and the growth hormone in 1989 were also included in the doping list [16].

In addition to the challenges faced by anti-doping laboratories, a new setback arose to anti-doping organizations with the suspicion of several state sponsored doping activities. Most of these suspicions were centred on the Eastern Bloc countries, such as the Soviet Union, Poland, Czechoslovakia and the German Democratic Republic (GDR), but allegations that other countries like England, Australia and Canada were covering up some of its athletes doping abuse cases were also emerging [16, 19]. Regarding the GDR, these suspicions have been substantiated with the finding of several studies concerning the development of ideal drug regimens to improve performance, after its collapse in 1990 [19].

In 1988 at the Seoul Olympic Games, the increasing public awareness that new measures should be employed was catapulted by another scandal; the 100-metres champion, Ben Johnson (see Figure I.2), tested positive for stanozolol, an anabolic steroid [3].

All these events led to the recognition that governmental participation and support were required in the fight against doping. The Anti-Doping Convention of the Council of Europe that opened for signature on the 16th November 1989, was the first step of the member states towards international harmonisation of the measures to be taken against doping [16, 20]. The Convention was also open to non-members states and it set up a number of common principles and regulations requiring each contracting state to undertake legislative, financial, technical and educational measures [16, 20].

Figure I.2 – Ben Johnson at the Seoul Olympic Games (Reproduced from Kristine Toohey and A. J. Veal, *The Olympic Games: a social science perspective*, CAB international, 2007).

Before the Anti-Doping Convention, the Council of Europe had already adopted the Anti-Doping Resolution in 1967, stating that doping was the opposite of sport values and that the sport organizations should be responsible for doping control, and draw the European Anti-Doping Charter for Sport, which had a recommendation status and contained guidelines for governments concerning anti-doping policies. The Anti-Doping Convention arose as a sign of governmental motivation to reduce and, if possible, eliminate doping from sports [16, 20].

I.2.5 THE WORLD ANTI-DOPING AGENCY (WADA)

In the 1990´s, the IOC medical commission established, as a condition to assign the IOC accreditation to anti-doping laboratories, the laboratory's previous accreditation by the International Organization for Standardization (ISO) [2]. This demand, by the IOC, was an important development within anti-doping laboratories; not only was it useful to standardize methods and procedures as it markedly strengthened the idea of high quality anti-doping laboratories.

Around this time, "out of competition" tests were introduced to avoid and detect, mostly, anabolic steroids abuse as training phase enhancers [2, 3]. These measures along with the development of more effective tests resulted in a significant decline in abuse of these compounds among elite athletes. Despite these improvements, at that time, no reliable method was yet available to detect blood doping and the misuse of emerging peptides hormones, such as the growth hormone and the erythropoietin (EPO) [1, 4, 21]. Only in the years 2000 and 2004 reliable tests were introduced for EPO and hGH, respectively [3, 21].

In 1998, one of the biggest scandals in the history of sports drove the anti-doping community toward the creation of the World Anti-Doping Agency (WADA).

Right before the beginning of the 1998 Tour de France, a large number of prohibited medical substances, in which were included several dosages of EPO and anabolic steroids, were found by French Customs police inside the car of the longtime masseur for the former Festina road-racing team, Willy Voet [4, 16, 22-24]. The Festina team was expelled from the race and other six teams quit during the Tour [4, 23]. Moreover, the arrestment of Voet uncovered one of the major doping schemes involving athletes, coaches, team physicians and other team staff [6, 16]. In the subsequent investigations, carried out by the French and Italian authorities, several other top teams and athletes were also implicated in that highly organized scheme [6].

Following these events and with a growing pressure on the IOC demanding new measures, a World Conference on Doping, proposed by the IOC, was held in February 1999 in Lausanne [1, 9, 16]. The need for an independent international agency for doping became clear, which led to the establishment of the World Anti-Doping Agency on November 10 of that same year [1, 3, 16]. WADA was established as a Swiss foundation [1].

The involved parts agreed that the WADA would be equally financed by sports organizations and governments [25]. Its main mission was to coordinate and promote the development of international standardized anti-doping rules, facilitate the coordination between sport organizations and governmental authorities, ongoing with doping control research, and promote doping prevention activities [1, 3, 16].

In 2002, WADA moved its headquarters to Montreal in order to distance itself from the IOC and mark its position as an independent body [1, 9]. The first major project of the WADA was the elaboration and implementation of the World Anti-Doping Code (WADC) that was adopted in the second World Conference on Doping in Copenhagen in 2003 [1, 9]. The Code was developed after intensive collaboration with several stakeholders and it became the first worldwide accepted document providing a set of harmonized anti-doping policies, rules and regulations within sport organisations and public authorities [1].

Figure I.3 – The WADA Code and current components of the WADA International Standards (Adapted from www.wada-ama.org).

The WADC was part of the World Anti-Doping Program (WADP) presented by WADA on that conference that included two other proceedings (see Figure I.3), the International Standards for the Prohibited List, for the Laboratories, for Testing and for Therapeutic Use Exemptions (TUE), and models of good practice and guidelines [1-3]. From the three levels of the WADP, only the models of the best practice and guidelines was not mandatory for all Code signatories [3]. The Code and the International standards entered into force on January 2004 in time for the 2004 Athens Olympic Games. At those Games, a significant change within the Prohibited List was the inclusion of gene doping as a prohibited method [1].

Despite the developments in the fight against doping, the beginning of the twenty-first century was also marked by another scandal involving the USA Corporation BALCO (Bay Area Laboratory Co-operative) [9]. BALCO was providing athletes with a new enhancing drug undetectable by the GC/MS screening methods used at that time [9]. That unknown substance was later isolated by the United States Anti-Doping Agency (USADA) from a used syringe and it was found to be a designed anabolic steroid which was called tetrahydrogestrinone (THG) [9]. Alongside with the development of new pharmaceutical enhancing drugs, BALCO also produced combined drugs containing the enhancing substance and its masking agent to prevent an adverse analytical finding [6].

Regarding the WADP, a legal problem still persisted; many governments could not formally take part in legal agreements with nongovernmental entities [1, 16, 26]. This situation was overcome in 2005, with the UNESCO Anti-Doping Convention that led to the first global treaty against doping in sport [16, 26]. The Convention guaranteed the effectiveness of the World Anti-Doping Code and supported the WADA activities, providing the legal framework for governments to act. The Convention came into effect in 2007 [1, 3, 16, 26].

In 2006 WADA started the revision process of the World Anti-Doping Code that was presented and adopted in the third Anti-Doping World Conference in November 2007, held in Madrid [3]. The revised World Anti-Doping Code and its International Standards were implemented in 2009 [2, 3].

As science advances, so the compounds and methods used by athletes that do not respect the spirit of sport advance. Consequently, the list of prohibited substances is forever changing and several compounds and methods are now included. Within these, the use of recombinant peptides, hormone antagonists and modulators, or gene doping seems to be the major future challenge in the fight against doping.

I.3 THE ROLE OF ENDOGENOUS AND SYNTHETIC AAS IN THE HUMAN BODY

The human body is an extremely complex structure that contains a variety of systems, each one playing a specific role. The perfect functioning of the human body implies that all these systems work together. This means that it has to have the ability to self regulate and that the different organs and tissues that constitute those systems also communicate between themselves. The main control mechanisms, which regulate all aspects of physical life, are the nervous and the endocrine system [27]. With regard to the endocrine system, this consists of glands that release chemical messengers, called hormones, through the blood to act in distant sites [27-29]. Compared with the time of action of the nervous system, the time of action of these compounds in the body is long and, therefore, they are responsible for long-lasting generalized physiological effects, such as growth, development, reproduction and metabolic rate [27, 28].

The hormones will generate different responses depending on the organs in which they operate and although they can be transported through the bloodstream to any cell in the body, only certain cells, called target cells, express highly specific receptors for hormone's recognition [27, 28]. After the signal recognition, several chemical reactions are triggered inside the target cells, leading to a modification in the output of those cells. A common modification is, for example, the synthesis of specific proteins [27].

Knowing that hormones play a key role in the regulation of the human body, it becomes clear that any alteration of these compounds in the organism will trigger a set of mechanisms that will change the physiological processes of the body.

Hormones can be divided in two classes according to their chemical composition, the steroids and the non steroids hormones that comprises, mainly, proteins and amines [28]. Their effects have long been recognized by athletes that, for several decades, use these compounds to enhance their athletic performance. Within steroids, the male sex hormones, or androgens, and their synthetic derivatives are responsible for the muscular development and strength.

This chapter pretends to illustrate the major aspects concerning the biochemistry of the androgenic anabolic steroids, its mechanism of action and its effects in the human body.

I.3.1 STRUCTURE

As derivatives of cholesterol, the androgens, in which is included the natural male hormone testosterone, possess the backbone of perhydrocyclopentanophenanthrene ring system [30]. The carbon atoms of the anabolic steroids are numbered and the tetracyclic rings are labelled as shown in Figure I.4 for testosterone. Naturally occurring androgens contain in their structure angular methyl groups, which are the methyl groups attached to the C-10 and C-13 carbon atoms, corresponding to the

ring junction [30]. Other common substitutions are the hydroxyl or carbonyl group at C-3 and C-17 carbon atoms. In addition, an alkyl side chain may be found at the C-17 carbon atom [30].
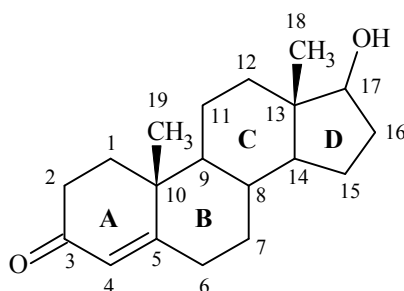
Figure I.4 – Steroid`s carbon atoms numbering and ring labelling system.

I.3.2 BIOSYNTHESIS AND SECRETION OF ANDROGENS

In the human body, the biosynthesis of most steroid hormones occurs mainly in a specific set of tissues, which are the adrenal cortex gland and the gonads that comprises the testes and ovaries. Within these specific tissues the biological pathways to produce the steroid hormones are common. Nevertheless, the secretion of steroid hormones is different in the different tissues, mainly due to the different distribution, level of expression and activities of the involved enzymes [28].

In normal men, the production rate of testosterone is approximately 3-10 mg/ day of which more than 95% is produced in the testes, whereas in women this value is about 10 times lower and it's mainly secreted in the adrenal cortex [1, 28].

Regarding other androgens, the steroid 5α-dihydrotestosterone (DHT), a more potent androgen than testosterone, is secreted in the testes but most of the circulating DHT is formed in the peripheral tissues by conversion of testosterone, catalysed by the enzyme 5α-reductase [1, 31]. The aromatisation of the A ring of testosterone, catalysed by the enzyme aromatase, to produce 17β-estradiol in the brain and in the male reproductive organs has important consequences in the sexual dimorphism [32, 33]. Epitestosterone, which is the epimer of testosterone, is also thought to be primarily formed in the testes [1].

Dehydroepiandrosterone (DHEA) and androstenedione that can be precursors of other androgens are mostly synthesised in the adrenal cortex as well as the 11β-hydroxyandrostenedione and its metabolites [1, 34, 35].

It is important to stress that steroidogenesis of some of these androgens has also been reported to take place in peripheral tissues, either by interconversion or by complete biosynthesis [34, 35].

I.3.2.1 BIOSYNTHESIS OF CHOLESTEROL

Cholesterol is the biochemical precursor of all steroid hormones [36]. In this chapter it is presented the *de novo* synthesis of cholesterol, although cholesterol may be obtained from other sources, such as low-density lipoprotein (LDL) and high-density lipoprotein (HDL) as well as from the hydrolysis of esterified cholesterol stored as lipid droplets [1, 37].

The elucidation of the biosynthetic pathway to cholesterol was achieved in the 1960s and it starts with the biosynthesis of mevalonic acid [30, 36]. The first step in the mevalonate pathway involves a Claisen-type condensation between two molecules of acetyl-CoA, catalysed by the enzyme acetoacetyl-CoA thiolase, to form acetoacetyl-CoA [36, 38]. The second step is catalysed by the enzyme hydroxymethylglutaryl-CoA synthase, and it involves an aldol-type reaction between acetoacetyl-CoA and another molecule of acetyl-CoA to produce the hydroxymethylglutaryl-CoA [36, 38]. Mevalonic acid is formed after hydroxymethylglutaryl-CoA reduction with two molecules of NADPH. This last step is catalised by the enzyme hydroxymethylglutaryl-CoA reductase and is the rate-limiting step in cholesterol biosynthesis [30, 36].

The next step in the Mevalonate pathway is the conversion of mevalonic acid, a six carbon compound, into the five carbon isopentenyl pyrophosphate (IPP), after successive phosphorylation and decarboxylation [30, 36, 38]. Following its formation, isopentenyl pyrophosphate suffers an enzymatic isomerisation that gives rise to dimethylallyl pyrophosphate (DMAPP), establishing an equilibrium that makes both compounds available to the cell (see Figure I.5).
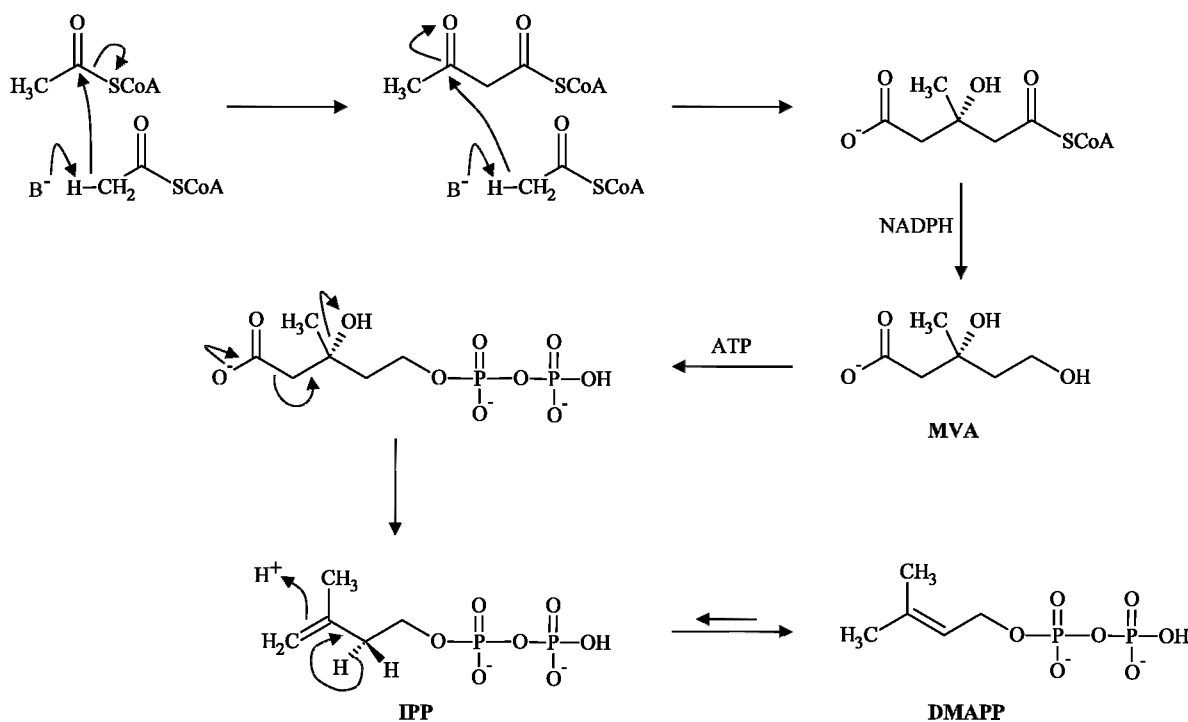
Figure I.5 – Biosynthetic pathway to form IPP and DMAPP.

The condensation of IPP and DMAPP produces a ten carbon compound called geranyl pyrophosphate [36]. Geranyl pyrophosphate can also undergo a subsequent condensation with IPP to produce the fifteenth carbon unit, farnesyl pyrophosphate (see Figure I.6). This reaction is catalysed by the enzyme prenyl transferase [30].
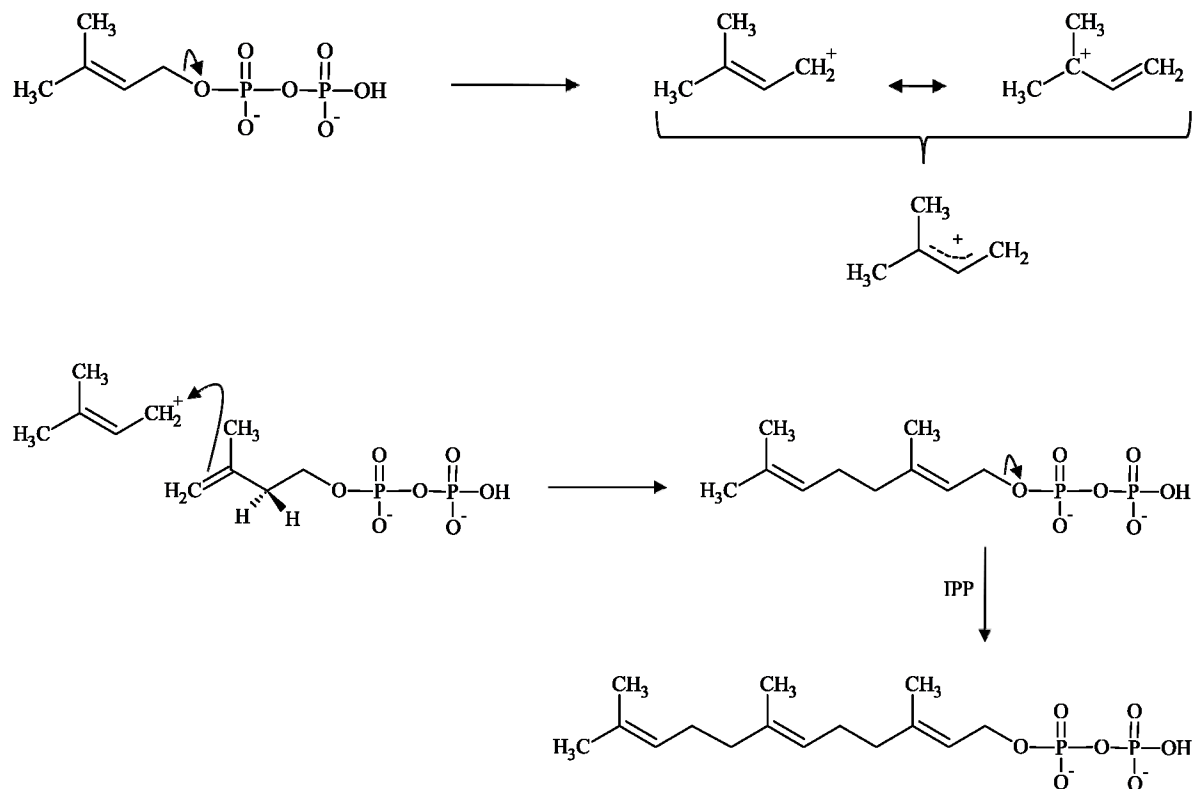
Figure I.6 – Biosynthetic pathway to form the C_{15} compound farnesyl pyrophosphate. The condensation of IPP and DMAPP starts with the enzymatic formation of an allylic cation from DMAPP.

The formation of the squalene, which is the direct precursor of cholesterol, occurs after the reductive condensation of two farnesyl pyrophosphate molecules [30, 36]. Squalene is subsequently epoxidized to yield 2,3-oxidosqualene, which undergoes a notable series of cyclisation to create the four fused rings structure of lanosterol [36, 38]. The enzymes involved in this step are the squalene epoxidase and the epoxysqualene cyclise [30].

The conversion of lanosterol into cholesterol involves the removal of three methyl groups and the introduction of a double bond in the carbon C-5 (see Figure I.7), through a series of reduction and oxidative demethylation reactions catalysed by enzymes of the cytochrome P450 family [30, 36, 38].
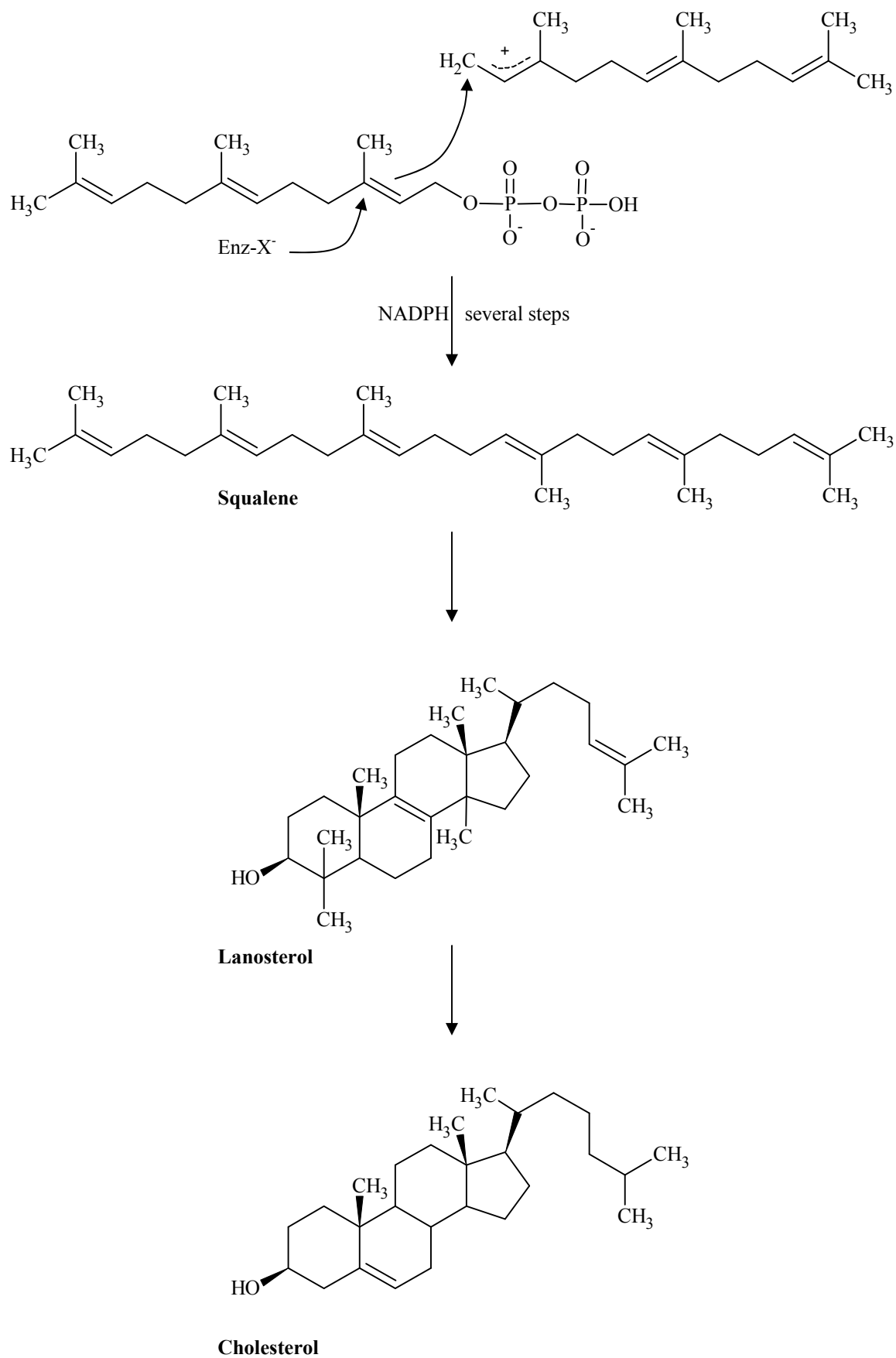
Figure I.7 – Biosynthetic pathway to form Cholesterol.

I.3.2.2 STEROIDOGENESIS OF ANDROGENS

The biosynthesis of steroid sex hormones, particularly of androgens, from cholesterol is well established and it is thought to start at the alkyl side chain attached to the C-17 carbon atom [36, 38]. The first steps are catalysed by a mitochondrial cytochrome P450 side-chain cleavage enzyme (P450scc), designated CYP11A1. P450scc catalyses three distinct reactions that include two hydroxylation reactions at the C-22 and C-20 carbon atoms and the subsequent cleavage of their carbon-carbon bond to form pregnenolone (see Figure I.8) [1, 36, 38].
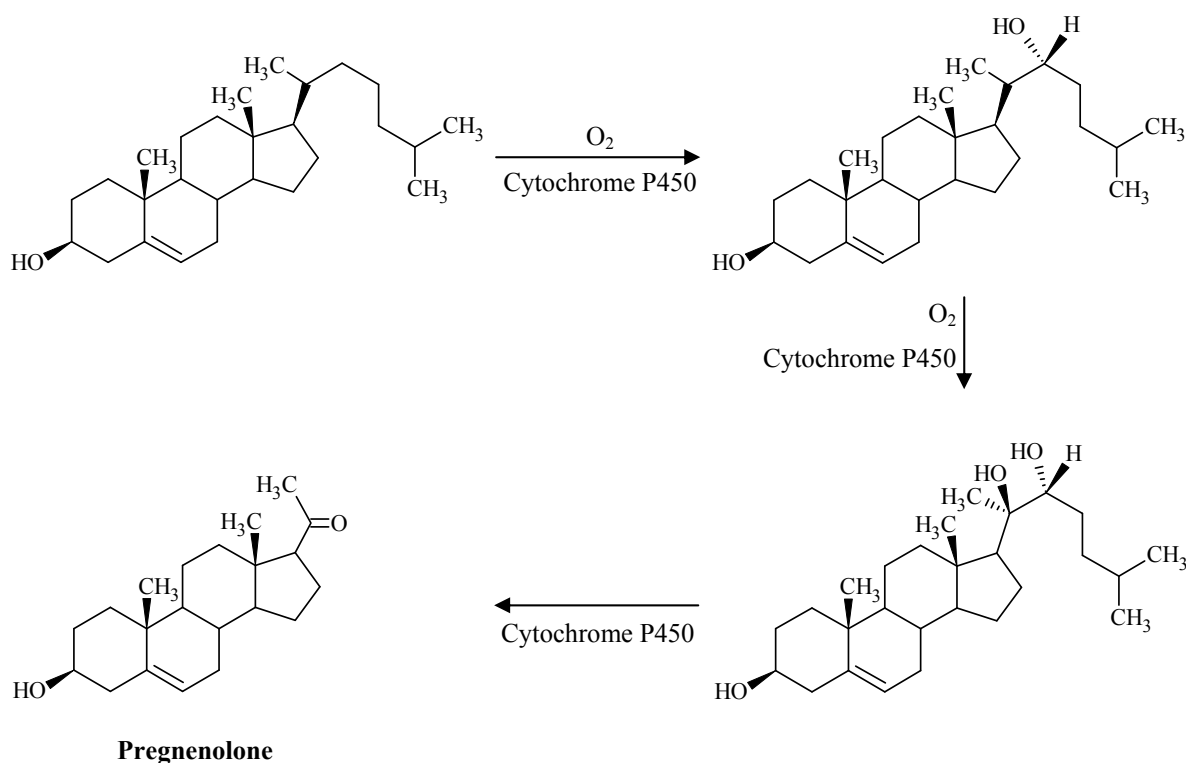
Figure I.8 – Biosynthetic pathway to form pregnenolone from cholesterol.

This reaction is the rate-limiting step in steroid hormones biosynthesis, mainly because cholesterol must be transported from the outer mitochondrial membrane to the inner mitochondrial membrane where P450scc is located [1, 39]. This process is mediated by the cholesterol-transport protein, steroidogenic acute regulatory protein (StAR). The synthesis of some androgens from pregnenolone is depicted in Figure I.9.

Regarding testosterone, the main male androgen, it is primary produced by Leydig interstitial cells present in the testes [40-42]. There are distinct pathways for its biosynthesis from pregnenolone. The main pathway in humans begins with the hydroxylation of the C-17 carbon atom and the

subsequent cleavage of the C-17 and C-20 carbon-carbon bond to form the C_{19} compound DHEA [1, 36, 38]. These reactions are catalysed by the enzyme CYP17 from the cytochromes P450 family [1]. The next step involves the enzyme 3β-hydroxysteroid dehydrogenase/isomerise (3β-HSD) that catalyses the conversion of the hydroxyl group at the C-3 carbon into a carbonyl group, leading to the formation of androstenedione that is readily converted to testosterone in the testis by 17β-hydroxysteroid dehydrogenase (17β-HSD) [1, 36].
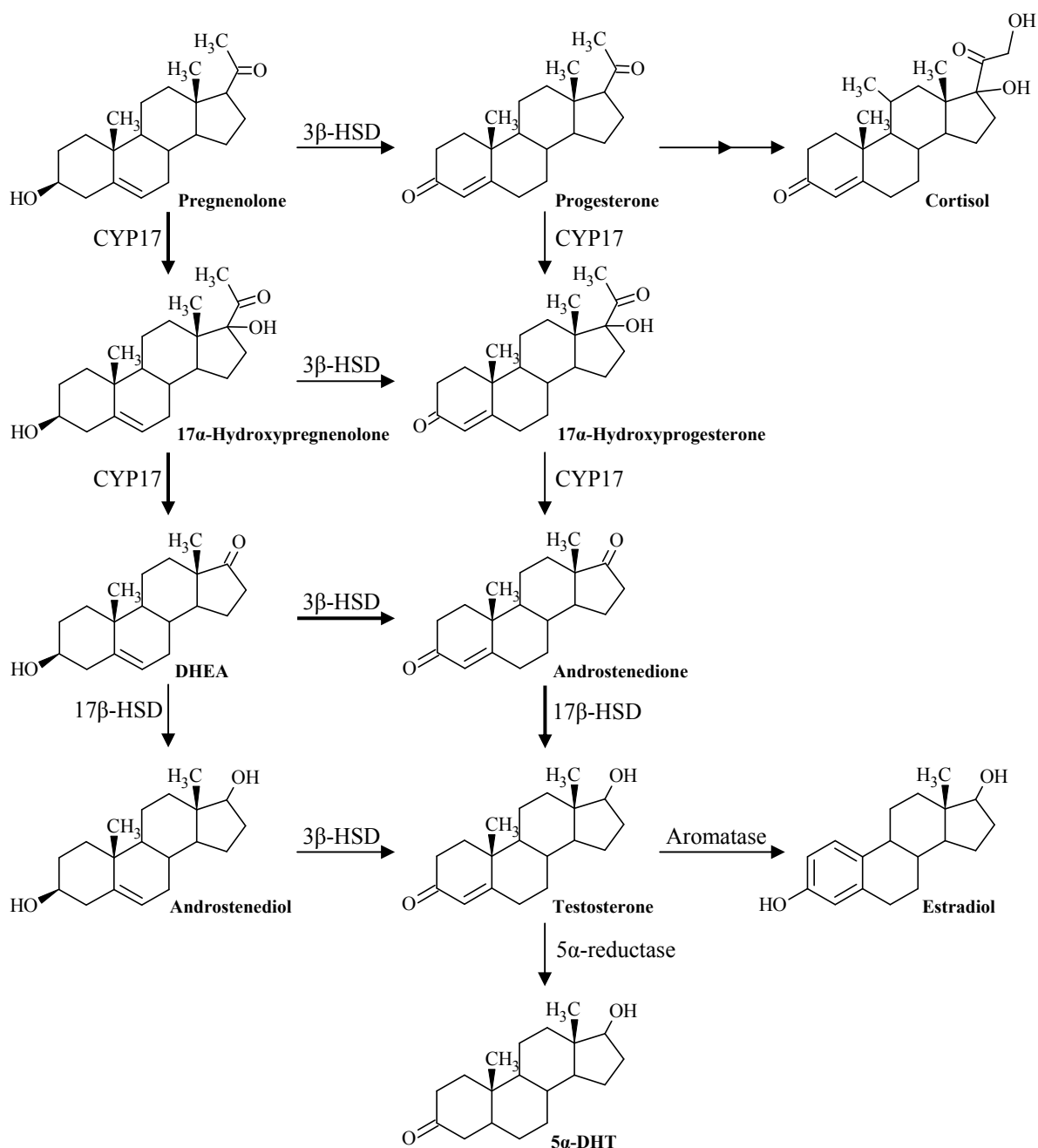
Figure I.9 – Biosynthetic pathway to form testosterone and other steroid metabolites. The larger arrows represent the preferred path to form testosterone.

I.3.3 ANDROGEN METABOLISM AND REGULATION

The amount and consequently the physiological effects of biologically active androgens in the human body depend on several factors. Their segregation rate from the endocrine glands is perhaps, the principal factor concerning their levels in the bloodstream [28]. The endocrine glands involved in the biosynthesis of androgens are unable to store more than a minimal quantity of newly formed steroid hormones and hence, the secretion of these hormones is straight related to their biosynthesis [43, 44]. As a consequence, regulation of hormonal release to ensure the normal physiological levels in the organism will act directly in the steroidogenic pathway.

Another factor affecting the amount of biologically active androgens in the bloodstream is deeply linked to their chemical structure. Androgens are hydrophobic compounds and require particular transport systems to travel through the bloodstream [45, 46]. Since only the free steroid is biologically active, the fraction of bound androgens to carrier proteins as well as the degree of binding specificity of those proteins to the steroid hormone, will dictate the hormone biological activity in the target cells [47]. Furthermore, it is thought that this transport system plays an important role in the regulation of androgens release into the target cells, acting like a buffer to supply these hormones, which balances the lack of storage capacity of the endocrine glands [28]. Moreover, some authors suggest that the carrier proteins prevent the premature elimination from the body of the small steroids by avoiding its passage into the renal tubules of the kidneys [28].

In addition to the rate of release into the blood and to the transport system, the rate of inactivation and elimination from the blood is also a critical feature that determines the level of active steroid hormones in the organism [28]. Inactivation of these compounds occurs mainly in the liver through a series of reactions that increase their solubility in water and therefore, facilitate their excretion.

I.3.3.1 TESTICULAR FUNCTION REGULATION

Testosterone is the major androgen in humans and it plays a vital role in a variety of functions in the human body. In the human male, testosterone is primary secreted by the Leydig cells [27, 41]. Testosterone secretion is controlled by a series of hormonal cascade reactions triggered by the "coordination center" of the human body, the hypothalamus [27, 48]. The hypothalamus is an endocrine gland located in the brain, whose principal function is to maintain homeostasis [27]. Both nervous and endocrine systems are integrated in the hypothalamus that coordinates and activates specific responses according to the stimuli received [27]. Testicular function is regulated by a series of feedback and feedforward mechanisms that involve the hypothalamus, the pituitary, and the testes [1, 48].

At puberty, the male hypothalamus reactivates the secretion of the pulsatile neurohormone, gonadotropin releasing hormone (GnRH) [49]. This hormone is a small peptide that acts on the pituitary gland and stimulates it to release the luteinizing hormone (LH) and the follicle-stimulating hormone (FSH) [48, 50, 51]. LH binds to specific receptors on the Leydig cells and stimulates the secretion of testosterone (see Figure I.10). This stimulation affects the rate-limiting step in testosterone biosynthesis [51]. LH increases the delivery of cholesterol to the side-chain cleavage enzyme P450scc, thus increasing its capacity to convert cholesterol to pregnenolone [52]. In addition, LH stimulates the gene expression and synthesis of a number of key enzymes in the steroid biosynthetic pathway. The role of LH is essential to maintain the bloodstream level of testosterone, since the quantity of secreted testosterone is in direct proportion to the levels of LH available [52]. Moreover, LH is also crucial to keep very high intra-testicular levels of testosterone that is fundamental for spermatogenesis [28]. FSH has its major effect on the Sertoli cells in the testes [52]. It stimulates the production of several proteins and plays an important role, together with LH, on the spermatogenic process [53].

In turn, the concentration level of testosterone and other peptides regulate LH and FSH secretion [54]. An elevated level of circulating testosterone, plus some of its derivatives, triggers a negative feedback inhibition of GnRH secretion as well as of the pituitary LH [48]. Low levels of testosterone in the blood activate the production of GnRH with subsequent increase of the pituitary hormone LH.
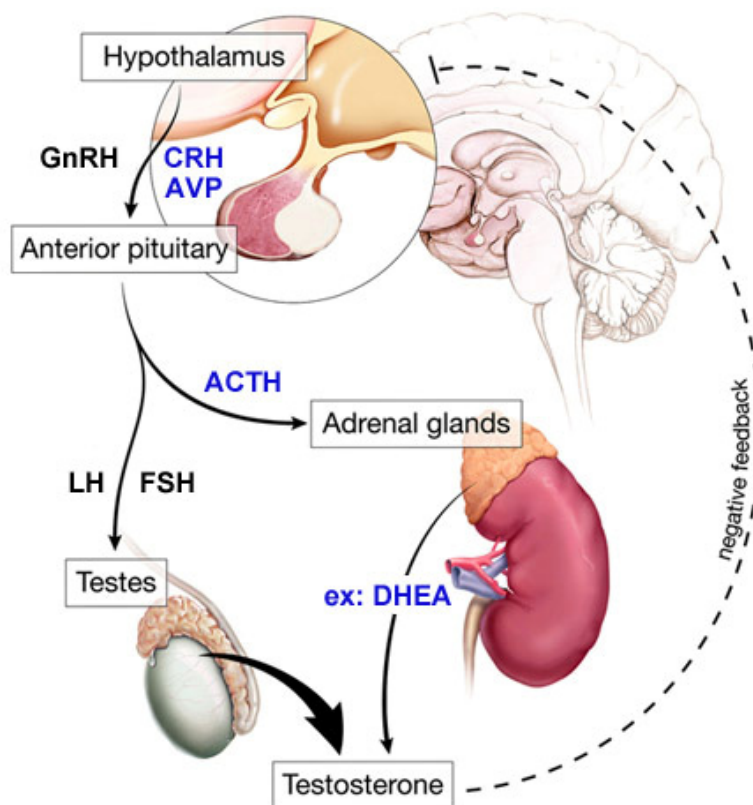
Figure I.10 – Hypothalamic-pituitary-testicular and hypothalamic-pituitary-adrenal axis (adapted from J.B. Aragon-Ching *et al.*, *Frontiers in Bioscience*, 12 (2007) 4957).

I.3.3.2 ANDROGENS: FROM BIOSYNTHESIS TO THE TARGET CELLS

After its production, testosterone is released into the bloodstream. The major fraction of testosterone that circulates in the blood is bound to plasma proteins, which allows a circulating concentration higher than its solubility [28, 55]. Only a small fraction present in the blood is unbound and hence in its biologically active form [55, 56]. The primary proteins involved in the transport of testosterone are the sex-hormone-binding globulin (SHBG) and albumin [52, 55, 57]. These hormones bound to testosterone with different degrees of affinity. SHBG is a glycoprotein produced in the liver that binds with high affinity to androgens. Its expression is regulated by a complex system that controls the bound fraction and so the availability of the active form [58]. The human serum albumin (HSA) is also produced in the liver, but unlike the SHBG, this protein is not specific for steroid hormones and binds with a wide variety of compounds [1, 56]. Although it has less affinity for androgens, HSA is responsible for the transportation of an important fraction of these compounds, mainly because the concentration of this protein circulating in the plasma is higher than the concentration of SHBG [1, 28]. Under physiological conditions, 45–70% of the androgens are transported by SHBG, 30–55% by HSA, and approximately 2% circulates unbound [52]. However, unlike the fraction bound to SHBG, the fraction bound to HSA dissociates readily in the capillaries and therefore can be considered to be biologically available [52].

It is important to stress that although the fraction bound to SHBG is considered to be unavailable to the androgen receptors in the target cells, recent studies have demonstrated the existence of alternative mechanisms involving membrane receptors that allow the androgens to act without entering the cell [58].

I.3.3.3 MECHANISM OF ANDROGENS ACTION

I.3.3.3.1 TESTOSTERONE

Testosterone acts at numerous levels in the body. It can act directly in several organs as a hormone and it may act indirectly as a prohormone after conversion into its active metabolites by the action of specific enzymes [28, 52]. It plays also an important role as a paracrine factor in spermatogenenis [52]. The main metabolites of testosterone are DHT and estradiol. The amount of testosterone that is converted into DHT in peripheral tissues is approximately 4% of its total secretion [52]. Although only a small amount of testosterone is transformed into estradiol, in adult men, that amount represents about 80% of the circulating estradiol [52]. In some tissues the action of testosterone is only mediated after conversion to its metabolites. The sexual differentiation of the brain, the formation and resorption of the trabecular bone and some sort of behaviors are few examples of testosterone effects mediated through its conversion to estradiol [28, 59]. Likewise, its effects on the prostate and sebaceous glands

of the skin are mediated through its conversion to DHT [28]. In table I.1 is shown the main androgen target organs.

Table I.1 - Major androgen target tissues

Reproductive organs
Prostate, seminal vesicles, epididymis
Testes
Penis
Brain
Pituitary gland
Breast
Skin
Hair follicles: sexual hair, scalp hair
Sebaceous gland
Upper respiratory septum
Larynx
Bone
Hematopoietic system
Muscle
Vascular system
Liver

I.3.3.3.2 ANDROGEN RECEPTOR

Androgens, particularly testosterone and 5α-dihydrotestosterone, travel through the bloodstream and bind to intracellular androgen receptors present in the target cells [28]. The androgen receptor is a member of the nuclear hormone receptors super-family that, like other nuclear receptors, functions as a ligand-dependent transcription factor [28, 31, 60].

The androgen receptor is a modular protein that consists of a ligand-binding domain, an N-terminal regulatory domain, a DNA binding domain and a hinge domain [31, 61]. The binding of androgens to the ligand-binding domain induces a conformational change in the androgen receptor that causes its phosphorylation, dimerisation and subsequent activation [35, 62]. In the target cells, the activation of the androgen receptor causes it to translocate from the cytoplasm to the nucleus [1, 32]. The hinge domain is responsible for the translocation of the activated receptor [52]. In the nucleus, the androgen-receptor complex regulate the target gene expression followed by protein synthesis by binding to specific DNA sequences, called hormone response elements, and by recruiting essential cofactors required for the expression of the regulated gene (see Figure I.11) [1, 61].

The two major androgens have different interaction degrees with the androgen receptor. DHT binds to the androgen receptor with the double affinity of testosterone, mainly because the DHT-androgen receptor complex shows higher thermostability and slower dissociation rate [28].
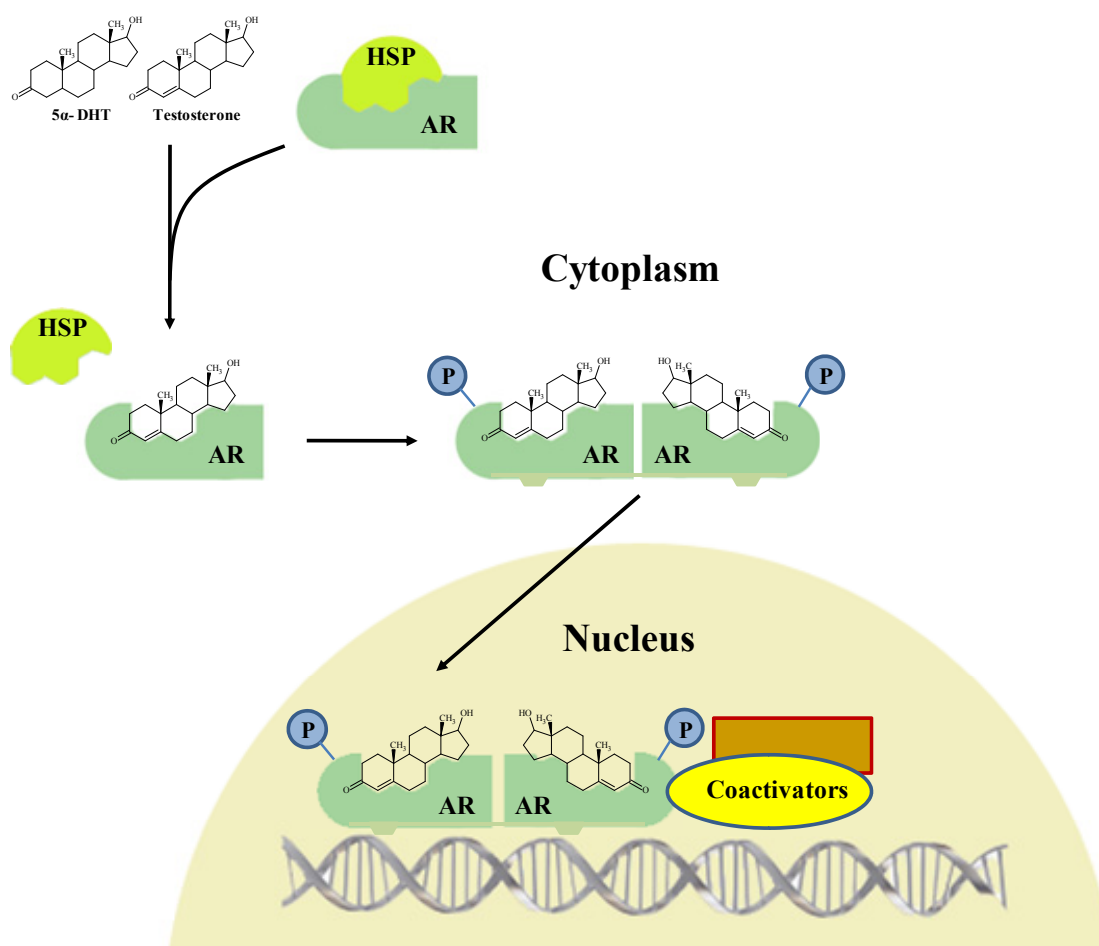
Figure I.11 – Regulation of androgen receptor action. In the presence of androgens, particularly testosterone and 5α-DHT, the androgen receptor (AR) releases the heat shock protein (HSP) complex, which is responsible for its inactivation form, and binds to the androgen moiety. The activated receptor translocates from the cytoplasm to the nucleus and binds to the DNA. The activated AR recruits essential coactivators and promotes the action of the RNA polymerase, which responsible for the target gene expression.

I.3.3.4 METABOLISM AND EXCRETION OF AAS

The inactivation and excretion of androgenic anabolic steroids involve a variety of enzyme systems that catalyse the transformation of these compounds to produce, in general, more polar metabolites, which are less toxic, more easily excreted and less likely to interact with cell membrane receptors [63, 64]. The biotransformation reactions involved in this process are generally divided in two groups, phase-I and phase-II metabolic reactions [63]. Phase-I metabolism consists of oxidative, reductive and hydrolytic enzymatic pathways that inactivate the drug and increase its polarity by adding or exposing functional groups [1, 64, 65]. Phase II metabolism can be characterised as the conjugation reactions

that modify the newly introduced functional group to form, mainly, O- and N- glucuronides as well as sulphate esters that change the physicochemical properties of the compounds and promote its elimination from the body [1, 63, 66]. The enzymes involved in this process are in high concentrations in the liver, but may be also present in a variety of other tissues, including the skin and kidneys [1, 63].

I.3.3.4.1 PHASE-I METABOLIC PATHWAYS

The primary reactions involved in the Phase-I metabolism of androgens comprise the reduction of double bonds and carbonyl groups, oxidation of hydroxyl groups, as well as aromatization, hydroxylation and epoxidation of the ring system [64, 67]. In Figure I.12, the primary metabolic pathways undergone by testosterone are presented. The main metabolites from testosterone inactivation are androsterone and etiocholanolone [1, 67]. These compounds are formed after enzymatic oxidation of the 17β-hydroxyl group, reduction of the double bond in the C-4 and C-5 positions and reduction of the C-3 carbonyl group [1]. Minor metabolic pathways lead to the formation of the mixture of 5α- and 5β-androstane-3α,17β-diol, after the reduction of the A-ring double bond and the C-3 carbonyl group.
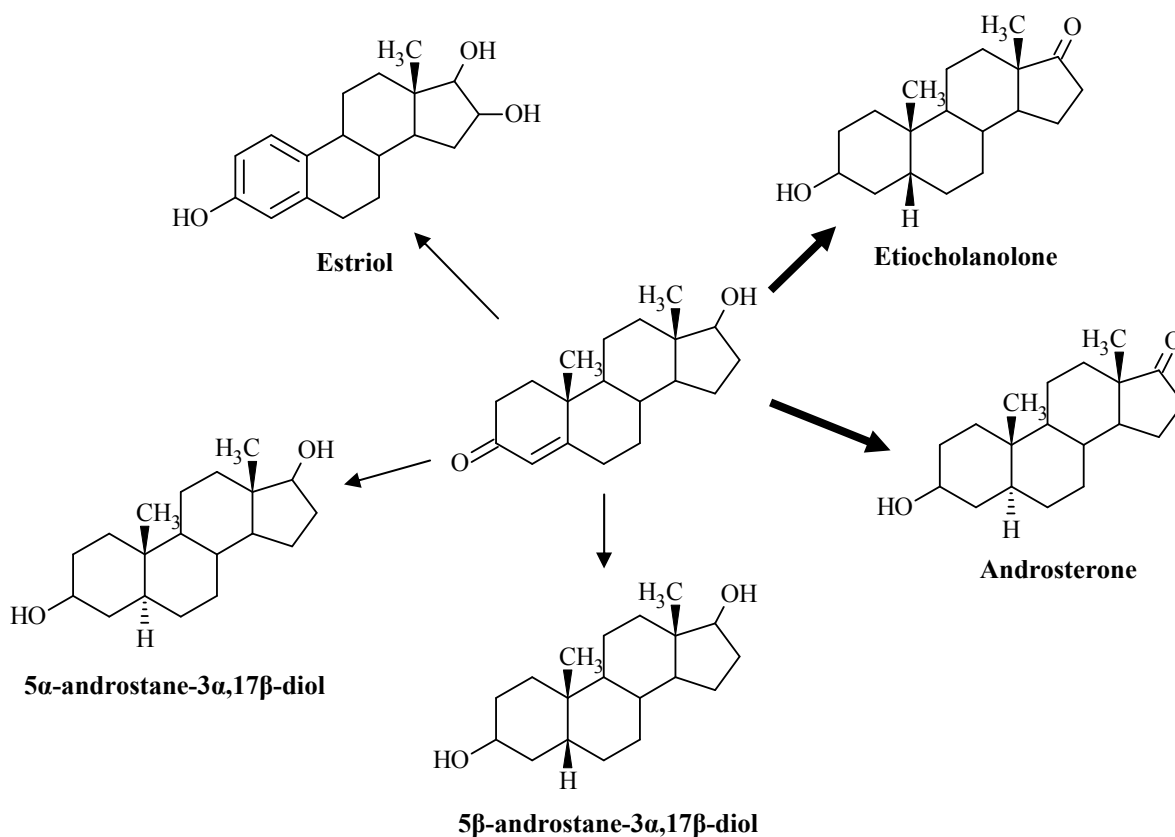
Figure I.12 – Phase I metabolism of testosterone [1]. Androsterone and Etiocholanolone represent the major metabolites of testosterone.

I.3.3.4.2 PHASE-II METABOLIC PATHWAYS

Contrasting to Phase-I metabolism, Phase-II has a great impact in the chemical and biological properties of the molecule, affecting largely its polarity and pharmacological activity [63]. The main Phase-II metabolic reactions that affect both endogenous and exogenous AAS are glucuronidation and sulphonation [64, 68, 69]. The resulting steroid conjugate presents higher polarity than the parent compound and is generally more easily excreted.

Conjugation of androgens by glucuronidation consists of a nucleophilic substitution involving the glucuronic acid from uridine diphosphoglucuronic acid and a functional group of the steroid molecule (see Figure I.13). This reaction is catalysed by enzymes of the uridine diphospho-glucuronosyltransferases (UGTs) family [63]. UGTs are membrane-bound enzymes that are anchored via the C-terminus to the endoplasmatic reticulum membrane [68]. At least 19 UGTs are known to be expressed in the human body. Based on the homology of the primary structures shared by this class of enzymes they have been divided in two families, UGT1 and UGT2, which are also divided in subfamilies according to their similarity [1]. Among these enzymes, the UGT2B17 isoform plays an important role in androgens conjugation [69].
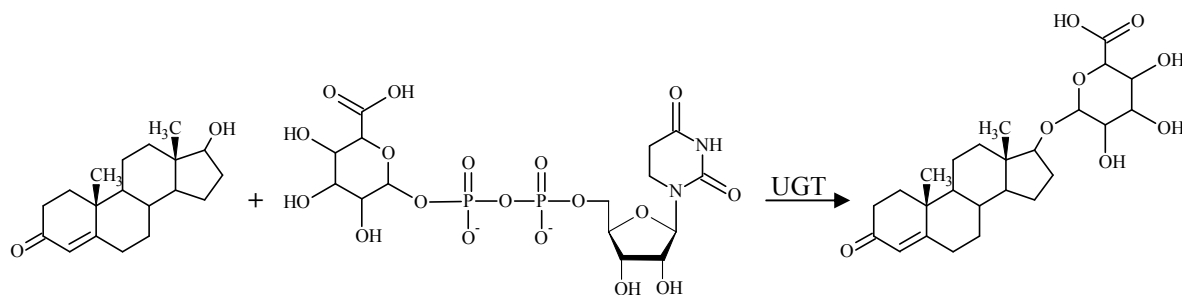
Figure I.13 – Phase II glucuronidation reaction of testosterone. Testosterone glucuronide is a minor metabolite of testosterone metabolic inactivation [1].

Regarding sulphonation, it is a common pathway before the metabolic excretion of endobiotic and xenobiotic compounds [68]. The enzymes responsible for the transfer of the sulphate moiety to the target compound are called sulphotransferases (SULT) and use the 3´-phosphoadenosine-5´-phosphosulfate as co-substrate [1]. Unlike the UGTs, the sulphotransferases enzymes may be found in the cytoplasm and also bound to membranes of the Golgi complex, however, only the cytoplasmatic fraction is active in the Phase-II metabolism [69].

The principal site of conjugation, in the AAS molecule, for both glucuronide and sulphate moieties is the hydroxyl group at the C_3 carbon [64]. Additionally, glucuronidation may occur at the hydroxyl group at the C_{17} carbon, whenever it is a secondary carbon [64]. Glucuronidation at the hydroxyl group of a tertiary C_{17} carbon is unlikely to occur due to steric hindrance [64]. Concerning

sulphonation, it may also occur at the hydroxyl group at the C_{17} carbon, either this is a secondary or tertiary carbon [64].

Within these two reactions, glucuronidation is the predominant metabolic pathway for AASs inactivation and therefore, the steroid glucuronides are the main excretion metabolites found in urine [64]. The exceptions are the 3β-hydroxylated steroids, such as DHEA, which is mainly excreted as sulphate [1, 64]. Moreover, the fraction of free androgens found in urine is around 3%, meaning that conjugation is vital for androgens elimination from the body [1].

I.3.3.5 TESTOSTERONE IN WOMEN

As mentioned above, testosterone circulating in women bloodstream is mostly originated in peripheral tissues by conversion of other weaker androgens, such as androstenedione and DHEA, which are produced in the adrenal gland and ovaries [70, 71]. In addition, testosterone is also formed inside both adrenal gland and ovaries [70, 71]. In the ovaries testosterone is produced in the theca cells as a response to LH [52, 72]. In the adrenal gland, it is produced in the cortex and is mainly formed as a by-product of the glucocorticoids biosynthesis [28]. Compared to men, the amount of testosterone produced by women is about 10 times lower; however, the effects of testosterone in women are not so less pronounced [28].

The secretion of glucocorticoids and consequently of testosterone by the adrenal gland is controlled by a series of hormonal cascade reactions triggered by the hypothalamus [52, 73, 74]. Likewise the regulation of testosterone secretion by the testes, the secretion of testosterone by adrenal gland in regulated by a series of feedback and feedforward mechanisms that involve the hypothalamus, the pituitary, and the adrenal gland [52, 73].

In response to a series of stimuli, the hypothalamus initiates the secretion of the corticotrophin releasing hormone (CRH) and the vasopressin (AVP) [52, 74, 75]. These hormones, mainly the CRH, act on the pituitary gland and induce it to produce the adrenocorticotropic hormone (ACTH) [52, 73-75]. ACTH is released in the circulation system and interacts with cells of the adrenal gland to produce both glucocorticoids and androgens [52].

In turn, the concentration level of glucocorticoids and androgens regulate the CRH and ACTH secretion [52]. Negative feedback occurs when the concentration of the adrenal compounds in the blood is elevated, thus inhibiting the ACTH and CRH secretion.

I.3.4 STRUCTURE AND BIOLOGICAL ACTIVITY

The androgens are a group of biologically active compounds responsible for the development and maintenance of the male sex characteristics [67]. In the male foetus, their action is related to the

development of the reproductive tissues, including prostate, seminal vesicle and testes [76]. During and after puberty, in addition to the development of the reproductive system, androgens are responsible for the development of the secondary male sex characteristics. At this stage, androgens promote both androgenic and anabolic activity depending on the tissue type in which they act [76]. The androgenic effects of androgens are generally considered as those associated with "masculinisation" or 'virilisation', being the major effects the deepening of the voice caused by the enlargement of the larynx, the growth of beard and other terminal hair, an augment in sebaceous gland activity and alteration of the emotional behaviour, particularly in aggressiveness and sexual libido [77].

The anabolic effects are those related with nitrogen retention and subsequent protein synthesis in muscle and bone, which allows the increase of muscle mass and strength [76]. The androgenic and anabolic activity of androgens has been evaluated by both in vivo and in vitro essays [78, 79]. The growth of specific organs along with protein anabolic activity, have been used to monitor both androgenic and anabolic effects [79]. The different androgens display different androgenic and anabolic effects suggesting a deep structure–activity relationship [1, 67]. It is important to stress that the evaluation of the most pronounced effect, androgenic or anabolic, is done using the anabolic index that is defined by the ratio of anabolic to androgenic activity. A value higher than one specifies a predominant anabolic nature of the compound, while a value lower than one indicates that the compound is predominantly androgenic [76].

I.3.4.1 ENDOGENOUS ANDROGENS

The contribution to enhance or diminish both androgenic and/or anabolic action by the different androgens has been evaluated through numerous structure-activity relationship studies [76, 80-82]. The functional groups present in the backbone structure of androgens play an important role in their anabolic/ androgenic activity. For instance, the D-ring 17β-hydroxyl group, present in both testosterone and 5α-DHT, is thought to be essential for the androgenic activity of the steroid molecule. The absence or oxidation of this group has shown a drastic decrease in the steroid androgenic activity [76].

Other studies, suggest that the presence of a double bond between the C-4 and C-5 carbon atoms, putatively reduces slightly the androgenic activity of the steroid. This feature is demonstrated by the higher affinity that 5α-DHT binds to the androgen receptor, compared to testosterone [76]. However, for 19-nortestosterone, a small metabolite produced by the human body in which lacks the C-19 methyl group when compared to testosterone, the reduction of the C-4 and C-5 double bonds has the opposite effect of that shown by 5α-DHT. This feature of the 19-nortestosterone is important for the dissociation of the androgenic and anabolic effects, since its effect is reduced in the androgenic

tissues after the action of the 5α-reductase, although it keeps its anabolic activity in the skeletal muscles, where the activity of the 5α-reductase is negligible [76, 77].

The A-ring carbonyl group at the C-3 position also assumes an important role in the androgenic activity [83]. A decrease in the androgenic properties has been demonstrated between testosterone and 5α-androstane-3β,17β-diol [76].

The introduction of a carbonyl group in the C-11 presents less affinity to the androgen receptor when compared to testosterone [76].

I.3.4.2 SYNTHETIC STEROIDS

Modern science advances have boosted the discovery of new pharmaceutical compounds based on endogenous androgen structures. A desirable dissociation of the anabolic and androgenic activity was achieved for some of these synthetic substances [76, 77]. These compounds emerged in medicine as therapeutic drugs to address androgen disorders. However, from its introduction as pharmaceutical drugs to its use as doping agents was a small step. Furthermore, several compounds started to be designed and produced clandestinely and released in a parallel market with the aim to surpass the doping tests [84].

Based on structure-activity studies performed with endogenous compounds it was possible to identify the essential groups related with both androgenic and anabolic activity, and therefore, synthesise powerful anabolic steroids [1]. The majority of these synthetic steroids resulted from structural modifications made to testosterone molecule with the aim to overcome its rapid biotransformation, increase its time of action, increase its anabolic activity and decrease the androgenic side effects [1, 77]. Figure I.14 shows the testosterone's significant sites where structural alterations have been introduced in an attempt to maximize the anabolic activity and some of the most used AAS showing these modifications [76].

(a)

| Bolasterone | Calusterone | Testosterone enanthate | Boldenone | Norethanrolone |
| Clostebol | Nandrolone | Mesterolone | Oxandrolone | Stanozolol |

(b)
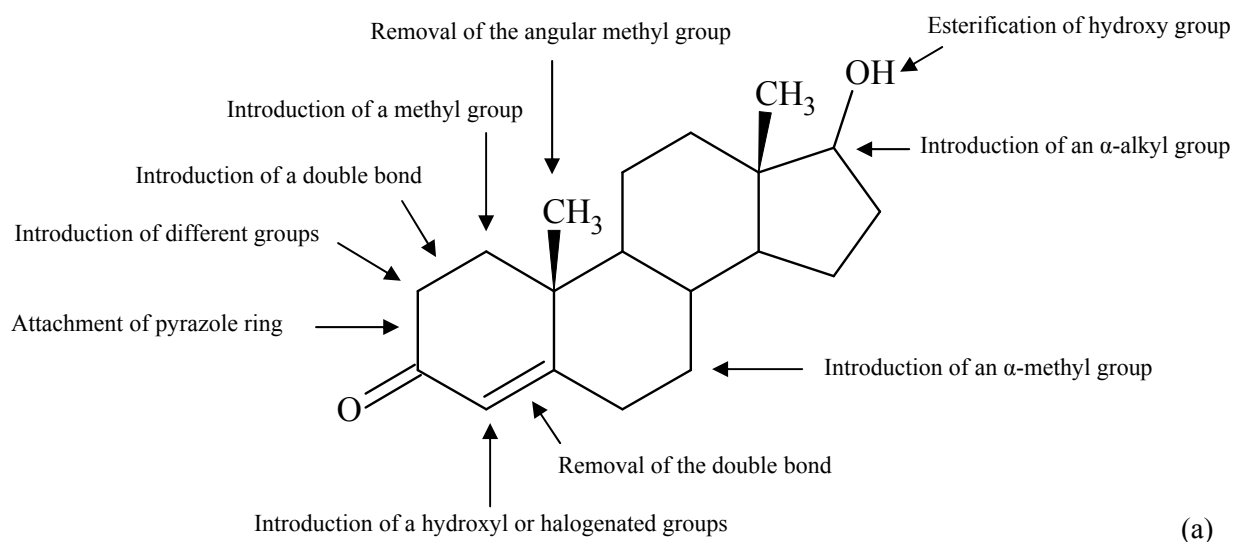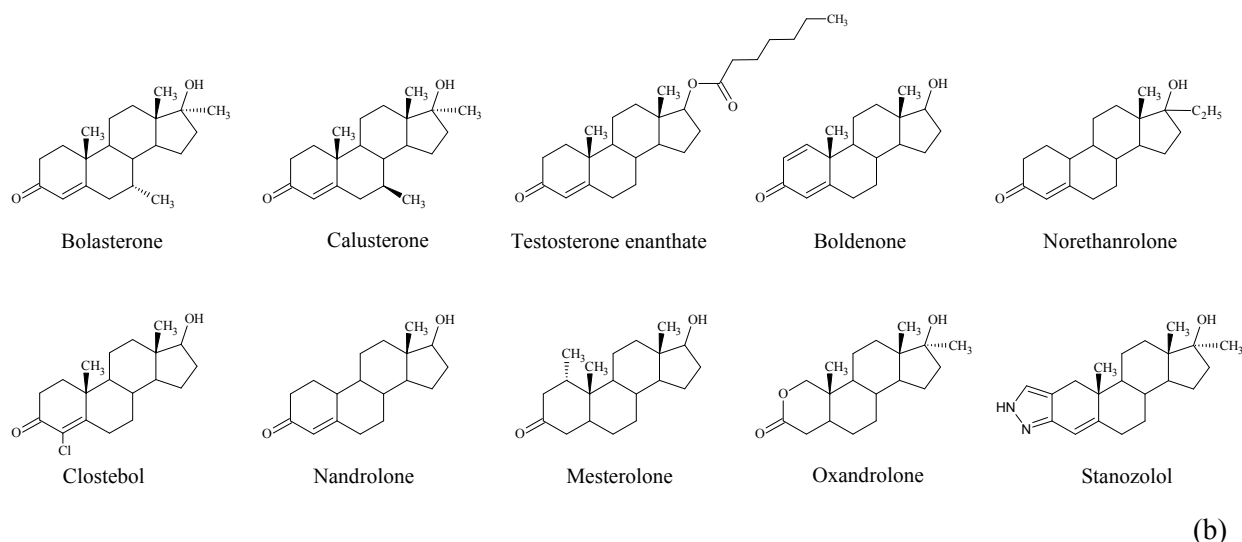
Figure I.14 – (a) Common structural alterations introduced to testosterone molecule. (b) Chemical structures of some examples of pharmaceutical and designer AAS [1].

I.3.5 PHYSIOLOGICAL EFFECTS OF AAS UNDER INTENSIVE TRAINING

Androgenic anabolic steroids exert multiple actions affecting both the physiology of the human body and the individual emotional behaviour. Besides its effects in the reproductive system and in the sexual characteristics and emotional behaviour, the metabolic action of androgens affects the regulation of several other hormones and metabolic pathways. Under intensive training, androgens induce the synthesis of proteins in muscle and bone, causing an accelerated growth of these organs [28, 85]. Furthermore, during acute endurance workout, as well as during competition, androgen`s action seems to be critical to enhance the performance capacity, since they affect the production of red blood cells and increase neural conduction [28]. In addition, after intense exercises, androgens are thought to prevent muscle catabolism and exhaustion and to speed up the recovery process [28, 85].

It is important to stress that the metabolic effects of androgens in muscle and bone hypertrophy must be associated with intense training. High levels of androgens in the blood, by itself, cannot produce a pronounced metabolic effect if the androgen specific receptors do not increase as well. It is known that intense training causes the up-regulation of androgen receptors [28, 57, 85].

I.3.6 BEHAVIOURAL EFFECTS OF AAS

Alongside with the AAS association with physical capacity and performance, they are also associated with behavioural characteristics, such as aggression, anxiety and motivation [77, 86]. Several studies have correlated the levels of testosterone and other AAS to an increase of dopamine synthesis and receptors, which is known to play an important role in several cognitive functions, such as motivation,

attention and memory [77, 87]. Other studies developed in rats have shown an increase in the aggressive behaviour after AAS intake [86]. High testosterone concentration has also been related to lower tolerance for frustration in adolescents [88].

I.3.7 ADVERSE EFFECTS OF AAS ABUSE

Abuse of anabolic androgenic steroids (AAS) can induce serious health problems. The major organs affected by the abuse of these compounds are the reproductive, the cardiovascular, the hepatic and the dermatologic organs [77, 89]. Endocrine and behaviour alteration is also attributed to the abuse of AAS[76]. Figure I.15 shows the major adverse effects of anabolic steroid abuse.
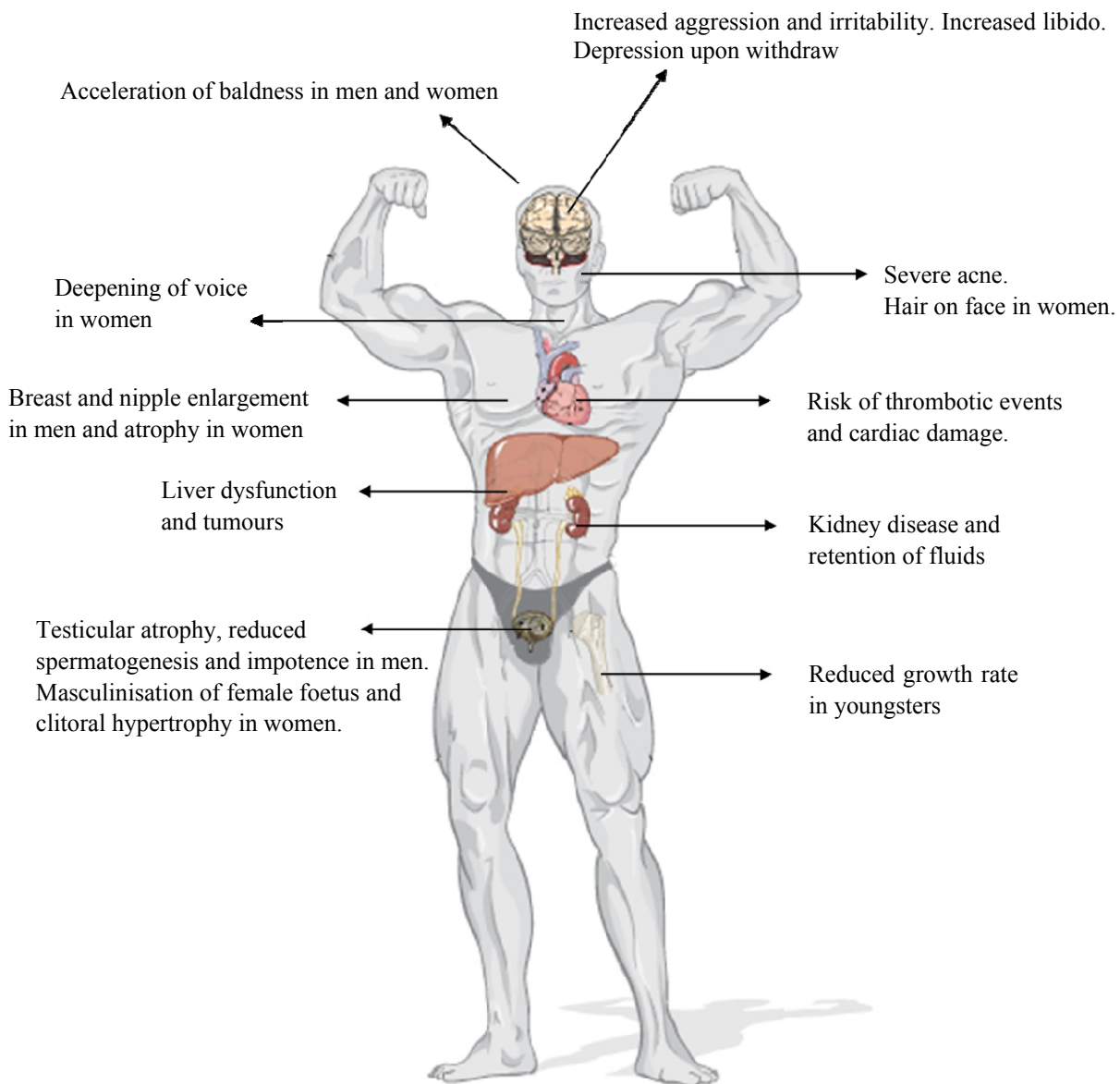
Figure I.15 – Schematic representation of the primary adverse effects of AAS abuse (adapted from Lynette Rushton, *The Endocrine System*, Chelsea House Publications, 2009)

Among the reproductive and endocrine systems, common adverse effects are the decrease of LH and FSH secretion that has direct effects in testicular atrophy and spermatogenesis. Fertility problems, impotence, prostate hypertrophy, prostatic carcinoma and gynecomastia were also associated to AAS use in men, whereas in women masculinization, menstrual irregularities and breast atrophy are the main effects [76]. Along with these alterations, common side effects in both men and women include libido changes, acne as a result of sebaceous glands stimulation, hair recession and alopecia [89, 90].

Several reports have linked the misuse of AAS to a variety of different cardiovascular side effects. AAS promote a lipid content alteration, causing the augment of the low density lipoprotein cholesterol and the decline of high density lipoprotein cholesterol along with apoprotein [1, 76]. This alteration in the lipid metabolism may be related to several coronary artery diseases. Other cardiovascular events comprise the risk of thrombosis, serious arrhythmia, alteration in both morphologic and functional characteristics of the heart and acute myocardial infarction, which is the primary cardiovascular event related to AAS abuse [1].

The AAS cause deep adverse effects on the liver, and common hepatic health issues include increasing levels of liver enzymes, cholestatic jaundice, peliosis hepatis and neoplasia [76].

As mentioned previously the use of AAS may cause some changes in the mental status and behaviour. The major effects are increasing aggressiveness, irritability and mood changes. Some abusers may also suffer from psychosis [89, 91].

I.4 ANALYSIS OF AAS IN DOPING CONTROL

I.4.1 HISTORICAL BACKGROUND

Effective detection of androgenic anabolic steroids is the outcome of a succession of events, mostly related to the availability and development of analytical technologies and methods able to detect their presence in the athlete's body.

The development of radioimmunoassays to detect some of these compounds in the mid 1970s, led to their inclusion in the list of prohibited compounds of the IOC in 1976, although their abuse by athletes was known for a long time [28]. About the same time, the introduction of mass spectrometry detectors (MS) coupled to gas chromatography (GC) was a major development in doping control, so that, in the 1980s, with the widespread availability of high resolution GC-MS systems, radioimmunoassays were completely abandoned [1, 92, 93]. The introduction of mass spectrometers in doping analysis made it possible to achieve distinctive "fingerprints" of each banned substance and, most important, of its metabolites [92].

In the beginning of the 1980s, a problem concerning the detection of anabolic steroids was still persisting; how is it possible to detect the abuse of endogenous steroids, such as testosterone? The answer was given by Professor Donike`s group that introduced a testosterone (T) to epitestosterone (E) ratio threshold, in which a measurement of a urinary T/E exceeding 6 should be considered a doping offence [1, 93]. This proposal was based on the fact that epitestosterone is not a metabolite of testosterone and therefore its concentration does not increase after administration of testosterone, meaning that an increased ratio of T/E would represent an abuse of testosterone [94].

As it was expected, it did not take long for athletes to find new formulations of testosterone with longer activity and requiring lower dosage [1]. At the same time, false positives were substantiated invoking physiological alterations due to metabolic and environmental factors [94]. To overcome these issues, doping laboratories started to detect the excreted testosterone metabolites along with the T/E ratio [1]. Furthermore, as a complementary tool, at the end of the 1990s, isotope ratio mass spectrometry (IRMS) was introduced and it became essential to detected metabolites of synthetic source [1].

Nowadays, a T/E ratio above 4 is considered abnormal and further analysis should be performed to confirm if it represents a doping offence.

I.4.2 DETECTION OF AAS IN DOPING CONTROL

It is important to stress that in order to ensure the accuracy of the analytical testing that is essential to guarantee the effectiveness and validity of the analytical result, doping tests are carried out following quality guidelines provided by the WADA International Standards for Laboratories. Consequently,

initial screening analysis is currently performed on a single aliquot integrated in batch groups that include appropriate control samples. Moreover, if a presumptive adverse analytical finding is detected, confirmatory analysis must be performed in triplicate using, if possible, more specific methods [95].

The detection of AAS misuse is normally performed using urine specimen as matrix. The AAS metabolism plays an important role in the methodologies used to detect these compounds. As it was explained in the last chapter, both endogenous and exogenous AAS suffer extensive biotransformation by Phase-I and Phase-II metabolism and are excreted to urine in a modified inactivated form. Since the main pathway for conjugation and inactivation of these substances in the human body is glucuronidation, in urine, most compounds are found as glucuronide conjugates. Therefore, the detection methods routinely employed focus primarily on those metabolites. Chromatographic-spectrometric techniques, mainly GC-MS techniques, are still the current method for both screening and confirmatory analysis of AAS. They include the detection of the characteristic exogenous AAS metabolites, whose presence is exclusively due to doping, and the accurate measurement of the steroid profile of endogenous steroids.

I.4.2.1 ANALYTICAL TECHNIQUES

In general, the normal proceeding for AAS determination includes chromatographic separation coupled to mass spectrometry detectors. As already mentioned, the use of GC-MS methodologies is the most employed strategy for AAS control. However, over the last years with the development of suitable LC-MS and LC-MS/MS systems, some AAS presenting poor chromatographic properties for GC-MS analysis, even after derivatisation, are being analysed by LC-MS(/MS) procedures.

I.4.2.1.1 GC-MS

GC-MS is probably the most important tool for the identification of volatile and semi-volatile compounds. This technique combines the ability of GC to separate volatile compounds with great resolution, with the detailed structural information provided by the MS detector. Briefly, after the GC run the analytes are eluted into the mass spectrometer, where they are ionised and fragmented, usually by electron impact (EI) ionisation. The fragments are accelerated into the mass analyser, where they are separated according to their mass/ charge ratio, and then into the detector. The resulting mass spectrum presents the characteristic molecular fragments of each analyte (detailed description is given in chapter I.5).

The most common mass spectrometers coupled with gas chromatographs contain the single quadrupole mass analyser, which is also the mass analyser currently used for the screening of AAS and its metabolites [96-101]. GC-MS systems can be used in full scan, for a comprehensive analysis,

or in selected ion monitoring (SIM), for a selective scan of the most characteristic and specific fragment ions of the target compounds [102]. However, due to the required lower limits of detection for some AAS, which can be as low as 1-2 ng/mL, routine screening analyses are usually performed in SIM mode. General screening methods are performed selecting up to four characteristic ions per analyte. Owing to the numerous AAS and their metabolites included in the screening methods, the number of monitoring ions per analyte is chosen in order to guarantee its correct identification and, at the same time, to avoid unnecessary repetitions of sample preparation and analysis.

A great improvement in doping analysis of steroids was the introduction of high resolution mass spectrometers that made possible to distinguish between two nominal masses with precise measures of the characteristic ions [103]. This is especially important for long-term metabolites that are present in urine at lower concentrations, and therefore, precise measurement of the characteristic ions provides higher sensitivity to the analytical method [103].

Recently, the introduction of tandem mass spectrometry allowed greater specificity for the identification of AAS due to the higher discrimination power, which is of great importance, especially for confirmatory purposes.

The initial screening analyses for AAS detection comprise both qualitative and quantitative measurements. For most AAS and its metabolites the qualitative evidence of the characteristic ions is sufficient for a presumptive adverse analytical finding, whereas for urinary endogenous AAS the precise measurement of excreted concentration is required.

Concerning the endogenous AAS, there are some issues regarding the use of T/E ratio alone, including the fluctuation that the steroid profile may suffer from inter-individual samples and the use of epitestosterone as a masking agent [104]. Therefore, complementary tools to detect the administration of endogenous steroids were introduced, of which isotope ratio mass spectrometry (IRMS) became essential in doping control [104].

It is important to stress that to be analysed by GC-MS techniques, the target analyte must be both volatile and thermal stable. Since most of the anabolic steroids, either in the conjugated or free form, do not fulfil this requirement, as a general rule, the analysis of such substances by GC-MS techniques requires extensive sample preparation.

I.4.2.1.2 GC-C-IRMS

The measurement of $^{13}C/^{12}C$ isotope ratio to provide confirmation for administration of synthetic endogenous hormones was proposed in 1990 to detect the misuse of testosterone [1, 104]. Although both synthetic and physiological steroids are chemically identical, differences in their $^{13}C/^{12}C$ isotope ratio can be found. The scientific basis supporting this technique is given by the kinetic isotope effects from the natural C3 and C4 pathways used by plants to fix CO_2 [105, 106]. Plants discriminate

differently against ^{13}C depending on the CO_2 assimilation pathway followed and consequently their organic compounds will present characteristic isotopic signatures depending on their origin [106].

The analytical method employed is sustained by the fact that the isotope ratio found in human endogenous metabolites is a result of their diet, which ultimately derived from a variety of nutrients with different sources, whereas the synthetic steroids derived from a single vegetal precursor and will present a distinct isotopic signature [104, 105].

Accurate carbon isotope analysis is carried out on GC/C/IRMS systems that couple gas chromatography with online combustion-isotope ratio mass spectrometry [106]. The CO_2 formed in the combustion interface is analysed by the IR mass spectrometer that continuously measures the isotopic ratio.

The carbon isotope ratios measured are given against an international reference and are expressed in terms of $^{13}\delta$ C values in units of per mille ($^0/_{00}$), where $^{13}\delta$ C is calculated according to the following equation [1, 105, 106]:

$$^{13}\delta\,C = \left(\frac{r_{sample}}{r_{reference}} - 1\right) \times 1000, \qquad r = \left(\frac{^{13}C}{^{12}C}\right)$$

Physiological values commonly vary between -25 to -16 $^0/_{00}$, while values lower than -27 $^0/_{00}$ are often achieved for synthetic compounds [107].

I.4.2.1.3 LC-MS(/MS)

The introduction of liquid chromatography coupled with MS or MS/MS systems for the analysis of AAS presenting marginal gas chromatography properties, even after derivatisation, emerged as an important solution for their detection [108]. The availability of soft ionisation interfaces such as atmospheric pressure chemical ionisation (APCI) and electrospray ionisation (ESI) expanded the use LC–MS(/MS) techniques and since then it has been used to complement numerous doping analysis [108, 109]. AAS such as trenbolone, gestrinone, stanozolol and related compounds that are thermolabile and/or present exceptional polarity are already being analysed by these techniques with great sensitivity. In fact, the marginal properties of these compounds for GC-MS have resulted in high detection limits and until the introduction of LC-MS strategies, athletes successfully misused some of these compounds, overcoming doping control [109]. Furthermore, the potential use of LC–MS(/MS) techniques to allow the direct analysis of conjugated AAS, which would significantly reduce both the time of the analysis and analyte losses during the sample preparation, represents a major encouragement for its implementation [110]. In addition, the direct measure of sulphate conjugates would be of great importance for doping control. Recently, several studies have reported the use of LC-MS/MS methodologies for a broader screening of AAS [111, 112].

Despite its potential and the attempts to cover more AAS in anti-doping screening methods, the single use of LC-MS(/MS) for the analysis of AAS and other banned substances still presents some limitations, of which the most evident is the cost of these equipments compared to GC-MS. Furthermore, there is still no reliable method to detect the origin of the endogenous steroids based on LC systems [110]. Concerning the direct analysis of conjugates, the lack of analytical standards remains the major challenge. Moreover, problems with AAS ionisation are known and may lead to losses in sensitivity for some compounds [108]. An option to overcome this issue is to perform a derivatisation step.

I.4.2.2 SAMPLE TREATMENT

Steroid detection in urine is still based on the method developed by Professor Donike in the early 1980s and it comprises three major steps: (i) enzymatic hydrolysis of the conjugated compound to produce the free parent compound, (ii) extraction of the AAS from the urine matrix and (iii) derivatisation of the free analyte to enhance its physical-chemical proprieties for GC-MS analysis [113-116].

An overview scheme of the GC-MS sample treatment for steroid determination is presented in Figure I.16. The first two steps can be carried out in alternating order, meaning that the enzymatic hydrolysis of the AAS conjugates can be performed either directly on the urine sample or on the extract solution. Special care must be taken regarding the first approach to ensure the complete or extensive deconjugation of key AAS.

SAMPLE TREATMENT

Step 1
2 mL of urine + 0,750 mL phosphate buffer (0,8M; pH 7) + 50 µL β-glucuronidase from *E.coli*.
Keep 1 hour at 55°C.

Step 2
Alkalinisation with carbonate buffer
Liquid-liquid extraction or solid phase extraction.
Evaporation to dryness.

Step 3
Add 50µL MSTFA/NH_4I/DTE
Keep 30 minutes at 78°C.
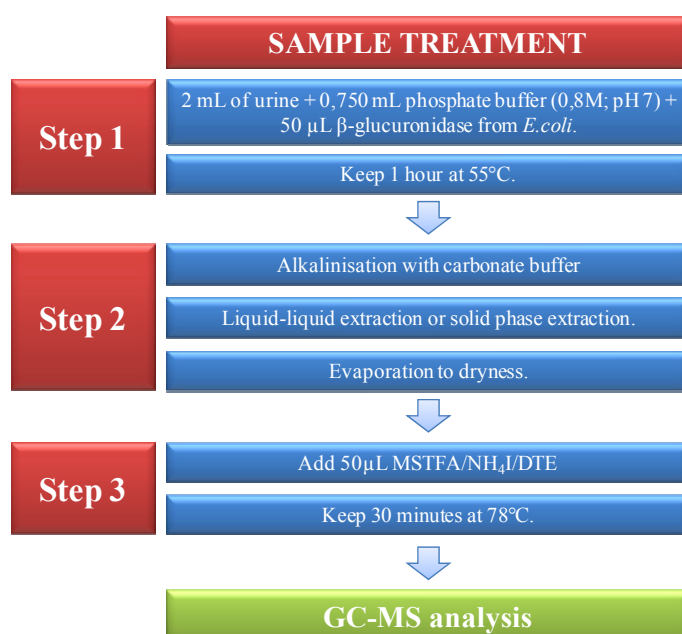
GC-MS analysis

Figure I.16 – Schematic sample treatment methodology for AAS analysis by GC-MS.

I.4.2.2.1 HYDROLYSIS

The hydrolysis of the AAS can be carried out by both enzymatic and chemical approaches; however, enzymatic hydrolysis is the primary choice within anti-doping laboratories, since chemical hydrolysis presents several disadvantages that comprise the degradation of some analytes, formation of several contaminants by other urine organic components degradation, subsequent increase of matrix interference and degradation of important working parts of the analysis equipment [94].

Regarding the enzymatic hydrolysis, different approaches can be followed depending on the target conjugated metabolites. Deconjugation enzymes with β-glucuronidase activity are purified mainly from bacterial sources, particularly from *Escherichia coli*, whereas the sulfatase activity that is responsible for cleavage of the sulphate moiety is purified mainly from molluscs, predominantly from *Helix pomatia* and *Patella vulgate [110, 117]*. It is important to stress that the purification of enzymes presenting single sulfatase activity is extremely difficult and a mixture of both sulfatase and β-glucuronidase activities are always present [117]. Moreover, enzymatic preparations from *Helix pomatia* are known to possess unwanted enzymatic activities that may cause the alteration of the urinary profile [117, 118].

Although the use of sulfatase activity for deconjugation of specific steroids that are found in urine mainly as sulphate conjugates could be of interest for doping purposes, efficient enzymatic preparations are yet inexistent [119]. Consequently, most anti-doping laboratories perform the hydrolysis of AAS, using solely purified β-glucuronidase from *E.coli*, which is highly specific for glucuronide conjugades and preserves the integrity of the steroid profile.

I.4.2.2.2 EXTRACTION AND CLEAN-UP OF AAS FROM URINE

In order to accomplish maximum specificity and sensitivity for the analysis of anabolic steroids from human urine, special attention must be focused on the clean-up and extraction steps. Typical sample treatment procedures employed for AAS determination include solid phase extraction (SPE) or liquid-liquid extraction (LLE) [120].

Liquid-liquid extraction is one of the oldest sample preparation techniques and is frequently employed prior to chemical analysis by chromatography [121]. Briefly, in LLE the target analyte is extracted from a liquid medium, normally an aqueous sample matrix, and dissolved in a solvent that is immiscible with that liquid medium, normally an organic solvent [121]. The solubility of the analyte in the two immiscible solvents will dictate the distribution between the two phases. An efficient extraction is accomplished if the analyte has favourable solubility for the extracting solvent [121].

The extraction of the unconjugated steroids and respective phase-I metabolites is accomplished after alkalinisation of the urine bulk, using mainly diethyl or tert-butyl methyl ether as solvents [111, 122-124]. This approach is widely used within anti-doping laboratories and it presents

high efficiency of extraction [111, 122-124]. Nevertheless, some limitations are attributed to LLE: high consumption of organic solvents, difficulties of automation, possible formation of emulsions and limited flexibility in terms of extraction chemistry [121, 125]. Although most of these limitations are easily avoided with low volumes of urine samples, improved sample treatment equipments and centrifugation, the low selectivity in terms of extraction chemistry, which is important, for instance, in confirmatory analyses, is difficult to surpass [125].

An alternative approach to extract the steroids from urine samples comprises solid phase extraction. SPE consists of a separation process in which the analyte of interest is normally isolated in a packaged disposable cartridge containing a solid sorbent and later eluted [121]. Briefly, the liquid medium passes through the SPE cartridge, previously equilibrated, and its constituents are retained in the cartridge according to their affinity to the solid phase. The extraction removes much of the sample matrix and allows extensive sample clean up with different solvents. At the end, the subsequent recovery of the analytes of interest occurs by elution from the solid phase with an appropriate solvent. SPE has proven to be suitable for the cleanup and extraction of AAS from urine samples and therefore is employed as much as LLE within anti-doping laboratories [126-128]. Specific solid sorbents for AAS extraction based on C18 have been developed by the analytical suppliers. The elution from the cartridge is performed using the same solvents used by LLE. The practical advantages of SPE are more evident concerning confirmatory analyses, since the use of different solid sorbents or different clean-up mixtures of solvents originate cleaner extracts and allow more selectivity.

In both LLE and SPE procedures the collected organic phase is evaporated to dryness and the dry residues may be then derivatised for GC–MS analysis or reconstituted in appropriate solvents for LC analysis.

I.4.2.2.3 DERIVATISATION

As it was mentioned above, GC-MS is the primary technique for both screening and confirmatory analyses of AAS misuse. However, the direct analysis of the "free" steroids is difficult, mainly due to the low volatility of AAS and to the presence of functional groups in the molecules, which are frequently unstable under the chromatographic conditions [1, 129]. GC-MS provides specificity and sensitivity for the analysis of AAS, but since AAS do not fulfil the standard requirement of volatility and thermal stability, a derivatisation step must be employed prior to the GC-MS analysis [110].

Derivatisation is by definition a procedure in which the analyte is chemically modified to change its characteristics and consequently its suitability for the intended purpose.
Besides increasing the volatility and thermal stability, derivatisation of the AAS also improves the symmetry of GC peaks, which facilitates quantitative evaluations [120, 130]. Most important, derivatisation is essential to improve the GC separation of the urinary AAS, which is particularly important for the separation of steroid epimers [130]. Moreover, derivatisation is also used to enhance

mass spectrometry properties by producing more favourable diagnostic fragmentation patterns [120, 130, 131].

The conversion into TMS derivatives is by far the most widely employed procedure for AAS derivatisation [96, 127, 132, 133]. There are various methods for preparation of TMS derivatives; nevertheless the most employed derivatisation reagent used by the WADA accredited laboratories is methyl-N-trimethylsilyltrifluoroacetamide (MSTFA), principally in combination with ammonium iodide and ethanethiol, which gives rise to *in situ* generated trimethyliodosilane (TMIS), a highly reactive trimethylsilylation reagent [129]. The major advantage of MSTFA over other TMS donors is the higher analyte volatility obtained [131].

Although extreme reaction conditions are not required, as most derivatisation reactions, silylation of AAS must be carried out in the absence of moisture. Consequently, the reaction is normally performed in close vessels and immediately prior to the analysis. Nevertheless, AAS derivatives have been reported to be stable for few days in the absence of moisture [130].

I.5 OTHER STRATEGIES FOR AAS DOPING CONTROL

The aim of the research programme presented in this thesis was primarily the development of a new screening method based on mass spectrometry (MS) using the soft ionisation technique matrix-assisted laser desorption/ionisation (MALDI). The major goals to be achieved were the development of an accurate, sensitive and robust methodology able to improve the screening of AAS for doping control in both analysis time and sample throughput. Additionally, the developed method should be capable to overcome the GC-MS limitation related to thermolabile and polar AAS, so that the initial screening method could be extended to all AAS included in the prohibited list.

In parallel with the development of a screening procedure based on MALDI-MS(/MS) techniques and applying the deep expertise of the research group on reaction enhancement by delivery energy based techniques, the improvement of the global sample preparation for the analysis of AAS by anti-doping control laboratories was also included in the research programme.

In this chapter a description of both analytical and sample treatment techniques employed to achieve the aforementioned goals are described.

I.5.1. MASS SPECTROMETRY: MALDI-MS(/MS) STRATEGIES

I.5.1.1 BASICS OF MASS SPECTROMETRY

Mass spectrometry (MS) is a powerful analytical technique that has become imperative in numerous fields of science. Nowadays, MS is perhaps the most versatile and comprehensive technique available for chemists and biochemists, as it allows both qualitative and quantitative analyses and presents a wide range of applications.

In mass spectrometry analysis, gaseous molecules are ionised by any suitable method, accelerated by an electronic field into the mass analyser, separated according to their mass-to-charge ratio (m/z) and detected in proportion to their abundance.

Inside the mass spectrometer, the occurrence of these processes requires, at the very least, four components, the sample inlet, the ion source, the mass analyser and the detector. Other essential features common to all mass spectrometers are a vacuum and data systems. It is important to stress that, with exception to the ionisation process that might be performed at atmospheric pressure depending on the ion source, all the other processes occur under high vacuum conditions to avoid molecular collisions, which may lead to fragmentation of the molecular ion as well as to the formation of different species [134]. Moreover, high vacuum is essential for ions to move freely and reach the detector with minimum losses in sensitivity.

There is no single approach for mass spectrometry analyses and numerous strategies of sample introduction, ionisation, separation and detection are available depending on the application field.

I.5.1.1.1 MODES OF IONISATION

The ionisation process is the first step in the analysis by mass spectrometry and it takes place inside the ion source. In mass spectrometry, the ionisation process is essential because, unlike neutral particles, the motion and direction of charged particles are easy to manipulate experimentally by application of electric and magnetic fields [134, 135]. This feature is crucial for the subsequent separation and detection processes [135].

A variety of ionisation techniques may be used with mass spectrometry. Before the introduction of recent soft ionisation techniques, most mass spectrometric analysis were restricted to low weigh compounds and the ionisation was achieved primarily by electron impact [134, 136]. In EI process, prior to ionisation the analytes are normally separated by hyphenated techniques, mainly GC, and must be transferred into the gas phase [134]. The evaporation of the analyte is mainly achieved by heating, which to some extent restricts this mode of ionisation to thermally stable and volatile compounds [134]. In the EI process, the neutral molecules are bombarded with a beam of energetic electrons with a kinetic energy of dozens of electron volts (eV) (see Figure I.17). The collision between the sample neutral molecules and the beam of electrons causes the ionisation of the molecules, predominantly, by expulsion of an electron, originating singly charged positive ions [135].
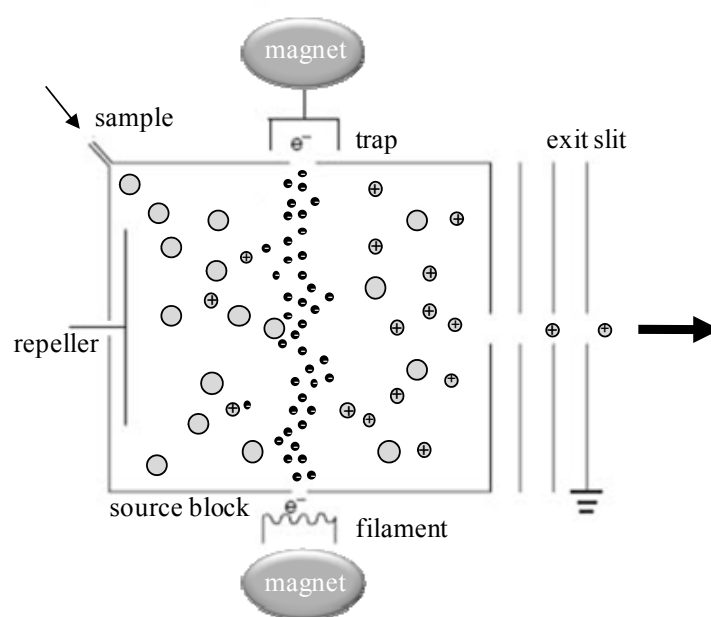
Figure I.17 – Common EI ion source (Chhabil Dass, *Fundamentals of Contemporary Mass Spectrometry*, John Wiley & Sons, 2007).

This mode of ionisation usually creates molecular fragments and subsequently the molecular ion is normally absent [137]. The fragmentation of the molecules occurs due to the energy delivery by the electron beam that extremely exceeds the ionisation energy of the sample molecule [137]. The fragmentation pattern obtained for a molecule is characteristic of that molecule for a specific kinetic energy of the electron beam. Kinetic energies of the electron beam within the range of 50 to 100 eV are required to achieve efficient ionisation and fragmentation patterns [134]. The fragmentation patterns obtained are used as fingerprints of the molecules. Moreover, since the kinetic energy of the electron beam used is conventionally 70 eV, EI allows the construction of libraries with mass spectrometric fingerprints of several organic compounds [134, 138].

Chemical ionisation (CI) is a technique that was introduced in the late 1960s as an alternative approach to overcome the extensive fragmentation of larger molecules or molecules with labile bonds, which cause their mass spectra complex to interpret [135]. This technique yields less fragmentation and the molecular ion is easily identified. In the CI ionisation process, the ionisation source is filled with a gas, for instance, methane, which after collision with a beam of highly energetic electrons is ionised and converted into reactive species that, through acid-base reactions, ionise the sample molecules [137]. For CI success, the pressure inside the ion source has to be sufficient to allow frequent collisions and an efficient pumping system has to be present after the ion source to ensure high vacuum conditions [134]. In this ionisation technique, the sample molecule ionisation occurs mainly through protonation and there is almost no fragmentation, therefore it might be considered as a soft ionisation technique.

At the end of the 20th century, two soft ionisation techniques allowing for sensitive mass spectrometric analysis of large biological molecules, namely electrospray ionisation (ESI) and matrix assisted laser desorption/ionisation (MALDI), revolutionised life sciences, in particular, the field of analytical biochemistry science [139-147]. Prior to the introduction of these two ionisation techniques, field desorption (FD), liquid secondary ion mass spectrometry (SIMS) and fast atom bombardment (FAB) played a vital role in extending mass spectrometry analysis to large molecules and dictated the scientific context for the development of upgraded MALDI and ESI techniques [135, 148].

A great feature of electrospray ionisation is that it is applicable to liquid phase samples. In ESI process, ionisation is produced by applying a strong electric field, under atmospheric pressure, to a continuous stream of diluted sample solution passing through a stainless steel capillary tube at a rate of few microlitres per minute [135]. The electric field is obtained by applying a potential difference to the tip of the capillary relatively to the walls of the surrounding area, called counter-electrode [135, 139, 149]. This potential difference induces a charge accumulation at the liquid surface located at the end of the capillary and allows the expansion of the liquid surface that disperses as a spray to form highly charged droplets [139]. To limit the spray dispersion in space a gas is injected coaxially at a low flow rate [134]. The transfer of the ions from the liquid-phase to the gas-phase is normally achieved by a hot flow of nitrogen that removes the last solvent molecules. Part of these evaporated ions is transported to the high vacuum mass analyser via a series of pressure-reduction stages [134]. A

scheme of ESI is showed in Figure I.18. Nowadays, ESI has become the predominantly ionisation technique coupled with LC, especially for analysis of biomolecules.
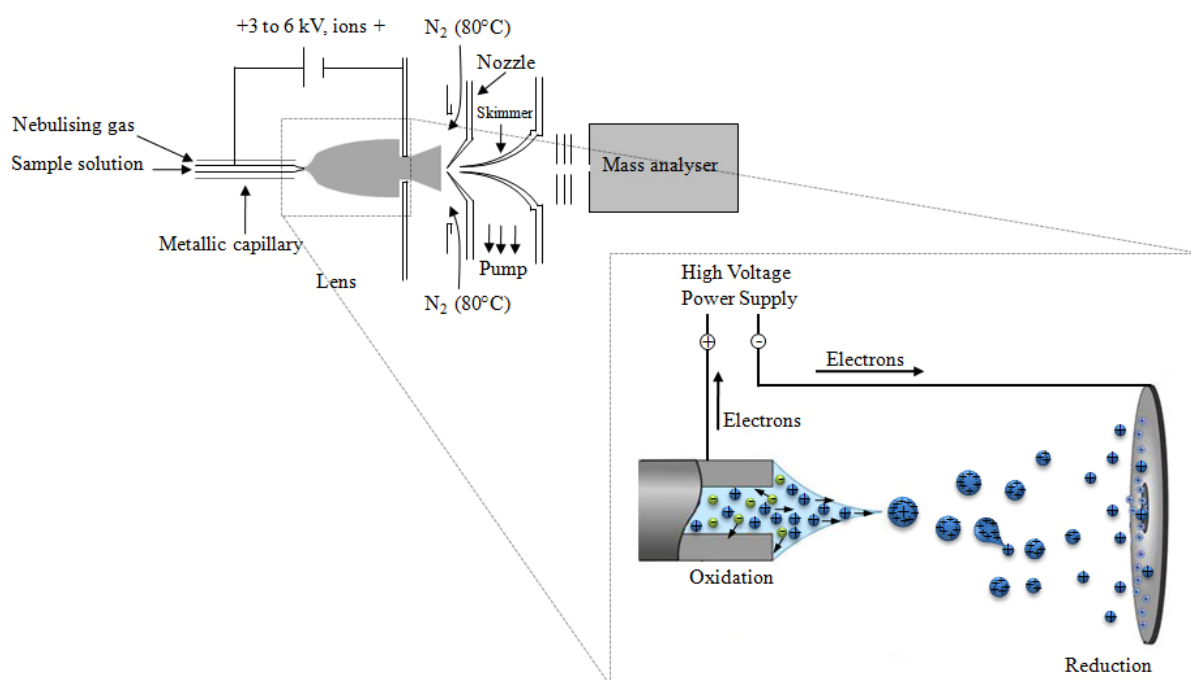
Figure I.18 – Schematic representation of the processes that occur in electrospray ionisation source.

Regarding the MALDI technique a detailed description is given in chapter I.5.1.2. Briefly, in MALDI process, ionisation is normally held by focusing a laser beam on a solid state mixture of the sample and a matrix with the ability to absorb the energy of the laser and transmit it to the sample.

Several other ionisation techniques are available today, however their use is less frequent than the ones mentioned and therefore are not included in this text.

I.5.1.1.2 MASS ANALYSERS

The mass analysers are the core of the mass spectrometer and are responsible for the separation of the ions formed in the ion source according to their mass-to-charge ratio. As it happens with the ion sources, there are several mass analysers commercially available and each has distinct characteristics that make them more or less suitable to a variety of applications. Accelerated ions can be distinguished from others based in differences in proprieties such as ion momentum, velocity and kinetic energy [135]. Consequently, their separation inside the mass analysers is performed using those properties.

The performance of a particular mass analyser is evaluated based on specific features that determinate the success of the analysis. The most important are the mass range, the resolution, the mass accuracy, the efficiency and analysis speed [135]. The mass range limits the m/z ratio suitable to analysis and determinates, for instance, the use of specific mass analysers for the analysis of large molecules instead of others. The resolution or the resolving power of a mass analyser plays a vital role in the success of analyte identification, since it represents the ability to separate distinct signals from two neighbouring mass ions. The mass accuracy represents the difference that is observed between the measure signal and the theoretical one; the more accurate is a measure the less difference is shown between the two *m/z* values. Resolution and accuracy are closely related, since high resolution is important to achieve accurate measures. The efficiency refers to the whole mass spectrometer and not only to the mass analyser and it takes into account the transmission of the mass analyser, which is the ratio between the number of ions that entered into the mass analyser and those who actually reached the detector, the duty cycle, which is the fraction of ions of a particular *m/z* ratio generated by the ion source that are in fact analysed, and the sensitivity of the detector. The efficiency of the mass spectrometer dictates its sensitivity and therefore the success of the analysis. Finally, the speed of the analysis or the speed of the mass scan is an essential feature in mass analysers and it represents the number of spectra acquire per unit time over a particular mass range. The speed of the scan represents a major role in MS applications, for instance, when coupled with chromatographic techniques, in which rapid scan is required to monitor the eluents, whereas for slow accurate measurements, less scan speed might be advantageous. The most common mass analysers as well as their principle of operation are presented in table I.2. The time-of-flight (TOF) mass analyser is described in detail in chapter I.5.1.2.3.

Table I.2- Common mass analysers (Reproduced from Gross, *Mass Spectrometry: A Textbook*, Springer 2004)

Mass Analyser	Acronym	Principle of operation
Time-of-flight	TOF	Time dispersion of a pulsed ion beam; separation by time-of-flight
Magnetic sector	B	Deflection of a continuous ion beam; separation by momentum in magnetic field due to Lorentz force
Linear quadrupole	Q	Continuous ion beam in linear radio frequency quadrupole field; separation due to stability of trajectories
Linear quadrupole ion trap	LIT	Continuous ion beam and trapped ions; storage and eventually separation in linear radio frequency quadrupole field due to stability of trajectories
Quadrupole ion trap	QIT	Trapped ions; separation in three-dimensional radio frequency quadrupole field due to stability of trajectories
Ion cyclotron resonance	ICR	Trapped ions, separation by cyclotron frequency (Lorentz force) in magnetic field
Orbitrap	OT	Trapped ions; separation by frequency of the axial ion motion

I.5.1.1.3 MASS DETECTORS

After separation within the mass analyser, the ions are detected and their abundance is proportionally transformed into an electrical signal. The information provided by the detector is displayed by the data system into a graph, called mass spectrum, which shows the electrical signal as a function of mass-to-charge ratio.

Most common detectors are based on the electric current produced when an arriving ion that hits a surface is neutralised or based on the kinetic energy transferred by arriving ions after collision with a surface that, as a result, produces an electronic current [134]. Since the number of ions arriving the detector is generally small the electric signal generated by the detector is amplified [134].

Ions detectors can be divided in two major categories, designated focal-point and focal-plane [134]. In focal-point detectors, single *m/z* ions are solely detected at one time, meaning that the ions arriving from the mass analyser are sequentially detected at one point [134]. In focal-plane detectors, all ions are detected simultaneously at all time when they arrive to a plane [134]. In this type of detectors, ions with the same *m/z* always hit the same place within that plane, which allows their identification.

I.5.1.1.4 MASS SPECTRUM

A mass spectrum is the result of a mass spectrometric analysis and it is a graph that represents the ions signal intensity against their mass-to-charge ratio. In a mass spectrum the most intense peak is designated by base peak and it is commonly normalised to correspond to 100% of intensity. All other peaks in the spectrum are presented with relative intensity to the base peak. In Figure I.19 is presented an example of a mass spectrum.
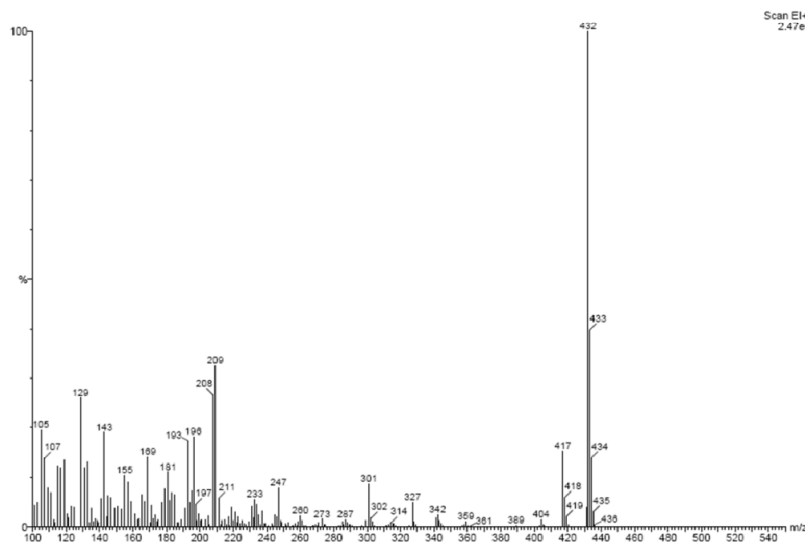
Figure I.19 – Mass spectrum of testosterone TMS derivative.

I.5.1.1.5 TANDEM MASS SPECTROMETRY

Tandem mass spectrometry (MS/MS) plays an essential role to achieve structural information about the analytes, which is extremely important not only for structural studies, but also for correct identification of unknown analytes. To perform MS/MS studies at least two stages of mass spectrometric analysis are employed, both in space or time [134]. Tandem mass spectrometry in space makes use of two mass analysers while in time the ions are stored or trapped and then selectively analysed [134].

Basically, for common in space equipments, in the first step of a MS/MS analysis, a target ion is separated and selected from all the ions produced in the ion source, using a first mass analyser system. Then, the selected ion or precursor ion undergoes fragmentation and the product ions are separated in a second mass analyser. A schematic image is shown in Figure I.20.
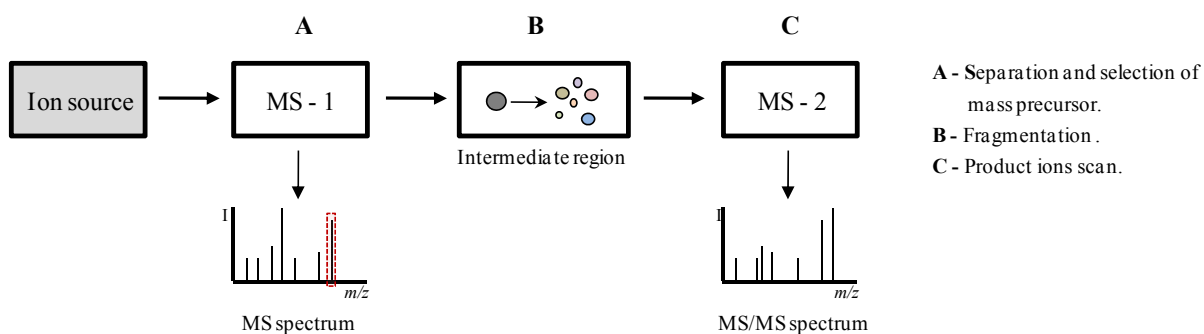
Figure I.20 – Schematic representation of an in space tandem mass spectrometry system.

Fragmentation of the precursor ion can be performed in several ways. Common fragmentation mechanisms are the collision-induced dissociation (CID), surface-induced dissociation (SID), absorption of electromagnetic radiations and electron capture dissociation (ECD) [134, 150-156]. Regarding the CID, which is probably the most used fragmentation method, it is achieved by collision of the accelerated ions with gas molecules inside a collision cell located between the two mass analysers [134]. Some limitations are related to CID fragmentation, such as lower fragmentation when applied to larger molecules as well as problems associated with the introduction of gas inside high vacuum systems. In the instruments using SID collision cell, fragmentation is achieved by collision with a solid surface [156]. The fragmentation yield depends mainly on the energy of the ions. This method avoids the introduction of gas inside the collision cell and consequently the compromising of the vacuum conditions. The other two fragmentation methods use photon or electrons beams and are typically associated with trap mass analysers.

I.5.1.2 MALDI-TOF-MS

I.5.1.2.1 PRINCIPLE OF MALDI

The technique of matrix assisted laser desorption/ionisation mass spectrometry is nowadays an indispensable tool in the analysis of biomolecules, in particular, high weigh molecules such as proteins [157-160]. In MALDI analysis, the sample solution is mixed with a matrix, normally, a small organic compound with the ability to absorb the ultraviolet (UV) energy from the laser beam. The mixture is applied onto a plate, especially designed for MALDI analysis, and let dry. As a result of solvent evaporation, the matrix crystallises on the top of the MALDI sample plate. It is important to stress that the matrix is in large excess relatively to the sample. The dried MALDI sample plate is introduced in the ion source of the MALDI instrument, which is under high vacuum, and irradiated with a pulsed laser beam. The exact mechanism involved in the MALDI process that leads to the vaporisation of the sample molecules is not yet elucidated [148]. Nevertheless, it is assumed that the high irradiance power of the laser is absorbed by the matrix molecules, which causes energy accumulation and subsequent rapid heating of the mixture matrix/sample crystals [148]. This process leads to the ablation of both matrix and sample molecules [148]. Figure I.21 shows high speed photographs of MALDI ablation.

The mechanism involved in the molecule`s ionisation process is also poorly understood [134]. An important feature concerning the ionisation process is that only a part of the vaporised molecules are transformed into ions, but interestingly the ratio between ions and neutral molecules is different for the matrix and sample molecules; the fraction ionised is significantly higher for the analyte molecules [148]. Several mechanisms have been suggested for the ionisation process, but only two are widely accepted. One model assumes that the ionisation of the neutral analyte molecules occur in the gas-phase triggered by photo-ionisation of the matrix molecules and subsequent charge transfer in the ablation process [148, 161, 162]. The second model assumes that the analyte ionisation process occurs before the ablation process by proton transfer while still in the solid phase or simply by retaining their solution charge [163]. The second model was recently proposed and it is supported by experiments carried out with infrared lasers that showed similar results than those obtained with conventional UV lasers. The energy supplied by IR lasers is not enough to originate photo-ionisation and therefore, this results support the ionisation model proposed [148]. Nevertheless, the ionisation efficiency was lower with the IR lasers, which may support the idea that both processes may occur [148].

MALDI is a soft ionisation technique that produces primarily singly charged ions, which are commonly the molecular ion of the analyte. The ions formed in the ion source are accelerated towards the mass analyser by the application of an electrostatic field.
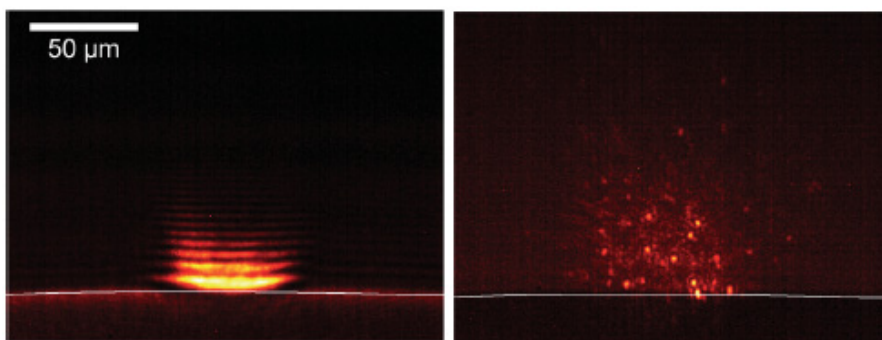
Figure I.21 - High-speed photographs of UV-MALDI plumes generated with a Nd:YAG laser operating at 266nm (Reproduced from Franz Hillenkamp and Jasna Peter-Katalinic, *MALDI-MS: A Practical Guide to Instrumentation, Methods and Applications*, WILEY-VCH, 2007).

I.5.1.2.2 MALDI MATRIX AND SAMPLE PREPARATION

MALDI ionisation process relies on the mixture of the sample to be analysed with a matrix. Co-crystallisation of this mixture is, for the majority of the analytes, essential for the success of the mass spectrometric analysis, since it is strictly related with the success of the ionisation process. So far, there is no unique MALDI matrix or sample preparation procedure suitable for all MALDI analytical applications and consequently there are numerous MALDI matrices commercially available [161, 164]. The choice of the matrix and the sample preparation are of great importance and they can determinate the success of the analysis, since both influence the ionisation behavior, as well as the formation of adducts and the stability of the analytes [148, 165].

Effective matrix compounds should absorb the energy from the laser beam and transfer it to the crystallised solid surface [134]. Moreover, it should act as a solvent in order to reduce the intermolecular forces between the analyte molecules and consequently avoid the formation of analyte clusters [134]. This purpose is achieved using a large molar excess of matrix comparatively to the analyte [136]. Additionally, the excess of matrix is important to avoid direct laser shots on the analyte, which would induce random fragmentation [148].

In general, the matrix compound indispensable characteristics are the ability to absorb UV radiation at the wavelength of the laser used, to be soluble in a wide range of solvents, to present good mixing with the analyte, to have long lifetime under vacuum conditions and to easily transfer protons during the ionisation process [166]. Furthermore, the matrix should sublimate at lower temperatures to facilitate the transfer to the gas phase [134].

Commonly used matrices are presented in Figure I.22. Despite the numerous matrices available, their use is mainly confined to the analysis of high molecular weight molecules. Regarding the analysis of small molecules, such as the anabolic androgenic steroids, a careful choice of the matrix is vital for the accomplishment of the analysis. Since most matrices are small organic

compounds, the presence of matrix ion interferences in the low-mass region of the MALDI mass spectra are likely to occur, as well as saturation of the detector [148, 166].

Dithranol	α-ciano-4-hydroxy cinnamic acid	Sinapinic acid
2-(4-hydroxyphenylazo)-benzoic acid	Ferulic acid	2,5-dihydroxy-benzoic acid
trans-3-indoleacrylic acid	3-hydroxy-picolinic acid	2,4,6-trihydroxyacetophenone
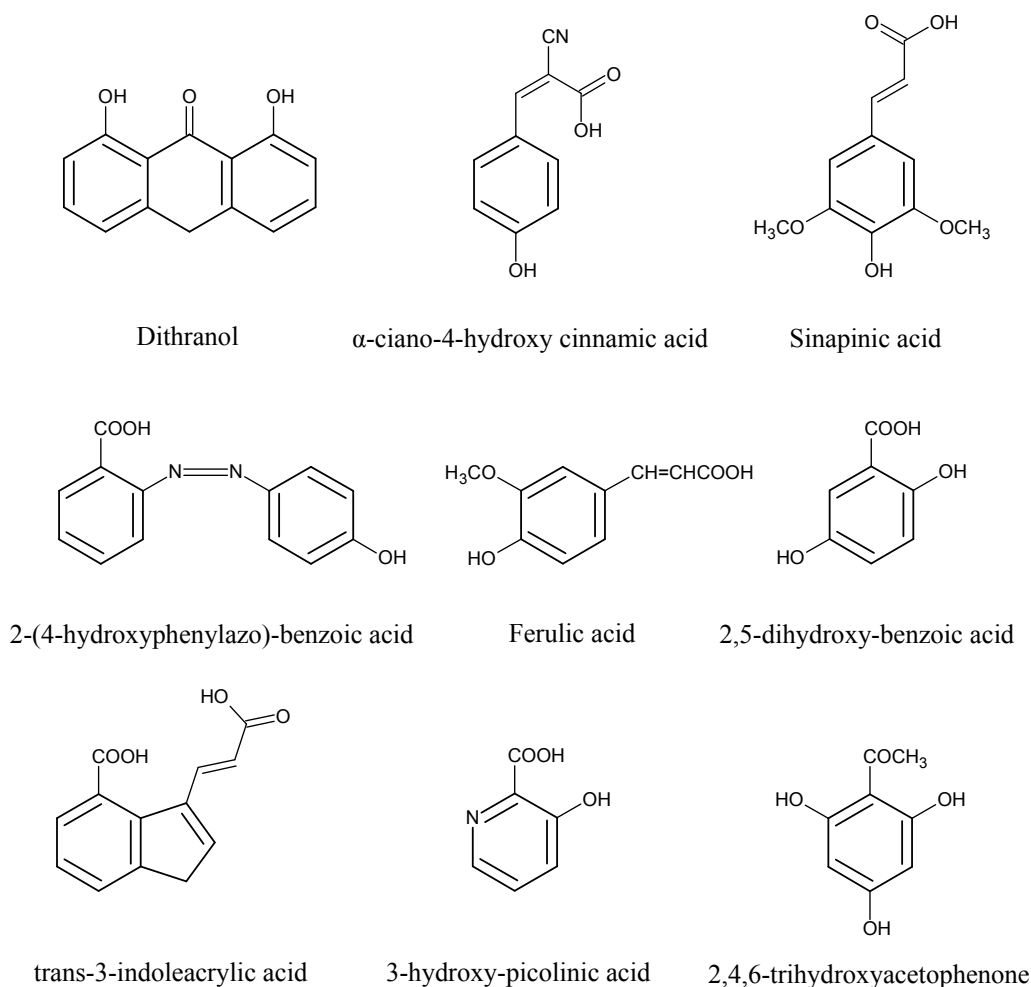
Figure I.22 – Structures of most common MALDI matrices.

Conventional MALDI matrices are mostly organic compounds that, commonly, possess aromatic rings in their structure [134]. Two major classes within these matrices are cinnamic acid and aromatic carbonyl derivatives [148]. A major problem of these matrices concerns the characteristic background in the low mass region and the formation of irregular matrix distribution in the MALDI sample plate and consequently inhomogeneous distribution of the analyte [134]. To overcome these problems several alternative matrices were developed [166-173]. These alternative approaches involve the use of liquid organic matrices, ionic liquids and inorganic compounds. However, the use of high laser fluences has been reported to be required to achieve good ionisation yields, which may lead to analyte fragmentation [148, 166]. Other drawbacks include low reproducibility.

Together with the matrix choice, sample preparation plays an important role in the MALDI analysis success. It can determinate the sensitivity and reproducibility of the analysis as it has influence

regarding the sample crystallisation [134]. A good sample preparation leads to small crystals formation and to homogeneity of the spot [136]. Moreover, it increases the distribution of the analyte through the spot and subsequently reduces the formation of sweet spots, which are high abundant analyte points inside the MALDI sample plate spot [148].

The most commonly used MALDI sample preparation technique is called *dried-droplet*. In this technique, the sample and the matrix are mixed prior to its application on the MALDI sample plate [134]. Evaporation of the solvent and consequent co-crystallisation of sample and matrix occur by slowly air-drying of the applied drop. Although its generalised use, this technique frequently results in heterogeneous distribution of the analyte [134, 174]. Drying the MALDI sample plate under vacuum improves the homogeneity of the spot and originates small crystals. Other approaches involve the formation of thin layers onto the MALDI plate. One of these techniques is called s*andwich matrix*. In this technique, three successive layers are formed. The first layer is formed by the matrix, to which follows the application of the sample and an additional application of matrix solution.

I.5.1.2.3 TIME-OF-FLIGHT MASS ANALYSER

Unlike most ionisation techniques, the ionisation by MALDI techniques presents distinct properties that influence the choice of the mass analyser. Typically, MALDI is a pulsed ionisation technique in which the ions are produced in a discontinuous process and in a short time [135]. Moreover, the ions produced in the MALDI source can present very large masses, up to 1 MDa, and therefore the mass analyser has to be able to measure within a wide range of masses. All these features are well suited for the time-of-flight analyser. The TOF analyser is by far the most used mass analyser for MALDI, although some trap mass analysers have also proven to be suitable [134]. Nevertheless, the "unlimited" mass range provided by TOF analysers confers a great advantage over other analysers. The concept behind the TOF analyser was described in 1946 by Stephens and it consists of a long field-free region, called flight tube, in which ions are separated on the basis of their velocity differences (see Figure I.23) [135].
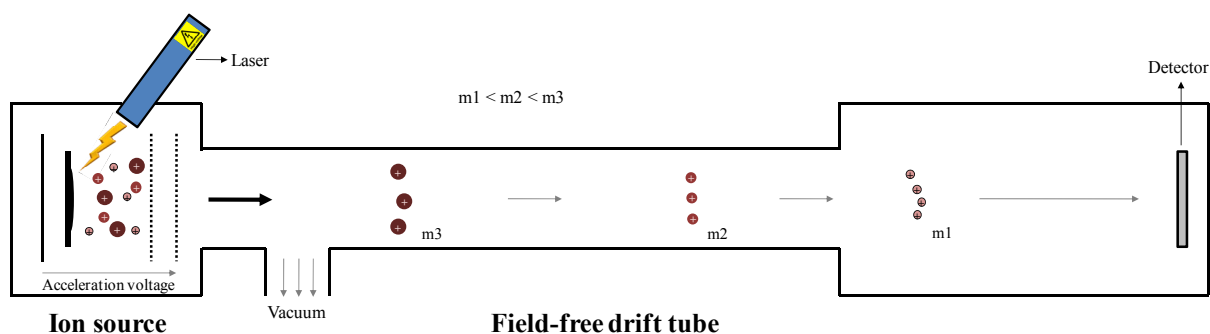
Figure I.23 – Schematic representation of the TOF analyser.

The ions produced in the MALDI source are accelerated towards the flight tube by a potential difference applied between the MALDI sample plate and a grid before the tube [148]. The potential difference applied to accelerate the ions is converted to kinetic energy [148]. Therefore, and based on the equation presented bellow, ions with the same charge will acquire the same kinetic energy,

$$E_K = zeU \qquad\qquad [134, 135, 148]$$

where z is the charge state of the ion, e the elementary charge and U the potential difference. In modern TOF analysers, U is normally 20kV.

Therefore, for all the ions with the same kinetic energy, different distributions on their masses will originate different velocities. The following equation demonstrate the relation between the mass, m, and velocity, v, of the ions,

$$E_K = \frac{1}{2}mv^2, \qquad or \quad v = \sqrt{\frac{2E_k}{m}} = \sqrt{\frac{2zeU}{m}}$$

After the initial acceleration, the ions travel through the fight tube at constant velocity. Light ions travel faster and reach the detector sooner than the heavy ions. The arrival time is dependent on the velocity and the tube length, L, and is given by,

$$t = \frac{L}{v}, \qquad or \quad t = L\sqrt{\frac{m}{2zeU}}$$

This equation can be inverted to

$$t^2 = \frac{m}{z}\left(\frac{L^2}{2eU}\right), \quad or \quad \frac{m}{z} = 2eU\left(\frac{t}{L}\right)^2$$

which shows that the *m/z* value can be calculated taking into account the time of the ions arrival to the detector.

When used with the MALDI source, two major features may cause a slightly error on the arrival time and, therefore, influence the mass resolution and accuracy. One of these features has to do with the fact that, during the ionisation process, the ions are transferred to the gas-phase with an initial velocity that might be different for the same ion species [134, 135, 148]. The other one concerns the fact that the solid surface of the MALDI plate is not homogeneous, which results in different starting points for the same ion species [134, 135, 148]. These features may cause peak broadening due to the arrival of the same ion species at different times and errors in the ion mass. To overcome these

drawbacks, modern MALDI-TOF instruments use a delayed time extraction, which is a short time between the ionisation process and the application of the potential difference, where no potential is applied. This strategy largely reduces the initial velocity problem amongst ions with the same *m/z*. The ions are first allowed to expand in the source according to their initial kinetic energy [134, 135, 148]. After the switching on of the potential field, for the ions with the same *m/z*, the slower ions and consequently those closer to the source will be subjected to a great potential difference and receive more kinetic energy [134, 135, 148]. Consequently, these ions are accelerated to a higher velocity and, by adjusting correctly the delay time, will reach the detector at the same time than the initially faster ions. Nevertheless, this time focusing approach is dependent on ion masses and the improvement of the mass resolution is only possible for a limited mass range [134, 135, 148].

Another strategy that can greatly minimise the effects of the initial velocity and spatial distributions and therefore improve resolution, is to use an electrostatic reflector, called reflectron [148]. The reflectron or ion mirror consists of grids and ring electrodes that create a retarding field at the end of the flight tube. This repelling potential slows down the ions and reflects them back to a second detector. For a particular *m/z* ion, the differences in the initial kinetic energy can be corrected in the reflectron. The higher energy ions will go further inside the reflectron compared to the lower energy ions, which makes them spend more time inside the refletron. By adjusting the reflectron voltages is possible to correct the slightly differences in the arrival time of the ions with the same *m/z* and re-focus the ions to the second detector with improved resolution (see Figure I.24). These two strategies play a vital role to achieve high resolution data with MALDI-TOF instrumentation.
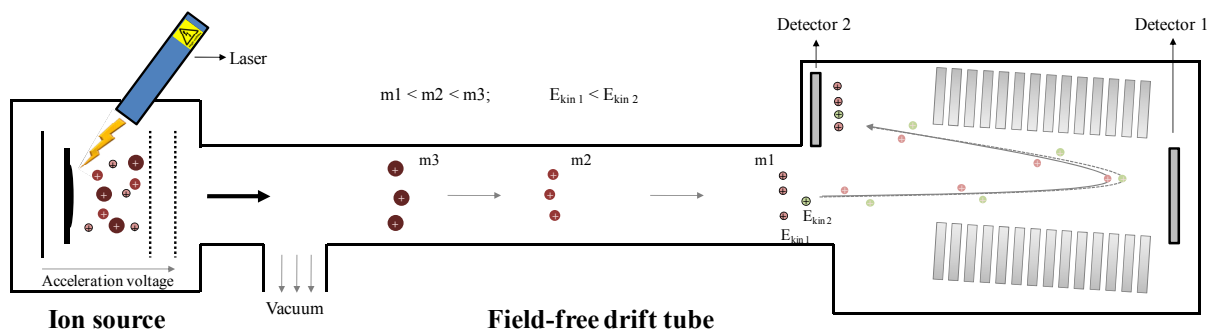
Figure I.24 – Schematic representation of the reflectron time-of-flight mass analyser.

I.5.1.2.4 TANDEM MASS SPECTROMETRY

Since MALDI is a soft ionisation technique, tandem mass spectrometry becomes an essential methodology for structural analysis and appropriate identification of molecules [175-178]. Traditionally, in MALDI instruments, tandem mass spectrometry is achieved by combination of two discrete TOF analysers, between which is a collision cell [148]. Ions are separated in the first drift tube

and an ion gate positioned in front of the collision cell allows only the target ion to pass through [148]. Inside the collision cell, the target ion fragments according to its kinetic energy and the potential held at the collision cell [134, 148]. After fragmentation, the collision cell will act as the ion source and the ions are accelerated into the second drift tube. A conventional MALDI-TOF/TOF-MS scheme is shown in Figure I.25.
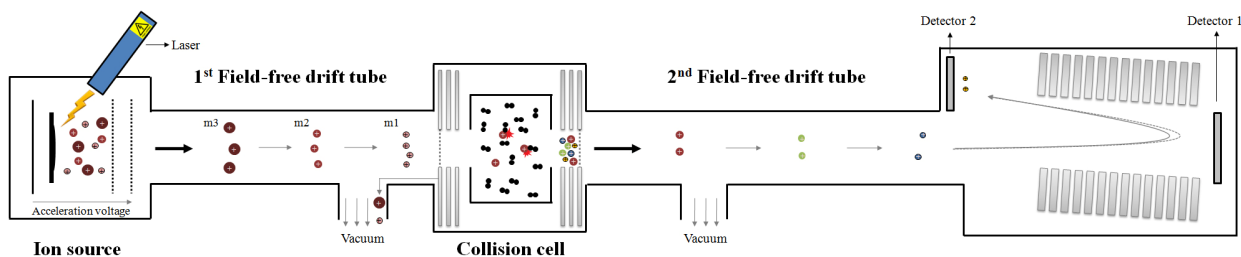
Figure I.25 – Schematic representation of the tandem time-of-flight mass analyser.

I.5.2. SAMPLE PREPARATION: THE ROLE OF ELECTROMAGNETIC AND ACOUSTIC IRRADIATION

The potential of both microwave and ultrasonic irradiation to transform current methodologies, in which are involved biochemical reactions, is of great importance. In the drive towards cleaner, faster, low cost, high yields and high throughput methodologies, the introduction of such technologies presents an enormous potential. Numerous studies have reported the use of these alternative energy sources to catalyse a variety of reactions and chemical processes.

I.5.2.1 ULTRASONIC IRRADIATION

I.5.2.1.1 ULTRASOUND

Ultrasound is defined based on the audible range of the human ear, which is able to detect sound frequencies between 20Hz to 20 KHz, considering a young, healthy person [179]. Therefore, ultrasound is a sound wave of a pitch above that frequency and it varies from 20 kHz to around 10MHz [180]. Figure I.26 presents the characteristic wavelengths and sound frequency ranges.
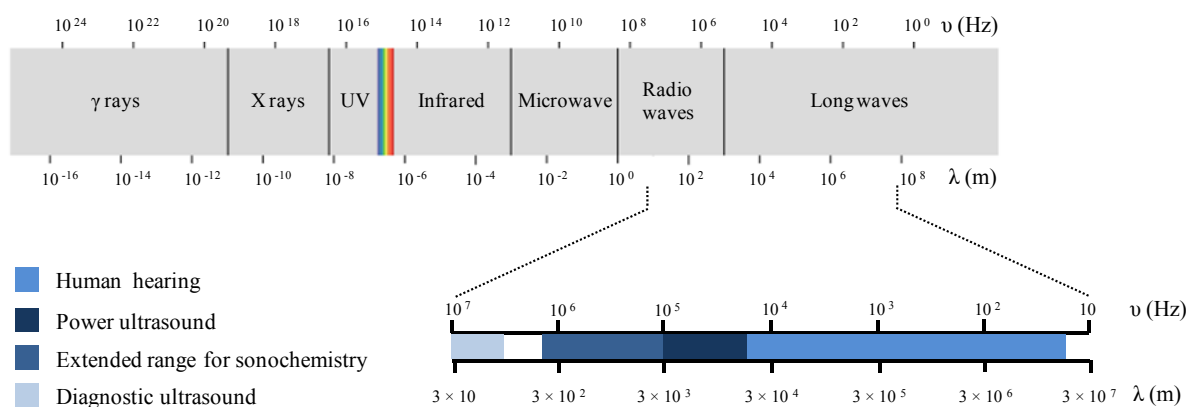
Figure I.26 – Electromagnetic and acoustic spectra (Adapted from T.J. Mason and J.P Lorimer, *Applied Sonochemistry: Uses of power ultrasound in chemistry and processing*, Wiley-VCH, 2002).

Because ultrasound is a wave, it transmits energy just like an electromagnetic wave or radiation [181]. However, unlike an electromagnetic wave, it requires a medium to travel or propagate [181]. Sound waves, in which are included ultrasound waves, are transmitted through a homogeneous medium by vibrational motion of the medium molecules [179]. The sound source creates a mechanical disturbance or vibrational motion that is transmitted to the medium molecules that are consequently pushed to a distance from its original position [181]. Before returning to their original position each molecule transmits that force to the adjacent molecule, creating a chain reaction [181].

Due to the wide range of ultrasound frequencies and consequently wavelengths, the interaction between the ultrasonic wave and the propagation medium (matter) changes as a function of the wavelength. Accordingly, the applications of ultrasounds are conventionally divided in two broad areas, power and diagnostic [179].

High frequency ultrasound waves, around 2 MHz and 10 MHz, are normally used for medical diagnostic, since they do not affect the chemistry of the propagation medium, but instead are changed by that medium (e.g. scattered, reflected, refracted, attenuated or absorbed) [182]. The physical effect of the medium on the wave is of great importance, for instance, in imaging diagnostic systems, where the short wavelengths are vital to detect small areas of phase change.

Power or high energy ultrasound concerns the waves produced at lower frequencies, around 20 kHz and 100 kHz. At these frequencies a phenomenon called acoustic cavitation occurs within liquid systems and may produce both chemical and physical changes in the propagation medium [183]. The application of ultrasounds in chemistry deals mainly with this sonic spectrum and it is called sonochemistry [183]. Recently, high power equipment has been developed capable of generating cavitation phenomena at higher frequencies, 2MHz [179]. Following its successful use in chemistry, high energy ultrasound is now being used for therapeutic purposes (e.g. destruction of blood clots).

I.5.2.1.2 SONOCHEMISTRY

As mentioned above, the high power of lower ultrasound frequencies in liquid media is concerned with the phenomenon of acoustic cavitation. The vibrational motion transmitted to the liquid medium induced by ultrasound waves causes alternately expansion and compression of the medium [179]. At sufficiently high power, the strength of the acoustic field may exceed the attractive forces of the particles in the expansion or rarefaction cycle and create cavities in the liquid medium [183, 184]. These cavities are called cavitation bubbles. Once formed, the cavitation bubbles grow during successive cycles by a process called rectified diffusion [185]. When oscillating pressures are transmitted on the liquid medium, small amounts of gas from the medium diffuse into the bubble during the expansion half cycle and out of the bubble during the compression half cycle [185]. However, the amount of gas expelled during the compression cycle is less than the amount that enters the bubble, which originates the growth of the bubble until it reaches an equilibrium size [185]. Under proper conditions, the bubble implodes violently in the compression half cycle, producing an extraordinary phenomenon known as "hot-spot" that originates temperatures of about 5,000 K and pressures around 1000 atm [183, 186-188]. Moreover, the formation of radicals from the medium components has been reported, which may contribute to promote specific reaction mechanisms [184, 189, 190].

Regarding heterogeneous systems involving both immiscible liquid-liquid or liquid-solid mediums, in addition to the formation of the hot-spot phenomenon, other sonochemical effects take place [179, 191]. When a cavitation bubble is formed and collapses close to a phase interface, the collapse is not symmetrical due to the resistance to liquid flow provided by that interface [179]. The result of this bubble deformation is the formation of liquid micro-jets that propagate, primarily, towards the interface at velocities greater than 100 ms^{-1} [186]. When these jets are formed near a liquid-liquid interface, they originate very fine emulsions that are more stable than those originated conventionally [184]. At a solid-liquid interface, the jets formed cause pitting and mechanical erosion of the solid surface that lead to disruption of the solid particles. This feature has great impact on the reaction catalysis, since it increases the surface area of the solid and facilitates the contact between the liquid medium and the solid particles [179]. This effect of asymmetric collapse is the reason why ultrasounds are extensively used for cleaning and extraction purposes.

I.5.2.1.3 FACTORS AFFECTING CAVITATION

Several factors affect the formation, growth and collapse of cavitation bubbles in liquid media and therefore determinate the success of ultrasounds to improve a particular reaction. Within these factors, the ultrasonic frequency, ultrasonic intensity, external temperature and pressure, solvent and nature of the dissolved gases are the most relevant [179, 184]. Other factor that has a great influence in the

success of the cavitation effects within the reaction medium is the shape of the reaction vial, which may change according to the ultrasonic device used.

I.5.2.1.3.1 FREQUENCY

The ultrasonic frequency applied to the reaction medium is determinant for the cavitation process. Increasing the ultrasonic frequency diminish the formation of cavitation bubbles [179]. The major explanation for this phenomenon regards the time of the expansion and compression cycles. At high frequencies, both cycles are very short to allow the bubble to grow to an adequate size and cause its violent collapse [179]. This is why most ultrasonic devices used in sonochemistry work between 20 kHz to 50 kHz [184].

I.5.2.1.3.2 INTENSITY

Increasing the ultrasonic intensity has an important influence on the cavitation phenomenon. The intensity affects both half cycles pressure and an increment will result in more violent bubble collapses [179]. This is the reason why some devices are now able to induce cavitation at higher frequencies, e.g. 1MHz. Despite the short cycle time at high frequencies that normally would require several cycles for the bubble to grow, with higher intensity, the pressure applied allows the bubble to grow more in each rarefaction half-cycle and most important, in the compression half cycle, the pressure applied is enough to promote the collapse of the bubble [179]. However, it is important to stress that this effect drops above a maximum value of intensity. One explanation to this cavitation drop effect might be the formation of very large bubbles in the rarefaction cycle and due to the short cycle time the pressure applied in the compression cycle is not enough to collapse the bubble [179]. Other explanation regards the formation of several cavitation bubbles close to the ultrasound source, acting like a wall that limits the cavitation effect inside the liquid medium [184].

I.5.2.1.3.3 TEMPERATURE AND PRESSURE

Temperature plays an important role in the cavitation process. Generally, higher temperature inside the liquid medium has a negative effect in cavitation, even though it allows an easier formation of cavitation bubbles [184]. This behaviour is linked with the increase of the vapour pressure inside the liquid and consequently inside the cavitation bubble, which results in a less violent bubble implosion [184].

The external pressure has two major consequences regarding the formation and implosion of the cavitation bubbles. The increasing of the external pressure hampers the formation and growth of the cavitation bubble [179, 184]. However, it increases the violence of the bubble collapse [179, 184]. Thus, if sufficient ultrasonic intensity is delivered to the system to form cavitation bubbles, an increasing pressure will result in increasing sonochemical effects.

I.5.2.1.3.4 SOLVENT

The effect of the solvent in the cavitation phenomenon is similar to the effect of the temperature, since different solvents will have different vapour pressures. The effect of cavitation will be harder to produce with low vapour pressure and high surface tension solvents, since the forces between molecules are stronger [179, 184]. However, at an adequate ultrasonic intensity, the effects resulting from the bubbles implosion will be greater [179, 184].

I.5.2.1.3.5 NATURE OF THE DISSOLVED GAS

Gases with higher specific heat capacity originate greater cavitational effects and, consequently, monoatomic gases are preferred to diatomic gases since they dissipate more energy upon cavitation [184].

I.5.2.1.4 ULTRASOUND SOURCE

The ultrasonic source that causes the mechanical disturbance in the liquid medium is called a transducer. The transducer converts mechanical or electrical energy into ultrasound waves. There are three major types of transducers, gas driven, liquid driven and electromechanical. Electromechanical transducers are the most commonly employed in sonochemistry devices [179]. Within this type of transducers, the use of piezoelectric or the magnetostrictive effects are the most explored. Although magnetostrictive transducer was historically one of the first transducers used, piezoelectric transducers are nowadays the most employed to generate ultrasound waves as well as detecting them [179]. The piezoelectric transducers make use of the piezoelectric properties of some materials to generate ultrasounds. Piezoelectric materials have the ability to convert mechanical stress into an electrical signal and the opposite, transform a potential difference into mechanical strain [181]. Another important feature is that upon mechanical strain, opposite charges are generated on each face of the piezoelectric material, which means that by alternating the polarity of the potential difference applied to the piezoelectric material it will expand or contract. Thus, by changing rapidly the polarity charge

applied to the piezoelectric material is possible to induce ultrasonic vibrational motion into a medium [179, 181]. Figure I.27 shows both direct and reverse piezoelectric effects.
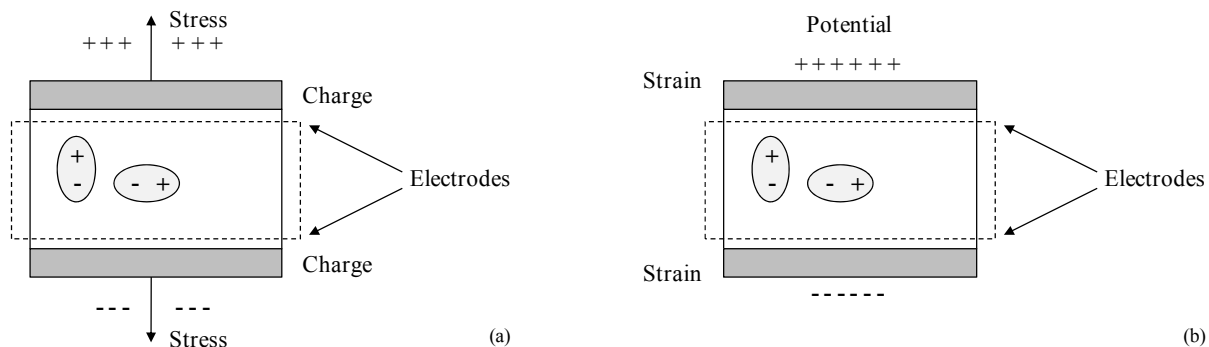
Figure I.27 – (a) Direct piezoelectric effect in which the application of mechanical stress generates a charge separation. (b) Reverse piezoelectric effect in which the application of a differential potential induces mechanical stress (Adapted from K. Kirk Shung, *Diagnostic Ultrasound: Imaging and Blood Flow Measurements*, Taylor & Francis, 2006).

I.5.2.1.5 APPLICATIONS

Ultrasounds have a wide range of applications in a variety of scientific fields. In medicine it is a valuable tool in medical imaging, providing a complementary method to X-ray and magnetic resonance diagnostic tools [179]. Concerning therapeutic purposes, it has been used, mainly, in massages, to improve blood circulation and in sporting injuries treatment. More recently, it is also being used to dissolve blood clots together with enzymatic catalysis and to enhance the action of chemotherapy agents in cancer [179]. In dental medicine, ultrasounds are being implemented as well for imaging purposes and more recently for dental tissue repair [192, 193]. In engineering, it is being used to assist drilling, gridding and cutting a variety of materials, such as stainless steel, ceramics, glass and carbide. In food industry, the use of ultrasounds is being implemented to destroy all kind of bacteria, fungi and viruses [179]. In geography, and this is perhaps the best known application of ultrasounds, it is used in navigation for localisation and communication purposes.

Regarding chemistry and biochemistry, several applications have been reported highlighting the role of cavitation bubbles to enhance a variety of reactions. In solid-liquid extraction reactions, the use of ultrasounds represents a great advance over conventional techniques [194]. The implosion of cavitation bubbles near the solid surface, generates micro jets capable to force the liquid to penetrate the solid surface and most important capable to disrupt the solid surface, which results in an increasing contact area between the liquid and the solid [179, 194]. As mentioned above, ultrasounds are an indispensable tool in cleaning processes and are being also implemented for a variety of processes,

such as filtration, mixing and formation of stable emulsions [195-197]. In cell extraction, ultrasonic energy provides efficient microbial cell wall disruption and, for that reason, is an essential tool to extract components from microorganisms to be used for both research and industrial aims [198, 199].

Despite its growing application in recent years, the use of ultrasounds to enhance chemical reaction dates back to 1938 [200]. From then, numerous authors, mainly organic chemists, have highlighted its effect in the reaction rate of a variety of organic synthesis [201-203]. In recent years the successful use of ultrasonic energy to enhance the reactivity of numerous chemical and biochemical reactions involved in analytical methodologies led to a growing interest of its application within the scientific community for which Professor Capelo *et al.* have greatly contributed with an extensive work comprising a broad field of ultrasonic applications [194, 204-212].

I.5.2.1.6 COMMON INSTRUMENTATION

The most common ultrasonic devices employed by sono-chemists comprises the conventional ultrasonic bath, which is the most common device providing ultrasonic irradiation in chemical and biochemical laboratories all over the world, the ultrasonic probe and the cup horn system [213]. These devices present distinct characteristics and hence the choice of the ultrasonic device must be done carefully in accordance with the intended application and purpose.

I.5.2.1.6.1 ULTRASONIC BATH

The conventional ultrasonic baths are, as mentioned above, the most available devices within chemical laboratories. These devices normally consist of a stainless steel container with one or more transducers on the bottom, depending on its volume. Due to their larger volume, the power density provided by these devices ranges between 1 and 5 Wcm^{-2}, which is a low intensity value [200, 213]. The temperature inside these devices is difficult to control and the frequency applied varies significantly with the supplier brand, which make it difficult to reproduce the same experimental conditions from laboratory to laboratory [179]. Furthermore, the position and shape of the vessel inside the tank, as well as the amount of water filling the tank, have influence on the amount of ultrasonic power that reaches the sample [179]. For this reason the majority of ultrasonic baths is essentially applied for cleaning and degassing purposes and fail to enhance chemical reactions.

Recently, new ultrasonic baths have been developed to overcome some of the issues mentioned above by including a heater, which allows temperature control, dual frequency, ultrasonic power regulation and different operation modes [213]. Nevertheless, its use for sonochemistry is very limited.

I.5.2.1.6.2 ULTRASONIC PROBE

The ultrasonic probe or sonic horn is a popular device amongst sono-chemists. Unlike, the ultrasonic baths, these devices are immersed on the liquid medium and are able to supply a large amount of ultrasonic power to the medium [179]. The majority of these systems work at fixed frequency, usually ranging from 20kHz to 40kHz, allowing the control of the vibrational amplitude of the tip, i.e. the ultrasonic power. In most commercially available ultrasonic probes, this function is controlled either by controlling the input power of the transducer or by changing the tip horn attached to the transducer. The tip horn works like an amplifier and so, changing its shape will have great influence on the power transmitted [179].

Despite its enormous potential, ultrasonic probes allow low throughput and since it must be immersed into the liquid medium, the possibility of contamination is higher, both from previous applications and from the metal material from the tip.

I.5.2.1.6.3 CUP HORN

An alternative to avoid the contamination issue associated with the ultrasonic probe is the cup horn. The cup horn is a high ultrasonic power system that allows indirect ultrasonication, which is a great advantage for analytical purposes, since the reaction vials are handled closed. Due to its characteristics, it may be seen as a small powerful ultrasonic bath. Like the ultrasonic probe, this device works at a predetermined frequency. The input power can be controlled as well as the cycle duty. In addition, this device can handle more than one sample at the same time, which results in higher throughput when compared with the ultrasonic probe.

Nevertheless, since the ultrasonic waves are transmitted through water and need to cross the wall of the vials, the ultrasonic power density provided by these devices is lower when compared to the probe. Furthermore, to achieve higher ultrasonic power, the container volume must be small, which imposes a limitation to the vial size. In Figure I.28 the relative power density provided by the three devices presented here are showed.
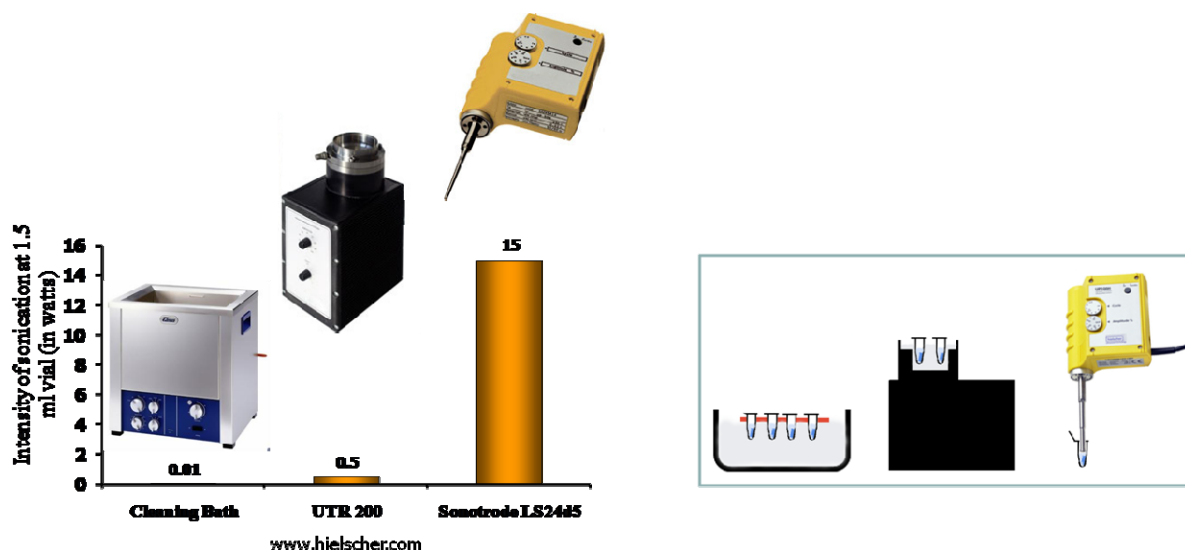
Figure I.28 – Relative intensities of the ultrasonic bath, cup horn and probe (Adapted from www.hielscher.com).

I.5.2.2 MICROWAVE IRRADIATION

I.5.2.2.1 MICROWAVE

Microwaves are electromagnetic radiation with characteristic wavelengths and frequency ranging from 0.1 to 100 cm and 0.3 to 300 GHz, respectively [121]. In the electromagnetic spectrum, microwaves lie between radio waves and infrared waves. Common domestic microwaves work at fixed frequency of 2.45 GHz, whereas scientific microwave reactors might work also at 0.9 GHz [121, 184, 214]. This feature of microwave ovens is an attempt to avoid interference with telecommunications applications and radar transmissions that make use of low frequency microwave.

The use of microwave irradiation to enhance chemical reactions was for the first time reported in the mid 1980s, few years after the introduction of the first microwave oven for scientific research by CEM [214, 215]. Since then, numerous reports emphasized the role of microwave to enhance chemical reactions and processes, but the mechanism of microwave catalysis is still a topic of discussion within the scientific community [215].

I.5.2.2.2 THERMAL EFFECTS

The mechanism thought to be primarily involved in microwave catalysis is concerned with the dielectric heating process originated by microwave irradiation to a liquid medium [215]. Unlike conventional heating, where thermal energy is transferred by conduction and convection, in

microwave heating, the microwave energy is converted into heat by polar molecules, mainly due to dipole rotation and ionic conduction [214].

When polar molecules or ions are irradiated with microwaves, their dipoles align with the electric field of the waves. If the molecules are in the gas phase the energy absorbed from microwaves makes them change their rotational states. However, if the molecules are in the liquid phase, they are not able to rotate freely and will dissipate energy in the form of heat through molecular friction and dielectric loss, in the effort to continuously align with the alternating electric field applied [216]. As a result of this phenomenon, the internal temperature of the liquid medium increases rapidly. When compared with conventional thermal heating, microwave irradiation produces an inverted temperature gradient, i.e. the temperature increases from the inside to the outside, whereas in conventional heating the temperature increases from the outside to the inside of the liquid medium. Moreover, the electromagnetic waves will also increase the molecular movement inside the liquid, which consequently results in faster interactions [121]. Nevertheless, this process relies on the ability of the analyte and solvent molecules to absorb the microwave energy, which is dependent of their dielectric properties [214].

The potential of a specific substance to transform electromagnetic energy into thermal energy is determined by the factor tanδ, known as the loss factor. The loss factor is defined as,

$$tan\delta = \frac{\varepsilon``}{\varepsilon`}$$

where ε" represents the dielectric loss coefficient that indicates the efficiency of conversion of electromagnetic energy into heat, and ε` represents the dielectric constant, which is a measure of the capacity of the molecules to be polarised by the electric field applied [121, 214]. Substances with an efficient absorption and conversion of microwaves into heat, will present high values of tanδ (tanδ > 0.5), whereas low efficient substances will present low values of tanδ (tanδ < 0.1) [214].

Within the thermal effects caused by microwave irradiation, overheating of the reaction medium above their conventional boiling points at atmospheric pressure has also been reported [217]. This superheating effect is considered to be linked with the inverted temperature gradient generated by microwave heating and has been reported to partially explain the improvement of reaction rates [217]. Furthermore, the generation of "hot-spots" of temperatures around 100°C to 200°C higher than the overall temperature inside the liquid medium have been reported by some authors, which have direct impact in reaction rates [218-220].

Based on the polarity properties of the solvents and analytes, microwave irradiation can be used as a selective mode of heating [220].

I.5.2.2.3 NON-THERMAL EFFECTS

Although some authors have suggested the existence of non-thermal effects, this is still a matter of great discussion [221, 222]. Non-thermal effects are supported by some results where an increase in the reaction yield was achieved using apolar solvents, which, due to its low dielectric properties, are "transparent" to microwaves and hence will show low thermal effects [214]. For this reason, some authors have suggested that these effects are more relevant with non polar solvents. Furthermore, it has been reported different reaction equilibrium positions when both conventional and microwave procedures were used, which should not happen if only thermal effects were generated [223].

The non thermal effects are considered as those resulting from the interaction of the electric field with specific molecules of the reaction medium. It has been argued that the presence of an electric field leads to orientation effects of dipolar molecules and consequently changes the activation energy of reaction [214]. Moreover, the electromagnetic waves have been reported to increase the molecular movement inside the liquid, which consequently results in faster interactions [121].

I.5.2.2.4 MICROWAVE SOURCE

Microwave energy in both domestic and scientific ovens is efficiently generated by a magnetron, which is a high-voltage system invented throughout the World War II as a component of a radar detection system [224]. The most common magnetron is the cavity magnetron that consists of a vacuum diode made up of circular resonant cavities around a hot cathode that emits electrons and has a negative potential with respect to the anode [224]. The cathode is under a perpendicular magnetic field that results in a force that deflects the motion of the electrons, which in the absence of the magnetic field would move in a straight path towards the ring anode [224]. It is this curved motion that allows oscillation of the cavities leading to the production of high-power microwave radiation. Figure I.29 shows a schematic image of a magnetron.
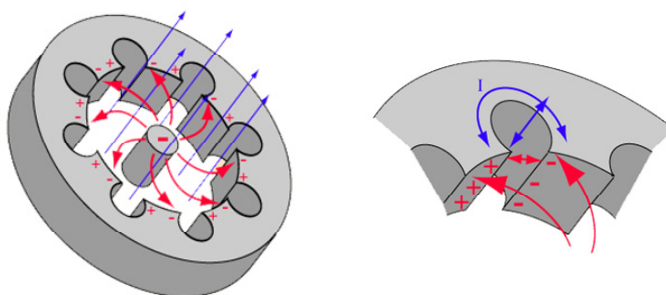
Figure I.29 – Schematic representation of a magnetron (Adapted from http://hyperphysics.phy-astr.gsu.edu/hbase/waves/magnetron.html).

I.5.2.2.5 APPLICATIONS

The most known application of microwaves concerns its use as a tool for domestic heating of food and liquids. In telecommunications, as mentioned above, microwaves also play an essential role, for instance, in cellular communications. In chemistry, the main application of microwaves is focused on its heating ability to enhance mainly organic reactions, either by increasing its reaction yield or reducing the time of reaction [214, 225].

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PART TWO

MALDI-MS(/MS) SCREENING METHODOLOGY

CHAPTER II

Comparative study of matrices for their use in the rapid screening of anabolic steroids by matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry

II.1 ABSTRACT

New data on sample preparation and matrix selection for the fast screening of androgenic anabolic steroids (AAS) by matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF-MS) is presented. The rapid screening of 15 steroids included in the World Anti-Doping Agency (WADA) prohibited list using MALDI was evaluated. Nine organic and two inorganic matrices were assessed in order to determine the best matrix for steroid identification in terms of ionisation yield and interference by characteristic matrix ions. The best results were achieved for the organic matrices 2-(4-hydroxyphenylazo)benzoic acid (HABA) and *trans*-3-indoleacrylic acid (IAA). Good signals for all the steroids studied were obtained for concentrations as low as 0.010 and 0.050 µg/mL on the MALDI sample plate for the HABA and IAA matrices, respectively. For these two matrices, the sensitivity achieved by MALDI is comparable with the sensitivity achieved by gas chromatography/mass spectrometry (GC/MS), which is the conventional technique used for AAS detection. Furthermore, the accuracy and precision obtained with MALDI are very good, since an internal mass calibration is performed with the matrix ions. For the inorganic matrices, laser fluences higher than those used with organic matrices are required to obtain good MALDI signals. When inorganic matrices were used in combination with glycerol as a dispersing agent, an important reduction of the background noise was observed. Urine samples spiked with the study compounds were processed by solid-phase extraction (SPE) and the screening was consistently positive.

II.2 INTRODUCTION

The use of anabolic steroids and hormones is a problem which generates social and environmental concern, since these substances are increasingly used, evading governmental control, for prohibited purposes such as enhancing athletic performance, fostering horse performance in equestrian sports and as illegal animal growth enhancers. Their use has spread in such a way that many nutritional supplement cocktails contain these substances without label warning, thus making the problem even worse [1-4]. The use of anabolic steroids and hormones in sports is forbidden by the International Olympic Committee (IOC) and the World Anti-Doping Agency (WADA), as well as by the main sports and horse racing authorities, at all times, in and out of competition [3, 5].

Furthermore, the release of such contaminants in the environment is becoming a public health issue, since they can act as 'endocrine disruptors' [6]. Traditionally, the standard technique used for the analysis of anabolic steroids is gas chromatography coupled to mass spectrometry (GC/MS) [7-10]. As a general rule, this technique requires prior derivatisation of the target analyte to obtain a thermally stable and volatile compound. More recently, due to the increasing complexity of doping analyses, mostly because of new substances that are being included every year in the WADA prohibited list, liquid chromatography coupled with tandem mass spectrometry (LC/MS/MS) is becoming an indispensible tool in doping control laboratories [11]. Although LC/MS/MS provides good sensitivity for the determination of steroids, long separation times and the previous development of chromatographic separation procedures are normally required [12-14]. Therefore, the need for an analytical technique allowing simplicity, speed and high throughput for the screening of the huge number of steroids and hormones currently available has led researchers to revisit the role of matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF-MS), in this context in view of the latest technological improvements in this analytical technique.

The application of MALDI to the analysis of small molecules, such as steroids and hormones, is still rare due to many factors, among which the most important are (i) the presence of matrix ion interferences in the low-mass region of the MALDI mass spectra (<m/z 500), (ii) detector saturation in the low mass region, (iii) difficulty in the differentiation of isobaric compounds, and (iv) strong competition from electrospray ionisation mass spectrometry (ESI-MS) techniques [15-17]. However, in recent years, due to technological advances in MS instrumentation and the development of several alternative methods to overcome the low-mass matrix interference problem, MALDI is becoming increasingly important for the determination of small molecules [17]. A wide variety of compounds, such as metal powders, porphyrins or 9-aminoacridine, are now being used as alternative MALDI matrices for the analysis of small molecules [18].

Another strategy to improve the MALDI signal for small molecules such as steroids is the use of derivatisation. The most important advantages of the derivatisation procedure prior to MALDI analysis are (i) improvement of the ionisation efficiency and (ii) displacement of the analyte signal to higher mass regions, minimising the interferences from the matrix.

The present work has tested the applicability of MALDI for the rapid screening of fifteen anabolic steroids, 17β-hydroxy-17-methylandrosta-1,4-dien-3-one (**1**), 17α-methyltestosterone (**2**), 4-androsten-3,17-dione (**3**), boldenone (**4**), nandrolone (**5**), 17α-trenbolone (**6**), noretiocholanolone (**7**), etiocholan-3α-ol- 17-one (**8**), mibolerone (**9**), 2α-methyl-5β-androstane-3α-ol-17-one (**10**), mesterolone (**11**), ethisterone (**12**), bolasterone (**13**), calusterone (**14**), and fluoxymesterolone (**15**) (for structures, see Supplementary Figure II.SM1, Supporting Information), focusing on the effect of sample preparation and matrix selection. In order to choose the optimum matrix for identification, MALDI spectra of the above-mentioned steroids were obtained in nine organic MALDI matrices (for structures, see Supplementary Figure II.SM2, Supporting Information): 2,5-dihydroxybenzoic acid (2,5-DHB), α-cyano-4-hydroxycinnamic acid (α-CHCA), sinapinic acid (SA), 2-(4-hydroxyphenylazo)benzoic acid (HABA), 3-hydroxypicolinic acid (3-HPA), dithranol, trans-3-indoleacrylic acid (IAA), ferulic acid (FA) and 2,4,6- trihydroxyacetophenone (THAP). In addition, two inorganic matrices, TiO_2 and Al_2O_3, were also evaluated for their suitability for the analysis of steroids by MALDI. Finally, the best conditions found were applied to the determination of steroids in human urine.

II.3 EXPERIMENTAL

II.3.1 APPARATUS

A model UNIVAPO 100H vacuum concentrator centrifuge (UniEquip, Martinsried, Germany) with a model Unijet II refrigerated aspirator vacuum pump (UniEquip) was used for (i) sample drying and (ii) sample pre-concentration. A Spectrafuge-mini minicentrifuge (Labnet, Madrid, Spain) and a Sky Line minicentrifuge-vortex (ELMI, Riga, Latvia) were used throughout the sample treatment, when necessary. A model Transsonic TI-H-5 ultrasonic bath (Elma, Singen, Germany) was used to facilitate steroid solubilisation. A Simplicity 185 system (Millipore, Milan, Italy) was used to obtain Milli-Q ultrapure water throughout all the experiments. The derivatisation procedure was performed in a 1.5mL microtube flat cap from Delta Lab (Barcelona, Spain). Separation of the steroid Girard T (GT) hydrazones from the unreacted Girard T reagent was carried out in a 2mL empty reversible solid-phase extraction (SPE) cartridge from Supelco (Belefonte, PA, USA) packed with a preparative C_{18} resin (125Å, 55-105μm; Waters, Barcelona, Spain). Urine clean-up was carried out in 3mL Oasis HLB cartridges (Waters).

II.3.2 STANDARDS AND REAGENTS

Standards of 17-α-methyltestosterone, 4-androsten-3,17-dione, boldenone and nandrolone were purchased from Riedel-de Haën (Seelze, Germany), 17-β-hydroxy-17-methylandrosta-1,4-dien-3-one was from Fluka (Buchs, Switzerland) and the standard Etiocholan-3α-ol-17-one was purchased from Sigma (Steinheim, Germany). The standards 17α-trenbolone, 2α-methyl-5β-androstane-3α-ol-17-one, mesterolone, fluoxymesterolone, bolasterone, calusterone, noretiocholanolone, ethisterone and mibolerone were kindly provided by the Portuguese National Anti-doping Laboratory. β-Glucuronidase from *Escherichia coli* was from Roche Diagnostic (Mannheim, Germany). Sodium phosphate dibasic was purchased from Sigma. α-CHCA, 2,5-DHB, SA, HABA, 3-HPA, IAA, FA and THAP were from Fluka and dithranol from Sigma, all of them *puriss* for MALDI-TOF-MS analysis, were used as organic matrices. The inorganic matrices TiO_2 and Al_2O_3 were purchased from Fluka and Merck (Darmstadt, Germany), respectively. Methanol (MeOH), acetonitrile (ACN) and the derivatisation reagent, Girard T (GT) hydrazine, used for sample and matrix preparation were purchased from Sigma; glacial acetic acid (>99.5%) was from Fluka; trifluoroacetic acid (TFA, 99%), diethyl ether and glycerol were from Riedel-de Haën.

Urine samples used in this work were obtained from healthy volunteers from the research team. The research ethical committee from the Science Faculty of Ourense approved the study protocol and all the volunteers gave their consent.

II.3.3 SAMPLE PREPARATION

II.3.3.1 STANDARD SOLUTIONS

Individual stock standard solutions of each anabolic steroid (ca. 100 mg/L) were prepared by weighing approximately 0.0025 g of analyte in a 25mL volumetric flask and making it to volume with methanol. These standard solutions were stored in the dark at –20°C and were stable for almost 6 months. Working standard solutions were prepared by dilution of the stock standard solutions in the appropriate volume of methanol.

II.3.3.2 URINE HYDROLYSIS PROCEDURE

Urine samples (5 mL) were hydrolysed at 60ºC for 90 min. with 100 µL of β-glucuronidase, after the addition of 1,5 mL of phosphate buffer (0.8M, pH 7) [18].

II.3.3.3 SPE CLEAN-UP OF HYDROLYSED SAMPLES

The clean-up procedure was carried out with an Oasis HLB SPE cartridge and was based on the protocol described in the Oasis SPE method for endocrine disruptors. The cartridge was conditioned with 3mL of diethyl ether, 3mL of methanol and 3mL of water. The urine samples (5 mL) were loaded onto the preconditioned cartridge and the cartridge was then washed with 3mL of methanol/water (30:70, v/v), reequilibrated with 3mL of water and washed a second time with 3mL of methanol/NH4OH/water (10:2:88, v/v/v). The target compounds were eluted with 2mL of methanol/ diethyl ether (10:90, v/v). Before the derivatisation step, the sample was dried.

II.3.3.4 DERIVATISATION STEP PROCEDURE

The procedure for the derivatisation of anabolic steroids to Girard T hydrazones was based on the protocol described by Wheeler [19]. In brief, the derivatisation was performed as follows: 1mL of the solution of oxosteroids in methanol, 8mg of Girard T hydrazine and 100 µL of glacial acetic acid were introduced into a 1.5mL microtube. The microtube was closed and heated at 60℃ for 30 min. After cooling, the solution was evaporated to dryness in a vacuum concentrator centrifuge and then reconstituted with 1mL of methanol/water (10:90, v/v).

II.3.3.5 SPE CLEAN-UP

After derivatisation, the steroid GT hydrazones were separated from unreacted GT hydrazine reagent by SPE in a C_{18} cartridge, according to the protocols described by Khan *et al*. and Griffiths *et al*. [20, 21]. Briefly, before use, the cartridges were conditioned with 5mL of methanol plus 10mL of MilliQ-water without allowing the cartridges to dry out. After loading the sample, the cartridge was washed with 2mL of methanol/water (10:90, v/v) in order to remove impurities from the cartridge and, finally, the steroid GT hydrazones were eluted from the cartridge with 1mL of methanol.

II.3.3.6 MALDI-TOF-MS ANALYSIS

The mass spectrometric analyses were performed with a model Voyager-DE PRO Biospectrometry Workstation MALDI-TOF-MS system (Applied Biosystems, Foster City, CA, USA) equipped with a nitrogen laser operating at 337 nm. MALDI mass spectra were acquired in positive ion reflectron mode, with an accelerating voltage of 20kV, an ion extraction delay of 80 ns, a grid voltage of 15 kV,

and a guide wire voltage of 40 V. The MALDI mass spectrum for each sample was based on the average of 600 laser shots.

Prior to MALDI analysis, the sample was mixed with an equal volume of the MALDI matrix solution and homogenised in a vortex instrument. The MALDI matrices used in this work were prepared as referred in the Applied Biosystems work book and are presented in Table II.1 [22]. An aliquot of the sample/matrix solution (1 mL) was handspotted onto the MALDI sample plate and the sample was allowed to dry.

Table II.1- Matrix concentration and matrix solution used to prepare the organic and inorganic matrices tested in this work.

Matrix	Matrix concentration (mg/mL)	Matrix solution
α-CHCA	10	ACN / H_2O/ TFA (50/49.9/0.1, v/v/v)
2,5-DHB	10	H_2O
SA	10	ACN / H_2O / TFA (50/49.9/0.1, v/v/v)
HABA	1.3	ACN / H_2O / MeOH (40/40/20, v/v/v)
3-HPA	50	ACN / H_2O (50/50, v/v)
IAA	18	MeOH
FA	10	MeOH / H_2O (50/50, v/v)
THAP	10	ACN / H_2O (50/50, v/v)
Dithranol	10	THF
TiO_2	2	H_2O
Al_2O_3	4	H_2O

ACN: acetonitrile; TFA: trifluoroacetic acid; MeOH: methanol; THF: tetrahydrofuran

II.4 RESULTS AND DISCUSSION

II.4.1 DIRECT ANALYSIS OF AAS

As a first attempt, the analysis of steroids by MALDI without derivatisation was investigated. The steroids 17β-hydroxy-17-methylandrosta-1,4-dien-3-one, 17α-methyltestosterone and 4-androsten-3,17-dione were selected for these studies. Selection of these depended on their availability at the time of this experiment. These three compounds are also some of the steroids that have been most studied

by MALDI. Standard solutions of steroids at different concentrations in methanol (ranging between 5 and 0.1 µg/mL) were mixed with an equal volume of the different matrix solutions. Aliquots of 1 µL (1:1 matrix/sample) were hand-spotted onto the MALDI sample plate using the dried-droplet method. For the highest analyte concentration assayed, 5µg/mL on the MALDI sample plate, positive identification of the three steroids was only possible with two of the nine matrices used, namely FA and 2,5-DHB (see Supplementary Figure II.SM3, Supporting Information). With the matrices HABA and α-CHCA, only two steroids were identified for the same level of concentration, namely 4-androsten-3,17-dione and 17α-methyltestosterone. In addition, the spectra depicted in Supplementary Figure II.SM3 in the Supporting Information clearly show the drawback caused by the interfering ions from the matrix. The best steroid peak to matrix ion ratios were obtained for the matrix 2,5-DHB. In addition, different ways of spotting the solutions, such as the 'thin layer' and the 'sandwich' methods, were applied in order to enhance the sensitivity in MALDI. Although we did try these approaches with the FA, 2,5-DHB, HABA and α-CHCA matrices, the results obtained were not better (data not shown). Focusing on the matrices FA and 2,5-DHB, an investigation to find the minimum amount of analyte detected was carried out. The results showed that the minimum concentration of steroids positively identified was 2.5µg/mL using FA as matrix, and 0.5µg/mL for 2,5-DHB. Griffiths and co-workers reported that compounds such as anabolic steroids are poorly ionised by MALDI [21, 23]. This occurs because these compounds are not readily protonated, since they lack functional groups with high proton affinity. In order to improve the ionisation of steroids and to obtain better detection limits by MALDI, their derivatisation to an acidic, basic or charged analogue has been suggested [20, 21].

II.4.2 ANALYSIS OF DERIVATISED AAS

A variety of derivatisation protocols have been reported, including the formation of sulphate esters, ferrocenecarbamates and pentafluorobenzyl derivatives from steroid alcohols and the formation of oximes, methyloximes, GP hydrazones and GT hydrazones from oxosteroids [21]. In spite of derivatisation being widely employed in GC/MS and ESI-MS, only a few studies regarding the use of derivatisation in MALDI have been reported [15].

For the purpose of this study, the anabolic steroids were treated with the Girard T hydrazine reagent to produce GT hydrazones. The GT hydrazone derivative is a quaternary ammonium ion that produces an intense $[M]^+$ ion signal in the MALDI mass spectrum, thus increasing the intensity of the steroid signal. Moreover, steroid ions are displaced by 114 *m/z* units to higher *m/z* values and, therefore, less interference from the matrix ions is expected. The theoretical *m/z* values after derivatisation for the steroids studied in this work are listed in Table II.2. Although Griffiths *et al.* have stressed the convenience of performing the derivatisation process using the Girard P rather than

the Girard T derivative to obtain higher ionisation yields, the Girard P derivative is no longer commercially available [21].

After derivatisation, the steroid GT hydrazones were purified by SPE according to the protocol described previously and then mixed with organic or inorganic MALDI matrices for MALDI-TOF-MS analysis.

Table II.2 – Molecular weights of the free steroids and MALDI-TOF-MS ions after steroids derivatisation with Girard T hydrazine.

Anabolic steroid	Molecular mass (Da)	*m/z* after derivatisation
17-α-methystestosterone	302.2246	416.3277
17-β-hydroxy-17-methylandrosta-1,4-dien-3-one	300.2089	414.3121
4-Androsten-3,17-dione	286.1933	400.2964
Boldenone	286.1933	400.2964
Nandrolone	274.1933	388.2964
Etiocholan-3α-ol-17-one	290.2246	404.3277
Trenbolone	270.1620	384.2651
2α-methyl-5α-androstan-3α-ol-17-one	304.2402	418.3434
Mesterolone	304.2402	418.3434
Fluoxymesterone	336.2101	450.3132
Bolasterone	316.2402	430.3434
Calusterone	316.2402	430.3434
Noretiocholanolone	276.2089	390.3121
Ethisterone	312.2089	426.3121
Mibolerone	302.2246	416.3277

II.4.3 INFLUENCE OF THE ORGANIC MALDI MATRIX

Initially, all the matrices were investigated for the analyses of the steroid GT hydrazones from the steroids 17α-methyltestosterone (*m/z* 302.22) and 17β-hydroxy-17-methylandrosta-1,4-dien-3-one (*m/z* 300.21) at a concentration of 1 μg/mL on the MALDI sample plate. These steroids were selected on the basis on their similar molecular mass, as their analysis could inform us about the influence of the MALDI matrix on the resolution obtained in the mass spectra. In addition, they are two of the more studied steroids by MALDI. Results obtained with these compounds could be extended to the other target steroids included in this work.

As it can be seen in Figure II.1, the derivatisation process enhanced the ionisation efficiencies compared with those of the non-derivatised steroids. This result was observed for all the steroids/matrix combinations studied. As an example, if the spectra obtained without (Supplementary

Figure II.SM3, Supporting Information) and with (Figure II.1) derivatisation, using the FA matrix, is compared, an enhancement in the ions of the steroids of about 650% is observed when derivatisation is performed (for a concentration, 2.5 times lower). In addition, the masses of the characteristic ions were displaced to higher *m/z* values, giving a better signal-to-noise ratio.

The worst results were obtained with the THAP matrix (data not shown). This matrix showed highly abundant ions in the mass range of the target steroids. Furthermore, some of the matrix ions were coincident with the theoretical m/z values of the target steroids after derivatisation (*m/z* values of 400.2 and 414.3).

For the 3-HPA, SA and dithranol matrices, although it was possible to detect the characteristic ions of the derivatised steroids, the relative signal intensities of the target compounds were low (data not shown). For these matrices, the derivatisation process was not enough to enhance ionisation appropriately.

For α-CHCA and 2,5-DHB (Figure II.1), the relative intensities obtained for the derivatised steroids were around 65% and 85% of their respective matrix base peaks.

The most promising results were obtained with FA, HABA and IAA. When the samples were mixed with these matrices, the base peaks of the spectra were the ions coming from the steroids under study. As it may be seen in Figure II.1, the best spectrum was obtained with the FA matrix, for which the relative signal intensity of the ions for both steroid was almost 100%. However, as shown below, the best results were obtained with the HABA matrix for low steroid concentrations.

It is also important to highlight the fact that the accuracy was always very good, independent of the MALDI matrix tested. This was because internal calibration was carried out for all the analyses using the characteristic ions of the matrix applied.

II.4.4 ANALYTICAL SENSITIVITY

To investigate the lower amount of steroids detectable, the five matrices that achieved the best results in terms of steroid ionisation were used, i.e. FA, HABA, IAA, α-CHCA and 2,5-DHB. For these experiments, a standard mixture solution of the following five steroids (17α-methystestosterone, 17β-hydroxy-17-methylandrosta-1,4-dien-3-one, 4-androsten-3,17- dione, boldenone and nandrolone) was derivatised at four concentration levels: 0.5; 0.125; 0.05 and 0.025 µg/mL. It is important to note that the steroids 4-androsten-3,17-dione and boldenone are isobaric compounds and, therefore, only a single ion at *m/z* 400.29 is observed for both compounds in the mass spectra.
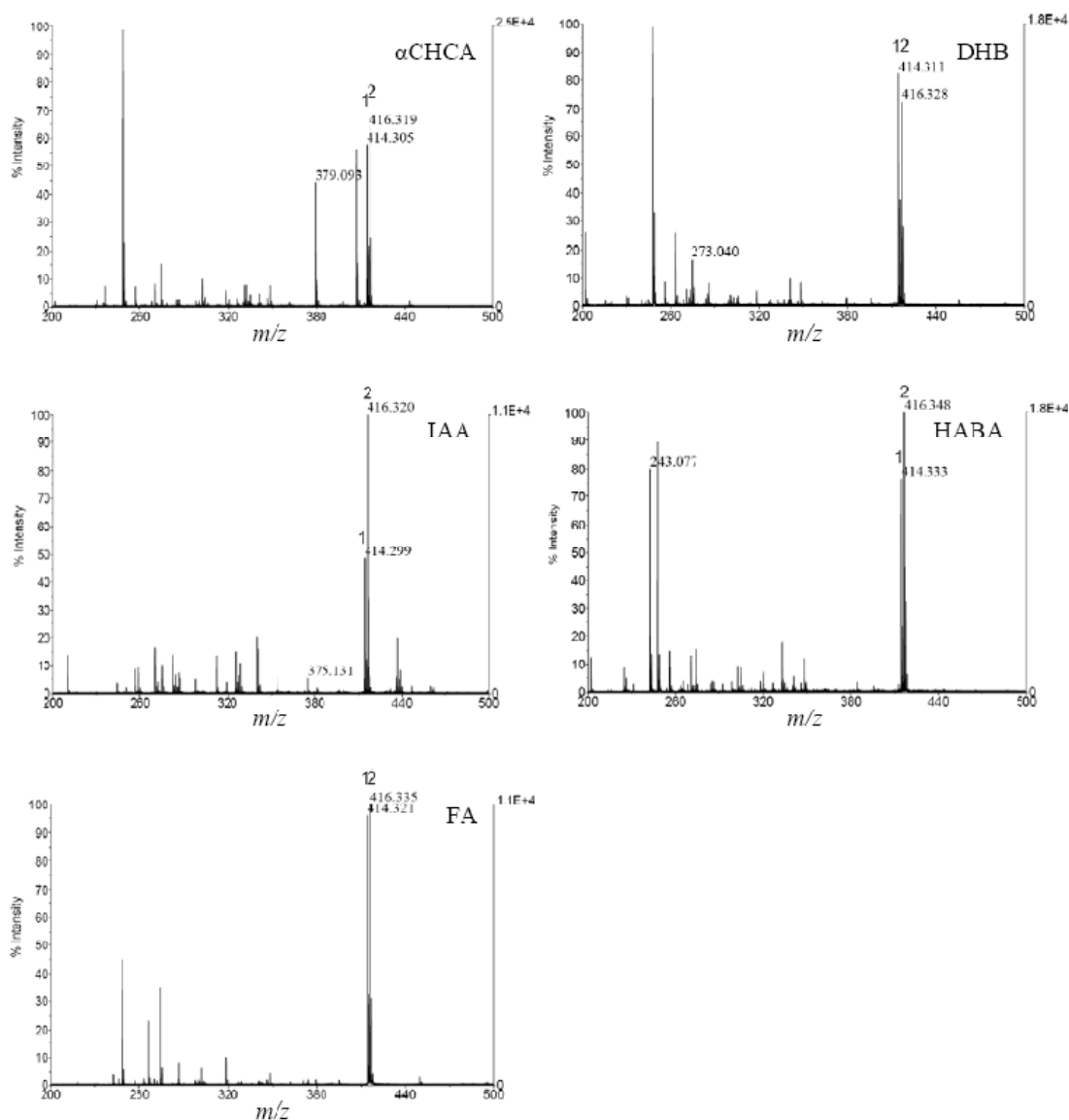
Figure II.1 - MALDI mass spectra of 17α-methyltestosterone and 17β-hydroxy-17-methylandrosta-1,4-dien-3-one GT hydrazones, at concentrations of 1 μg/mL on the MALDI sample plate, obtained with different organic matrices. Ion identification: **1**: 17β-hydroxy-17-methylandrosta-1,4-dien-3-one; and **2**: 17α-methyltestosterone.

It is also important to stress that the identification of isobaric compounds by MALDI-TOF-MS is only possible with a separation procedure prior to the analysis or by the use of post-source decay (PSD) product-ion analysis [20]. Nevertheless, the study presented in this report deals with the identification of the most appropriate matrix for MALDI analysis of steroids. This is of great importance, since the results achieved can also be applied to MALDI-TOF-TOF-MS.
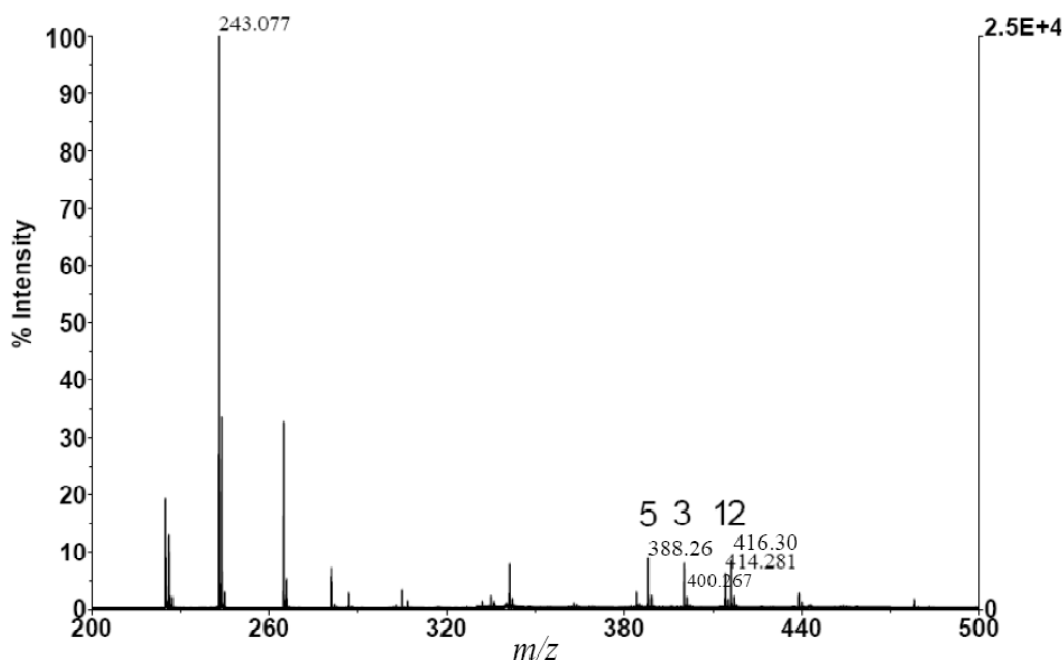
Figure II.2 - MALDI mass spectra of the target steroid GT hydrazones, at concentrations of 0.025 μg/mL on the MALDI sample plate, obtained with the HABA matrix. Ion identification:**1**: 17β-hydroxy-17-methylandrosta-1,4-dien-3-one; **2**: 17α-methyltestosterone; **3** (and **4**): sum of 4-androsten-3,17-dione and boldenone; and **5**: nandrolone.

The data presented in Figure II.2 demonstrates that only when the HABA matrix was used was it possible to positively identify the steroids at their lower concentration values (0.025 μg/mL). As expected, positive identification was also possible for the other matrices assayed, for all or some of the steroids depending on the concentration level, since the degree of ionisation obtained varied for the different matrices applied, as shown in Supplementary Figure II.SM4 in the Supporting Information. As an example, for 2,5-DHB, FA, HABA and IAA matrices, positive identification of all the compounds under study was possible when a sample concentration of 0.125μg/mL was applied to the MALDI sample plate. The best signal in terms of intensity was obtained with the HABA matrix which showed relative intensities ranging between 85% and 100%, followed by the FA matrix with relative intensities ranging between 20% and 55%, then the IAA matrix with relative intensities ranging between 20% and 35%, and finally the 2,5-DHB matrix with relative intensities lower than 15%. When the α-CHCA matrix was used, only the ion corresponding to the sum of 4-androsten-3,17-dione and boldenone was detected, at a concentration of 0.125 μg/mL on the MALDI sample plate. For lower concentrations, none of the steroid GT hydrazones were detected (data not shown).

Table II.3- Concentration of the matrix HABA after dilution.

Dilution factor	Matrix concentration (mg/mL)
1	1.3
4/5	1.0
3/5	0,78
2/5	0,52
1/5	0,26

Subsequently, the influence of the ratio sample/matrix on the sensitivity of the method was investigated. For this purpose and using the HABA matrix, different ratios of matrix to sample concentration were assayed, trying to accomplish a reasonable steroid ionisation yield while maintaining the signal intensity of the interfering matrix ions as low as possible. The concentrations of the HABA matrix applied are presented in Table II.3. In this experiment, the fifteen anabolic steroids were tested. As shown in Figure II.3, when the concentration of the matrix was lower than 1.3 mg/mL, positive identification of the characteristic ions of all the compounds studied was possible for a sample concentration of 0.010 μg/mL on the MALDI sample plate. The best results, in relative intensity, occurred for a concentration of 0.52mg/ mL of the HABA matrix. With this concentration of matrix, it was possible to increase the laser fluences to achieve higher ionisation yields of the sample, without saturating the spectrum with the matrix. For the isobaric steroids used in this study, the analysis of each compound alone was carried out to show that they can be positively identified at a concentration of 0.010 μg/mL on the MALDI sample plate. Positive identification was possible for all compounds (see Supplementary Figure II.SM5, Supporting Information).

Therefore, the sequence of MALDI matrices as a function of their performance from best to worse was as follows: HABA > FA = IAA > 2,5-DHB > α-CHCA >> SA = dithranol > 3-HPA > THAP. At this point it is also important to note that the sensitivity achieved with the HABA matrix for the detection of AAS after derivatisation is similar to that achieved by GC/MS, which is around 4–10 ng/mL in the single ion monitoring mode.
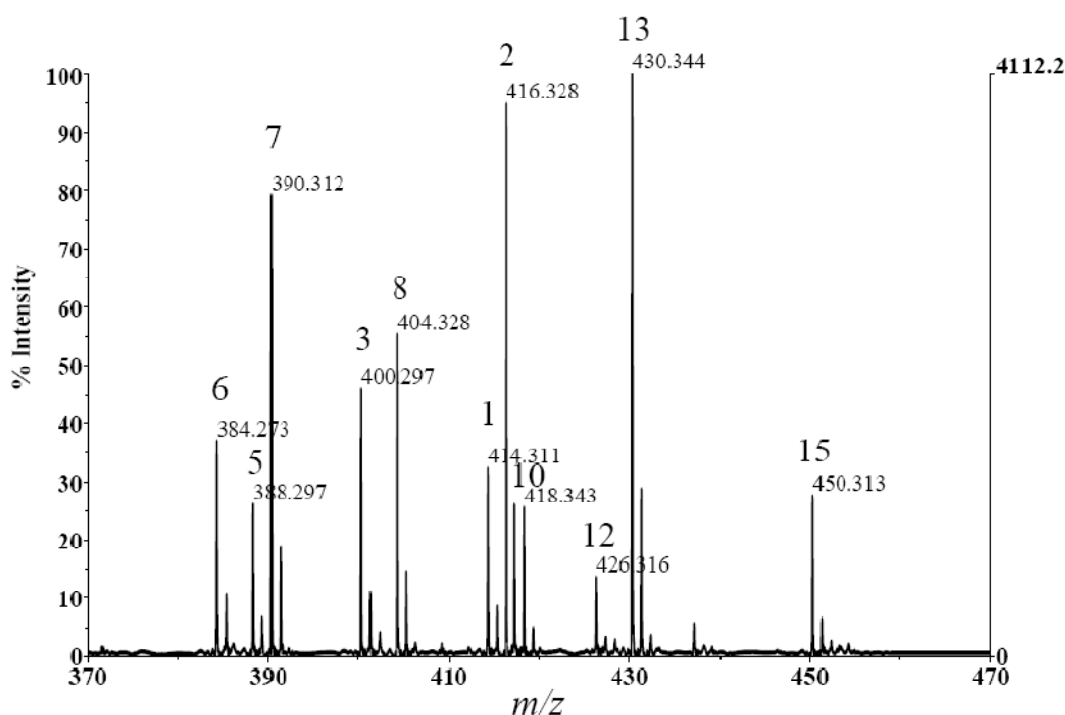
Figure II.3 - MALDI mass spectra of the target steroid GT hydrazones, at concentrations of 0.010 µg/mL on the MALDI sample plate, obtained with the HABA matrix diluted 2:5. Ion identification: **1**: 17β-hydroxy-17-methylandrosta-1,4-dien-3-one; **2** (and **9**): sum of 17α-methyltestosterone and mibolerone; **3** (and **4**): sum of 4-androsten-3,17-dione and boldenone; **5**: nandrolone; **6**: 17α-trenbolone; **7**: noretiocholanolone; **8**: etiocholan-3α-ol- 17-one; **10** (and **11**): sum of 2α-methyl-5β-androstane-3α-ol-17-one and mesterolone; **12**: ethisterone; **13** (and **14**): sum of bolasterone and calusterone; **15**: fluoxymesterolone.

II.4.5 INFLUENCE OF THE INORGANIC MALDI MATRIX

In order to overcome the background noise limitation of organic matrices, inorganic particles have been used as MALDI matrices by some workers [24]. Inorganic particles have low molecular weights and they are stable during the laser desorption process. Therefore, a low background noise was expected below m/z <500. Kinumi and co-workers proposed the use of different metals (Al, Mn, Mo, Si, Sn, W and Zn) or metal oxide (SnO_2, TiO_2, WO_3 and ZnO) powders as inorganic matrices for the analysis of small molecules such as poly(ethylene glycol), PEG 200 (average mass 200 Da) or methyl stearate ($[M+H]^+$ m/z 299) [24]. The authors observed that TiO_2 powder suspended in a dispersing solution was the best matrix, giving the lowest background and the highest signal intensity. Nevertheless, some authors (such as Brombacher *et al.*) have used TiO_2 of different particle sizes for the analysis of steroids without success [25].

In this work, TiO$_2$ and Al$_2$O$_3$ were tested as inorganic MALDI matrices for steroid analysis. The sample treatment was the same as that described above for organic matrices. Different sample concentrations of the derivatised steroids 17β-hydroxy-17-methylandrosta-1,4-dien-3-one, 17α-methyltestosterone, 4-androsten-3,17-dione, boldenone and nandrolone (0.5; 0.125; 0.05; and 0.025μg/mL) were analysed. In general, high laser intensities were required for these compounds in order to obtain good signal-to-noise ratios. The lowest concentration of steroid GT hydrazones that it was possible to identify either with TiO$_2$ (see Supplementary Figure II.SM6c, Supporting Information) or with Al$_2$O$_3$ (see Supplementary Figure II.SM6d, Supporting Information) was 0.25 μg/mL, corresponding to 0.25 ng of each analyte on the MALDI sample plate.

In order to improve the results obtained with inorganic matrices, Tanaka *et al.* and Sunner *et al.* proposed the use of glycerol as a dispersing solution to fix and disperse the analyte in the inorganic matrix [26, 27]. The procedure followed by us was based on the one described by Kinumi and co-workers [24]. Briefly, the inorganic matrix and sample were applied to the MALDI sample plate. After being air-dried, 1mL of the glycerol solution (10% in methanol) was added and the MALDI sample plate was vacuum-dried. Our results showed that the addition of glycerol enhanced the performance of the analysis. On the one hand, 10% less laser intensity was needed than when glycerol was not used. However, on the other hand, the background was greatly reduced (see Supplementary Figs. VIa and VIIa, Supporting Information). Unfortunately, this improvement was not enough to obtain a lower amount of steroid positively identified.

II.4.6 ANALYSIS OF REAL SAMPLES

Urine from a healthy young male was used in order to show the applicability of the HABA-based method developed. The analyses were carried out in duplicate. The blank urine samples were spiked with the steroids, 17α-methyltestosterone, nandrolone, 17α-trenbolone, bolasterone and fluoxymesterolone, up to a level of 10 ng/mL, which is the Minimum Required Performance Limit (MRPL) recommended by the WADA organisation, for the majority of steroids [28]. It must be stressed here that the selection of these five steroids depends on the fact that their characteristic ions appear at significant different *m/z* values, making it possible to evaluate the signal in a wide *m/z* range.
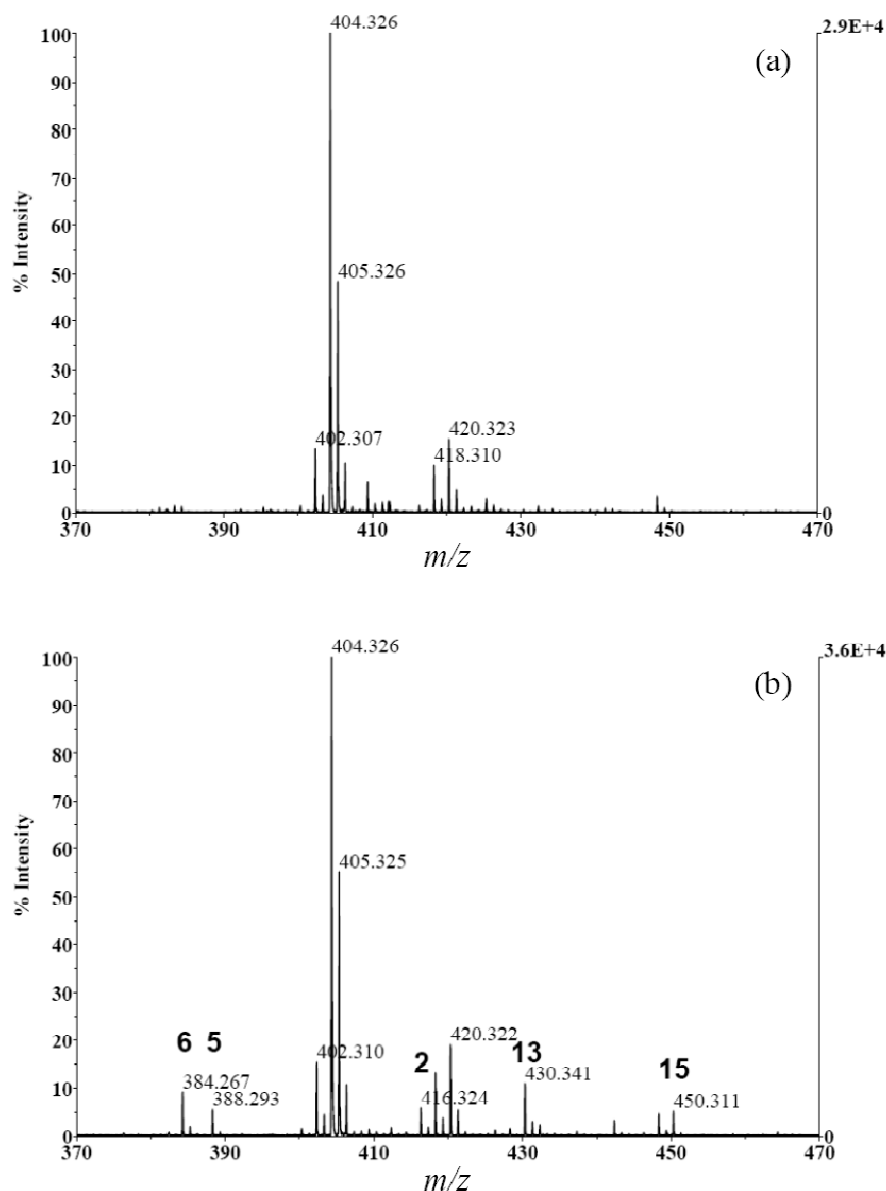
Figure II.4 - MALDI mass spectra obtained after the analysis of human male urine with (b) and without spike (a) of five exogenous steroids. Steroid GT hydrazones ion identification: **2**: 17α-methyltestosterone; **5**: nandrolone; **6**: 17α-trenbolone; **13**: bolasterone; and **15**: fluoxymesterolone.

Most steroids are eliminated from the body as conjugated anabolic steroids, which have higher polarity and are more readily excreted in urine than steroids in their free form [29]. Although it is possible to determine the conjugated anabolic steroids, there are no available standards for most of them. Hence, an enzymatic hydrolysis step was required to transform the conjugated anabolic steroids into their free form. Briefly, urine samples (5 mL) with and without spikes were hydrolysed according to the protocol described in the Experimental section. After cooling at room temperature, the urine samples were cleaned up with an Oasis HLB SPE cartridge and then evaporated to dryness. After derivatisation, the sample was mixed with the HABA matrix at 0.52mg/mL and hand-spotted onto the MALDI sample plate.

As it is shown in Figure II.4, all the anabolic steroids spiked into the urine were detected. It is, however, important to note that the other ions that appear in the MALDI mass spectra correspond to the endogenous steroids. For instance, the most abundant ion at *m/z* 404.326 is characteristic of androsterone and etiocholanolone, while the ion at *m/z* 402.307 is characteristic for testosterone, epitestosterone and dehydroepiandrosterone (DHEA). Moreover, the ion observed at *m/z* 420.323 corresponds to 11-keto-etiocholanolone.

The results obtained showed that the clean-up and pre-concentration processes in conjunction with the HABA based matrix method constitute a good approach for the fast screening of steroids in samples such as urine.

II.5 CONCLUSION

Of the nine organic matrices tested in this study, HABA was found to be the most robust for the fast screening of steroids. Using this matrix we have demonstrated that MALDI can be used as a robust method for the fast screening of steroids with similar sensitivity to the traditional GC/MS techniques that are commonly used for this purpose. Although the minimum steroid concentration detected in urine samples was 10 ng/mL, the pre-concentration factor of 5 used in this work could be increased, thus allowing lower detection limits.

The use of inorganic matrices has been described as a good approach for the determination of small molecules by MALDI-TOF-MS. In our case, the use of higher laser fluencies to achieve good signal intensity in the MALDI mass spectra results in higher matrix fragmentation and, consequently, in an increment of the background noise. Positive identification of the target steroid GT hydrazones was possible at concentrations of 0.25μg/mL. The use of a dispersing solution such as glycerol allowed the reduction of the laser fluences required to obtain the MALDI mass spectra and, therefore, the background noise was reduced. Nevertheless, no modification in the detection limits was observed by glycerol addition. In addition, the use of MALDI-TOF-TOF-MS to create a mass fingerprint library of all the steroids in the WADA prohibited list using the HABA-matrix based method is currently in progress.

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II.7 SUPPLEMENTARY MATERIAL
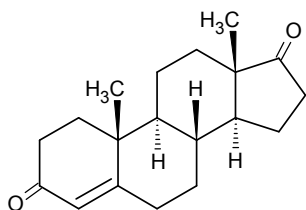
4-androstene-3,17-dione
(M=286.19 g/mol)
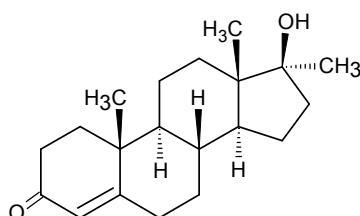
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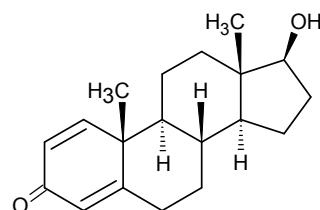
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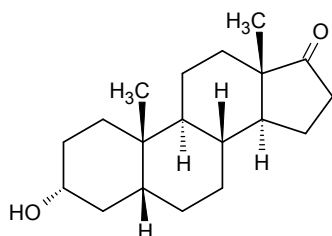
Etiocholan-3α-ol-17-one
(M=290.22 g/mol)
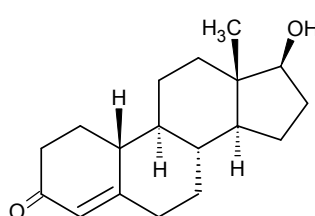
Nandrolone
(M=274.19 g/mol)
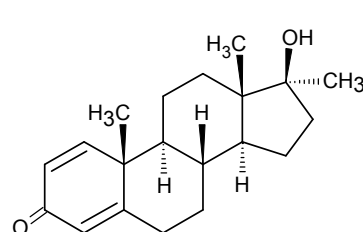
17β-Hydroxy-17-methylandrosta-1,4-dien-3-one
(M=300.21 g/mol)
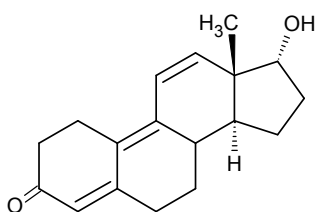
17α-trenbolone
(M=270.16 g/mol)
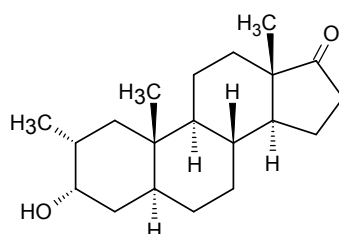
2α-methyl-5α-androstan-3α-ol-17-one
(M=304.24 g/mol)
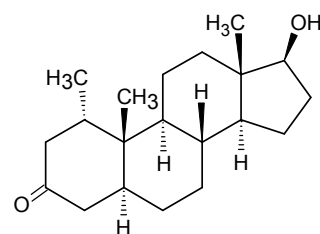
Mesterolone
(M=304.24 g/mol)
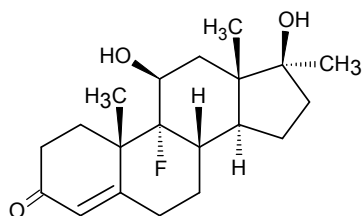
Fluoxymesterone
(M=336.21g /mol)
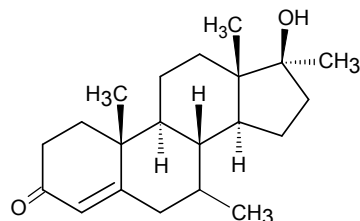
Bolasterone
(M=316.24 g /mol)

Calusterone
(M=316.24 g /mol)

Mibolerone
(M=302.22 g /mol)

Noretiocholanolone
(M=276.21 g /mol)
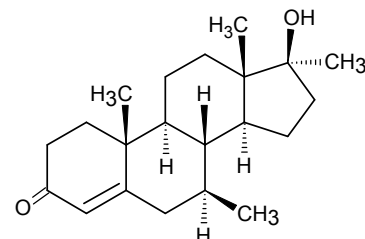
Ethisterone
(M=312.21 g /mol)
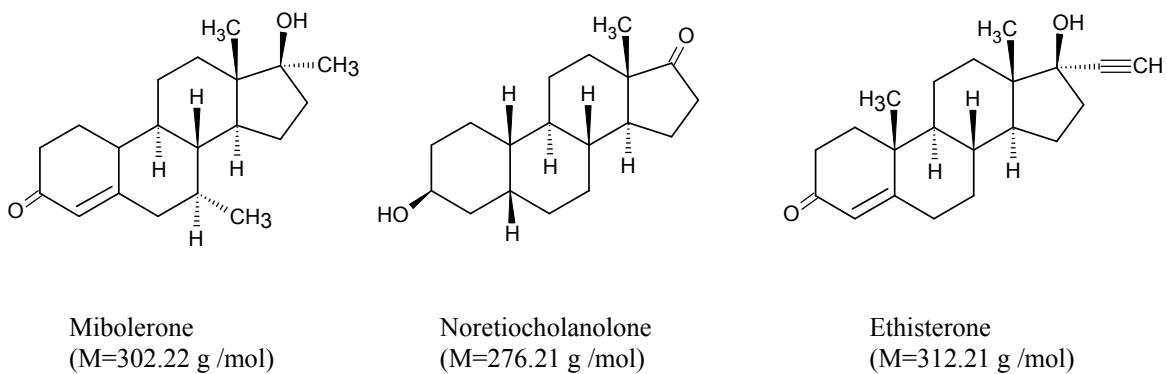
Figure II.SM1 - Chemical structures of the fifteen anabolic steroids analysed.

Dithranol

αCHCA

SA

HABA

3-HPA

2,5-DHB

IAA

FA

THAP
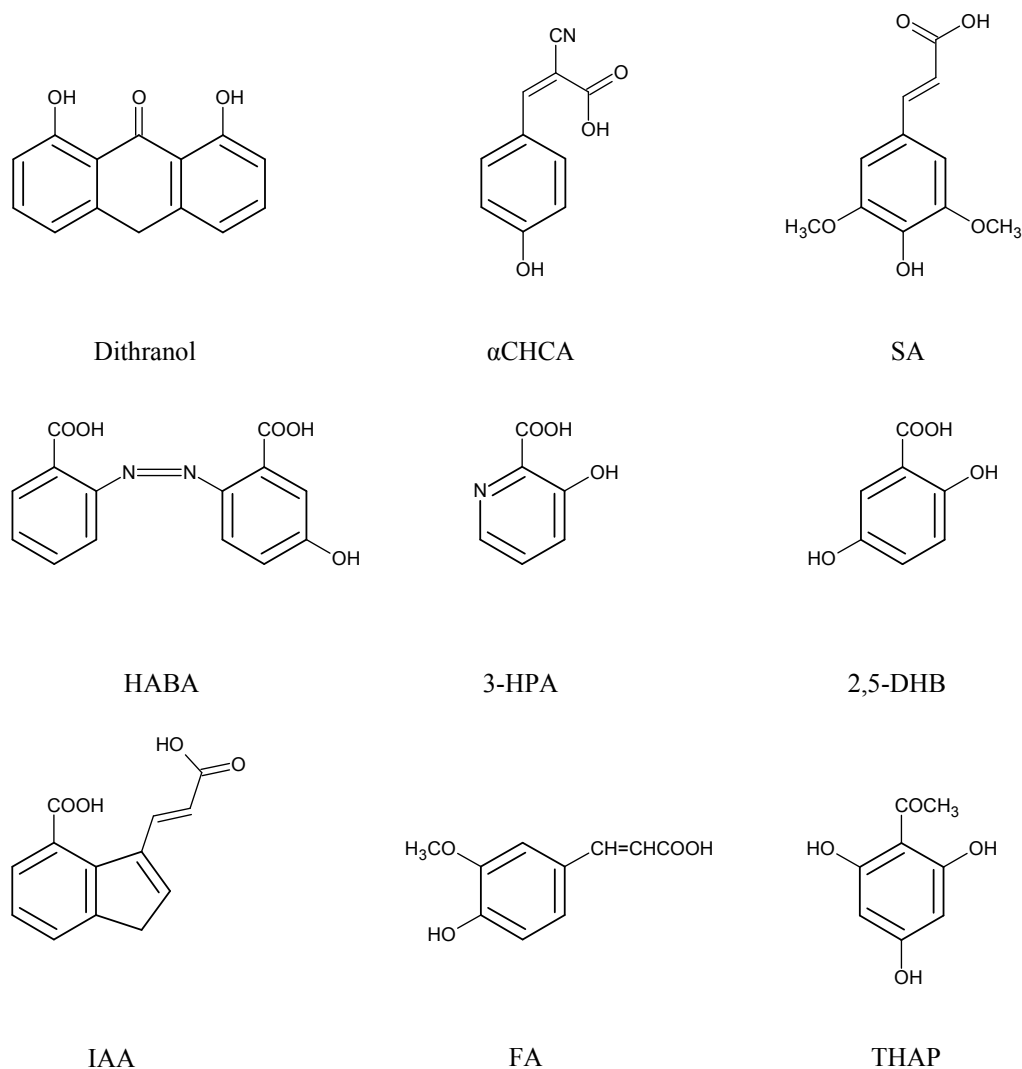
Figure II.SM2 - Chemical structures of the nine organic matrices applied.
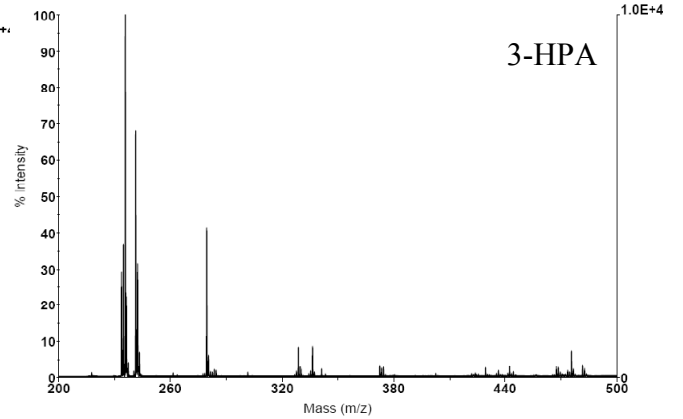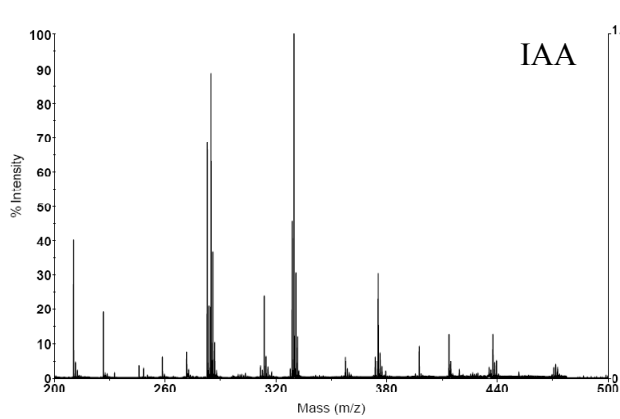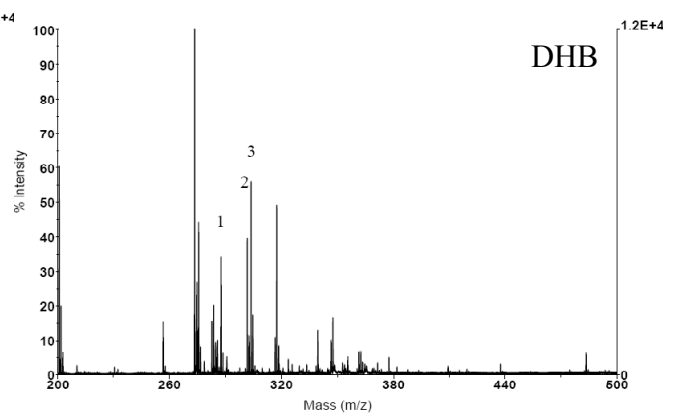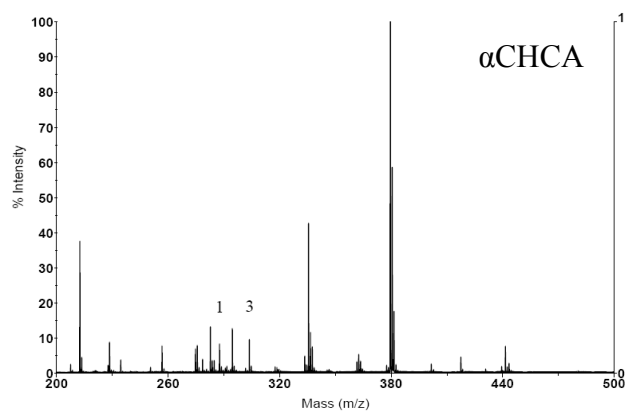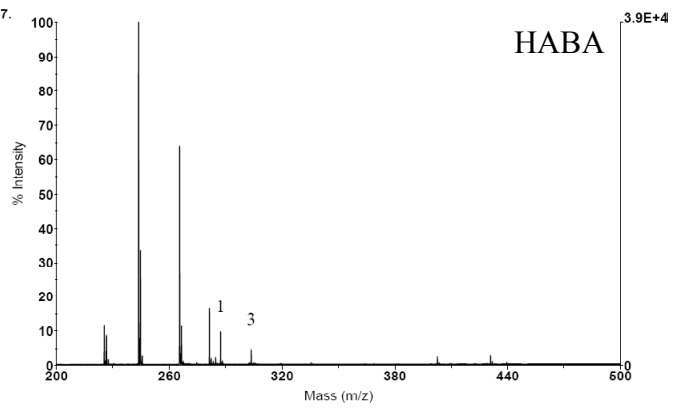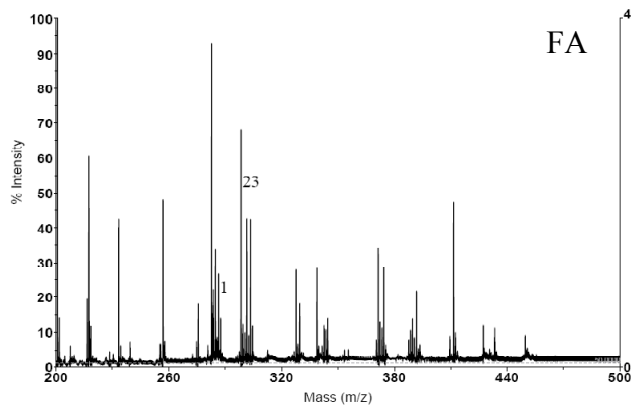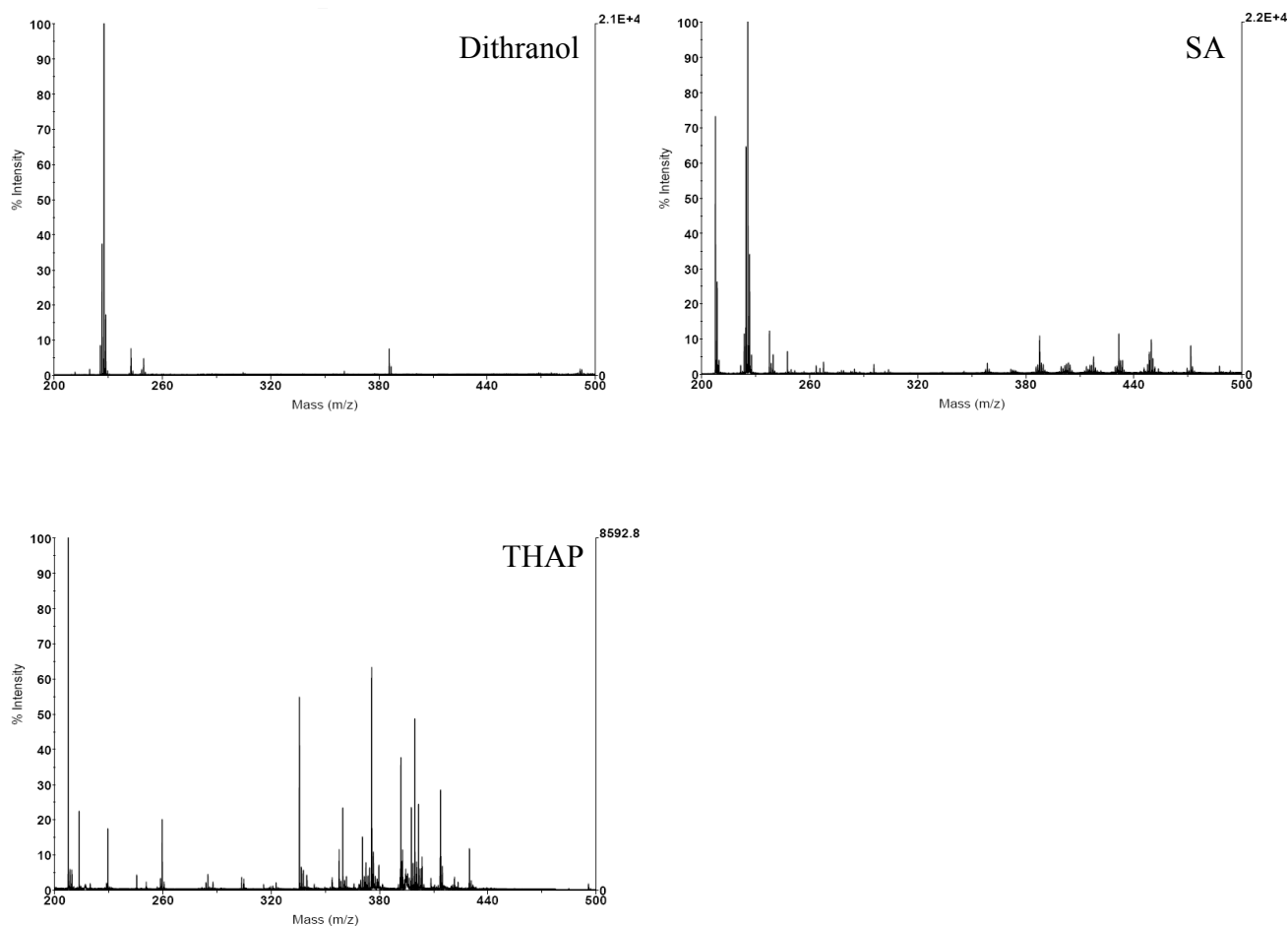
Figure II.SM3 - MALDI mass spectra of 17-β-hydroxy-17-methylandrosta-1,4-dien-3-one, 17-α-methyltestosterone and 4-androsten-3,17-dione at a concentration of 5 μg/mL in the MALDI sample plate, obtained with different organic matrices. Peak identification: **1**: 17-β-hydroxy-17-methylandrosta-1,4-dien-3-one; **2**: 17-α-methyltestosterone; and **3**: 4-androsten-3,17-dione.
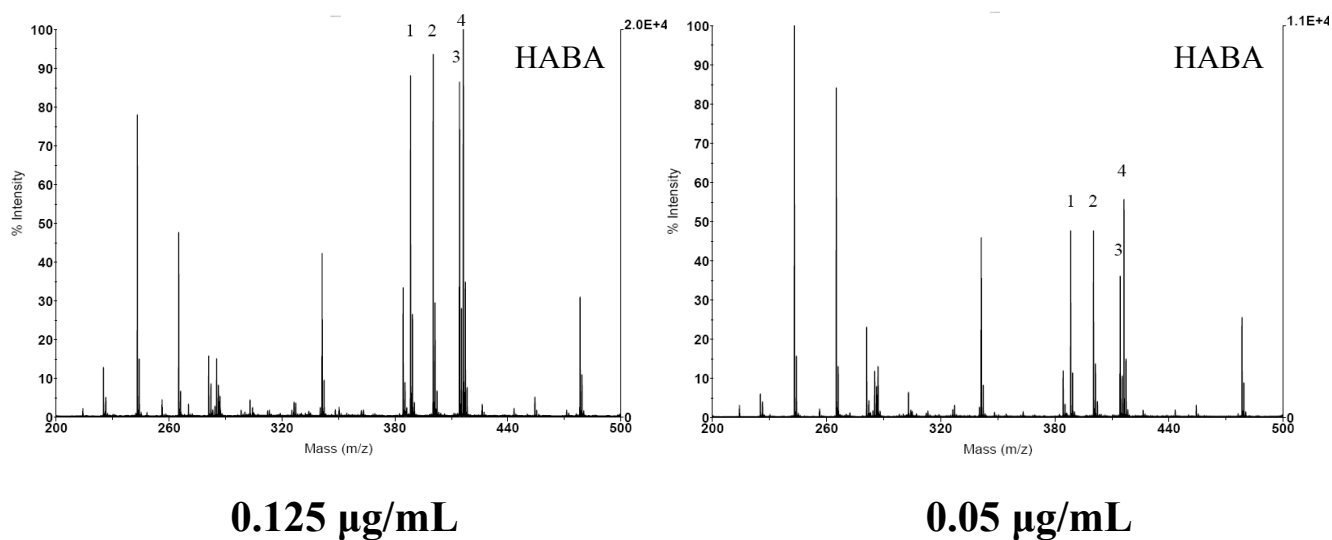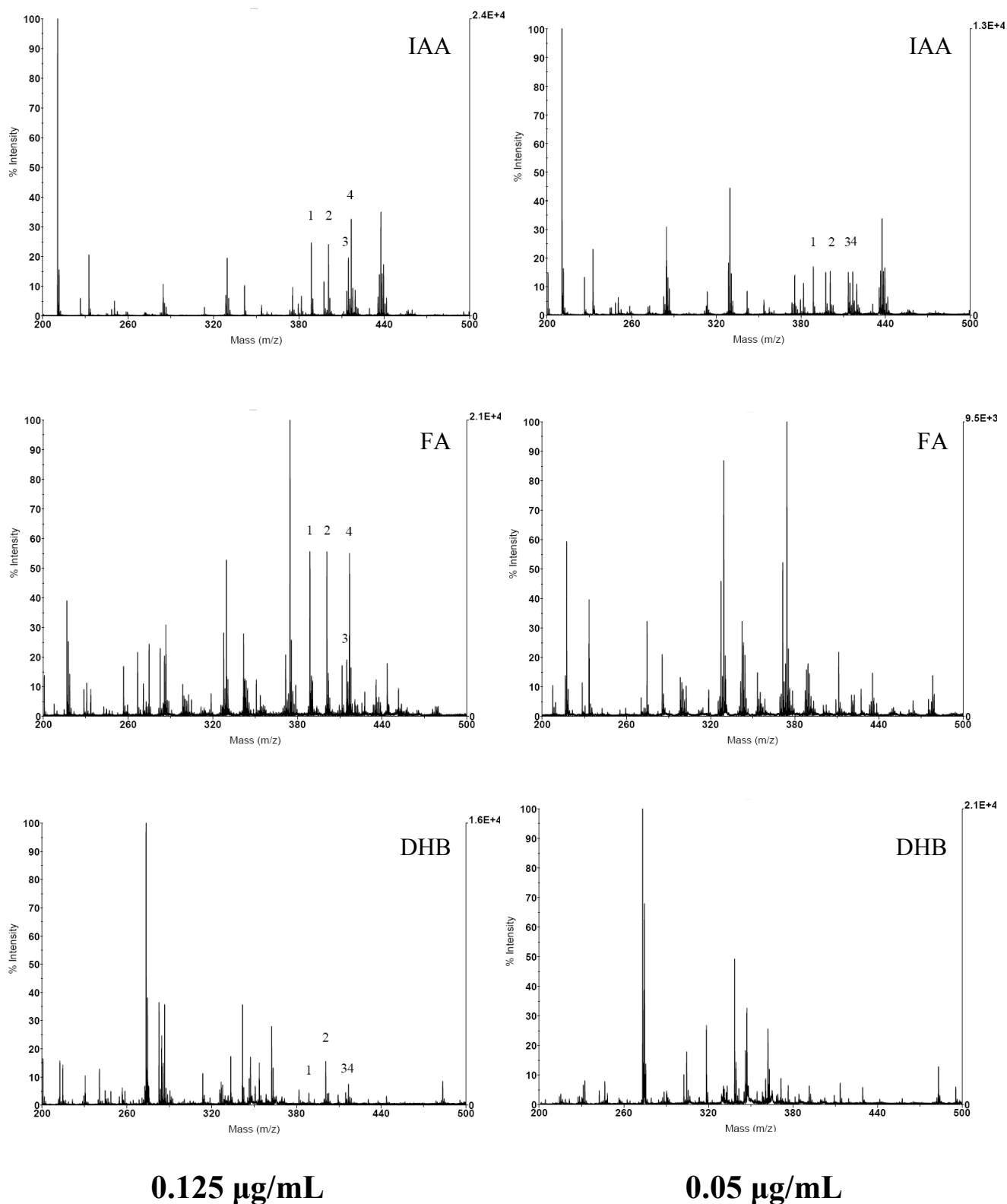
0.125 μg/mL   **0.05 μg/mL**

0.125 µg/mL

0.05 µg/mL

Figure II.SM4 - MALDI mass spectra of the target steroid GT hydrazones, at concentrations of 0.125 and 0.05 µg/mL in the MALDI sample plate, obtained with HABA, IAA, FA and 2,5-DHB matrices. Peak identification: **1**: nandrolone; **2**: sum of 4-androsten-3,17-dione and boldenone; **3**: 17-β-hydroxy-17-methylandrosta-1,4-dien-3-one; and **4**: 17-α-methyltestosterone.
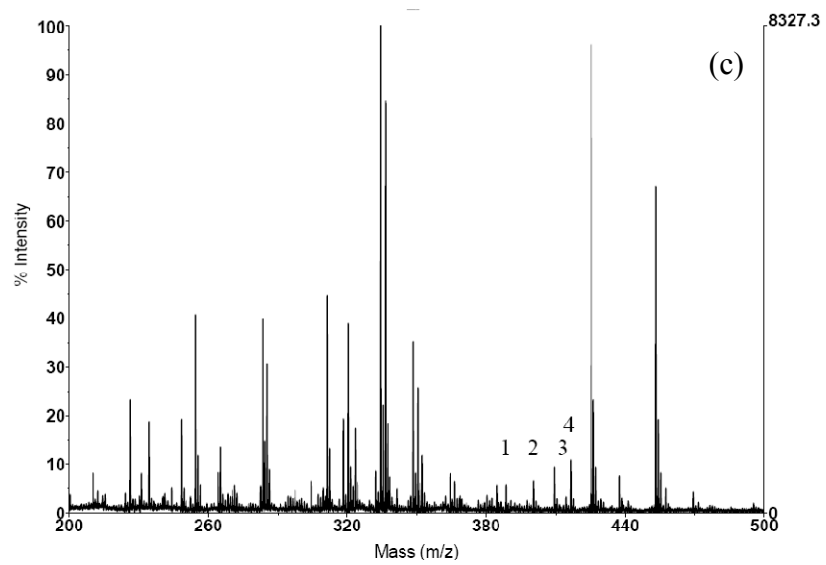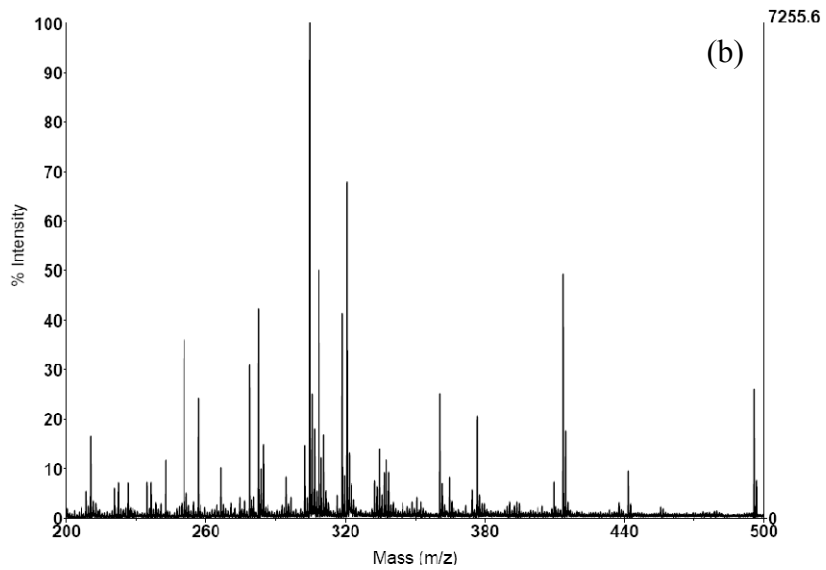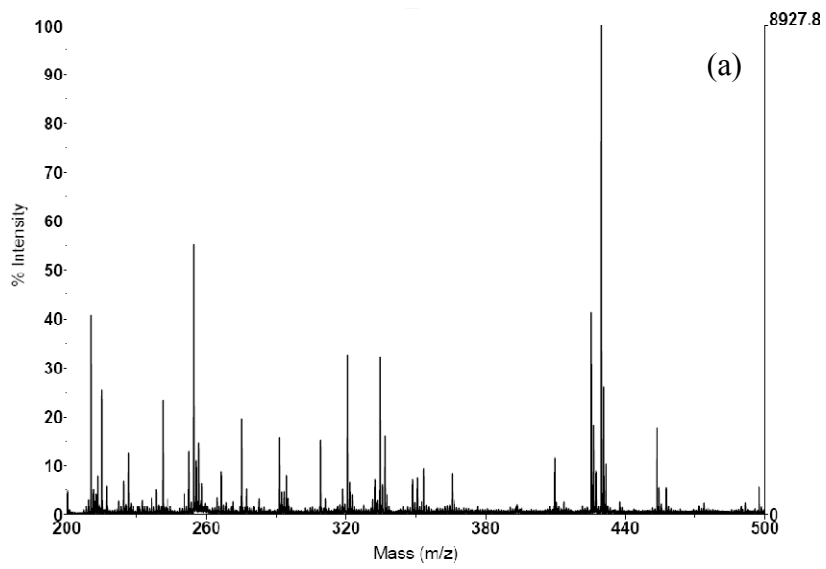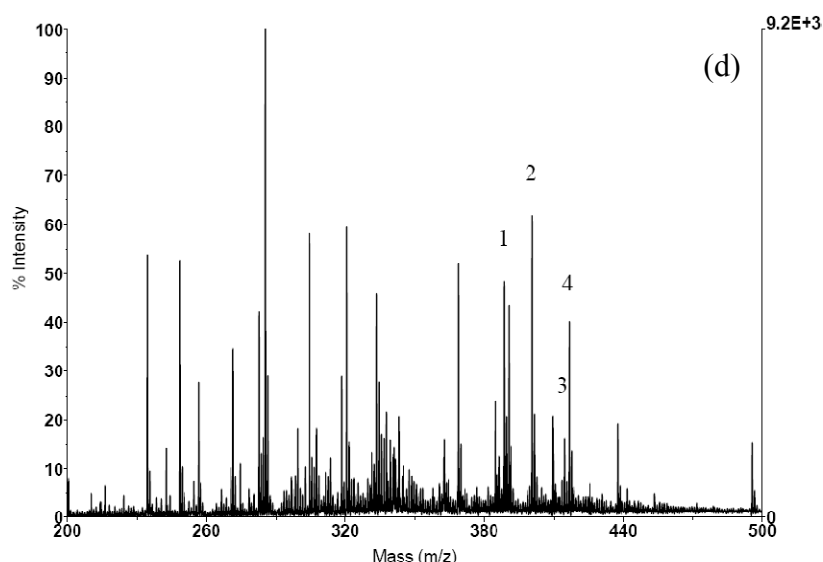
Figure II.SM5 - MALDI mass spectra of: (a) the inorganic TiO_2 matrix; (b) the inorganic Al_2O_3 matrix; (c) the target steroid GT hydrazones (0.250 µg/ml) obtained with the TiO_2 matrix; (d) the target steroid GT hydrazones (0.250 µg/ml) obtained with the Al_2O_3 matrix. Peak identification: **1**: nandrolone; **2**: 4-Androsten-3,17-dione and boldenone; **3**: 17-β-hydroxy-17-methylandrosta-1,4-dien-3-one; **4**: 17-α-methyltestosterone.
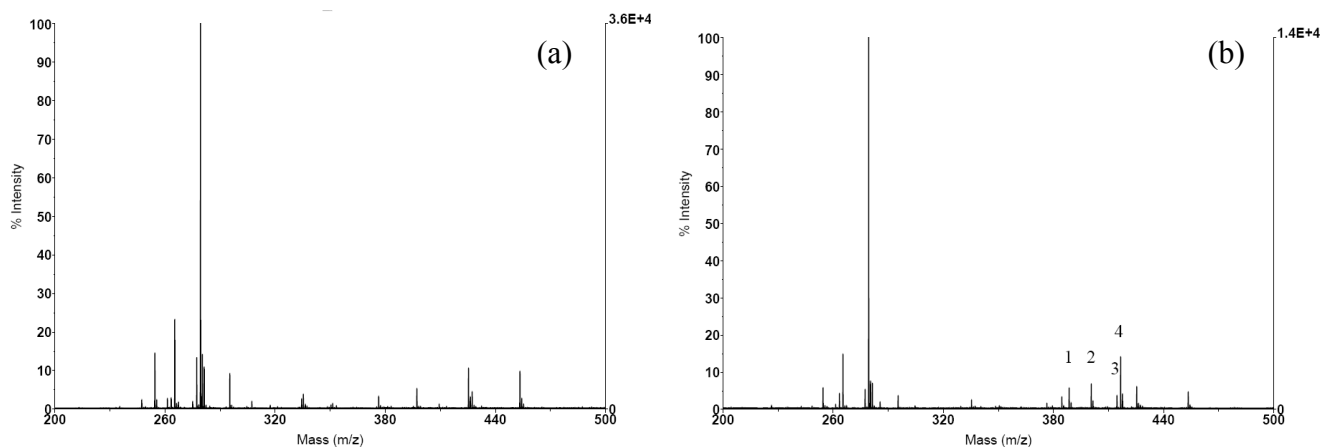
Figure II.SM6 - MALDI mass spectra of: (a) the inorganic TiO_2 matrix with the dispersing solution; (b) the target steroid GT hydrazones (0.250 µg/ml) obtained with the TiO_2 matrix with the dispersing solution. Peak identification: **1**: nandrolone; **2**: 4-Androsten-3,17-dione and boldenone; **3**: 17-β-hydroxy-17-methylandrosta-1,4-dien-3-one; **4**: 17-α-methyltestosterone.

CHAPTER III

MLibrary: a free software tool for doping screening by matrix assisted laser desorption ionization-based strategies

PUBLISHED IN:

III.1 ABSTRACT

This work presents a novel database search engine – MLibrary - designed to assist the user in the detection and identification of androgenic anabolic steroids (AAS) and its metabolites by matrix assisted laser desorption/ionization (MALDI) strategies. The detection of the banned compounds in the samples analyzed was accomplished by searching (i) the mass spectrometric (MS) spectra against the library developed to identify possible positives and (ii) by comparison of the tandem mass spectrometric (MS/MS) spectra produced after fragmentation of the possible positives with a complete set of spectra that have previously been assigned to the software. With the help of the MLibrary software application, the use of MALDI techniques for doping control is simplified and the time for evaluation and interpretation of the results is reduced. To do so it takes as input several MALDI-TOF-MS and MALDI-TOF-MS/MS spectra and aids the researcher in an automatic mode by identifying in which samples are possible positives and then to confirm it by comparison of the tandem mass spectra results. Furthermore, the software program can, potentially, be further expanded to other banned compounds.

The overall applicability of the MLibrary tool is shown by the analysis of spiked urine samples. All positive samples were successfully identified.

III.2 INTRODUCTION

The detection of doping offences by world antidoping agencies is a matter of increasing complexity. Although the attempts made by antidoping organizations and the national antidoping laboratories at detecting doping practices in and out of competition, the increasing workloads and consequent

delaying reports and raising costs, remain an important limitation to which we must add the continuously introduction of new banned substances and methods to antidoping regulations.

The use of forbidden anabolic steroids and hormones to enhance athletic performance has important health and social implications. Their use was first introduced in sports as agents supporting the athlete recuperation after extreme stress and fatigue, but rapidly became the main group in doping abuse [1].

Nowadays, this class of drugs is a major group included in the prohibited list of the world anti-doping agency (WADA) as well as of major sports authorities [2-5]. In the WADA statistic report for 2009, the androgenic anabolic steroids (AAS) represented 64.9 % of all adverse analytical findings reported by WADA accredited laboratories [6]. Although this data might be likely and largely overestimate, because of the well known problems in the detectability of other doping agents (e.g. peptide hormones).

The use of AAS to increase muscle mass and strength is not a behaviour strictly related to elite athletes, as their use is increasing amongst amateur athletes as well as outside sports as an expression of an improved life style [7, 8]. The illicit AAS use is an increasing trend in western societies and the emergent AAS dependence is a matter of growing public health concern [9].

Quickly following the development of mass spectrometry (MS) detectors, its use coupled to gas chromatography (GC) has become the standard technique for AAS control and currently, most methods for routine detection of these compounds and their metabolites, comprising both screening and confirmatory analysis, are still based in GC-MS techniques [10-14]. More recently, due to the increasing complexity of doping analyses and in order to enhance the detection of this group, liquid chromatography coupled with MS/MS is gaining ground within antidoping laboratories [15-17]. Although it avoids the derivatisation step required by GC-MS and provides good sensitivity for the determination of steroids, long separation times are normally required [18, 19].

The combination of these two factors, which are the long separation times of chromatographic techniques and the increasing workloads within antidoping laboratories, expose an urgent need for an analytical technique allowing simplicity, speed and high throughput for the screening of the huge number of banned compounds, particularly the AAS.

Recently, the use of matrix-assisted laser desorption/ionization (MALDI) for the analysis of small molecules, has grown as a potential technique, which is reflected by the increasing number of studies reported in literature [20-24]. Moreover, it appears extremely promising for high-throughput, which is a major demand for future antidoping methods.

In light of the latest technological improvements of this analytical technique we have recently study the applicability of a wide variety of commercial MALDI matrices for the rapid screening of AAS [25]. The matrix 2-(4-hydroxyphenylazo)-benzoic acid (HABA) was found to be the most robust for the analysis of anabolic steroids after a derivatisation step with the reagent Girard T hydrazine. In the work developed we demonstrated the use of MALDI-TOF-MS as a fast and robust method for the fast screening of AAS. The positive identification of the characteristic peaks for all the compounds

studied was possible for a sample concentration of 10 ng/mL in the MALDI sample plate. The sensitivity achieved with the HABA matrix after derivatisation was similar to that achieved by GC/MS – around 4–10 ng/mL in the single ion monitoring mode.

In the present work, the use of MALDI-TOF-TOF-MS to create a mass library for the AAS figuring in the WADA prohibit list was studied with the purpose to facilitate its application inside antidoping and public health laboratories. However, to be successfully implemented not only the analytical method has to have high throughput, but also the data interpretation step has to be easy and fast. For that reason, a novel database search engine – MLibrary - has been developed to assist the user in the detection and identification of these compounds and its metabolites. The present manuscript describes in detail the software tool MLibrary, explaining through a real example how to use it. This software is freely source code available, and it can be run as a multiple platform.

III.3 EXPERIMENTAL

III.3.1 APPARATUS

A model UNIVAPO 100H vacuum concentrator centrifuge (UniEquip, Martinsried, Germany) with a model Unijet II refrigerated aspirator vacuum pump (UniEquip) was used for (i) sample drying and (ii) sample pre-concentration. A Spectrafuge-mini minicentrifuge (Labnet, Madrid, Spain) and a Sky Line minicentrifuge-vortex (ELMI, Riga, Latvia) were used throughout the sample treatment, when necessary. A Simplicity 185 system (Millipore, Milan, Italy) was used to obtain Milli-Q ultrapure water throughout all the experiments. The derivatisation procedure was performed in a 1.5mL microtube flat cap from Delta Lab (Barcelona, Spain). Separation of the steroid Girard T (GT) hydrazones from the unreacted Girard T reagent was carried out in a 2mL empty reversible solid-phase extraction (SPE) cartridge from Supelco (Belefonte, PA, USA) packed with a preparative C18 resin (125Å, 55-105µm; Waters, Barcelona, Spain).

III.3.2 STANDARDS AND REAGENTS

Standards of 17-α-methyltestosterone, 4-androsten-3,17-dione, boldenone and nandrolone were purchased from Riedel-de Haën (Seelze, Germany), 17-β-hydroxy-17-methylandrosta-1,4-dien-3-one was from Fluka (Buchs, Switzerland) and the standard Etiocholan-3α-ol-17-one was purchased from Sigma (Steinheim, Germany). The standards 17α-Trenbolone, 2α-methyl-5β-androstane-3α-ol-17-one, Mesterolone, Fluoxymesterolone, Bolasterone, Calusterone, Noretiocholanolone, Ethisterone, Mibolerone, testosterone, epitestosterone and androsterone were kindly provided by the Portuguese National Anti-doping Laboratory and the Italy National Anti-doping Laboratory. A solution of β-

glucuronidase from *Escherichia coli* K12 with a specific activity approximately of 140U/mg at 37ºC and pH 7 with nitrophenyl-β-D-glucuronidase as substrate (1 mL contains at least 140U) was purchased from Roche Diagnostic (Mannheim, Germany). Sodium hydrogen phosphate, sodium phosphate dibasic, tert-butylmethyl ether, methanol (MeOH), acetonitrile (ACN) and the derivatisation reagent, Girard T (GT) hydrazine, used for sample and matrix preparation were purchased from Sigma; glacial acetic acid (>99.5%) was from Fluka; trifluoroacetic acid (TFA, 99%) was from Riedel-de Haën.

Urine samples used in this work were obtained from healthy volunteers from the research team. The research ethical committee from the Science Faculty of Ourense approved the study protocol and all the volunteers gave their consent.

III.3.3 SAMPLE PREPARATION

III.3.3.1 STANDARD SOLUTIONS

Individual stock standard solutions of each compound (500 mg/L) were prepared by weighing 0.0125 g of analyte in a 25 mL volumetric flask and making it to volume with methanol. These standard solutions were stored in the dark at -20 ºC. Working standard solutions were prepared by dilution of the stock standard solutions in the appropriate volume of methanol.

III.3.3.2 URINE HYDROLYSIS PROCEDURE

Urine samples (2 mL) were hydrolysed with 50 µL of the commercial solution of β-glucuronidase, after the addition of 0,750 mL of phosphate buffer (0.8M, pH 7). The hydrolysis was performed at 55 ºC during 60 min.

III.3.3.3 LIQUID-LIQUID EXTRACTION OF TARGET ANALYTES

After cooling to room temperature, 0.5 mL of carbonate buffer (pH 9) was added to alkalinize the hydrolyzed solution. Liquid–liquid extraction was carried out by agitation with 5 mL of tert-butyl methyl ether for around 10 min; after centrifugation, the organic phase layer was transferred to a new vessel.

III.3.3.4 DERIVATISATION PROCEDURE

The procedure for derivatisation with Girard T hydrazine was performed based on the protocol described by Wheeler [26], as follows: The collected organic phase was dried under a gentle nitrogen stream at 40ºC. After the addition of 500 µL of a methanolic solution with 10% glacial acetic acid and 4 mg of Girard T hydrazine, the vial was closed and the derivatisation reaction was then performed at 60 ºC during 30 min. After cooling, the solution was evaporated to dryness in a vacuum concentrator centrifuge and then reconstituted with 1mL of methanol/water (10:90, v/v).

III.3.3.5 SPE CLEAN-UP

After derivatisation, the steroid GT hydrazones were separated from un-reacted GT hydrazine reagent by SPE in a C_{18} cartridge, according to the protocols described by Khan *et al.* [21] and Griffiths *et al.* [22]. Briefly, before use, the cartridges were conditioned with 5mL of methanol plus 10mL of MilliQ-water without allowing the cartridges to dry out. After loading the sample, the cartridge was washed with 2mL of methanol/water (10:90, v/v) in order to remove impurities from the cartridge and, finally, the steroid GT hydrazones were eluted from the cartridge with 1mL of methanol.

III.3.3.6 MALDI-TOF-MS AND MALDI-TOF-TOF-MS ANALYSIS

The mass spectrometric analyses were performed on Applied Biosystems 4700 Proteomics Analyzer with TOF/TOF™ Optics system (Applied Biosystems, Foster City, CA, USA) equipped with and a diode pumped Nd:YAG laser with 200 Hz repetition rate. The instrument was operated for detection in positive ion reflectron mode. The MS spectrum for each sample was based on the average of 1000 laser shots; for the MS/MS up to 4000 shots were accumulated. MS/MS mode was operated with 1 kV collision energy; air was used as the collision gas such that nominally single collision conditions were achieved. For MS analysis, laser desorbed ions were accelerated from the source at 20 kV. For MS/MS analysis, ions were accelerated from the source at 8.0 kV. Both modes employed delayed ion extraction for improved ion focusing. The MS/MS data was acquired using the instrument default calibration, whilst the MS was acquired using the peaks from the HABA matrix for internal calibration. Prior to MALDI analysis, the sample was mixed with an equal volume of the MALDI matrix solution and homogenised in a vortex instrument. The HABA MALDI matrix used in this work was prepared according to the developed method, 0.52 mg in 1mL of a solution of $ACN/H_2O/MeOH$ (40/40/20, v/v/v) [25]. An aliquot of the sample/matrix solution (0.5 µL) was hand-spotted onto the MALDI sample plate and the sample was allowed to dry. Figure III.1 presents a scheme of the sample treatment followed in this study.
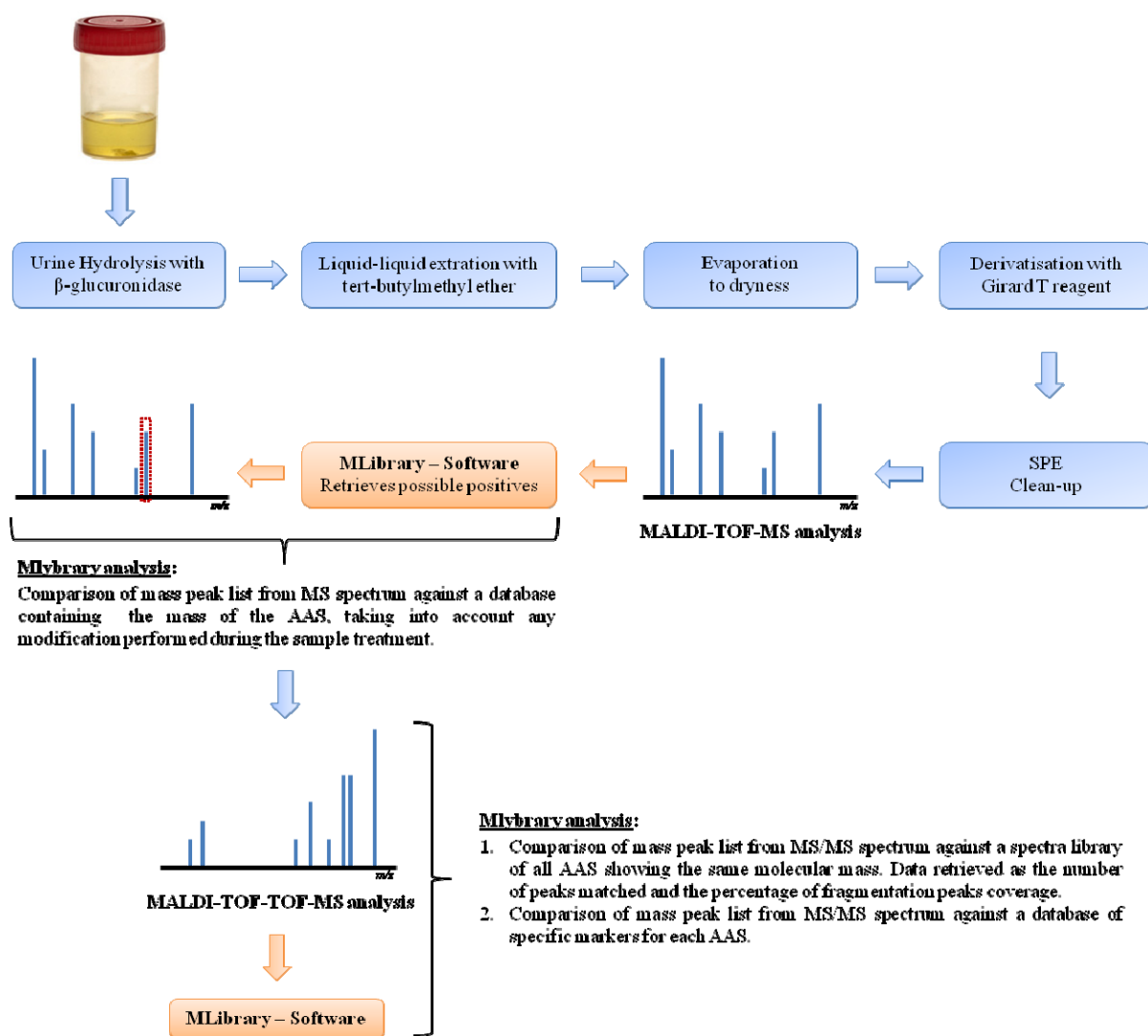
Figure III.1 – Schematic diagram of the MALDI-MS(/MS) strategy to analyse AAS using the MLibrary software.

III.3.3.7 MLIBRARY SOFTWARE

Current versions of the software and their supporting user manuals are freely available for download and use, without restriction, via internet at http://sing.ei.uvigo.es/MLibrary. The program was developed based on previous work on a tool for accurate protein quantification [27].

MLibrary operates on excel comma-separated-values, CSV, files with centroid mass and relative intensity data extracted from the instrument software (Data Explorer^{TM} Software, version 4.5). This data can be analyzed and compared with the compound data stored in the MLibrary repository, which contains the characteristic mass values of the molecular ion and the fragmentation ions for each target AAS. For each database created and stored in the MLibrary repository, the user may include standard modification mass variations corresponding to specific derivatisation reagents and the

consequent MS/MS spectra for each AAS presenting that particular modification. The MLibrary database comprises also MS/MS compound markers. The MLibrary repository is stored in a single standard XML file, which can be easily modified with any plain text editor. More information about how to edit this file can be found in the software web page.

III.4 RESULTS AND DISCUSSION

The MLibrary software is explained in detail in the following sections. First we describe the use of MLibrary to detect the presence of possible AASs in the target samples and then its use to confirm the identity of the compound.

III.4.1 MS MODE

III.4.1.1 CONSTRUCTION OF MS DATABASE

The MS database in the MLibrary software contains the characteristic mass values for each target AAS. Additionally, the mass values of AAS glucuronides, which are the main excretion metabolites of AAS in the human body, were also introduced in the MLibrary database. In our previous work, the analysis of AAS by MALDI techniques was performed after derivatisation with Girard T hydrazine and therefore only this specific modification was introduced in the database. Nevertheless, the introduction of specific modifications to the database is easily performed by typing the name and the mass variation in the modifications correspondent line within the XML file.

III.4.1.2 DETECTING THE PRESENCE OF POSSIBLE AASs

After the SPE clean-up procedure the collected sample solution was mixed with the MALDI matrix HABA and spotted onto the MALDI sample plate. The MALDI ion source is a soft ionisation technique and therefore the MALDI-TOF-MS analysis measure, primarily, singly charged ions that correspondent to the molecular ions of the sample solution species as well as to the MALDI matrix characteristic ions. The mass spectrum obtained after the MALDI-TOF-MS analysis is exported as a list of peak to a CSV file. Only the centroid mass and relative intensity of each peak is used by the MLibrary software (see Figure III.2). When the MLibrary program is started the interface shown in Figure III.3 appears in the screen. By clicking in the "Load MS spectrum data" button we are asked to input the CSV excel file previously created. In this step, the user can submit the entire spectrum or select the peaks above a horizontal line that corresponds to a percentage of the base peak.

	A	B	C
1	Centroid Mass	Relative Intensity	
2	199.989334	0.06	
3	200.214874	0.04	
4	201.628937	0.03	
5	202.540497	0.04	
6	207.518555	0.03	
7	208.030182	0.04	
8	214.165939	3.13	
9	242.881165	0.13	
10	242.950806	0.42	
11	243.077316	37.37	
12	399.991333	0.13	
13	400.13974	0.16	
14	400.304291	20.59	
15	416.043976	0.21	
16	416.336853	53.2	
17	430.357758	1.4	
18	430.535797	0.19	

Figure III.2 – CSV file showing centroid mass and relative intensity of each peak.

Alternatively, the spectrum can be processed inside the respective MS data software and only then transferred to a CSV file. It is important to stress that the MLibrary software permits the loading of multiple CSV files corresponding to several spectra by clicking in the "Load a set of MS spectra data".
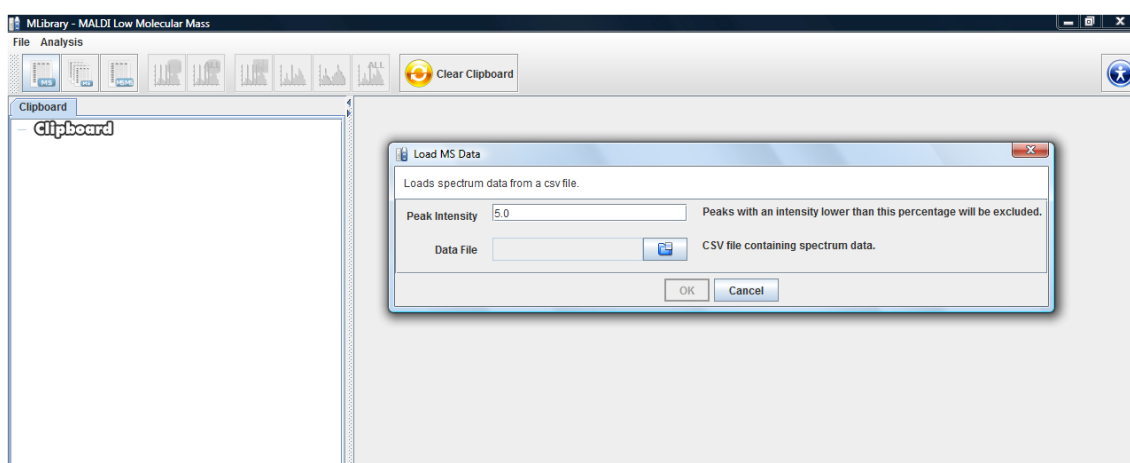
Figure III.3 – MLibrary interface for MS spectrum data load.

After the file input, the MLibrary software compares the list of peaks within the CSV file against a database. Concerning the database, the user can create and upload its own search database.

The database contemplates several parameters that potentiate the software with a desirable flexibility. The database encloses parameters such as the class of compound and all the modifications possible to occur during the sample treatment procedure. These modifications take into account if the compound is in its "conjugated" or "free" form and if it was modified with a derivatisation agent. The user can introduce in the database as many modifications as required and can set running the search according to the sample`s characteristics. The search is performed taking into account the error window between the database and the experimental mass values. This error window is given by the mass tolerance parameter and it can be selected as percentage, parts per million (ppm) or absolute mass units (amu) (see Figure III.4). Since the AAS mass values are close to the matrix characteristic ion mass values, it is possible to perform an internal calibration for each MALDI plate spot using the matrix peaks as reference. Consequently, the experimental mass values obtained are very accurate and precise, showing a very low error window.
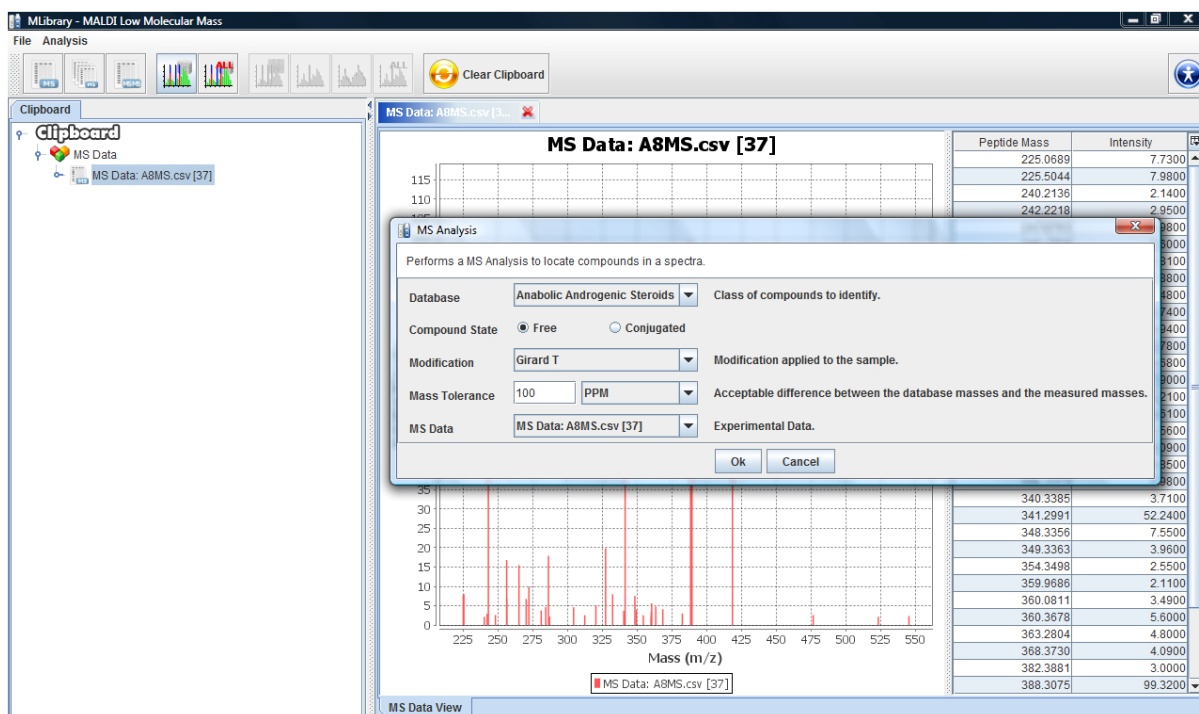
Figure III.4 – MLibrary MS spectrum analysis search window. **Input information:** Database, compound state, modification and mass tolerance.

The search retrieves the mass values that matched, showing both experimental and theoretic values, as well as, the name of the compound. Moreover, the experimental peak intensity for each matched peak is given (see Figure III.5).
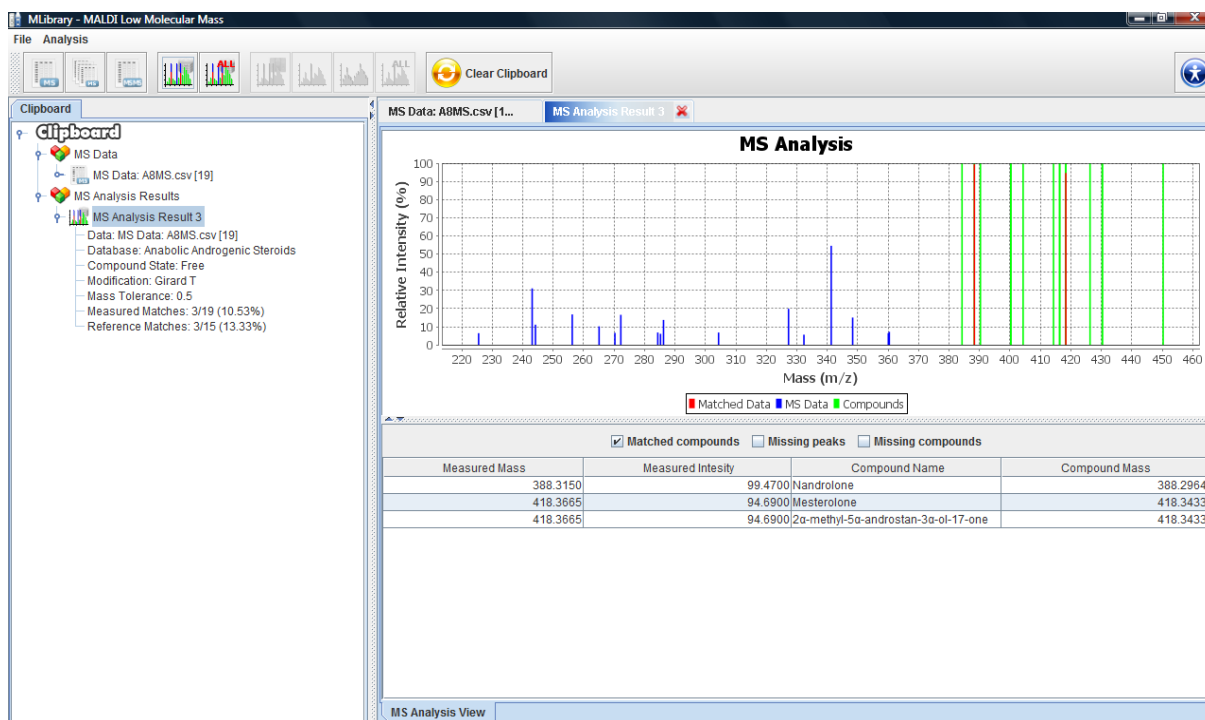
Figure III.5 – MLibrary MS spectrum analysis display window. **Data identification: Blue line:** experimental spectrum mass peaks; **Green line:** database reference masses; **Red line:** experimental mass that matched with the reference mass.

III.4.2 MS/MS MODE

In the first step of the analysis, the Mlibrary software detected the ions that matched with the theoretic mass values of AAS within the MS database. In the second step, the ions detected by the MLibrary are selected for fragmentation in a second round of MS analysis, in which MS/MS spectra are acquire. Each AAS compound presents a characteristic fragmentation pattern that will be used by the MLibrary as a signature of that compound. Likewise the MS mode, the mass spectrum obtained after the MALDI-TOF-TOF-MS analysis is exported as a list of peak to a CSV file.

III.4.2.1 CONSTRUCTION OF MS/MS DATABASE

The MS/MS mass spectral library in the MLibrary software contains the characteristic fragmentation ions for each compound. The construction of the database is easily performed using standard solutions of AAS. To ensure reliable spectral data, the software allows the input of infinite replicates corresponding to each standard. With these data, the MLibrary software generates a list of common fragmentation mass values for all replicates, as well as, a list of the media value for the ions relative

intensity. In addition, the software allows the user to choose the discriminate power of the generated list, meaning that the user can decide to include within this list, the mass values that are present, for instance, at least in 95% percent of the spectral mass data inputted for each compound.

The construction of the mass spectral library in the MLibrary software is an ongoing process that is simple to achieve and updated by any qualified user, with the advantage that it can be easily adapted to numerous experimental conditions and compounds.

III.4.2.2 CONFIRMING THE PRESENCE OF AASs

At this step, the CSV file corresponding to the MS/MS spectrum acquired for the working sample is loaded into the MLibrary search engine for MS/MS confirmation by clicking in the "MS/MS load data" button (see Figure III.6).
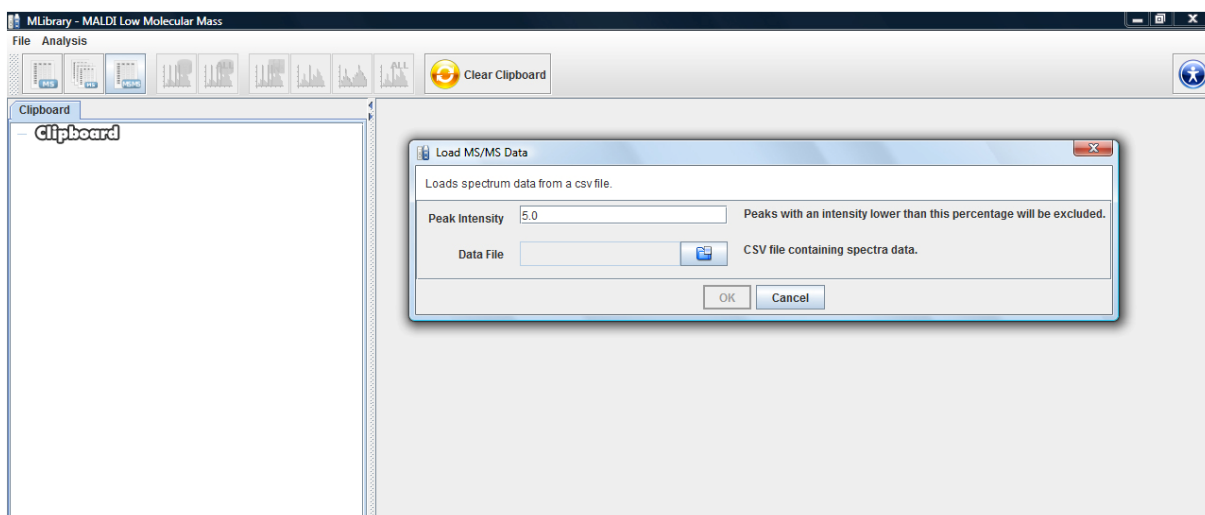
Figure III.6 – MLibrary interface for MS/MS spectrum data load.

In the same manner than for the MS mode, the input file window allows the user to select the peaks above a horizontal line that corresponds to a percentage of the base peak. After loading the sample CSV file, the user can initiate the search engine by clicking in the "MS/MS analysis" button, which will retrieve an input window (see Figure III.7). At this stage the user has to select the database, the modifications performed in the sample treatment procedure, the mass value of the precursor molecular mass and the mass tolerance permitted for each mass peak. Additionally, the user has to select the mass tolerance within the database mass spectra and its discriminate power. The MLibrary search engine uses these data to narrow the searchable mass spectral records. This command retrieves a list of all compounds within the MS/MS library that fit the search criteria, ranked by similarity to the inputted file. The similarity is attained regarding the number of mass values that matched and the

relative intensity of all peaks. To ensure reliable results, the search score concerns only to a limited number of mass values, defined previously by the user. For instance, the user may limit the search to the 20 most intense peaks within the inputted file and the library data (see Figure III.8). This tool is essential to avoid misinterpretation of the results due to the fact that different spectra of the same sample, generally present distinct overall number of mass peaks.
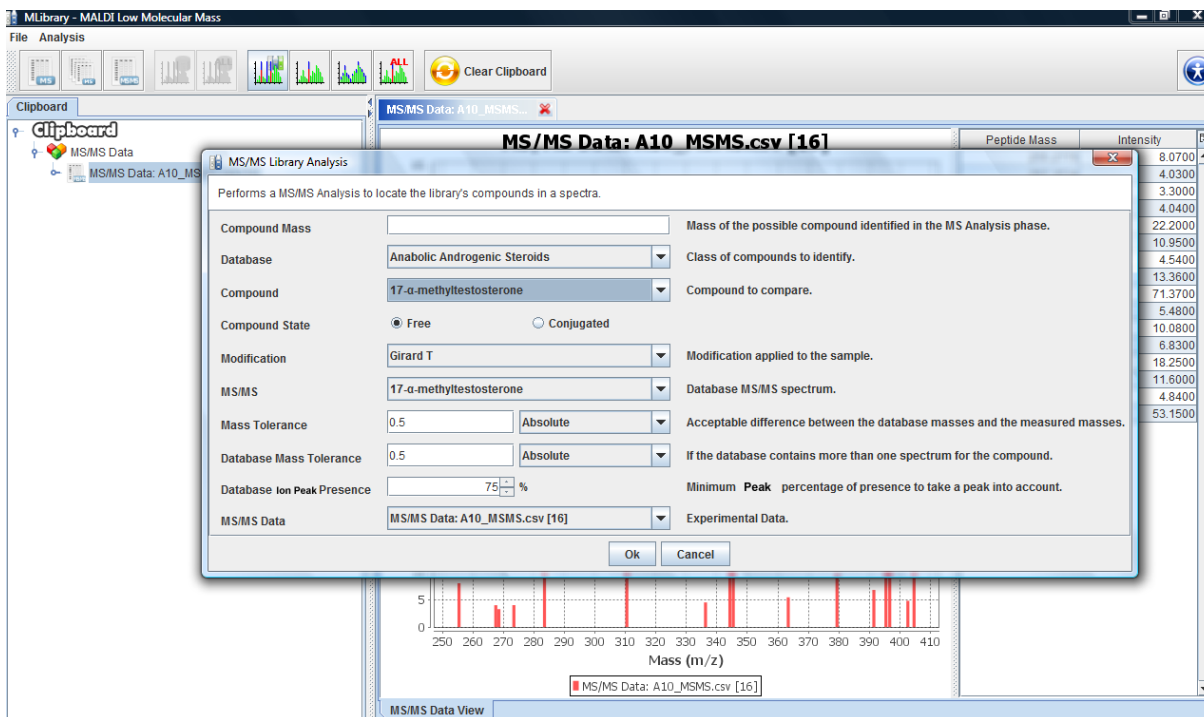
Figure III.7 – MLibrary MS/MS spectrum analysis search window. **Input information:** Database, compound mass or compound name, compound state, modification, mass tolerance concerning the experimental and reference mass values, mass tolerance within the database spectra and discriminate power.

Another important tool within the MLibrary software is the MS/MS experimental spectra comparison. With this tool the user is able to compare two experimental spectra, which is extremely important if we are working at different conditions than the one recorded in the MLibrary MS/MS database. With this tool, the user can confirm the identity of a specific compound by adding a standard solution of that compound to the MALDI analysis and then compare the two spectra using the MLibrary (see Figure III.9). The results appear in the same way that showed in Figure III.8.
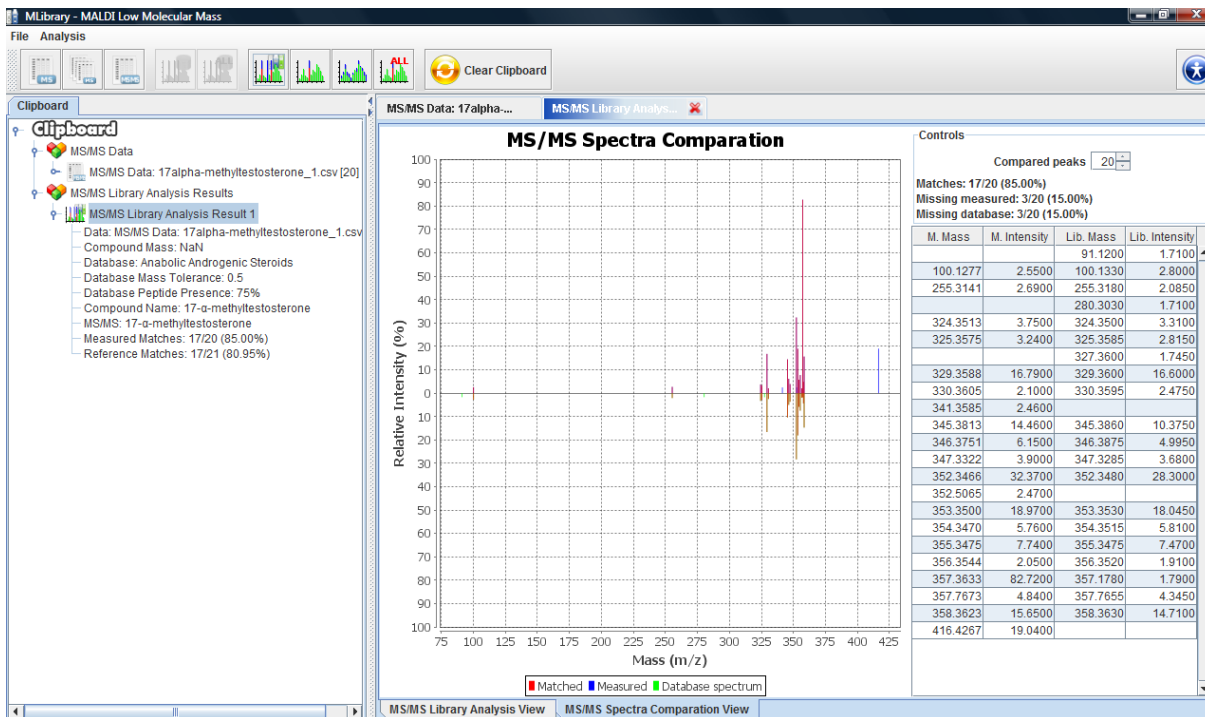
Figure III.8 – MLibrary MS/MS spectrum analysis display window.
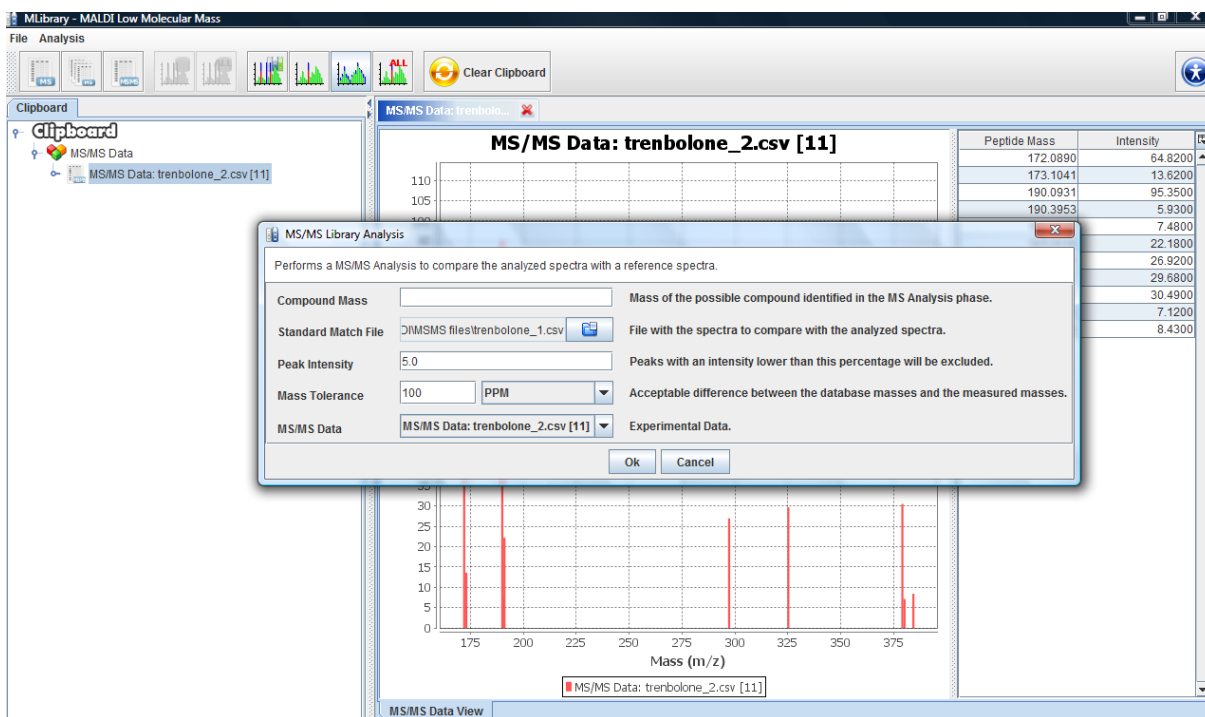
Figure III.9 – MLibrary MS/MS spectra comparison analysis window.

III.4.3 CASE STUDY

Figure III.10 presents the results obtained with the data acquired from a real urine sample spiked with the AAS trenbolone and 17α-methyltestosterone, following the methodology described in the sample preparation section. As it may be seen, all spiked compounds were detected as possible positives.
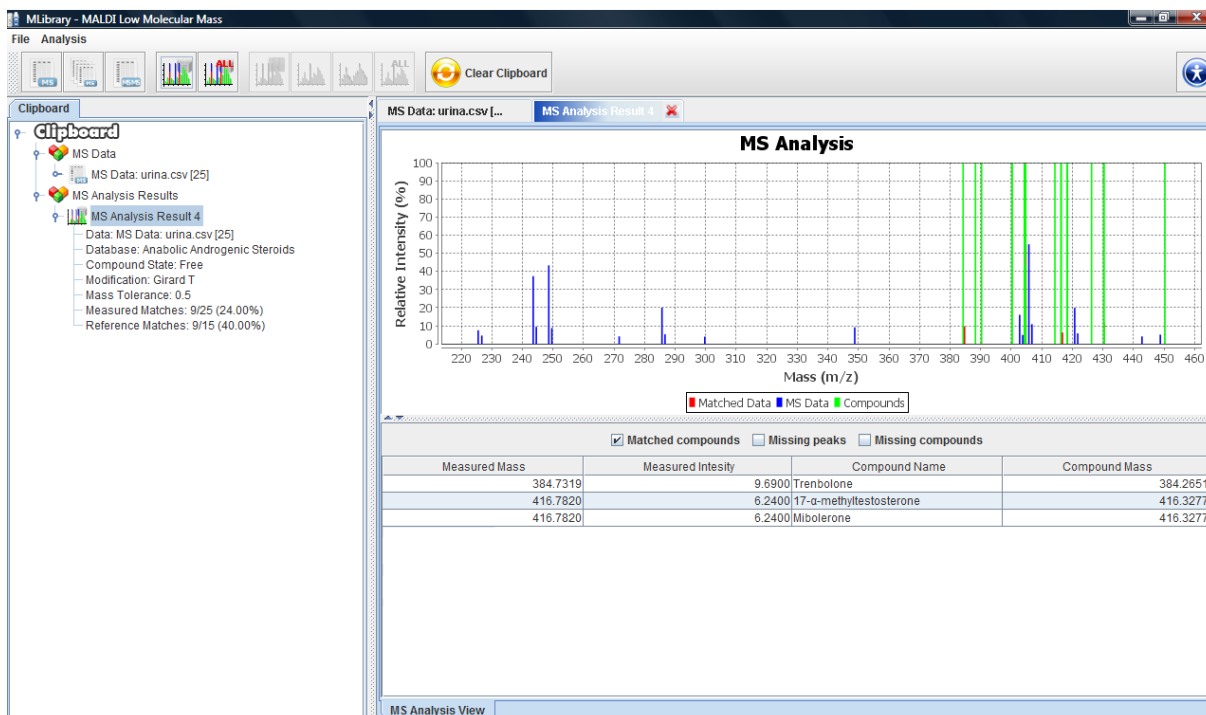
Figure III.10 – MLibrary urine sample MS spectrum analysis display window. **Data identification: Blue line:** experimental spectrum mass peaks; **Green line:** database reference masses; **Red line:** experimental mass that matched with the reference mass.

Additionally, mibolerone, which has the same molecular mass that 17α-methyltestosterone, was also considered as a possible positive. As mentioned above, following the detection of the possible presence of the AAS in the urine sample, a MALDI-TOF-TOF-MS analysis was performed to the ions retrieved as possible positives. The MLibrary software was used to confirm the identity of the compounds. Figure III.11 shows the results obtained for the three compounds. As it may be seen in Figure III.11a, trenbolone is easily identified by MLibrary MS/MS data analysis, showing 90% of positive match to the reference database spectra within the 10 most intense peaks. Moreover, all the mass ions present in the MS/MS spectrum matched with MLibrary reference mass values for trenbolone.

(a)
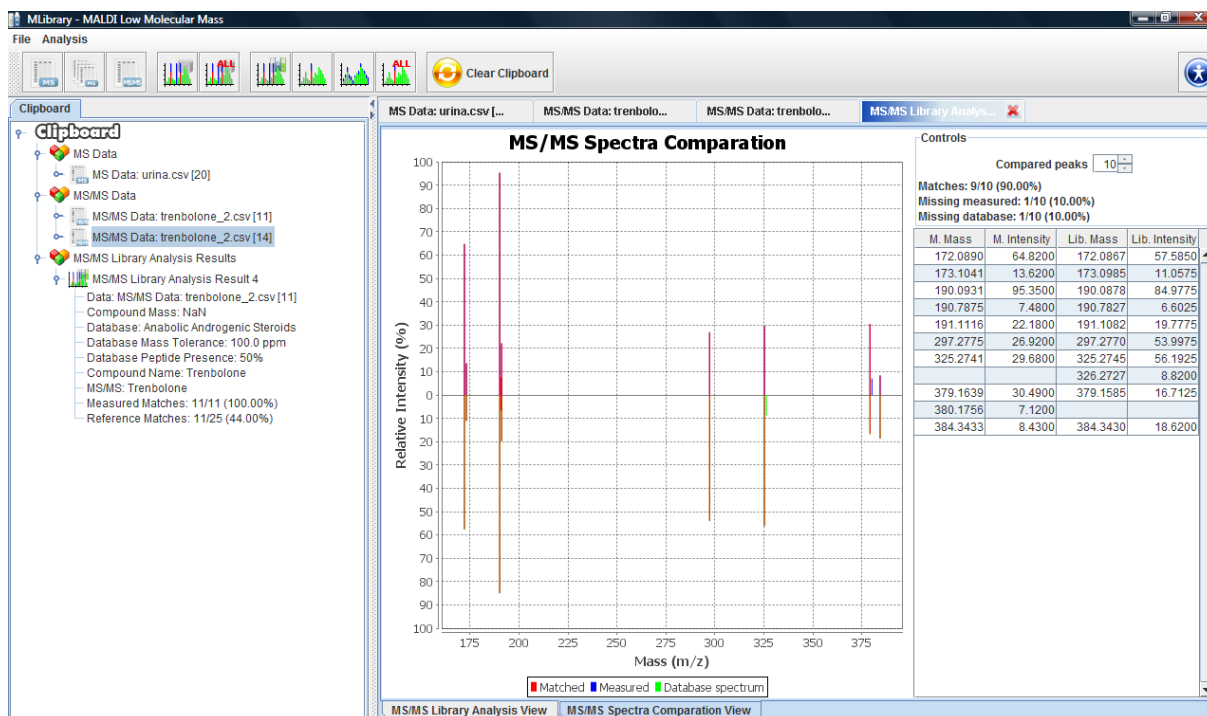
(b)
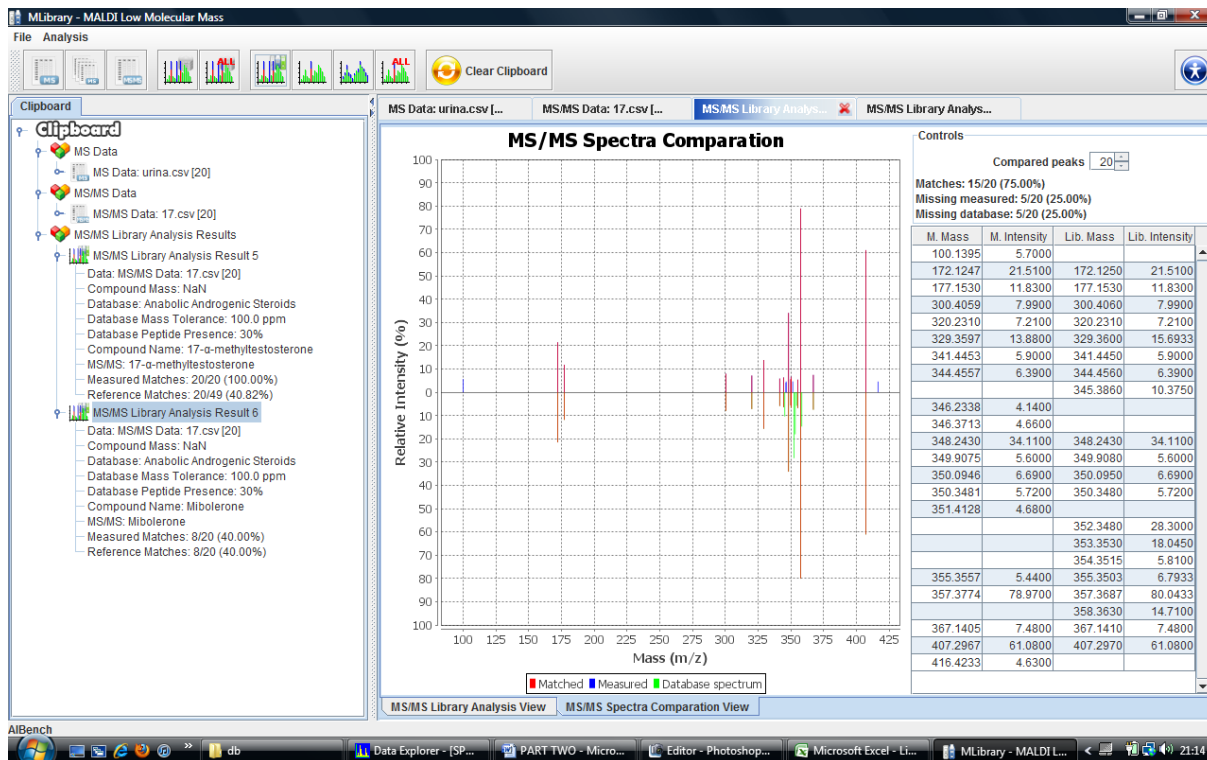
(c)
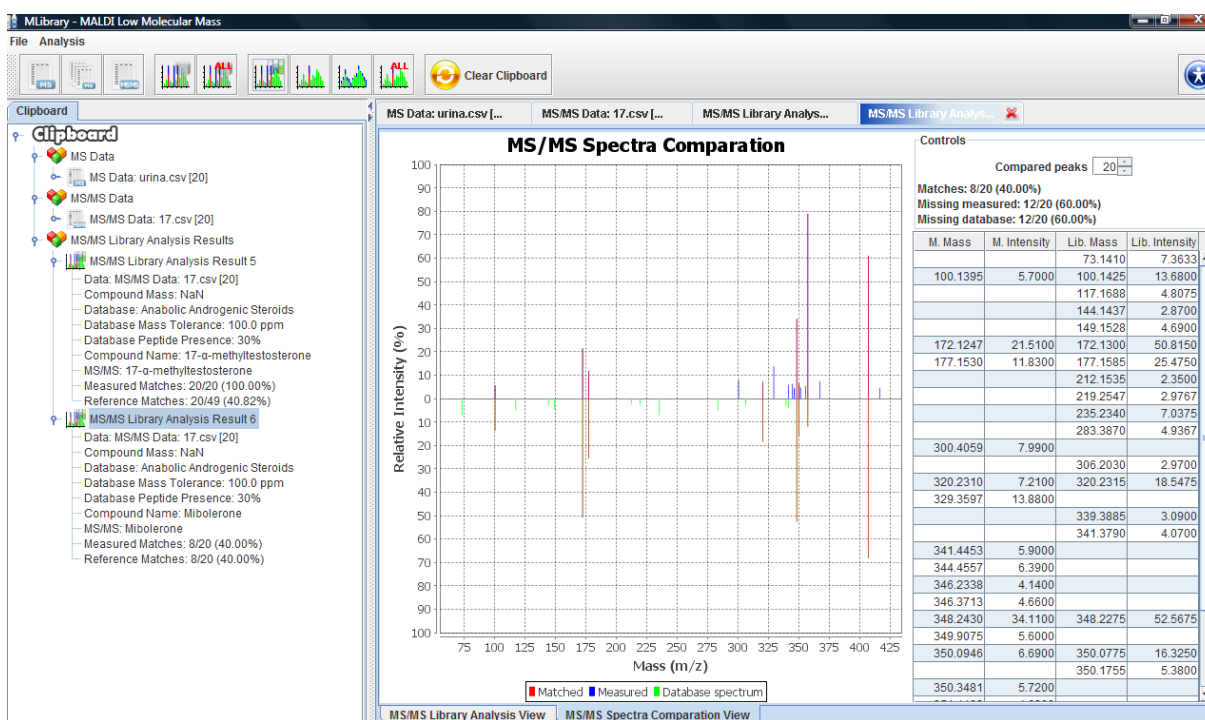
Figure III.11 – MLibrary MS/MS spectrum analysis display window for (a) trenbolone, (b) 17α-methyltestosterone and (c) Mibolerone.

Regarding 17α-methyltestosterone and mibolerone, the MLibrary MS/MS data analysis clearly confirms the presence of 17α-methyltestosterone. Although both 17α-methyltestosterone and mibolerone showed similar fragmentation patterns, MLibrary software retrieves a higher percentage of mass values matching within the 20 more intense peaks; 75% against 40%. Furthermore, all the mass ions present in the MS/MS spectrum matched with MLibrary reference mass values for 17α-methyltestosterone.

III.5 CONCLUSION

We have developed friendly software to help in an automated mode to detect and identify the presence of AAS in urine samples. MLibrary software allows the user to perform robust and accurate screening and confirmation for AAS in both MS and MS/MS mode after matrix assisted laser desorption ionization time of flight mass spectrometry. The software presented here is a very versatile tool that can be simply adapted to future modifications carried on the developed sample treatment procedure, as well as, be easily applied to other compounds. The MLibrary software saves times and it is a simple

tool to work with, by any analyst. Additionally, MLibrary software has a wizard easy to follow for its installation.

The installation wizard is available from the MLibrary web site as an executable file that depends on the final user operating system: Windows, Linux or MAC. By executing the setup file, the installation wizard will be automatically launched. If the user does not have the required Java Runtime Environment (JRE) installed in the computer, the installation wizard will first install this component, and then it will continue with the Mlibrary installation. The user has to simply follow the instructions on the screen to successfully complete the installation.

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PART THREE

IMPROVING SAMPLE TREATMENT

CHAPTER IV

Improved ultrasonic-based sample treatment for the screening of anabolic steroids by gas chromatography / mass spectrometry (GC-MS)

PUBLISHED IN:

IV.1 ABSTRACT

A rapid sample treatment procedure for the gas chromatography/mass spectrometry (GC/MS) determination of anabolic steroids in human urine has been developed. The new procedure makes use of ultrasonic energy to reduce reaction times and increase the overall sensitivity. The following variables affecting the performance of the ultrasonic treatment were optimised: (i) time, (ii) device, (iii) frequency, and (iv) temperature. It was found that, under an ultrasonic field, the hydrolysis of conjugated steroids with β-glucuronidase from *Escherichia coli* K12 was possible with a treatment time of 10 minutes. The accuracy and precision of the ultrasonic method were found to be in agreement with those achieved with the conventional thermal conductivity procedure (Student´s t-test; $p=0.05$, n=10). After the enzymatic hydrolysis, the derivatisation of the target compounds with trimethylsilyl (TMS) reagent, methyl-N-trimethylsilyltrifluoro acetamide (MSTFA)/ NH_4I/ dithioerythritol (DTE) (1000/2/4, v/w/w), was also accelerated using ultrasonic energy. In order to test the applicability of the use of ultrasonic energy in the acceleration of the derivatisation reaction with TMS, the classic method of thermal conductivity was applied for comparative purposes to a pool of 35 androgenic anabolic steroids (AAS) and/or their metabolites. The results demonstrated that after 3 min of sonication in a Sonoreactor device (50% amplitude), 19 of the 35 compounds studied showed similar reaction yield to those obtained with the classic procedure requiring 30 min (Student´s t-test; $p=0.05$, n=5); 13 increased to higher silylation yields; and for the steroids 1-testosterone, danazol and etiocholanolone-D5, the same results were obtained using a sonication time of 5 min.

The overall applicability of the ultrasonic-based sample treatment method is shown by the analysis of five urine samples. The results are similar to those achieved by the routine procedure. The new method is fast, robust, and allows high sample throughput

IV.2 INTRODUCTION

The use of forbidden anabolic steroids and hormones to enhance athletic performance has important health and social implications. Androgenic anabolic steroids (AAS) are a major group included in the prohibited list of the World Anti-doping Agency (WADA) as well as of major sports authorities [1-4].This class of drugs represented 59% of all adverse analytical findings reported by WADA-accredited laboratories, as stated in the WADA statistical report for 2008 [5]. The AAS are a class of hormones that include the natural male sex hormone, testosterone, and its many synthetic derivatives [6-9]. Their use has spread in such a way that many nutritional supplement cocktails contain these substances without adequate indication, thus making the problem even worse [10-13].

Doping control of AAS is based on the detection of these compounds and their metabolites in urine samples from athletes [14]. Most methods for routine detection of anabolic steroids and their metabolites, comprising both screening and confirmatory analysis, are based on gas chromatography/ mass spectrometry (GC/MS) techniques [15-20]. The urinary steroid profile procedure is still based on the profiling method developed in 1983 by Donike *et al.* for the determination of testosterone misuse in sports, which was later extended to other endogenous steroids [21-23]. In the human body, most anabolic steroids are absorbed and then transformed by phase I and phase II metabolic reactions. The formation of AAS glucuronate conjugates is the main metabolic pathway of conjugation and inactivation of these compounds [24-26]. Therefore, for the majority of AAS, the steroid will be excreted in urine as glucuronide conjugates.

To be analysed by GC/MS, the analyte of interest must be both volatile and thermally stable. Since neither free nor conjugated steroids fulfil this requirement, before analysis conjugated steroids must be (i) hydrolysed to produce the free parent compound and (ii) derivatised to provide thermal stability and volatility [27, 28]. Derivatisation is also used to enhance mass spectrometry properties by producing more favourable diagnostic fragmentation patterns. These sample treatment pre-requisites of the GC/MS analyses of AAS are the main drawbacks of the method, since the sample treatment is time-consuming and labour-intensive. Traditionally, the cleavage of the glucuronide moiety to produce the free steroid is performed at 55°C for 1 h with the β-glucuronidase enzyme from *E. coli* that is highly specific to β-glucuronides [29]. After hydrolysis, the incorporation of a trimethylsilyl (TMS) group in the free analyte is by far the most widely employed procedure for AAS derivatisation [30, 31]. However, some problems may arise, since several anabolic steroids have in their structure more than one reaction centre for the TMS group and, for a few of them, the formation of more than one final product can occur, resulting in sensitivity losses and low reproducibility. This work introduces the use of ultrasonic energy for AAS sample treatment prior to their analysis by GC/MS. Several studies have reported the use of high intensity ultrasound to accelerate chemical and enzymatic reactions [32-34]. For β-glucuronidase, Sánchez and co-workers recently reported the use of ultrasonication to enhance the hydrolysis reaction of female steroids from urine [35]. In this preliminary study, they were able to reduce the hydrolysis time to half an hour with β-glucuronidase

from *Helix pomatia* [35]. Recently, some work on the ultrasonic enhancement of estrogens derivatisation was published by Vallejo *et al* [36]. When ultrasonic waves are applied to a liquid, the generation of tiny gas bubbles, called cavitation bubbles, occurs [37]. Once formed, cavitation bubbles absorb the energy from the sound waves and grow until they reach an unstable size and collapse violently. As a result of cavitational bubble implosion, extreme temperatures and pressures are generated. These are thought to release sufficient energy to affect chemical reactions [37, 38]. The main aim of this study was to develop a fast, low-labour intensive, sample treatment for anti-doping control with high throughput, based on the enhancement of chemical reactions produced by ultrasonic energy. Thus, ultrasonic irradiation was applied to the two critical steps of androgenic anabolic steroids sample treatment, i.e., the hydrolysis of the conjugated analyte and the derivatisation reaction with a TMS reagent. The effects of (i) time, (ii) amplitude, (iii) frequency, and (iv) temperature on the performance of the ultrasonic sample treatment were studied and the results compared with those achieved with the conventional sample preparation process.

IV.3 EXPERIMENTAL

IV.3.1 APPARATUS

A model UNIVAPO 100H vacuum concentrator centrifuge from UniEquip (Martinsried, Germany) with a refrigerated aspirator vacuum pump model Unijet II was used for (i) sample drying and (ii) sample pre-concentration. A minicentrifuge, model Spectrafuge-mini, from Labnet (Madrid, Spain), and a minicentrifuge-vortex, model Sky Line, from ELMI (Riga, Latvia), were used throughout the sample treatment. A Simplicity 185 from Millipore (Milan, Italy) was used to obtain Milli-Q water. An model Transsonic TI-H-5 ultrasonic bath from Elma (Singen, Germany), a model UTR200 cup horn sonoreactor (SR), and a model UP 50H (UP) ultrasonic cell disruptor, both from Dr. Hielscher (Teltow, Germany), were used to accelerate the enzymatic hydrolysis and the derivatisation procedure. The enzymatic hydrolysis of conjugated steroids and the subsequent derivatisation reaction were performed in 10mL glass vessels from 3V Chimica (Rome, Italy). Separation of the free steroids after hydrolysis was performed in a 2mL empty reversible solid-phase extraction (SPE) cartridge from Supelco packed with a preparative C_{18} resin (125Å, 55–105μm) from Waters (Barcelona, Spain).

IV.3.2 STANDARDS AND REAGENTS

The standards of 17-α-methyltestosterone (MT), 4-androsten-3,17-dione (AD), boldenone (Bol) and nandrolone (Nan) were purchased from Riedel-de Haën (Seelze, Germany), 17β-hydroxy-17-methylandrosta-1,4-dien-3-one (MA) was from Fluka (Buchs, Switzerland), etiocholan-3α-ol-17-one

(Etio), etiocholan-3α-ol-17-one glucuronide (Etio G) and dehydroisoandrosterone 3-glucuronide (DHEA G) were purchased from Sigma (Steinheim, Germany). The standards testosterone, epitestosterone, androsterone, 5α-androstane-3α17β-diol, 5β-androstane-3α17β-diol, DHEA, 11keto-etiocholanolone, pregnanediol, 11-hydroxy-androsterone, 11-hydroxy-etiocholanolone, 17α-ethynyl-17β-hydroxyandrost-4-eno[2,3-d]isoxazole (danazol), 1α-methyl-5α-androstan-3α-ol -17-one (mesterolone metabolite 1), furazabol and 16β-hydroxyfurazabol (furazabol metabolite 1), 4-chloro-6β,17β-dihydroxy-17α-methyl-1,4-androstadien-3-one (chlormetandienone metabolite 1), 17β-hydroxy-7β,17α-dimethylandrost-4-en-3-one (calusterone), 17β-hydroxy-7α,17α-dimethylandrost-4-en-3-one (bolasterone), 4-chloro-4-androsten-3α-ol-17-one (clostebol metabolite 1), androsta-1,4-dien-17β-ol-3-one (β-boldenone), 9α-fluoro-18-nor-17,17-dimethyl-androst-4,13-dien-11β-ol-3-one (fluoxymesterone metabolite 1), 9α-fluoro-17α-methyl-4-androsten-3α,6β,11β,17β-tetra-ol (fluoxymesterone metabolite 2), 6β-hydroxyfluoxymesterone (fluoxymesterone metabolite 3), 17α-methyl-5β-androstane-3α,17β-diol (17α-methyltestosterone metabolite 2), 17α-hydroxy-17α-methyl-2-oxa-5α-androstan-3-one (epioxandrolone), 17β-hydroxy-17α-methyl-2-oxa-5α-androstan-3-one (oxandrolone), 7α,17α-dimethyl-19-nortestosterone (mibolerone), 17α-hydroxyestran-4,9,11-trien-3-one (epitrenbolone), 17α-ethyl-5β-estrane-3α,17β,21-triol (norethandrolone metabolite 1), 17β-hydroxy-1β-methyl-5α-androst-1-en-3-one (metenolone), 13β,17α-diethyl-3α,17β-dihydroxy-5α-gonane (norboletone metabolite 1), 13β,17α-diethyl-3α,17β-dihydroxy-5β-gonane (norboletone metabolite 2), 3´-hydroxystanozolol (stanozolol metabolite), 17β-hydroxyandrost-1,4-ene-3one (1-testosterone), 17β,4-hydroxyandrost-3-one (4-hydroxytestosterone), 4-hydroxy-19-nortestosterone (oxabolone), 5β-estran-3α-ol-17-one (nandrolone metabolite 2), 5β-androstane-3α17β-diol, 1-methyltestosterone, methyldienolone, 2-hydroxymethyl-17α-methylandrostadiene-11α,17β-diol-3-one (formebolone metabolite 1), formestane, 6β-hydroxy-methandienone (methandienone metabolite 2), 17α-methyl-5βandrostane-3α,17β-diol (methandienone metabolite 3), testosterone D3 and etiocholanolone D5 were purchased from NARL (Pymble, Australia).

Individual stock standard solutions of each anabolic steroid (500 mg/L) were prepared by weighing 0.0125 g of analyte in a 25 mL volumetric flask and making it to volume with methanol. These standard solutions were stored in the dark at -20 ºC. Working standard solutions were prepared by dilution of the stock standard solutions in the appropriate volume of methanol.

A solution of β-glucuronidase from *Escherichia coli* K12 with a specific activity approximately of 140U/mg at 37ºC and pH 7 with nitrophenyl-β-D-glucuronidase as substrate (1 mL contains at least 140U) was purchased from Roche Diagnostic (Mannheim, Germany). Sodium hydrogen phosphate was purchased from Sigma. Methanol was from Scharlau Chemie (Barcelona, Spain) and diethyl ether was from Riedel-de Haën. The derivatisation reagent N-methyl-N-trimethylsilyltrifluoro acetamide (MSTFA) was from Fluka and from Macherey-Nagel (Düren, Germany) via the Italian distributor (Delchimica Scientific, Napoli, Italy). Ammonium iodide (NH$_4$I) and dithioerythritol (DTE) were supplied from Sigma-Aldrich. The derivatisation reagent was a mixture of MSTFA/NH$_4$I/DTE (1000/2/4, v/w/w) that was stored at 4ºC for a maximum of 2 weeks.

IV.3.3 GC/MS INSTRUMENTATION AND OPERATING CONDITIONS

Two different GC-MS systems were used during this study as it was carried out in two different laboratories.

IV.3.3.1 GC/MS SYSTEM AND PARAMETERS USED FOR THE OPTIMISATION STUDY

Gas chromatographic (GC) analyses were carried out on a Trace GC Thermo Finnigan gas chromatograph (Rodano, Italy) equipped with a PolarisQ ITMS ion trap mass spectrometer, interfaced to a PC running the XCalibur 1.4 software, from Thermo Electron Corporation (Waltham, MA, USA). Chromatographic separations were done by using a BPX5 fused-silica capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness) from SGE (Darmstadt, Germany). A PTV injector operating in the splitless mode (3 min) was used for the 1 μL injection volume into a silcosteel liner without packing (120 mm × 2 mm i.d.). The temperature programmed in the PTV injector was 250 ºC. To avoid carry over, after 3 minutes, a split/column ratio equal to 20 mL/min was used. The GC was set to constant pressure of 100 kPa. The oven temperature was programmed as follows: 100 °C; ramped at 17 °C/min to 240 °C; ramped again at 2 °C/min to 300 °C; and finally ramped at 10 °C/min to 320 °C and held for 2 min. The transfer line and the ionization source temperatures were 280 and 250 °C, respectively. Mass detection was performed in full scan mode (*m/z* 50-500) and in single ion monitoring mode (SIM) using specific fragment ions listed in the Table IV.1, with a solvent delay time of 10 min. The compounds were quantified in relation to 17-α-methyltestosterone, which was used as internal standard.

IV.3.3.2 GC/MS SYSTEM AND PARAMETERS USED FOR THE VALIDATION STUDY

Gas chromatographic analyses were carried out on a HP6890 gas chromatograph coupled to a 5973 quadrupole mass spectrometer (Agilent Technologies, Milan, Italy). Chromatographic separations were carried out on a phenyl-methylsilicone column (HP1, 17m×0.2mm i.d., 0.11μm film thickness) from Agilent Technologies. The injection was made in split mode with a split ratio of 1:10 and the injection volume was 1 μL. Helium was used as the carrier gas at a constant pressure of 75 kPa. The injection port was set at 280°C. The oven temperature was programmed as follows: initial temperature 188°C, held for 2.5 min; ramped at 3°C/min to 211°C and held for 2 min; ramped again at 10°C/min to 238°C; and finally ramped at 40°C/min to 320°C and held for 3 min. The transfer line and the ionisation source temperatures were 250 and 230°C, respectively. Mass detection was performed in

SIM mode using specific fragment ions (see Table IV.1), with a solvent delay time of 2.6 min. The quantification of all AASs was carried out using the ''diagnostic'' ions shown in the first column of Table IV.1 for each compound, whereas the ''diagnostic'' ions in the second column were used to confirm the identity of the substance. Quantitative measurement of AASs was performed by response factor calibration (RFC). The urinary concentrations of the exogenous AAS were calculated using the ratio of the areas of the target ions and of 17α-methyltestosterone, which was used as the internal standard (ISTD). The concentrations of the endogenous AAS were determined in relation to the respective deuterated standards.

IV.3.4 URINE SAMPLES

Urine samples were collected in clean plastic disposable containers and kept at –20°C until analysis. Urine samples used in this work were obtained from healthy volunteers of our research team.

IV.3.5 SAMPLE PREPARATION

IV.3.5.1 URINE HYDROLYSIS PROCEDURE

Urine samples (2 mL) were hydrolysed with 50 µL of the commercial solution of β-glucuronidase from *E. coli*, after the addition of 0.750mL of phosphate buffer (0.8M, pH 7). The hydrolysis was performed using three different systems as follows: (a) conventional heating at 55°C for 60 min; (b) using ultrasonic energy provided by a cup horn sonoreactor; and (c) using ultrasonic energy provided by an ultrasonic probe, both operating at 60% of sonication amplitude. These amplitudes were chosen based on our previous experience with ultrasonic devices [38]. We emphasise that the UP is capable of delivering ultrasonication intensity higher than the SR. In contrast, the SR allows indirect sonication (there is no need to introduce a probe inside the sample). Consequently, higher sonication times were used with the SR (10 min vs. 5 min for SR and UP, respectively). The differences in performance between those devices have been recently reviewed [39].

IV.3.5.2 SPE CLEAN-UP OF URINE SAMPLES

The urine clean-up procedure was performed with a C_{18} SPE cartridge, conditioned before use with 5mL of methanol and 10mL of water. The urine samples were loaded onto the preconditioned cartridge and the cartridge was then washed with 5mL of a methanol/water mixture (1:9, v/v). The target compounds were eluted with 2mL of a methanol/diethyl ether mixture (1:9, v/v).

Table IV.1 - Diagnostic ions (*m/z*) used in SIM mode for AAS detection.

AAS compounds	Derivative	Diagnostic ions (*m/z*)	
		Quantification ion	*Qualification ion*
Androsterone	bis-O-TMS	419	434
Bolasterone	bis-O-TMS	445	315
Boldenone β	bis-O-TMS	206	430
Calusterone	bis-O-TMS	445	315
Chlormetandienone m1	bis-O-TMS	143	315
Clostebol m1	bis-O-TMS	451	453
Danazol	bis-O-TMS	481	452
DHEA	bis-O-TMS	237	327
Epioxandrolone	mono-O-TMS	143	308
Epitestosterone	bis-O-TMS	432	417
Epitrenbolone	bis-O-TMS	307	322
Etiocholanolone	bis-O-TMS	419	434
Etiocholanolone D5	bis-O-TMS	439	424
Fluoxymesterone m1	bis-O-TMS	462	208
Fluoxymesterone m2	tetra-O-TMS	143	462
Fluoxymesterone m3	tetra-O-TMS	640	605
Formebolone m1	tri-O-TMS	367	222
Formestane	tri-O-TMS	518	503
Furazabol	bis-O-TMS	143	387
Furazabol m	bis-O-TMS	218	490
Metandienome m2	tri-O-TMS	517	532
Metandienome m3	bis-O-TMS	206	444
Metenolone	bis-O-TMS	195	446
Methyldienolone	bis-O-TMS	430	415
Methyltestosterone m2	bis-O-TMS	143	255
Mesterolone m1	bis-O-TMS	433	448
Mibolerone	bis-O-TMS	301	446
Nandrolone m2	bis-O-TMS	405	420
Norboletone m1	bis-O-TMS	157	435
Norboletone m2	bis-O-TMS	157	435
Norethandrolone m1	bis-O-TMS	157	331
Oxabolone	bis-O-TMS	506	491
Oxandrolone	mono-O-TMS	143	308
Pregnanediol	bis-O-TMS	117	269
Stanozolol m1	N-TMS, bis-O-TMS	254	545
Testosterone	bis-O-TMS	432	417
Testosterone D3	bis-O-TMS	435	420
1-methyltestosterone	bis-O-TMS	446	431
1-Testosterone	bis-O-TMS	194	179
4-hydroxy-testosterone	tri-O-TMS	520	505
5αAndrostane-3α17β-diol	bis-O-TMS	241	256
5βAndrostane-3α17β-diol	bis-O-TMS	241	256
11-hydroxy-etiocholanolone	bis-O-TMS	522	417
11-hydroxy-androsterone	bis-O-TMS	522	417
11Keto-Etiocholanolone	bis-O-TMS	505	415
17α-methyltestosterone	bis-O-TMS	301	446

IV.3.5.3 DERIVATISATION PROCEDURE

The procedure for derivatisation of anabolic steroids with the TMS derivatisation reagent was as follows: The collected eluate sample was dried under a gentle nitrogen stream at room temperature. After the addition of 50 µL of a mixture of MSTFA/NH$_4$I/DTE (1000:2:4, v/w/w), the vial was closed and the derivatisation reaction was then performed by means of four different systems: the conventional heating procedure at 60°C for 1 h; the heating process used in the WADA-accredited Laboratorio Antidoping di Roma at 78°C for 30 min; or using ultrasonic energy provided by a SR operating at 50% of sonication amplitude or the common ultrasonic bath device operating at two different frequencies (35 and 130 kHz) and 100% amplitude. Note: the SR can deliver an intensity of ultrasonication 50 times higher than the ultrasonic bath. Hence, higher sonication times were selected for the ultrasonic bath (15 min for the ultrasonic bath vs. 3 min for the SR).

IV.3.6 EXPERIMENTAL DESIGN

IV.3.6.1 OPTIMISATION PROCEDURE

The optimisation procedure was carried out at the Science and Technology Faculty-UNL, Monte de Caparica, Portugal, using the standards available in our laboratory.

For the enzymatic hydrolysis optimisation study, two target steroids, DHEA and etiocholanolone glucuronates, were used. The experimental procedure was followed as described in the sample preparation section but, instead of urine, spiked water with 50ng/mL of each steroid was used to compare the ultrasonic-based sample treatment with the conventional thermal conductivity procedure. It is important to stress that, after the enzymatic hydrolysis, the derivatisation procedure was carried out by the conventional method for both hydrolysis procedures.

The optimisation of the TMS derivatisation study was performed with four target steroids, nandrolone, 4-androsten-3,17-dione, boldenone and 17β-hydroxy-17-methylandrosta-1,4-dien-3-one. The derivatisation procedure described in the sample preparation section was applied to 0.5mL of a methanolic solution with the four target compounds at a concentration of 50 ng/mL.

The steroid 17α-methyltestosterone was used as internal standard (ISTD) for both experimental studies. It is important to stress that for the derivatisation study the ISTD was derivatised by the conventional procedure and added to all samples at the end of both the accelerated and the conventional method. The final concentration of the ISTD in each sample was 200 ng/mL.

IV.3.6.2 ASSESSMENT OF THE ULTRASONIC PROCEDURE

The assessment of the ultrasonic procedure was carried out at the Laboratorio Antidoping di Roma in Italy. To assess the ultrasonic performance in the hydrolysis step, urine samples from healthy volunteers were used. The natural endogenous steroids were used to assess the method. Deuterated AAS were used as internal standards. Once the hydrolysis was complete, the derivatisation procedure was always carried out by the conventional protocol.

To assess the TMS derivatisation procedure the ultrasonic based treatment was applied to a methanolic solution with several AAS and compared with the routine method. The steroid 17α-methyltestosterone was used as the ISTD. The final concentration of the ISTD in each sample was 200 ng/mL.

IV.3.6.3 APPLICATION TO REAL SAMPLES

Five human urine samples were used to verify that the new procedure was as effective as the routine one. The urine samples were spiked with 50mL of a standard solution with several "free" exogenous AAS. The concentration in urine of the spiked AAS ranged from 5 to 375 ng/mL. The natural conjugated endogenous AAS of each urine sample were also analyzed in this experiment. Each sample was prepared in triplicate. Both the hydrolysis and the derivatisation step were accelerated using the ultrasonic protocol and compared with the routine procedure.

IV.3.7 STATISTICAL ANALYSIS

In order to test if the difference between the ultrasonic method and the conventional method was significant, the t-test was calculated using a statistical equation that allowed the comparison of the two experimental methods [40].

IV.4 RESULTS AND DISCUSSION

The conventional sample treatment commonly used for the quantification of steroids in urine samples is depicted in Figure IV.1. Here the two key steps of the sample procedure are in evidence. The first step entails the enzymatic hydrolysis process, where the glucoronide conjugate is transformed into the free parent compound. This reaction takes place at 55°C for 1 h. Once the free steroids have been extracted and pre-concentrated through evaporation to dryness, in the fifth step, they are derivatised by

adding MSTFA. The reaction is usually enhanced by heating at 60°C for 1 h. The aforementioned steps of the sample handling were the objectives of our study, in which we focused on achieving a higher yield of reaction in a shorter time by applying ultrasonication.
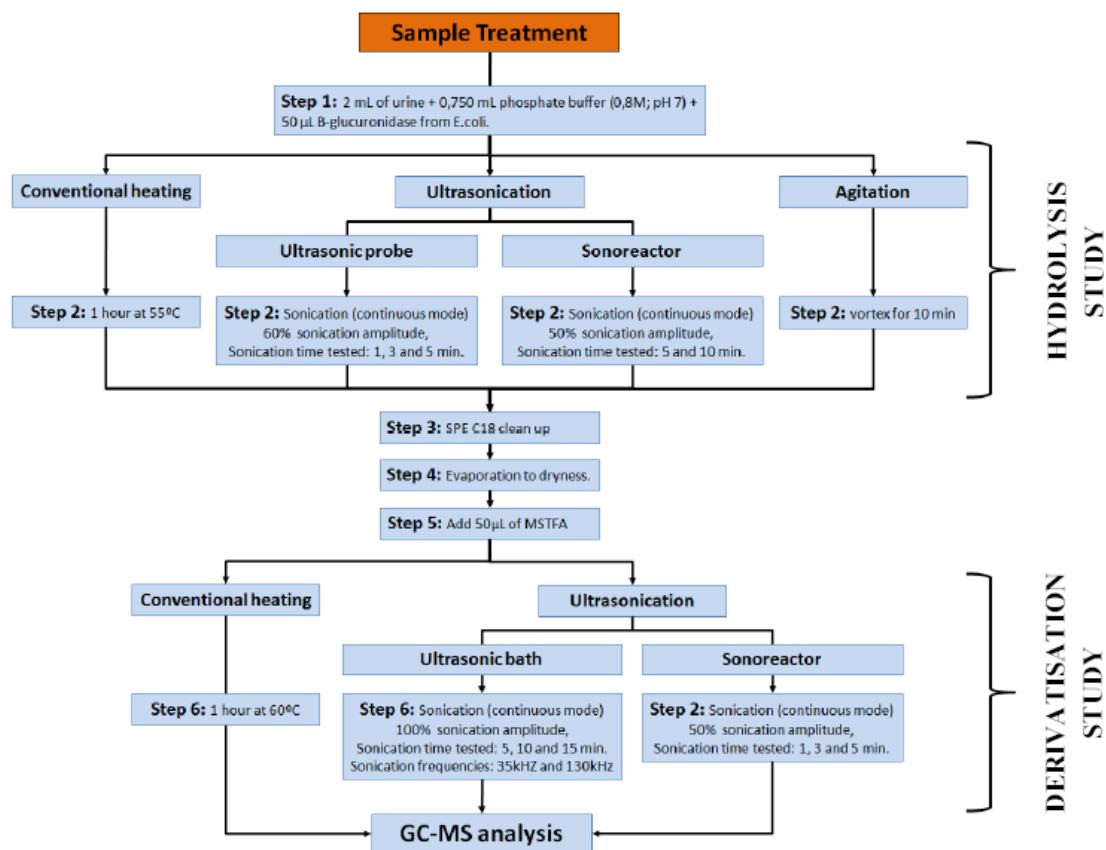
Figure IV.1 – Comprehensive scheme of the classic and the ultrasonic protocols used in the optimization study for the determination of androgenic anabolic steroids.

IV.4.1 EFFECT OF ULTRASSONICATION IN THE ENHANCEMENT OF THE ENZYMATIC HYDROLYSIS

To assess the performance of ultrasonication in the sample treatment, the first set of experiments was carried out maintaining all the sample handling steps described in Figure IV.1, unaltered except for the second step, in which the enzymatic reaction takes place. Two target steroids, DHEA and etiocholanolone glucuronates, were used in this preliminary study. For the optimisation experiments these compounds were spiked into water blanks. Ultrasonication can be applied in the analytical laboratory using different devices [39]. It must be emphasised that the intensity of sonication provided by each device is different and is in the order: UP>SR>ultrasonic bath. Literature on the acceleration

of enzymatic reactions using ultrasonication reveals that only the SR and the UP can efficiently boost the enzymatic reactions in short application times of generally<10 min [41]. For this reason, only the UP and the SR were used in this study.

IV.4.1.1 ULTRASONIC PROBE

As a general rule, two variables affect the performance of ultrasonication, amplitude and time. Several warnings must be heeded concerning the UP. First, high sonication amplitudes leading to high intensities of ultrasonication should be avoided, since ultrasonication can boost but can also inactivate enzymes [42]. For the same reason long application times must be avoided. Second, the form of the vessel must be conical, to avoid so-called "dead zones", zones where the ultrasonic field produced by the probe is without effect [32, 37]. The conical form maintains the distance between the horn tip and the wall container as short as possible, ensuring optimal ultrasonic performance. Based on our experience gained in working with ultrasound and enzymes, we decided to set the ultrasonication amplitude to a medium level 60% and set the ultrasonication time to 1, 3 or 5 min [42]. The results of this set of experiments are shown in Figure IV.2. Clearly, the yields of hydrolysis increase as the ultrasonication time increases, especially for the etiocholanolone steroid. Five minutes of ultrasonication was sufficient to enhance the enzymatic reaction to yields comparable with those obtained with the conventional method of heating at 55°C during 1 h.
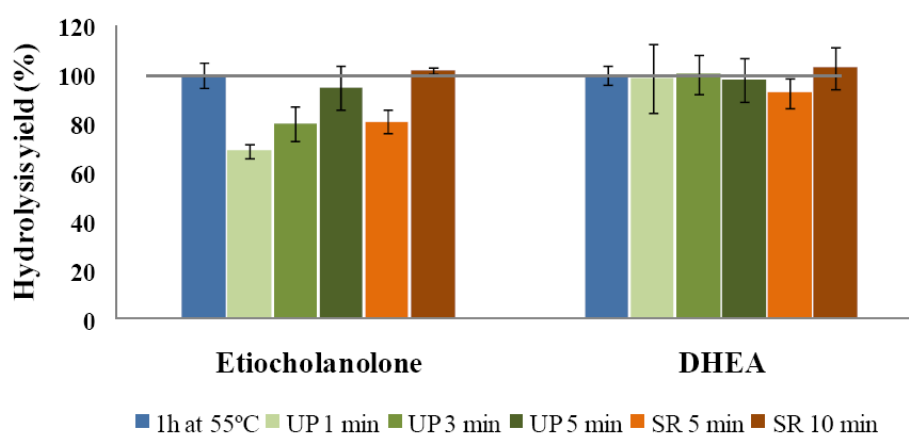
Figure IV.2 – Effect of the ultrasonication on the hydrolysis yield of dehydrolisoandrosterone 3-glucuronide (DHEA) and etiocholanolone glucuronide using two devices, an ultrasonic probe (UP) at 60 % of ultrasonic amplitude and a sonoreactor (SR) at 50% of ultrasonic amplitude (n=5). The results are given in percentages (100% = result via the classic method).

IV.4.1.2 SONOREACTOR

In practical terms, the sonoreactor can be considered as a powerful mini-ultrasonic bath that can deliver up to 50 times more energy than a normal ultrasonic bath [38]. The ultrasonication amplitude selected in this set of experiments was 50%, but the applications times were increased as the SR is 50 times less powerful than the UP. The results are presented in Figure IV.2, where they may be compared with the results obtained with the UP and with the classical heating procedure. As was expected, to obtain yields of hydrolysis equivalent to the heating method, the ultrasonication time required was slightly higher than when the UP was used: 10 min vs. 5 min. Nevertheless, the results obtained were consistent, showing relative standard deviations (RSDs) below 9% and yields higher than 95%. The total time in this step was reduced six-fold. It must be stressed that, when a solution is ultrasonicated, a slow but constant increase in the bulk temperature occurs and, depending on the solvent used, the physical characteristics of the liquid media may change with time, reducing the effects resulting from cavitational collapse. For this reason the water in the SR bath was changed after 5 min of ultrasonication. Moreover, to confirm that the increase of the hydrolysis reaction rate was due to the ultrasonication and not to sample heating, a set of experiments was devised in which the hydrolysis was carried out on etiocholanolone for 10 min at 37°C, which was around the temperature reached in the samples after ultrasonication. The hydrolysis yield obtained was $36 \pm 2\%$ lower (n=5) that achieved with ultrasonication.

IV.4.1.3 APPLICATION TO FURTHER STEROIDS

Since the hydrolysis reaction yield is influenced by the type of steroid, it is important to apply optimised ultrasonic conditions to as many compounds as possible to ensure that, independently of the steroid structure, at least, similar hydrolysis yield to the thermal conductivity procedure is achieved. For this study, only the SR device was used to avoid the risk of contamination that arises from the use of UPs. It is important to stress that for this experimental set, instead of spiked water, real urine samples were used.

The results obtained are shown in Table IV.2. It is demonstrated that, except for the endogenous 11β-hydroxyandrosterone and 11β-hydroxyetiocholanolone, the efficiency of hydrolysis using the ultrasonic method was similar to that in the conventional method. Statistically, the results obtained for the endogenous 5β-androstane-3α,17β-diol (95±3) and 11-ketoetiocholanolone (109±9) are not comparable with those achieved by the standard method, taking into account the confidence test used. Further experiments were carried out with the SR at higher amplitudes and increasing the time to check whether this result could be improved. It was found that at 60% intensity there was a ca. 10% improvement of yield for both for the 11β-hydroxy steroids, and for 5β-androstane-3α,17β-diol, and the result became comparable with that of the standard method (97±5; |tcal|<2.11). For amplitudes

higher than 60% the yields obtained decreased rapidly, probably due to the inactivation of the enzyme caused by the ultrasonic energy. When the time applied was increased from 10 to 15 min, at 60% amplitude, the recovery yield of these two compounds was >95%. For 11β-hydroxyandrosterone and 11β-hydroxyetiocholanolone, the hydroxy group at the C_{11} position may play a role in the hydrolysis efficiency, since it is the only structural difference between these and the other steroids. However, the information from these compounds is not significant in the endogenous profile of the athlete and for further experiments the time was fixed at 10 min.

A parallel set of hydrolysis experiments of the target conjugated compounds was carried out to test if 10 min at 55°C was sufficient time to obtain similar results to those obtained with the SR. As expected, in general, 10 min at 55°C was insufficient to achieve the same hydrolysis yield. Only for testosterone, epitestosterone and DHEA, 10 min at 55°C was enough to hydrolyse the steroid glucuronides to their respective free compounds, with 100% yield (data not shown). For the other glucuronides, the hydrolysis yield was very low, ranging from 50 to 75% for etiocholanolone, androsterone and 11-ketoetiocholanolone, and <35% for 11β-hydroxyandrosterone and 11β-hydroxyetiocholanolone (data not shown). Furthermore, a set of experiments was conducted to assess the single effect of ultrasonication (without enzyme) in the hydrolysis of the conjugated AAS. The overall procedure described in the sample preparation section was applied to two experimental sets of the same urine sample (without enzymatic hydrolysis). One of the samples was submitted to ultrasonication before the SPE procedure. The results were similar for all steroids in both experimental sets, meaning that only the 'free' AAS were detected and that the ultrasonic energy by itself is not sufficient to hydrolyse the conjugated AAS.

Table IV.2- Hydrolysis yields (US procedure/ T conductivity×100) of AAS glucuronides from urine. Comparison of the thermal conductivity procedure (55ºC; 1 hour) and the ultrasonic procedure (sonoreactor at 50% of amplitude, 10 min).

AAS glucuronides	Conventional Procedure (ng/mL; n=9)	Ultrasonic Method (ng/mL; n=10)	Reaction yield (US method / Conventional method ×100, X±SD)	$|t_{cal}|$*
Androsterone	2722 ± 74	2687 ± 80	99 ± 3	0.99
DHEA	25 ± 1	25 ± 1	99 ± 5	0.47
Etiocholanolone	2548 ± 92	2509 ± 100	98 ± 4	0.88
Epitestosterone	72 ± 1	72 ± 2	100 ± 2	0.24
Pregnanediol	424 ± 42	431 ± 31	102 ± 7	0.43
Testosterone	19 ± 1	19 ± 1	100 ± 2	0.24
5αAndrostane-3α17β-diol	66 ± 1	67 ± 1	102 ± 2	1.87
5βAndrostane-3α17β-diol	228 ± 11	216 ± 8	95 ± 3	2.83
11Ketoetiocholanolone	1213 ± 101	1325 ± 107	109 ± 9	2.35
11Hydroxyandrosterone	853 ± 37	682 ± 61	80 ± 7	7.25
11Hydroxyetiocholanolone	173 ± 9	129 ± 10	75 ± 6	9.64

n = number of technical replicates

*t_{cal} is a calculated t-test for the comparison of two experimental means

$t_{theoretic} = 2.11$; $P = 0.05$

IV.4.2 EFFECT OF ULTRASSONICATION IN THE ENHANCEMENT OF THE DERIVATISATION PROCEDURE

Among the several derivatisation reactions described in the literature, the production of TMS derivatives is by far the most widely employed due to its simplicity, high reaction yields and robustness under controlled conditions (e.g. low moisture). To investigate the effect of ultrasonic energy on the TMS derivatisation of anabolic steroids, we choose the ultrasonic bath and the SR. Two main reasons drove this selection. On one hand, the low sample volume to be ultrasonicated made the use of an UP useless. The minimum amount of sample that can be sonicated with an UP is as low as 10 µL. However, work with low volumes and probes requires constant attention by the operator since it must be carried out manually. As an example, the aerosol formation resulting from cavitation spreads out the sample towards the wall of the container, making it necessary to stop the treatment and centrifuge the container to allow the sample to settle down. Therefore, the automation of ultrasonication with low volumes (ca. 50µL) is difficult. In addition, the TMS reagent is a very strong silylating agent and it may cause health problems when inhaled. Treatments involving a probe are carried out in open vessels although they can be closed when the ultrasonic bath or the SR are used.

Despite the low intensity provided by the ultrasonic bath, it was used in this set of experiments mainly because similar work has been published on the synergic effect of ultrasonication and temperature to enhance specific chemical reactions [43].

IV.4.2.1 ULTRASONIC BATH

In the ultrasonic bath the ultrasonic amplitude was set to 100% to maintain the ultrasonication intensity as high as possible. The ultrasonication times tested were 5, 10 and 15 min. The frequency of ultrasonication was also studied by selecting the options of 35 or 130 kHz. In addition, the combined effect of temperature and ultrasonication on the derivatisation procedure was also evaluated. Two sets of experiments were developed in parallel, and for comparative purposes the conventional heating process at 60°C for1 h was also used.

IV.4.2.1.1 EFFECT OF ULTRASONICATION COMBINED WITH TEMPERATURE

As may be seen in Figure IV.3, when the ultrasonic bath is used, the combination of heating and ultrasonication is critical to obtain results comparable with those from conventional heating at 60°C for 1 h. This is especially true for nandrolone (Nan), 4-androsten-3,17-dione (AD) and boldenone (Bol). The highest yields of derivatisation were obtained after 15 min of ultrasonication. However, for MA the best result obtained (130 kHz, 60°C, 15 min) was only 85% of the derivatisation yield obtained with the conventional treatment.

IV.4.2.1.2 EFFECT OF FREQUENCY

Without warming in combination with ultrasonication, the best results were obtained after 15 min of ultrasonication at a frequency of 35 kHz. However, when the ultrasonic bath was thermostated at 60°C the best results were obtained after 15 min of ultrasonication at 130 kHz. This was especially true for MA, for which the derivatisation yield achieved was closer to that by the conventional method (60°C; 1 h).

We hypothesise that a synergic effect between temperature and frequency prevails in this step. Therefore, we conclude that the combination of ultrasonication and warming performs better at an ultrasonic frequency of 130 kHz.

IV.4.2.2 SONOREACTOR

The starting ultrasonication amplitude chosen was 50% and the sonication times tested were 1, 3 and 5 minutes. Remarkably, the derivatisation process was complete with the SR in 1 min with the same derivatisation yield as with the conventional process. This surprising result, with similar accuracy and precision, was repeated for the four target steroids used in the optimisation process. Obviously, the higher cavitation efficiency of the SR than the UB plays an important role in the acceleration of the process, thus showing the effectiveness of ultrasonic energy in enhancing the reaction.
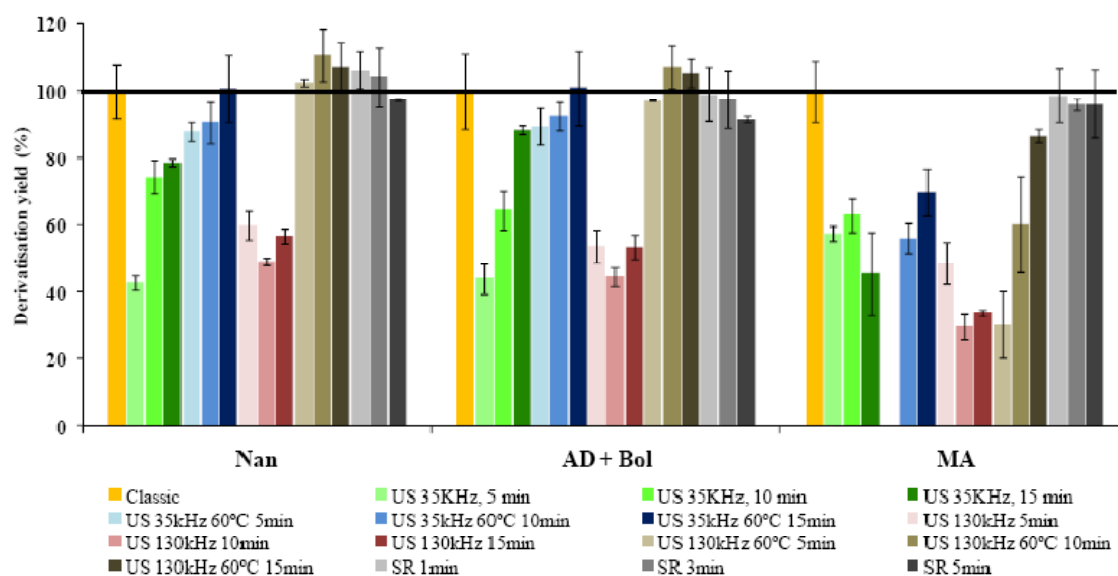
Figure IV.3 – Effect of the ultrasonication in the derivatisation yield of nandrolone (Nan), 4-androstes-3,17-dione (AD), boldenone (Bol) and 17β-hydroxy-17-methylandrosta-1,4-dien-3-one (MA), by means of two devices, a sonoreactor (SR) at 50% of ultrasonic amplitude and an ultrasonic bath (US) at 100 % of ultrasonic amplitude with two frequencies, 35kHz and 130kHz (n=5). The classic treatment consists of heating at 60°C for 1h. The results are given in percentages (100% = result via the classic method).

IV.4.2.3 APPLICATION TO FURTHER STEROIDS

As carried out for the hydrolysis step, the new treatment was employed on a large number of other steroids. This was carried out in the WADA-accredited Laboratorio *Antidoping di Roma*, Italy. In addition, and for comparative purposes, the heating process used in this step by the laboratory in Rome, 78°C for 30 min, was also employed on a pool of 35 steroids. The results demonstrated that,

although 1 min of sonication time was enough to achieve the same yields or higher than those obtained at 60°C for 1 h, it was not enough to reach the yields achieved at 78°C for five of the 35 steroids studied, namely, 1-testosterone (68±9%), 1-methyltestosterone (78±6%), b-boldenone (67±5%) and the two metabolites of methandienone, methandienone met. 2 (73±5%) and methandienone met. 3 (69±7%). Therefore, further experiments were carried out in which the thermal conductivity method at 78°C for 30 min was compared with the ultrasonication protocol using the SR at 50% amplitude for times of 3 and 5 min. As can be seen in Table IV.3, the derivatisation yields, after 3 min of ultrasonication, are excellent and comparable, in terms of precision and accuracy, with those obtained by the heating method at 78°C. The results show that of the 35 compounds studied 16 are not within the confidence test used, as for at least 13 of the steroids studied, an improvement of the reaction yield is clearly achieved (increments of about 10–48%; |tcal|>2.11). By increasing the sonication time to 5 min, for steroid 1- testosterone (<90%; |tcal|>2.11), similar results to those from the conventional procedure were obtained. However, for the other compounds the yields were not significantly improved by increasing the ultrasonication time from 3 to 5 min.

IV.4.3 ANALYTICAL APPLICATION

In order to study the reliability of the method, the ultrasonic accelerated procedure was applied to five control urine samples from healthy young people. The urine samples were spiked with banned androgenic anabolic steroids. Both the hydrolysis and the derivatisation step were accelerated using the ultrasonic protocol (hydrolysis accelerated with a SR operating at 60% amplitude for 10 min and derivatisation accelerated with a SR operating at 50% amplitude for 3 min) and the results compared with those for the same control sample processed by the routine procedure (hydrolysis at 55°C for 1 h and derivatisation at 78°C for 30 min). It is important to stress that the number of individual steroids spiked in the urine samples was reduced for the volunteers 2, 3, 4 and 5 to simplify the data analysis. The results are shown in Table 4 and as can be seen that they are similar for both sample treatment procedures. Furthermore, the precision and accuracy of the ultrasonic procedure indicate that the method shows sufficient robustness for quantitative analysis under routine conditions.

Table IV.3 - Derivatisation yield (US procedure/ T conductivity×100) of AAS with MSTFA. Comparison of the thermal conductivity procedure (78ºC; 30 min) and the ultrasonic procedure (sonoreactor at 50% of amplitude, 3min).

AAS glucuronides	Conventional Procedure (ng/mL; n=9)	Ultrasonic Method (ng/mL; n=10)	Reaction yield (US method / Conventional method ×100, X±SD)	$\|t_{cal}\|^*$
Bolasterone	299 ± 16	322 ± 11	107 ± 4	2.57
Boldenone β	299 ± 9	312 ± 20	105 ± 7	1.40
Calusterone	299 ± 15	307 ± 11	103 ± 4	0.94
Chlormetandienone m1	900 ± 61	869 ± 76	97 ± 8	0.70
Clostebol m1	296 ± 15	278 ± 14	94 ± 5	1.94
Danazol	899 ± 18	814 ± 55	91 ± 6	3.25
Epioxandrolone	903 ± 70	1120 ± 76	124 ± 8	4.70
Epitrenbolone	600 ± 54	703 ± 65	117 ± 11	2.74
Etiocholanolone D5	201 ± 10	184 ± 8	91 ± 4	3.07
Fluoxymesterone m1	303 ± 6	344 ± 20	113 ± 6	4.42
Fluoxymesterone m2	304 ± 21	410 ± 31	135 ± 10	6.28
Fluoxymesterone m3	301 ± 25	444 ± 40	148 ± 13	6.84
Formebolone m1	504 ± 44	456 ± 28	90 ± 6	2.08
Formestane	101 ± 8	107 ± 10	106 ± 10	1.12
Furazabol	396 ± 32	473 ± 41	119 ± 10	3.31
Furazabol m	602 ± 45	702 ± 72	117 ± 12	2.62
Metandienome m2	302 ± 12	345 ± 31	114 ± 10	2.93
Metandienome m3	301 ± 4	317 ± 36	106 ± 12	1.02
Metenolone	299 ± 16	287 ± 21	96 ± 7	1.01
Methyldienolone	896 ± 71	945 ± 79	105 ± 9	1.03
Methyltestosterone m2	304 ± 26	301 ± 17	99 ± 6	0.21
Mesterolone m1	303 ± 27	288 ± 9	95 ± 3	1.19
Mibolerone	297 ± 21	296 ± 11	100 ± 4	0.14
Nandrolone m2	150 ± 10	150 ± 4	100 ± 3	0.07
Norboletone m1	297 ± 15	324 ± 13	109 ± 4	3.05
Norboletone m2	150 ± 4	159 ± 7	106 ± 4	2.47
Norethandrolone m1	298 ± 17	299 ± 12	100 ± 4	0.08
Oxabolone	448 ± 34	522 ± 29	117 ± 7	3.67
Oxandrolone	1498 ± 60	1924 ± 143	128 ± 10	6.15
Stanozolol m1	181 ± 16	185 ± 15	102 ± 9	0.39
Testosterone D3	398 ± 31	443 ± 72	111 ± 18	1.28
1-methyltestosterone	597 ± 34	556 ± 57	93 ± 10	1.37
1-Testosterone	602 ± 33	515 ± 61	85 ± 10	2.81
4-hydroxy-testosterone	597 ± 30	600 ± 24	100 ± 4	0.15
5βAndrostane-3α17β-diol	202 ± 12	210 ± 14	104 ± 7	0.85

n = number of technical replicates

$*t_{cal}$ is a calculated t-test for the comparison of two experimental means;

$t_{theoretic}$ = 2.31; P = 0.05

Table IV.4 - Comparison of the classic and the ultrasonic procedures. The recoveries are calculated as the ratio yield between the ultrasonic method and the classic method and are expressed as percentages.

AAS	Yield (US method / classic method ×100, X±SD); |t$_{cal}$|									
	Volunteer 1 (male)		Volunteer 2 (male)		Volunteer 3 (male)		Volunteer 4 (male)		Volunteer 5 (female)	
Androsterone G	87 ± 5	2.30	104 ± 9	0.49	112 ± 8	1.77	108 ± 4	2.12	94 ± 3	1.29
Bolasterone	96 ± 3	1.13	94 ± 10	0.84	108 ± 6	1.99	104 ± 3	1.34	98 ± 4	0.42
Calusterone	94 ± 6	1.98	106 ± 5	0.79	114 ± 9	2.29	103 ± 2	1.60	95 ± 4	1.04
Clostebol m1	94 ± 4	1.77	113 ± 12	1.71	107 ± 9	1.02	99 ± 2	0.46	96 ± 3	0.86
Danazol	-	-	108 ± 8	1.33	110 ± 9	2.08	93 ± 1	1.44	93 ± 9	1.16
DHEA G	95 ± 1	2.16	100 ± 8	< 0.1	92 ± 6	1.90	102 ± 5	0.70	91 ± 6	2.01
Epixandrolone	95 ± 6	1.65	-	-	-	-	-	-	-	-
Epitrenbolone	149 ± 8	6.13	-	-	-	-	-	-	-	-
Epitestosterone G	100 ± 2	0.02	116 ± 12	2.29	96 ± 10	0.66	113 ± 7	2.25	95 ± 7	0.92
Etiocholanolone G	97 ± 1	1.72	88 ± 10	1.44	111 ± 9	2.20	97 ± 5	0.85	99 ± 4	0.21
Etiocholanolone D5	104 ± 2	2.22	-	-	-	-	-	-	-	-
Fluoxymesterone m1	96 ± 11	0.72	110 ± 6	2.24	108 ± 11	1.36	106 ± 10	1.34	98 ± 1	0.44
Methyldienolone	95 ± 6	1.37	-	-	-	-	-	-	-	-
Methyltestosterone m2	97 ± 2	1.34	91 ± 9	1.01	113 ± 8	2.27	109 ± 5	1.80	95 ± 3	0.86
Mesterolone m2	99 ± 5	0.40	-	-	-	-	-	-	-	-
Mibolerone	95 ± 7	0.71	105 ± 3	1.10	105 ± 5	1.22	106 ± 2	2.13	100 ± 5	< 0.1
Nandrolone m2	105 ± 2	2.18	-	-	-	-	-	-	-	-
Norboletone m1	96 ± 1	1.75	-	-	-	-	-	-	-	-
Noboletone m2	115 ± 11	2.28	-	-	-	-	-	-	-	-

Table IV.4 (continuation)

AAS	**Yield** (US method / classic method ×100, X±SD); $|t_{cal}|$									
	Volunteer 1 (male)		Volunteer 2 (male)		Volunteer 3 (male)		Volunteer 4 (male)		Volunteer 5 (female)	
Norethandrolone m1	109 ± 4	1.91	-	-	-	-	-	-	-	-
Oxabolone	101 ± 3	1.03	-	-	-	-	-	-	-	-
Oxandrolone	95 ± 3	1.35	109 ± 7	1.47	103 ± 9	0.60	107 ± 4	2.01	90 ± 6	1.92
Pregnanediol G	97 ± 1	1.48	-	-	-	-	-	-	-	-
Testosterone G	99 ± 1	0.65	111 ± 10	2.26	108 ± 6	2.11	-	-	103 ± 2	0.92
Testosterone D3	109 ± 7	2.26	-	-	-	-	-	-	-	-
1-methyltestosterone	95 ± 7	1.23	-	-	-	-	-	-	-	-
1-Testosterone	92 ± 4	2.06	-	-	-	-	-	-	-	-
4-hydroxy-testosterone	106 ± 6	1.82	-	-	-	-	-	-	-	-
5αAndrostane-3α17β-diol G	91 ± 1	2.11	91 ± 7	1.64	97 ± 13	0.33	106 ± 9	1.14	104 ± 9	0.71
5βAndrostane-3α17β-diol G	93 ± 7	1.83	106 ± 6	1.23	101 ± 6	0.17	100 ± 3	< 0.1	105 ± 8	1.01
11-hydroxy-androsterone G	72 ± 7	6.32	82 ± 8	3.63	84 ± 9	2.81	88 ± 7	2.27	83 ± 8	3.16
11-hydroxy-etiocholanolone G	74 ± 2	6.85	88 ± 7	2.86	81 ± 8	3.36	90 ± 10	2.00	86 ± 4	3.50

n = 5 technical replicates.

*t_{cal} is a calculated t-test for the comparison of two experimental means;

$t_{theoretic}$ = 2.31; P = 0.05

IV.5 CONCLUSIONS

This work reveals a new method based on ultrasonic energy to improve sample treatment in the determination of anabolic steroids for doping control. The developed method reduces the total time of the AAS determination from around 150 min, which is the approximate time spent in the overall conventional sample treatment plus the time required for the GC/MS analysis, to around 85 min for most compounds and to 90 min if 11β-hydroxyandrosterone and 11β-hydroxyetiocholanolone are present. The new approach is less time - consuming, is robust, and has the same reproducibility as the traditional method. In addition, for thirteen of the anabolic steroids studied the new method was able to improve the derivatisation yields obtained. The new sample treatment proposed here can be easily adapted to robotic platforms for high-throughput analysis and water-refrigerated systems can also be adapted to avoid water overheating during ultrasonication, a crucial requirement for control agencies in the screening of doping substances in sport.

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CHAPTER V

Accelerated sample treatment for the screening of Banned Doping Substances by GC-MS: Ultrasonication versus Microwave energy

V.1 ABSTRACT

A comparison between ultrasonication and microwave irradiation as tools to achieve a rapid sample treatment for the analysis of banned doping substances in human urine by means of gas chromatography–mass spectrometry (GC–MS) was performed. The following variables were studied and optimised: (i) time of treatment, (ii) temperature, (iii) microwave power and (iv) ultrasonic amplitude. The results were evaluated and compared with those achieved by the routine method used in the World Anti-Doping Agency (WADA) accredited Antidoping Laboratory of Rome. Only under the effect of the ultrasonic field was it possible to enhance the enzymatic hydrolysis reaction rate of conjugated compounds. Similar reaction yield to the routine method was achieved after 10 min for most compounds.

Under microwave irradiation, denaturation of the enzyme occurs for high microwave power. The use of both ultrasonic or microwave energy to improve the reaction rate of the derivatisation of the target compounds with trimethyliodosilane/methyl-N-trimethylsilyltrifluoroacetamide (TMSI/MSTFA/NH$_4$I/2-mercaptoethanol) was also evaluated. To test the use of the two systems in the acceleration of the reaction with TMSI, a pool of 55 banned substances and/or their metabolites were used. After 3 min of ultrasonication, 34 of the 55 compounds had recoveries similar to those obtained with the classic procedure that lasts for 30 min (Student'st test, n=5), 18 increased to higher silylation yields, and for the compounds 13β,17α-diethyl-3α,17β-dihydroxy-5α-gonane (norboletone metabolite 1), metoprolol and metipranolol the same results were obtained increasing the ultrasonication time to 5 min. Similar results were obtained after 3 min of microwave irradiation at 1.200 W. In this case, 30 of the 55 compounds had recoveries similar to the classic procedure (Student's t test, n=5) whilst 18 had higher silylation yields. For the compounds 3α-hydroxy-1α-methyl-5α-androstan-17-one (mesterolone metabolite 1), 17α-ethyl-5β-estrane-3α,17β,21-triol (norethandrolone metabolite 1), epioxandrolone, 4-chloro-6β,17β-dihydroxy-17α-methyl-1,4-androstadien-3-one (chlormetandienone metabolite 1),

carphedon, esmolol and bambuterol the same results were obtained after 5 min under microwave irradiation.

V.2 INTRODUCTION

The global fight against doping is becoming a problem of increasing complexity. To protect safe and fair competition, antidoping organisations together with the national antidoping laboratories are making a huge effort to put into practice in and out of competition programs for the control of banned substances. Furthermore, every year, new substances are added to the World Anti-Doping Agency (WADA) list of banned substances and, consequently, laboratory analyses are becoming more complex, which may result in increasing workloads, delaying reports and raising costs of each test.

Doping control of androgenic anabolic steroids (AAS) and other banned substances, such as diuretics, stimulants, β2-agonists and beta-blockers, is based on the detection of such compounds and their metabolites in urine samples from athletes [1–3]. In urine, these compounds are mostly in their conjugated form [4, 5]. The main pathway for conjugation and inactivation of these substances in the human body is glucuronidation [5, 6]. Therefore, most compounds will be excreted in urine as glucuronate conjugates. Currently, most methods for routine detection of these compounds and their metabolites, comprising both screening and confirmatory analysis, are based on chromatographic-spectrometric techniques, mainly GC-MS techniques [2, 7–11]. It is important to stress that to be analysed by GC–MS techniques, the target analyte must be both volatile and thermally stable. Since most of the banned substances mentioned above, either in the conjugated or free form, do not fulfil this requirement, as a general rule, the analysis of such substances by GC–MS techniques requires a (i) previous enzymatic hydrolysis of the conjugating groups to produce the free parent compound, and (ii) the derivatisation of the free analyte to enhance its volatility, thermal stability and mass spectrometry properties [12, 13]. These two steps of the sample treatment play a critical role in the analysis of doping substances, especially in terms of time and, more importantly, sensitivity. Both steps are enhanced by conventional heating. The cleavage of the glucuronide moiety to produce the free compound is performed at 55 °C for 1 h with the β-glucuronidase enzyme from E. coli, which is highly specific for β-glucuronides [5]. After hydrolysis, the incorporation of a trimethylsilyl (TMS) group in the free analyte is performed at 78 °C for 30 min with methyl-N-trimethylsilyltrifluoroacetamide (MSTFA), the most popular derivatisation reagent routinely used by the WADA accredited laboratories [14, 15]. These experimental conditions have been reported by Ali Shareef and co-workers as the optimum conditions to achieve maximum derivatisation yield; yet for a few compounds, generally those with more than one position available for the TMS group, the formation of more than one final product can occur, resulting in sensitivity losses and low reproducibility [16].

Recently and due to some drawbacks mentioned above for the GC–MS analysis of AAS and other banned compounds, several methodologies that use liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS) are being rapidly introduced as a better alternative [17–21]. These strategies allow the direct determination of the conjugate analytes and overcome the marginal GC–MS properties of some compounds, even after derivatisation. Nevertheless, the detection of conjugated banned substances is still confined to a small number of compounds because only few conjugated standards are available. Furthermore, concerning the determination of the free AAS, even for LC–MS/MS, derivatisation to enhance their ionization properties is generally carried out due to the limited capability of electrospray to ionize these compounds [20].

Modern sample preparation methods using ultrasonic (US) and microwave energy (MW) are receiving growing interest as an alternative to conventional heating methods, particularly as they offer enhancement of reaction rates and, occasionally, increment in their yields.

When the ultrasonic waves cross a liquid solution different chemical and physical phenomena take place. The reason why ultrasonic energy can affect chemical reactions is linked to the formation of cavitation bubbles [22, 23]. This phenomenon occurs by means of compression and decompression as ultrasonic waves are continuously applied to a liquid medium [22, 23]. These bubbles can grow, oscillate, split and implode. As a result of cavitational bubble implosion, extreme temperatures and pressures are generated, acting like microreactors inside the liquid media [24–27]. Moreover, the formation of highly reactive chemical radicals may also take place inside the liquid medium [23]. Several studies have highlighted the use of ultrasonic energy to enhance numerous physic-chemical applications and more recently to improve enzymatic reactions, which is a remarkable advance in the use of ultrasonic energy for sample treatment in analytical chemistry [28–30]. Although the mechanism by which ultrasonication accelerates the enzymatic kinetics is not well understood, some authors have pointed out that the contact area between phases is increased due to cavitation, allowing a reduction of mass transfer limitations in the enzyme–substrate system [23, 24].

The cup horn sonoreactor and the ultrasonic probe are common devices used nowadays to efficiently boost enzymatic reactions kinetics [31, 32]. A minimum knowledge of the properties of such devices is required otherwise unexpected effects can be obtained. As an example, the intensity of sonication, which ultimately dictates the properties of cavitation, is considerably different among the above-mentioned systems. Additionally, it must be taken into account that the ultrasonic probe needs to be inserted into the sample and its applications are done in open vessels, which can be inconvenient for some applications where cross-contamination may occur if the tip is not correctly cleaned and the experimental handling carefully done. All these problems are avoided by using the cup horn sonoreactor, which, in practical terms, can be considered as a powerful mini-ultrasonic bath.

Concerning microwave energy, several authors have described its use in different fields of chemistry to increase reaction yields and kinetics [33–36]. The microwave heating is produced when microwaves interact with some molecules that have the ability to transform electromagnetic energy into heat [37]. Therefore, in contrast to the conventional heating, the microwave heating process is

generated internally through material–microwave interaction, making the heating process very fast [36]. Nevertheless, the so-called microwave effect cannot be explained by the fast heating phenomenon alone, and some theories based on specific radiation effects have been described [36].

In the present work, we study and compare the effects caused by the introduction of microwave and ultrasonic energy in both hydrolysis and derivatisation steps of the routine sample treatment for the determination of banned doping substances in human urine. Although the assessment of ultrasonication for the analysis of AAS has been recently performed in our research group, in the present work, we will evaluate its use in the workflow of two screening methods applied in WADA antidoping laboratories, which includes other banned substances, such as diuretics, stimulants, β-blockers and β$_2$-agonists. The effect of microwave energy will be for the first time, to the best of our knowledge, evaluated for the overall sample treatment procedure. The effects of time of treatment, heating, ultrasonic amplitude and microwave power were investigated and compared to the conventional heating procedure.

V.3 EXPERIMENTAL

V.3.1 APPARATUS

An ultrasonic cup horn sonoreactor (SR), model UTR200 (200 W, 24 kHz), from Dr. Hielsher (Teltow, Germany) and a microwave oven MARS 3 from CEM Corporation (North Carolina, USA) were used to accelerate the enzymatic hydrolysis and the TMS derivatisation procedure. A Simplicity 185 from Millipore (Milan, Italy) was used to obtain Milli-Q water. The enzymatic hydrolysis of conjugated compounds and the subsequent derivatisation reaction were performed in 10-mL glass vessels from 3 V Chimica (Rome, Italy).

V.3.2 STANDARDS AND REAGENTS

The standards testosterone, testosterone D3, epitestosterone, androsterone, etiocholanolone, etiocholanolone D5, 5α-androstane-3α,17β-diol, 5β-androstane-3α,17β-diol, dehydroisoandrosterone (DHEA), 11-keto-etiocholanolone, 11-hydroxy-etiocholanolone, 11-hydroxy-androsterone, 5β-estran-3α-ol-17-one (nandrolone metabolite 2), 9α-fluoro-17,17-dimethyl-18-norandrosta-4,13-diene-11β-ol-3-one (fluoxymesterone metabolite 1), 9α-fluoro-17α-methyl-4-androsten-3α,6β,11β,17β-tetra-ol (fluoxymesterone metabolite 2), 6β-hydroxyfluoxymesterone (fluoxymesterone metabolite 3), 3α-hydroxy-1α-methyl-5α-androstan-17-one (mesterolone metabolite 1), 17β-hydroxy-7α,17α-dimethylandrost-4-en-3-one (bolasterone), 17β,4-hydroxyandrost-3-one (4-hydroxytestosterone), 17α-methyl-5β-androstene-3α,17β-diol (17α-methyltestosterone metabolite 2), 17β-hydroxy-17α-methyl-2-

oxa-5α-androstan-3-one (oxandrolone), 17α-hydroxy-17α-methyl-2-oxa-5α-androstan-3-one (epioxandrolone), 4-chloro-4-androsten-3a-ol-17-one (clostebol metabolite 1), 17α-ethyl-5β-estrane-3α,17β,21-triol (norethandrolone metabolite 1), methyldienolone, 13β,17α-diethyl-3α,17β-dihydroxy-5α-gonane (norboletone metabolite 1), 13β,17α-diethyl-3α,17β-dihydroxy-5β-gonane (norboletone metabolite 2), 4-hydroxy-19-nortestosterone (oxabolone), 4-chloro-6β,17β-dihydroxy-17α-methyl-1,4-androstadien-3-one (chlormetandienone metabolite 1), 17α-ethynyl-17β-hydroxyandrost-4-eno[2,3-d]isoxazole (danazol), 2-hydroxymethyl-17α-methylandrostadiene-11α,17β-diol-3-one (formebolone metabolite 1), formestane, 6β-hydroxy-methandienone (methandienone metabolite 2), 17α-methyl-5β-androstane-3α,17β-diol (methandienone metabolite 3), triamterene, probenecid, pemoline, oxilofrine, octopamine, indenolol, pholedrine, etilefrine, etamivan, norfenefrine, 4-phenylpiracetam (carphedon), 6-hydroxy-bromantan, benzoylecgonine, 4-hydroxy-amphetamine, timolol, bisoprolol, betaxolol, salmeterol metabolite, procaterol, penbutolol, nebivolol, metoprolol, metipranolol, esmolol, carteolol, bambuterol and acebutolol were purchased from NARL (Pymble, Australia). The steroid 17α-methyltestosterone was purchased from Riedel-de Haën (Seelze, Germany).

Individual stock standard solutions of each compound (500 mg/L) were prepared by weighing 0.0125 g of analyte in a 25 mL volumetric flask and making it up to volume with methanol. These standard solutions were stored in the dark at -20 °C. Working standard solutions were prepared by dilution of the stock standard solutions in the appropriate volume of methanol.

A solution of β-glucuronidase from *Escherichia coli K12* with a specific activity approximately of 140 U/mg at 37 °C and pH 7 with nitrophenyl-β-D-glucuronidase as substrate (1 mL contains at least 140 U) was purchased from Roche Diagnostic (Mannheim, Germany). All chemicals (sodium bicarbonate, sodium phosphate, sodium hydrogen phosphate, tert-butylmethyl ether) were from Carlo Erba (Milano, Italy). The derivatisation reagent N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) was from Macherey-Nagel (Düren, Germany) via the Italian distributor (Delchimica Scientific, Napoli, Italy). Ammonium iodide and dithioerythritol (DTE) were supplied by Sigma-Aldrich (Milano, Italy). The derivatisation agent was a mixture of MSTFA/NH$_4$I/DTE (1000:2:4, v/w/w) stored at 4 °C for a maximum of 2 weeks.

V.3.3 SAMPLE PREPARATION

V.3.3.1 URINE HYDROLYSIS PROCEDURE

Urine samples (2 mL) were hydrolysed with 50 μL of the commercial solution of β-glucuronidase, after the addition of 0.750 mL of phosphate buffer (0.8 M, pH 7). The hydrolysis was accelerated by means of three different systems as follows: heating at 55 °C for 60 min, or using ultrasonic and microwave energy provided by a sonoreactor and microwave reactor, respectively.

V.3.3.2 LIQUID-LIQUID EXTRACTION OF TARGET ANALYTES

After cooling to room temperature, 0.5 mL of carbonate buffer (pH 9) was added to alkalinize the hydrolyzed solution. Liquid–liquid extraction was carried out by agitation with 5 mL of tert-butyl methyl ether for around 10 min; after centrifugation, the organic phase layer was transferred to a new vessel.

V.3.3.3 DERIVATISATION PROCEDURE

The procedure for derivatisation with MSTFA was performed as follows: The collected organic phase was dried under a gentle nitrogen stream at 40 °C. After the addition of 50 μL of MSTFA, the vial was closed and the derivatisation reaction was then performed by means of three different systems as follows: heating at 78 °C for 30 min, or using ultrasonic and microwave energy provided by a sonoreactor and a microwave reactor, respectively.

V.3.4 GC-MS INSTRUMENTATION AND OPERATING CONDITIONS

Gas chromatographic (GC) analyses were carried out on an HP6890 gas chromatograph coupled to a 5973 mass spectrometric detector (Agilent Technologies Italia, Milan, Italy). Chromatographic separations were carried out in a phenyl-methylsilicone column (HP1, 17 m×0.2-mm i.d., 0.11-μm film thickness) from Agilent Technologies Italia. The injection was done in split mode with a split ratio of 1:10 and the injection volume was 1 μL. Helium was used as carrier gas at a constant pressure of 75 kPa. The injection port was set at 280 °C. Two different screening methods were used to determine all banned substances employed in this study: *Screening method 1* - the oven temperature was programmed as follows: initial temperature 188 °C, held for 2.5 min; 3 °C/min ramp to 211 °C, held for 2.0 min; 10 °C/min ramp to 238 °C; and 40 °C/min ramp to 320 °C and held for 3 min. The transfer line temperature was 250 °C, and the ionization source temperature was 230 °C. *Screening method 2* - the oven temperature was programmed as follows: initial temperature 150 °C; 10 °C/min ramp to 200 °C, held for 0.5 min; 20 °C/min ramp to 230 °C, held for 0.5 min; 12 °C/min ramp to 290 °C; and 30 °C/min ramp to 320 °C and held for 1 min. The transfer line temperature was 250 °C, and the ionization source temperature was 230 °C.

For both screening methods the mass detection was performed in selected ion monitoring (SIM) mode using specific fragment ions (see Table V.1), with a solvent delay time of 2.6 min. The dwell time in both screening methods was 20 ms. The quantification of all compounds was carried out using the "diagnostic" ions presented in the "Quantification ion" column of Table V.1, whereas the

"diagnostic" ions in the "Qualification ion" column were used to confirm the identity of the substance. Quantitative measurement of all compounds was performed by response factor calibration (RFC). The values of the urinary concentration of the exogenous compounds were calculated by using the ratio of the areas of the target ions and 17-α-methyltestosterone, which was used as internal standard (ISTD). The concentrations of the endogenous AAS were calculated in relation to the respective deuterated standards.

Table V.1 - Diagnostic ions (*m/z*) used in SIM mode for detection of banned substances.

	Derivative	Diagnostic ions (*m/z*)		GC-MS method
		Quantification ion	*Qualification ion*	
AAS compounds				
Testosterone	bis-O-TMS	432	417	1
Epitestosterone	bis-O-TMS	432	417	1
Androsterone	bis-O-TMS	419	434	1
Etiocholanolone	bis-O-TMS	419	434	1
5αAndrostane-3α17β-diol	bis-O-TMS	241	256	1
5βAndrostane-3α17β-diol	bis-O-TMS	241	256	1
DHEA	bis-O-TMS	237	327	1
11Keto-Etiocholanolone	bis-O-TMS	505	415	1
11-hydroxy-etiocholanolone	bis-O-TMS	522	417	1
11-hydroxy-androsterone	bis-O-TMS	522	417	1
Nandrolone m2	bis-O-TMS	405	420	1
Fluoxymesterone m1	bis-O-TMS	462	208	2
Fluoxymesterone m2	tetra-O-TMS	143	462	2
Fluoxymesterone m3	tetra-O-TMS	640	605	2
Mesterolone m1	bis-O-TMS	433	448	1
Bolasterone	bis-O-TMS	445	315	1
4-hydroxy-testosterone	tri-O-TMS	520	505	1
Methyltestosterone m2	bis-O-TMS	143	255	1
Oxandrolone	mono-O-TMS	143	308	1
Epioxandrolone	mono-O-TMS	143	308	1
Clostebol m1	bis-O-TMS	451	453	1
Norethandrolone m1	bis-O-TMS	157	331	1
Methyldienolone	bis-O-TMS	430	415	1
Norboletone m2	bis-O-TMS	157	435	1
Norboletone m1	bis-O-TMS	157	435	1
Oxabolone	bis-O-TMS	506	491	1
Chlormetandienone m1	bis-O-TMS	143	315	1
Danazol	bis-O-TMS	481	452	1
Formebolone m1	tri-O-TMS	367	222	2
Formestane	tri-O-TMS	518	503	2
Metandienome m2	tri-O-TMS	517	532	2
Metandienome m3	bis-O-TMS	206	444	2
17α-methyltestosterone	bis-O-TMS	301	446	1
Diuretics				
triamterene	tri-N-TMS	432	417	2
probenecid	mono-O-TMS	432	417	2

Table V.1 (continuation)

	Derivative	Diagnostic ions (*m/z*)		GC-MS method
		Quantification ion	*Qualification ion*	
Stimulants				
pemoline	tri-N,O-TMS	178	392	2
oxilofrine	bis-O-TMS	58	267	2
	tri-O-TMS	130	382	2
octopamine	tetra-N,O-TMS	174	426	2
pholedrine	mono-O-TMS	58	179	2
	bis-O-TMS	130	294	2
etilefrine	bis-O-TMS	268	310	2
	tri-N,O-TMS	130	382	2
etamivan	mono-O-TMS	223	294	2
norfenefrine	tetra-N,O-TMS	174	426	2
carphedon	bis-N-TMS	272	257	2
6-hydroxy-bromantan	mono-N-TMS	393	395	2
Benzoylecgonine	mono-O-TMS	82	240	2
4-hydroxy-amphetamine	bis-N,O-TMS	116	280	2
	tri-N,O-TMS	188	352	2
Beta-blockers				
timolol	mono-O-TMS	373	186	2
bisoprolol	bis-N,O-TMS	72	405	2
betaxolol	mono-O-TMS	72	263	2
	bis-N,O-TMS	144	264	2
salmeterol metabolite	bis-O-TMS	260	369	2
indenolol	mono-O-TMS	72	319	2
	bis-O-TMS	144	204	2
penbutolol	mono-O-TMS	86	348	2
nebivolol	tri-N,O-TMS	116	368	2
metoprolol	mono-O-TMS	72	223	2
	bis-N,O-TMS	144	224	2
metipranolol	mono-O-TMS	152	265	2
	bis-N,O-TMS	144	266	2
esmolol	mono-O-TMS	251	352	2
	bis-N,O-TMS	144	424	2
carteolol	bis-N,O-TMS	235	241	2
acebutolol	tri-N,O-TMS	350	365	2
Beta-2 agonists				
procaterol	tri-N,O-TMS	407	100	2
bambuterol	mono-O-TMS	86	439	2

V.3.5 URINE SAMPLES

Urine samples were collected in clean plastic disposable containers and kept at −20°C until analysis.

Urine samples used in this work were obtained from healthy volunteers of our research team.

V.3.6 EXPERIMENTAL DESIGN

The routine sample treatment used for the analysis of anabolic steroids and other substances detectable as TMS derivatives by the WADA accredited Anti-Doping Laboratory of Rome is depicted in Figure V.1.
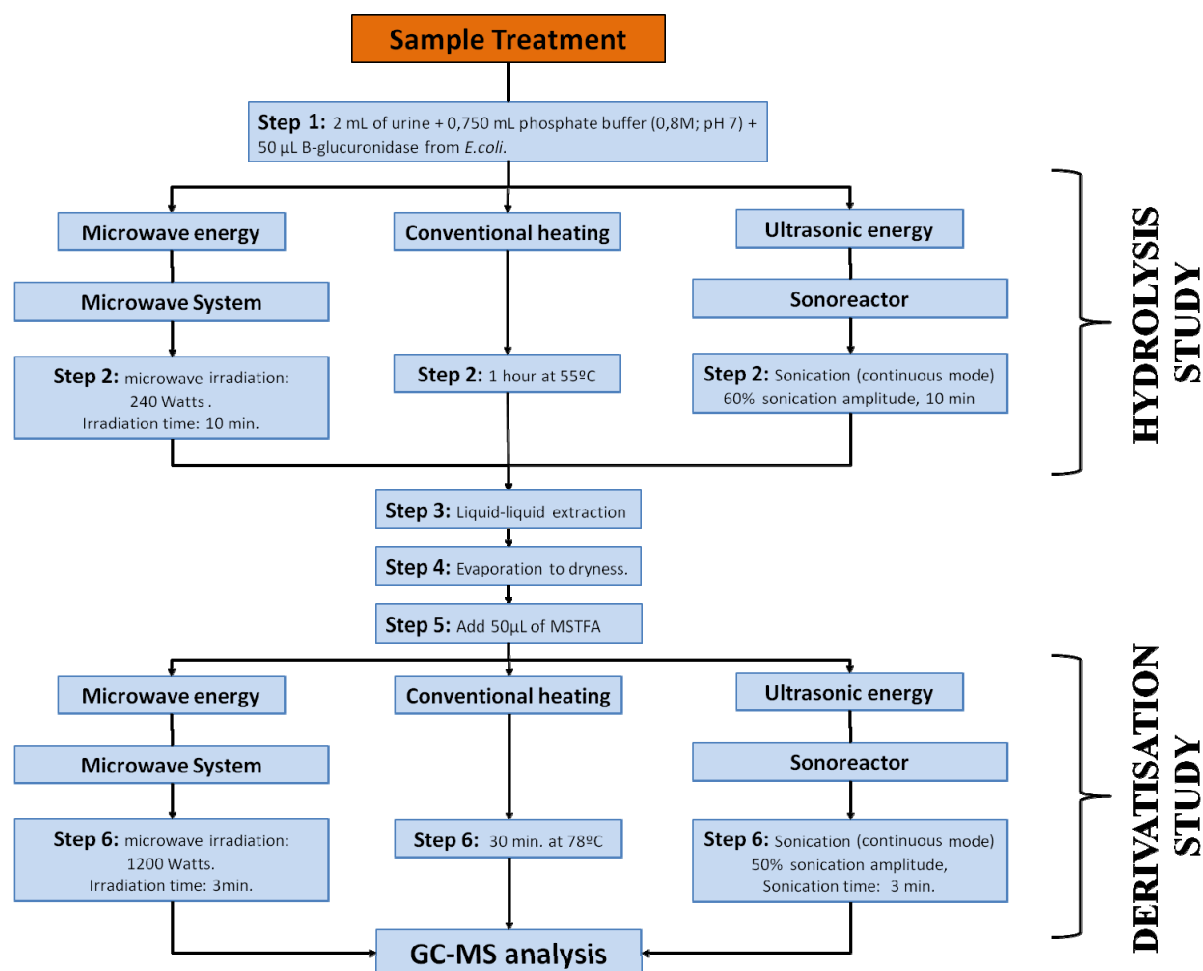
Figure V.1 - Comprehensive scheme of the classic, ultrasonic and microwave protocols for the determination of the banned compounds presented in table V.1.

V.3.6.1 OPTIMISATION OF HYDROLYSIS PROCEDURE

For the enzymatic hydrolysis optimisation study, the natural endogenous steroids androsterone, etiocholanolone, 11-keto-androsterone, 11-hydroxy-etiocholanolone and 11-hydroxy-androsterone were used. The experimental procedure was followed as described in Sect. "Sample preparation". Deuterated AAS were used as internal standards. It is important to notice that after the hydrolysis study, the derivatisation procedure was carried out by conventional means for all experiments.

After optimisation, both ultrasonic andmicrowave methods were applied to the same control sample and compared with the conventional heating procedure. Ten target steroids were analysed in this study: testosterone, epitestosterone, androsterone, etiocholanolone, DHEA, 5α-androstane-3α,17β-diol, 5β-androstane-3α,17β-diol, 11keto-etiocholanolone, 11-hydroxy-etiocholanolone and 11-hydroxy-androsterone.

V.3.6.2 OPTIMISATION OF DERIVATISATION PROCEDURE

The optimisation of the TMS derivatisation study was performed with 25 target banned substances. The experimental procedure described in Sect. "Derivatisation procedure" was applied to 0.5 mL of a methanolic solution spiked with the 25 compounds. The concentration value ranged from 150 to 600 ng/mL.

The effectiveness of the MSTFA derivatisation procedure assisted by microwave energy depends mainly on two critical experimental variables: microwave power and reaction time. To investigate and optimize the effect of the microwave power on the derivatisation yield, as a first approach, the irradiation time was set constant at 3 min and the irradiation power was studied in the range 360 to 1200 W. Subsequently, the time of irradiation was optimized for the microwave power presenting better results.

After optimisation, both accelerated procedures were applied to a methanolic solution with 55 target compounds comprising 30 anabolic steroids, 2 diuretics, 11 stimulants, 10 beta-blockers and 2 β2-agonists. The results were compared with the routine procedure. In this set of experiments the optimum conditions to achieve maximum derivatisation yield with the microwave and ultrasonic energy procedure were applied to the same control sample. The steroid 17-α-methyltestosterone was used as ISTD. It is important to stress that for the derivatisation study the ISTD was derivatised by the conventional procedure and added to all samples at the end of both accelerated and conventional procedures. The final concentration of ISTD in each sample was 200 ng/mL.

V.3.6.3 APPLICATION TO REAL SAMPLES

The overall applicability of both accelerated procedures was tested in a real urine sample. The results were compared with the routine procedure. The natural conjugated endogenous AAS from each urine sample were used in this experiment. Each sample was prepared in triplicate.

V.3.7 STATISTICAL ANALYSIS

In order to test if both the ultrasonic and the microwave methods are comparable to the conventional method, the t-test for the comparison of two experimental means was used [38].

V.4 RESULTS AND DISCUSSION

In the comprehensive scheme depicted in Figure V.1, the two critical steps in which the ultrasonic and microwave energy were applied are highlighted. The first step entails the enzymatic hydrolysis process, where the glucuronate conjugates are transformed into the free parent compound. This reaction takes place at 55 °C for 1 h. Once the free steroids have been extracted and pre-concentrated through evaporation to dryness, in the sixth step, they are derivatised by adding MSTFA. The reaction is enhanced by heating at 78 °C for 30 min. It is important to stress that the results obtained by using the analytical approaches proposed here have been compared with those obtained with the routine sample treatment. The GC–MS analyses were carried out using two different screening methods, described in Sect. "GC–MS instrumentation and operating conditions", from the WADA accredited Anti-Doping Laboratory of Rome.

V.4.1 EFFECT OF ULTRASONIC AND MICROWAVE ENERGY IN THE ENHANCEMENT OF ENZYMATIC HYDROLYSIS REACTION

To evaluate the performance of the ultrasonic and microwave energy in the sample treatment, the first set of experiments was devised maintaining all the handling as described in Figure V.1 unaltered except the step where the enzymatic reaction takes place. For the enzymatic hydrolysis study, the ultrasonic and microwave energy were applied to steroid glucuronates. The following five target steroid glucuronates, known to have the longest reaction times for enzymatic hydrolysis, were used in this preliminary study: androsterone, etiocholanolone, 11-keto-androsterone, 11-hydroxy-etiocholanolone and 11-hydroxy-androsterone.

V.4.1.1 EFFECT OF MICROWAVE ENERGY

To perform the microwave-assisted hydrolysis, the time of treatment and the microwave power were optimised. The temperature of the samples during the treatment was followed by using a thermocouple. The optimisation of the microwave-assisted procedure was done keeping the reaction temperature below 60 °C, to avoid thermal denaturation of the enzyme.

To optimise the microwave power the hydrolysis time was set at 10 min, whilst the output power varied between 60 and 480 W. Above this power value, the temperature after 10 min exceeded the 60 °C upper limit. Results shown in Figure V.2 show that as the energy produced by the microwave oven increases from 60 to 240 W so the recovery yields are increased till a maximum value is achieved. Above 240 W the yields decreased constantly. The worst reaction yield was observed for the hydrolysis of the steroid glucuronates 11-hydroxy-etiocholanolone and 11-hydroxy-androsterone, whose recoveries were around 45% of the conventional hydrolysis procedure.
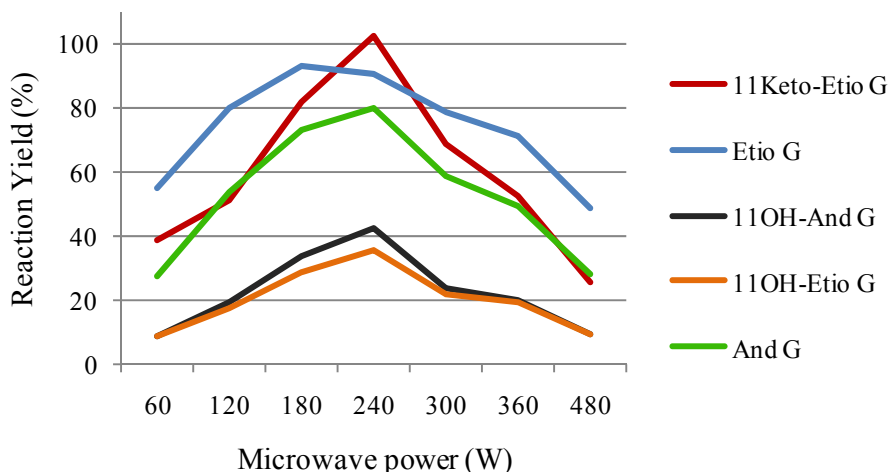
Figure V.2 - Effect of the microwave power on the hydrolysis yield of the natural endogenous steroids androsterone, etiocholanolone, 11-keto-androsterone, 11-hydroxy-etiocholanolone and 11-hydroxy-androsterone (10 minutes of treatment; n=3). The results are given in percentages (100% = result via the classic method).

These results are consistent with a previous study developed in our laboratory, which stresses the role of the hydroxy group at the C_{11} position in the hydrolysis efficiency, since it is the only structural difference between these and the other steroids studied [39]. For the other compounds, the reaction yields ranged between 80 and 100%. Results shown in Figure V.2 clearly indicate that the protocol using 10 min of irradiation with microwave energy to accelerate the hydrolysis reaction performs worse than the conventional heating protocol, because the hydrolysis yields are lower for the majority of the target steroids studied. The low efficiency of microwave energy in accelerating the enzymatic hydrolysis of steroids is in agreement with literature that highlights partial enzyme inactivation as consequence of conformational changes promoted by microwave irradiation [40].

To study the effect of time, we developed a set of experiments in which the microwave power was set at 180 W and 240 W, whilst the time of irradiation was increased from 10 to 20 min. Results shown in Figure V.3 suggest that the hydrolysis yields can be increased to almost the same levels as those obtained with the conventional procedure for almost all target steroids. Nevertheless, 11-

hydroxy-etiocholanolone and 11-hydroxy-androsterone were hydrolysed in low yields, 50% and 60% respectively. Yadav et al. recently pointed out that microwave energy and conventional heating interact in a similar manner when polar solvents are employed, and therefore the results should be nearly the same when both methods of heating are used [41]. The results obtained in the above described set of experiments suggest that no great improvement arises from the use of microwave energy in the hydrolysis step, since the time required approaches the time of the conventional procedure.

Figure V.3 - Effect of the microwave time of treatment on the hydrolysis yield of the natural endogenous steroids androsterone, etiocholanolone, 11-keto-androsterone, 11-hydroxy-etiocholanolone and 11-hydroxy-androsterone (Microwave power: **A-** 180W, **B-** 240W; n=3). The results are given in percentages (100% = result via the classic method).

V.4.1.2 EFFECT OF ULTRASONIC ENERGY

The use of ultrasonic energy to enhance the enzymatic hydrolysis of steroid glucuronates was evaluated in an earlier study developed in our laboratory [39]. The experimental conditions were optimised there by varying the time of treatment and the amplitude of ultrasonication, and a similar result to the conventional hydrolysis procedure, for the majority of the steroids studied, was achieved in 10 min with the sonoreactor device at 60% of amplitude [39].

V.4.1.3 COMPARISON BETWEEN MICROWAVE AND ULTRASONIC ENERGY

To evaluate and compare the effect of both microwave and ultrasonic energy in the hydrolysis step, the two procedures were applied to the same control sample and compared with the conventional heating procedure. Ten target steroids from human urine were determined in this study: testosterone, epitestoste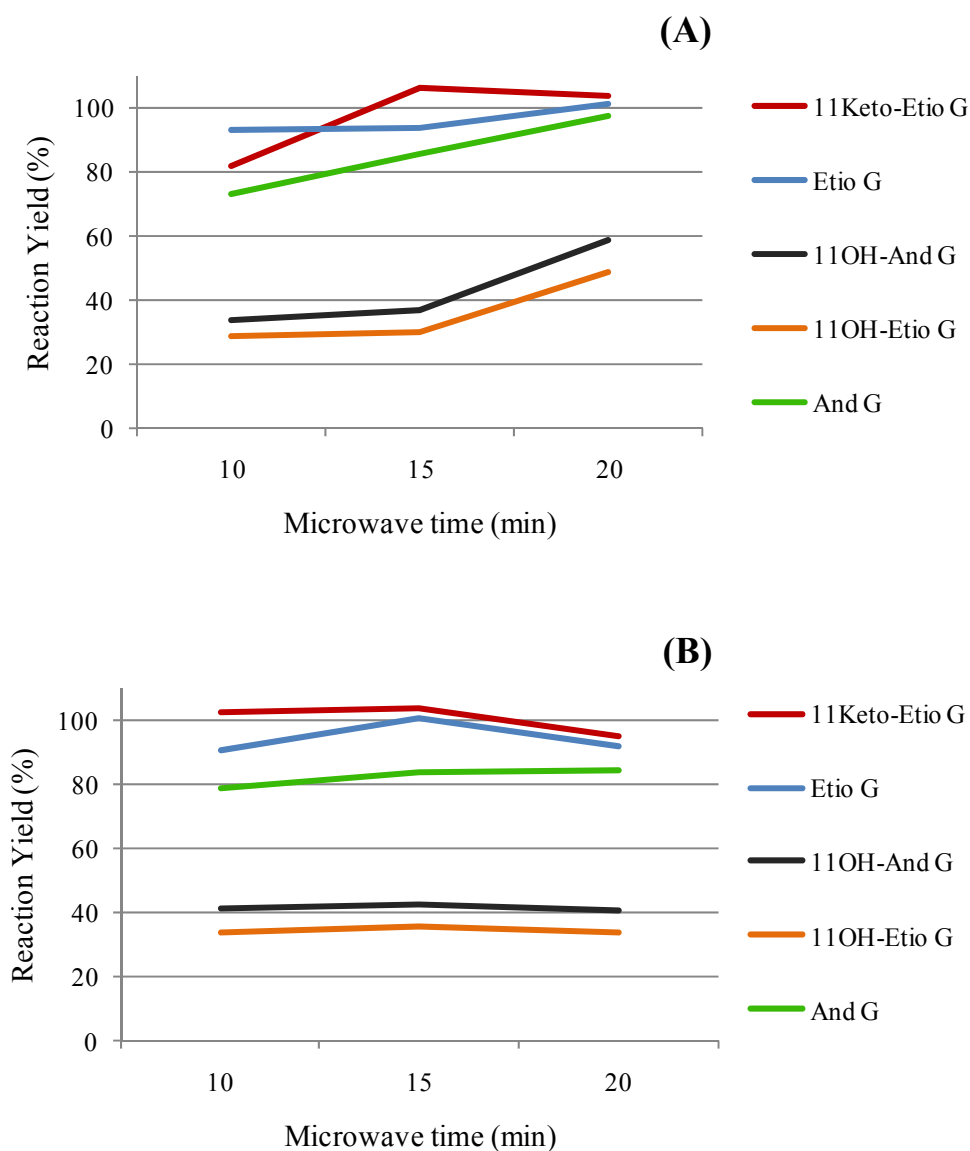rone, androsterone, etiocholanolone, DHEA, 5α-androstane-3α,17β-diol, 5β-androstane-3α,17β-diol, 11-keto-etiocholanolone, 11-hydroxy-etiocholanolone and 11-hydroxy-androsterone.

The results obtained are shown in Table V.2. As expected, except for the endogenous steroids 11β-hydroxy-androsterone and 11β-hydroxy-etiocholanolone, the efficiency of the hydrolysis when the ultrasonic energy was applied was comparable to the conventional heating method. The information taken from all studied compounds is important to the endogenous profile of the athlete. In any case, the 11β-hydroxy group present in these two compounds inhibits the steroid–receptor interaction by steric interference, thereby decreasing both androgenic and anabolic potencies when compared with the other ones under study, which makes them less significant to the endogenous profile of the athlete. Concerning the microwave hydrolysis, the reaction yield is not comparable to the conventional hydrolysis for two other important steroids, androsterone and 5β-androstane-3α,17β-diol.

A parallel set of hydrolysis experiments of the target conjugated compounds was devised to test if 10 min at 55 °C was enough to obtain similar results to those obtained with the sonoreactor. In general, 10 min at 55 °C was insufficient to achieve the same hydrolysis yields. Only for testosterone, epitestosterone and DHEA was 10 min at 55 °C enough to hydrolyse the steroid glucuronides to their respective free compound, with 100%of yield when compared to 1 h at 55 °C (data not shown). For the other glucuronides the hydrolysis yield was very low, ranging from 50 to 75% for etiocholanolone, androsterone and 11-keto-etiocholanolone, and below 35% for 11β-hydroxy-androsterone and 11β-hydroxy-etiocholanolone (data not shown).

Table V.2 - Hydrolysis yield of AAS glucuronides from urine. Comparison of the thermal conductivity procedure (1hour at 55ºC) with the ultrasonic procedure (60% of amplitude, 10min) and the microwave procedure (240 Watts of power, 10min).

	Conventional Procedure		Microwave Method			Ultrasonic Method		
				Reaction yield			Reaction yield	
	(ng/mL; n=6)	RSD	(ng/mL; n=5)	(MW / Conventional method ×100, X±SD)	$\lvert t_{cal}\rvert^{*}$	(ng/mL; n=5)	(US / Conventional method ×100, X±SD)	$\lvert t_{cal}\rvert^{*}$
Testosterone	32 ± 1	1	32 ± 1	100 ± 2	0.25	32 ± 1	100 ± 2	0.51
Epitestosterone	121 ± 3	2	118 ± 4	97 ± 3	1.70	121 ± 1	100 ± 1	0.40
Androsterone	4213 ± 100	2	3683 ± 186	87 ± 4	6.06	4230 ± 98	100 ± 2	0.28
Etiocholanolone	2215 ± 65	3	2146 ± 127	97 ± 6	1.17	2236 ± 67	101 ± 3	0.53
5α-Androstane-3α17β-diol	122 ± 3	2	124 ± 3	102 ± 3	1.04	125 ± 3	102 ± 2	1.42
5β-Androstane-3α17β-diol	245 ± 14	6	211 ± 5	86 ± 2	5.22	226 ± 16	92 ± 6	2.11
DHEA	50 ± 2	4	51 ± 1	102 ± 1	1.23	49 ± 3	98 ± 7	0.67
11Keto-etiocholanolone	2388 ± 180	8	2234 ± 196	94 ± 8	1.36	2608 ± 286	109 ± 12	1.56
11-hydroxy-androsterone	1372 ± 72	5	681 ± 64	50 ± 5	16.64	1072 ± 82	78 ± 6	6.47
11-hydroxy-Etiocholanolone	266 ± 17	6	118 ± 13	44 ± 5	15.81	197 ± 11	74 ± 4	7.78

V.4.2 EFFECT OF ULTRASONIC AND MICROWAVE ENERGY IN THE ENHANCEMENT OF DERIVATISATION REACTION

The second part of this work focused on the sixth step of the sample treatment, the derivatisation process, as shown in Figure V.1. Among the several derivatisation reactions described in the literature, the production of TMS derivatives is by far the most employed due to its simplicity, high reaction yields and robustness under controlled conditions. However, some of the studied compounds have more than one reaction position available for the TMS group, which may result in the formation of more than one final product leading to losses in sensitivity and reproducibility. Therefore, the formation of the TMS derivative is a critical stage of the analytical procedure.

V.4.2.1 EFFECT OF MICROWAVE ENERGY

To investigate and optimise the effect of the microwave power on the derivatisation yield, 25 banned compounds were used and the irradiation time was set constant at 3 min. The irradiation power was studied in the range 360 to 1200 W. The sample's temperature after the microwave heating treatment varied from 60 to 85 °C with the increasing of the microwave power. Figure V.4 shows that the derivatisation yield after 3 min of irradiation at 1200 W is comparable to the yield of the routine procedure that lasts for 30 min. Moreover, for diTMS and triTMS derivatives the yield at 1,200 W is higher than those obtained by the routine thermal heating method (78 °C for 30 min) employed in the Rome antidoping laboratory—increments of reaction yield up to 29% higher. This improvement is most likely because of the harsher conditions generated by the microwave energy. The results presented are concordant with the work recently published by Bowden et al. who investigated the effects of microwave energy applied to different derivatisation solvents for GC–MS analysis of steroids. Microwave heating with MSTFA afforded the same yield of derivatisation as the conventional heating for the studied steroids. However, since only monoTMS and diTMS steroid derivatives were studied by Bowden et al., their conclusions were simply focused on the reduction of the reaction time and failed to notice a great improvement in the reaction yield [42].

To optimize the microwave time, the derivatisation reaction was performed at 1200 W of intensity within five different times, 30 s, 60 s, 90 s, 120 s and 180 s. Figure V.5 shows that 30 s was enough to achieve the same hydrolysis yields as with 30 min at 78 °C for most compounds, especially those with just one reaction position for the TMS group. For the compounds with more than one reaction position for the TMS group higher treatment times are needed; therefore for further experiments the time used was 180 s.
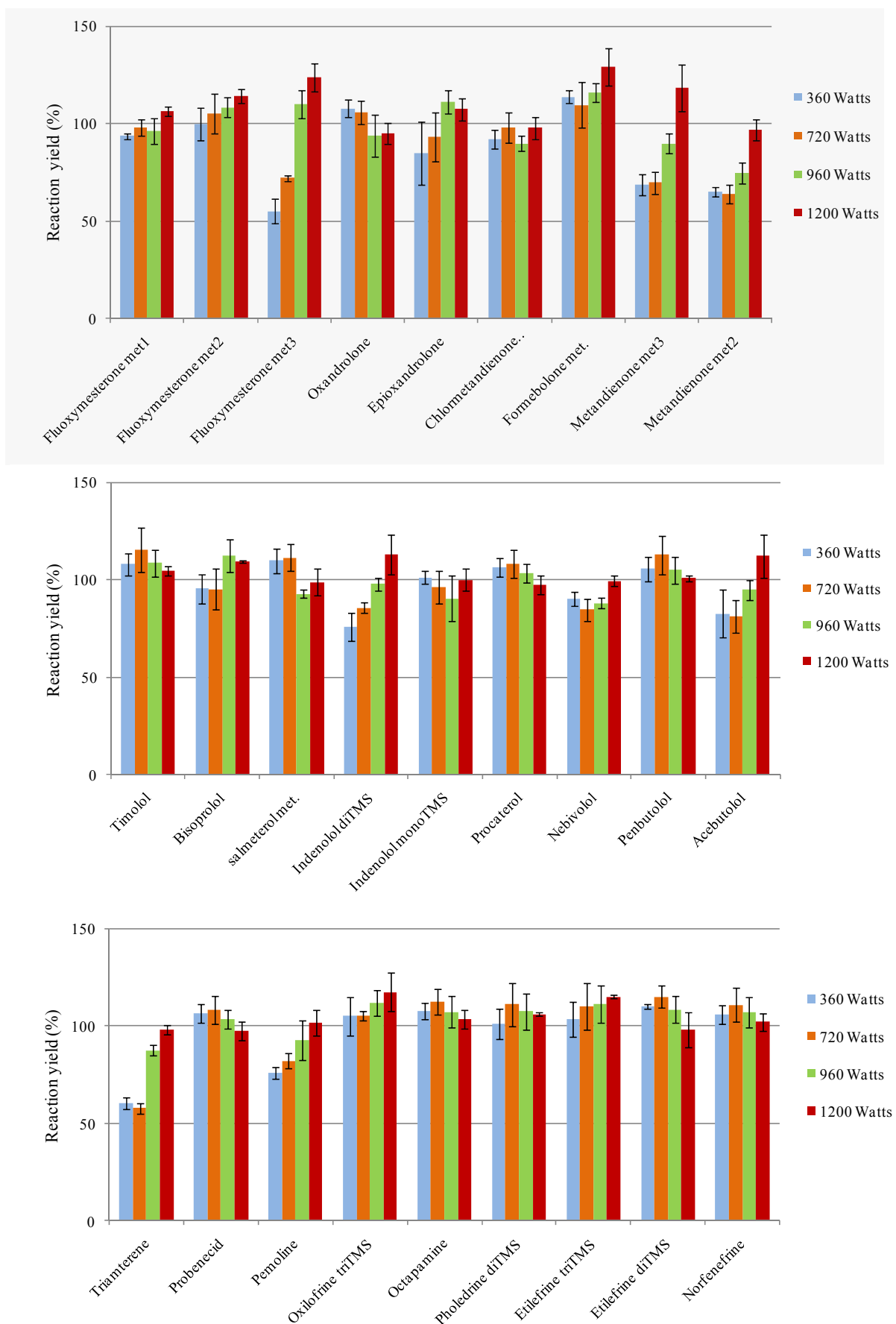
Figure V.4 - Effect of microwave power in the derivatisation yield of 25 banned substances (3 min of treatment; n=3). The results are given in percentages (100% = result via the classic method).
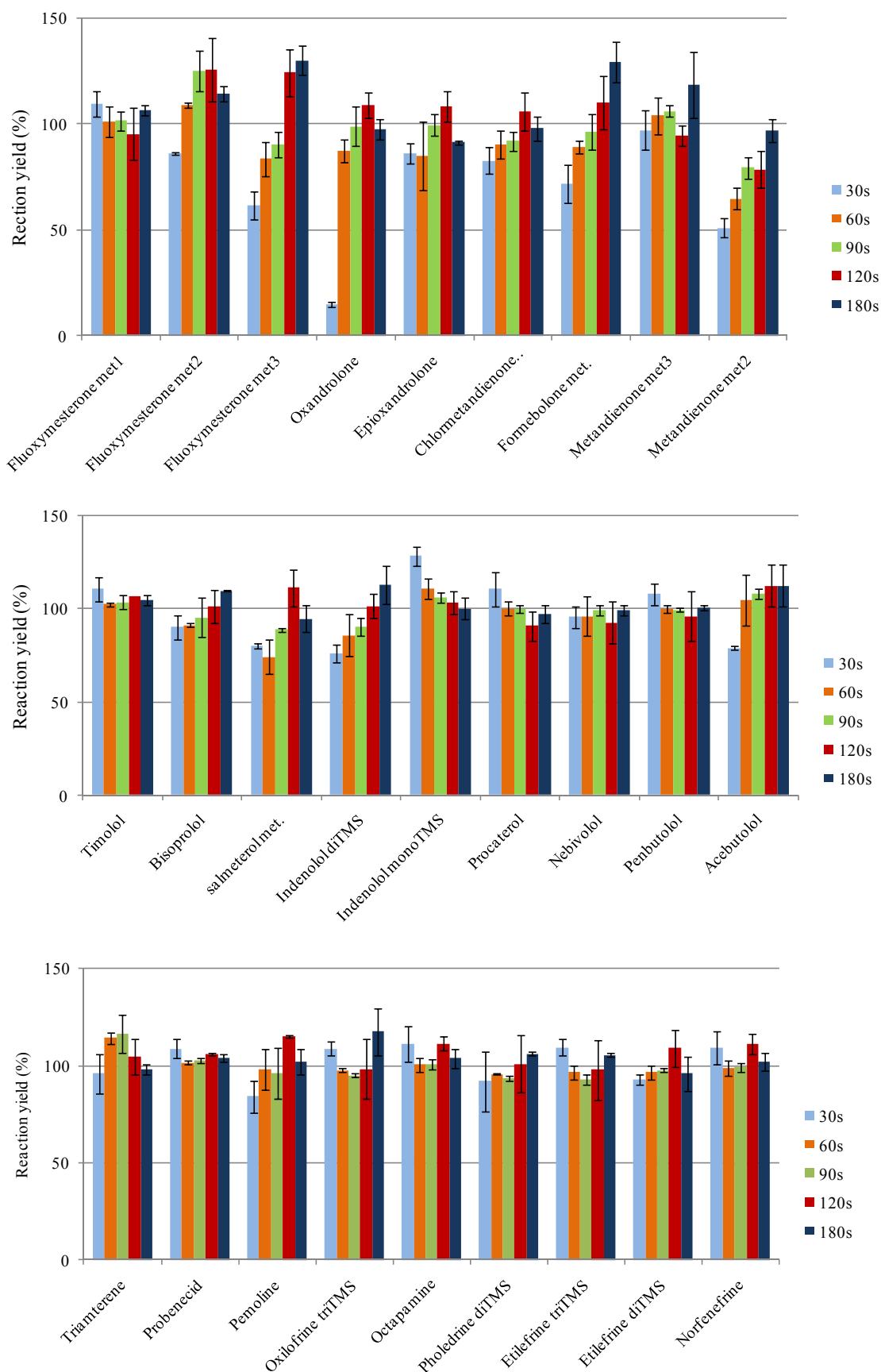
Figure V.5 - Effect of microwave time in the derivatisation yield of 25 banned substances (microwave power 1200 W; n=3). The results are given in percentages (100% = result via the classic method).

V.4.2.2 EFFECT OF ULTRASONIC ENERGY

As mentioned above for the hydrolysis study, the effect of the ultrasonic energy in the derivatisation reaction of AAS was previously optimised. The yields of AAS derivatisation after 3 min of ultrasonication with the sonoreactor at 50% of amplitude were comparable, in terms of precision and accuracy, to those obtained by the heating method at 78 °C [39]. Furthermore, for tri- and tetra-TMS derivatives, an improvement of the reaction yield was clearly achieved.

V.4.2.3 COMPARISON BETWEEN MICROWAVE AND ULTRASONIC ENERGY

In this set of experiments the optimum conditions to achieve maximum derivatisation yield with the microwave and ultrasonic energy procedure were applied to the same control sample and the results compared with the routine procedure.

As may be seen in Table V.3, the yields of derivatisation after 3 min of reaction with both microwave and ultrasonic procedures are excellent. Regarding the ultrasonic energy based procedure, of the 55 banned substances, 34 showed results comparable, in terms of precision and accuracy, to those achieved by the routine procedure. For the microwave energy based procedure, of the 55 compounds, 30 presented similar results to the routine procedure.

Moreover, for several compounds studied, an improvement of the reaction yield was clearly achieved for both procedures; higher reaction yields were achieved for 18 compounds for both the microwave and the ultrasonic procedure. It must be pointed out that for those substances, relative increments of about 10–30% for 8 compounds and 10–48% for 14 compounds were achieved when the microwave and the ultrasonic procedure were employed, respectively. These results are even more impressive if we take into account that not all WADA accredited laboratories employ 78 °C in the TMS derivatisation reaction; some perform this reaction at 60 °C for 0.5 h [43]. For the compounds norboletone metabolite, metoprolol and metipranolol the results achieved were comparable to the conventional procedure after 5 min of ultrasonication. Regarding the microwave procedure, similar results to the conventional procedure were also achieved after 5 min of reaction for the compounds mesterolone metabolite, norethandrolone metabolite, norboletone metabolite, chlormetandienone metabolite, carphedon, esmolol and bambuterol. For the other compounds the yields were not significantly improved by increasing the ultrasonication and microwave time from 3 to 5 min. In any case, for further experiments the time was set at 3 min for both methods.

Table V.3 - Derivatisation yield of TMS derivatives formation with MSTFA. Comparison of the thermal conductivity procedure (30 min at 78 °C) with the ultrasonic procedure (50% of amplitude, 3 min) and the microwave procedure (1,200 W of power, 3 min).

	Conventional Procedure		Microwave Method			Ultrasonic Method		
	(A_{AAS}/A_{ISTD})	RSD	(A_{AAS}/A_{ISTD})	Reaction yield (MW / Conventional method ×100, X±SD)	$|t_{call}|*$	(A_{AAS}/A_{ISTD})	Reaction yield (US / Conventional method ×100, X±SD)	$|t_{call}|*$
Oxabolone	$(29 \pm 3) \times 10^{-4}$	10	$(33 \pm 4) \times 10^{-4}$	113 ± 10	1.55	$(43 \pm 1) \times 10^{-4}$	148 ± 2	10.33
Chlormetandienone met.	$(73 \pm 3) \times 10^{-4}$	4	$(64 \pm 6) \times 10^{-4}$	88 ± 8	3.06	$(68 \pm 7) \times 10^{-4}$	93 ± 9	0.71
Danazol	$(32 \pm 5) \times 10^{-4}$	15	$(31 \pm 3) \times 10^{-4}$	98 ± 9	0.29	$(31 \pm 2) \times 10^{-4}$	97 ± 7	0.45
Formebolone met.	$(39 \pm 3) \times 10^{-4}$	9	$(51 \pm 4) \times 10^{-4}$	130 ± 10	5.07	$(35 \pm 2) \times 10^{-4}$	91 ± 4	2.13
Formestane	$(152 \pm 14) \times 10^{-3}$	9	$(137 \pm 7) \times 10^{-3}$	90 ± 5	2.10	$(150 \pm 10) \times 10^{-3}$	98 ± 6	0.32
Metandienome m2	$(42 \pm 3) \times 10^{-4}$	8	$(43 \pm 5) \times 10^{-4}$	102 ± 12	0.33	$(47 \pm 6) \times 10^{-4}$	112 ± 15	1.63
Metandienome m3	$(113 \pm 2) \times 10^{-3}$	1	$(134 \pm 18) \times 10^{-3}$	119 ± 16	2.64	$(109 \pm 4) \times 10^{-3}$	97 ± 4	2.00
Pemoline	$(38 \pm 2) \times 10^{-2}$	6	$(39 \pm 3) \times 10^{-2}$	102 ± 7	0.45	$(48 \pm 3) \times 10^{-2}$	126 ± 7	6.21
Oxilofrine triTMS	$(163 \pm 5) \times 10^{-1}$	3	$(192 \pm 27) \times 10^{-1}$	118 ± 16	2.36	$(172 \pm 17) \times 10^{-1}$	105 ± 11	1.08
Octopamine	20 ± 1	5	21 ± 1	104 ± 5	1.20	22 ± 1	112 ± 11	2.24
Pholedrine diTMS	$(43 \pm 2) \times 10^{-1}$	4	$(46 \pm 1) \times 10^{-1}$	106 ± 1	3.72	$(43 \pm 5) \times 10^{-1}$	101 ± 11	0.17
Etilefrine triTMS	$(99 \pm 3) \times 10^{-1}$	3	$(104 \pm 1) \times 10^{-1}$	105 ± 1	3.66	$(104 \pm 12) \times 10^{-1}$	105 ± 12	0.93
Etamivan	$(121 \pm 2) \times 10^{-2}$	2	$(118 \pm 2) \times 10^{-2}$	98 ± 2	2.10	$(136 \pm 16) \times 10^{-2}$	112 ± 13	1.95
Norfenefrine	$(104 \pm 5) \times 10^{-1}$	5	$(107 \pm 5) \times 10^{-1}$	102 ± 4	0.73	$(117 \pm 12) \times 10^{-1}$	112 ± 11	2.25
Carphedon	$(40 \pm 1) \times 10^{-2}$	2	$(33 \pm 2) \times 10^{-2}$	81 ± 4	9.00	$(36 \pm 4) \times 10^{-2}$	89 ± 11	2.29
6-hydroxy-bromantan	$(82 \pm 3) \times 10^{-2}$	4	$(87 \pm 1) \times 10^{-2}$	106 ± 2	3.10	$(92 \pm 1) \times 10^{-2}$	111 ± 2	6.03
Benzoylecgonine	$(192 \pm 4) \times 10^{-2}$	2	$(206 \pm 5) \times 10^{-2}$	107 ± 3	4.57	$(220 \pm 17) \times 10^{-2}$	115 ± 9	3.52
4-OH-amphetamine triTMS	$(12 \pm 1) \times 10^{-2}$	7	$(14 \pm 1) \times 10^{-2}$	119 ± 9	3.88	$(14 \pm 2) \times 10^{-2}$	125 ± 16	3.17
4-OH-amphetamine diTMS	$(35 \pm 1) \times 10^{-1}$	4	$(36 \pm 1) \times 10^{-1}$	104 ± 3	1.94	$(37 \pm 5) \times 10^{-1}$	107 ± 14	1.14
Triamterene	$(347 \pm 8) \times 10^{-3}$	2	$(341 \pm 8) \times 10^{-3}$	98 ± 2	1.29	$(335 \pm 40) \times 10^{-3}$	96 ± 11	0.70
Probenecid	$(344 \pm 8) \times 10^{-2}$	2	$(358 \pm 6) \times 10^{-2}$	104 ± 2	2.97	$(382 \pm 28) \times 10^{-2}$	111 ± 8	2.83

Table V.3 - Derivatisation yield of TMS derivatives formation with MSTFA. Comparison of the thermal conductivity procedure (30 min at 78 °C) with the ultrasonic procedure (50% of amplitude, 3 min) and the microwave procedure (1,200 W of power, 3 min).

	Conventional Procedure		Microwave Method			Ultrasonic Method		
	(A_{AAS}/A_{ISTD})	RSD	(A_{AAS}/A_{ISTD})	Reaction yield (MW / Conventional method ×100, X±SD)	$\|t_{cal}\|^*$	(A_{AAS}/A_{ISTD})	Reaction yield (US / Conventional method ×100, X±SD)	$\|t_{cal}\|^*$
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Norfenefrine	$(104 \pm 5) \times 10^{-1}$	5	$(107 \pm 5) \times 10^{-1}$	102 ± 4	0.73	$(117 \pm 12) \times 10^{-1}$	112 ± 11	2.25
Carphedon	$(40 \pm 1) \times 10^{-2}$	2	$(33 \pm 2) \times 10^{-2}$	81 ± 4	9.00	$(36 \pm 4) \times 10^{-2}$	89 ± 11	2.29
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Benzoylecgonine	$(192 \pm 4) \times 10^{-2}$	2	$(206 \pm 5) \times 10^{-2}$	107 ± 3	4.57	$(220 \pm 17) \times 10^{-2}$	115 ± 9	3.52
4-OH-amphetamine triTMS	$(12 \pm 1) \times 10^{-2}$	7	$(14 \pm 1) \times 10^{-2}$	119 ± 9	3.88	$(14 \pm 2) \times 10^{-2}$	125 ± 16	3.17
4-OH-amphetamine diTMS	$(35 \pm 1) \times 10^{-1}$	4	$(36 \pm 1) \times 10^{-1}$	104 ± 3	1.94	$(37 \pm 5) \times 10^{-1}$	107 ± 14	1.14
Triamterene	$(347 \pm 8) \times 10^{-3}$	2	$(341 \pm 8) \times 10^{-3}$	98 ± 2	1.29	$(335 \pm 40) \times 10^{-3}$	96 ± 11	0.70
Probenecid	$(344 \pm 8) \times 10^{-2}$	2	$(358 \pm 6) \times 10^{-2}$	104 ± 2	2.97	$(382 \pm 28) \times 10^{-2}$	111 ± 8	2.83

V.4.3 ANALYTICAL APPLICATION

In order to study the reliability of the method, both accelerated procedures were applied to a control urine sample. For this experimental assay only the endogenous AAS from urine were used because no more than a few conjugated standards are commercially available. Both hydrolysis and derivatisation steps were accelerated by using the microwave and ultrasonic methods. Concerning ultrasonication, the hydrolysis step was accelerated with the sonoreactor operating at 60% of amplitude for 10 min and the derivatisation accelerated with the sonoreactor operating at 50% of amplitude for 3 min. Concerning the microwave energy, the hydrolysis reaction was carried out inside the microwave system operating at 240 W for 10 min and the derivatisation was accelerated at 1200 W for 3 min. The results were compared with the conventional procedure (hydrolysis at 55 °C for 1 h and derivatisation at 78 °C for 30 min) and are shown in Table V.4. Only with the ultrasonic procedure were the results obtained comparable with the conventional method. Furthermore, the precision and accuracy of the ultrasonic procedure indicates that this method shows sufficient robustness for quantitative analysis under routine conditions. Regarding the microwave method, the hydrolysis yields are still far from those of the conventional method for compounds of great importance such as androsterone and 5β-androstane-3α,17β-diol, not to mention the 11-hydroxy-etiocholanolone and 11-hydroxy-androsterone, whose yields are around 50%.

V.5 CONCLUSION

This work shows that both ultrasonic and microwave energy can be used to improve and simplify the sample treatment in routine analysis of many banned substances for doping control. Under ultrasonic energy the two key steps of the sample treatment were improved. The new approach is less time consuming and has the same reproducibility in terms of accuracy as the traditional method. Moreover, for 18 of the banned compounds studied the ultrasonic assisted method was able to improve the derivatisation yields. Concerning the microwave energy assisted method this study demonstrates that it can also be successfully used to improve the derivatisation reaction time and, most importantly, the analytical sensitivity of some compounds, since for 18 of the studied substances the derivatisation yield was improved. Regarding the derivatisation reaction, both accelerated methods showed similar results; however, the microwave energy assisted method presents higher sample throughput. Nevertheless, the microwave energy fails in efficiently enhancing the enzymatic hydrolysis of the majority of the steroids tested.

Overall, this study suggests that the use of ultrasonic energy could be very effective as a tool to accelerate doping control tests carried out at major international events, where one of the main tasks for the analytical laboratory is to perform analyses as quickly and efficiently as possible.

Table V.4 - Comparison of the microwave and ultrasonic procedures with the classic procedure. The recoveries are calculated as the ratio yield between the microwave and ultrasonic method and the classic method and are expressed as percentages.

	Conventional Procedure		Microwave Method			Ultrasonic Method		
	(ng/mL; n=5)	RSD	(ng/mL; n=5)	Reaction yield (MW / Conventional method ×100, X±SD)	$\|t_{cal}\|$*	(ng/mL; n=5)	Reaction yield (US / Conventional method ×100, X±SD)	$\|t_{cal}\|$*
Testosterone	78 ± 3	4	77 ± 1	99 ± 1	0.65	77 ± 1	99 ± 1	0.65
Epitestosterone	94 ± 2	2	95 ± 1	101 ± 1	0.60	94 ± 2	100 ± 2	0.02
Androsterone	3923 ± 135	3	3095 ± 234	79 ± 6	6.85	3625 ± 256	92 ± 7	2.30
Etiocholanolone	2837 ± 68	2	2633 ± 257	93 ± 9	1.72	2755 ± 46	97 ± 2	2.24
5αAndrostane-3α17β-diol	103 ± 8	8	99 ± 8	96 ± 8	0.78	93 ± 6	91 ± 6	2.17
5βAndrostane-3α17β-diol	225 ± 8	3	192 ± 9	85 ± 4	6.05	210 ± 16	93 ± 7	1.83
DHEA	41 ± 2	5	36 ± 1	89 ± 2	4.48	39 ± 1	95 ± 2	2.16
Pregnanediol	468 ± 20	4	459 ± 14	98 ± 3	0.78	455 ± 3	97 ± 1	1.48
11-hydroxy-etiocholanolone	924 ± 26	3	417 ± 55	45 ± 6	18.55	677 ± 51	73 ± 6	9.62
11-hydroxy-androsterone	240 ± 9	4	125 ± 16	52 ± 7	14.21	174 ± 9	72 ± 4	11.76

*t_{cal} is a t-test that is calculated by comparison of the two experimental means obtained from the two different methods. The difference between both methods is significant when $|t_{cal}|$ is higher than the critical value given by the theoretic t-test at a determinate Probability distribution.

n = number of replicates.

$t_{theoretic}$ = 2,31; p = 0,05

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CHAPTER VI

Unravelling the role of ultrasonic energy in the enhancement of enzymatic kinetics

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VI.1 ABSTRACT

The enzymatic hydrolysis of anabolic androgenic steroids excreted to urine as glucuronide conjugates has been recently reported to be improved by applying ultrasonic energy to the reaction medium. The hydrolysis time using β-glucuronidase from *Escherichia coli K12* was reduced by a factor of six when ultrasonic energy was employed to enhance the enzymatic kinetic.

In this study, the effect of ultrasonic energy on the enzymatic hydrolysis kinetic parameters, as well as on the enzymatic activity of β-glucuronidase from *Escherichia coli K12* was assessed. The study was done using the compounds 4-nitrophenyl-β-D-glucuronide and 4-nitrophenol. Experimental data suggested that the reaction follows the Michaelis–Menten kinetic`s type. In addition it was found that the ultrasonic energy affects the initial velocity of reaction, which is higher when ultrasound waves are employed when compared to the classical method of incubation at 55ºC. Moreover the values of V_{max} and k_{cat} are higher for the ultrasonic essay ($V_{max(US)}$ = 17.1 ± 0.8 $\mu M min^{-1}$; $k_{cat\ (US)}$ = 340523 min^{-1}; $V_{max\ (55ºC)}$ = 14.8 ± 0.7 $\mu M\ min^{-1}$; $k_{cat\ (55ºC)}$ = 295187 min^{-1}) whilst the Michaelis–Menten constant obtained for the two methodologies showed similar values ($K_{M\ (US)}$ = 94.7 ± 7.2 μM; $K_{M(55ºC)}$= 92.5 ± 6.6 μM). Denaturation of the enzyme was also observed under the effects of ultrasonic energy, which was found particularly evident for experimental conditions with excess of substrate. This research work seems to support the idea that the use of ultrasonic energy affects the transition state of the enzymatic reaction and that mass transfer processes are also most likely enhanced.

VI.2 INTRODUCTION

The administration of anabolic steroids has been the most frequent offence to fair competition inside sports. Their use was first introduced as agents supporting the athlete recuperation after extreme stress and fatigue, but rapidly became the main group in doping abuse [1]. In the World Anti-Doping Agency (WADA) statistic report of 2009, the anabolic agents, in which is included the group of androgenic anabolic steroids (AAS), represented 64.9 % of all adverse analytical findings reported by WADAs´ accredited laboratories [2].

Due to their non-polar character, most AAS are modified in the human body by phase-I and phase-II metabolic reactions, prior to their excretion in urine [3]. Phase-I metabolism consists in enzymatic reactions (e.g., oxidation, reduction, or hydroxylation) that inactivate the drug and increase its polarity by adding or exposing functional sites into the AAS structure, where phase II metabolism can subsequently occur [1, 3, 4]. The Phase II metabolism can be characterized as the conjugation reactions that change the physicochemical properties of the compounds in order to promote its elimination from the body [1, 5]. The main phase-II reactions are the conjugation with glucuronic acid or sulfate moiety [1, 3]. However, for the AAS, the formation of glucuronate conjugates is the major metabolic pathway of conjugation and inactivation of these compounds [1, 3, 6, 7]. The result of both phase-I and phase-II reactions is the synthesis of hydrophilic compounds that are more easily excreted. Doping control of AAS is based on the detection of such compounds and its metabolites in urine samples from athletes [8, 9]. Currently, the routine procedure for the detection of these compounds, comprising both screening and confirmatory analysis, is typically carried out by gas chromatography – mass spectrometry (GC-MS) methodologies [10-13]. It is important to stress out that to be analysed by GC-MS techniques, conjugated steroids must be hydrolysed and derivatised to fit GC analysis pre-requisites and improve its mass spectrometric characteristics.

Of crucial importance, is the step of the enzymatic hydrolysis of the steroid glucuronides that is carried out directly on the urine sample with the enzyme β-glucuronidase (GUS, EC 3.2.1.31) from E. coli, which is a hydrolase highly specific to β-linked D-glucuronides [14]. Within anti-doping laboratories, the cleavage of the glucuronide moiety to produce the free compound is normally performed by conventional warming at 55ºC for 1 hour, which was found to be the optimum temperature to achieve maximum yield [15-19].

Over the past years, the increasing complexity of doping control, mainly due to the continuously introduction in anti-doping regulations of new banned substances and methods and also due to the increasing workloads inside anti-doping laboratories, has led scientists to develop new strategies for fast and high throughput sample treatments and analysis.

Modern approaches to sample preparation show a growing interest in ultrasonic energy as an alternative to conventional methods. The ability of ultrasonic irradiation methods to accelerate and, occasionally, to increase the chemical reactions yield have attracted the scientific community for its use over the last decades. Despite the widespread use of ultrasonic energy in various research

disciplines, only recently the synergetic use of ultrasonication and enzymes to enhance reactions has been described [20-23].

Briefly, the vibrational motion transmitted to the liquid medium induced by ultrasound waves causes alternately expansion and compression of the medium [24]. At sufficiently high power, the strength of the acoustic field may exceed the attractive forces of the particles in the expansion or rarefaction cycle and create cavities in the liquid medium called cavitation bubbles, which can grow, oscillate, split and implode [25-28]. It is this phenomenon, of cavitational bubble implosion, that produces the powerful shearing, causing the molecules in the liquid to become intensely agitated, as extreme temperatures and pressures are generated, acting like micro-reactors inside the liquid media [25, 26]. Moreover, the formation of highly reactive chemical radicals may also take place inside the liquid media [25, 26].

Although the improvement of chemical reactions is easily explained by the cavitation phenomena, the understanding of the synergetic effect between ultrasounds and enzymes is still far from being completely understood. Nevertheless, some authors have pointed out that those phenomena created by ultrasonic energy, increases the contact area between phases, which allows a reduction of mass transfer limitations in the enzyme–substrate system [29].

In recent years, our research group has focused on the development of ultrasonic-assisted enzymatic reactions as a tool to enhance laborious and tedious reactions. In this sense and in collaboration with the WADA accredited anti-doping laboratory of Rome, in Italy, it has been recently demonstrated that high intensity ultrasonic energy enhances the enzymatic hydrolysis of AAS with β-glucuronidase [30]. Althrough this is a remarkable finding, there is still a lack of fundamental research about the effects of ultrasonic energy on the enzyme β-glucuronidase catalytic activity.

In the present work the effects of ultrasonic energy on the kinetics of β-glucuronidase enzyme were evaluated using the compound p-nitrophenyl–β-glucuronide, which is commonly used for β-glucuronidase enzymatic essays. A simple theoretical kinetic model, base on Michaelis-Menten equation, is introduced in order to investigate the variations in the equation kinetic parameters. The results of the ultrasonic based enzymatic studies were compared with those obtained by the routine warming incubation at 55ºC.

VI.3 EXPERIMENTAL

VI.3.1 APPARATUS

A cup horn sonoreactor (SR), model from Dr. Hielscher (Teltow, Germany), was used to accelerate the enzymatic hydrolysis procedure. A minicentrifuge, model Spectrafuge-mini, from Labnet (Madrid, Spain), and a minicentrifuge-vortex, model Sky Line, from ELMI (Riga, Latvia) were used throughout the sample treatment. A Simplicity 185 from Millipore (Milan, Italy) was used to obtain Milli-Q

water. The enzymatic hydrolysis of 4-nitrophenyl-β-D-glucuronide was performed in 2 mL microtube flat cap from Delta Lab (Barcelona, Spain).

VI.3.2 STANDARDS AND REAGENTS

The standards of 4-Nitrophenyl-β-D-glucuronide and 4-Nitrophenol were purchased from Sigma-Aldrich (Steinheim, Germany). Sodium hydrogen phosphate and sodium phosphate dibasic were purchased from Sigma-Aldrich. A commercial solution of β-glucuronidase from *Escherichia coli K12* with a specific activity of 185 U/mg at 37ºC and pH 7 with nitrophenyl-β-D-glucuronidase as substrate (1 mL contained 1.47 mg of protein) was purchased from Roche Diagnostic (Mannheim, Germany). Methanol (MeOH) was purchased from Sigma-Aldrich and trifluoroacetic acid (TFA, 99%) was from Riedel-de Haën.

Individual stock standard solutions of each compound with 0.05 M were prepared in 10 mL volumetric flasks with methanol. These standard solutions were stored in the dark at -20 ºC. Working standard solutions were prepared by dilution of the stock standard solutions in the appropriate volume of methanol.

VI.3.3 CHROMATOGRAPHIC SYSTEM AND OPERATING CONDITIONS

To analyze the hydrolysis of the 4-nitrophenyl-β-D-glucuronide, measurements were carried out by HPLC, using a Waters 600E gradient metering pump model equipped with a Waters dual channel UV-visible detector. Samples were injected through a 20μL loop injector valve (Rheodyne Model 7120) and the chromatographic separation was performed on reversed phase 5 μm particle size, L × I.D. 15 cm × 4.6 mm Discovery® C18 column from Supelco (Bellefonte, Pennsylvania). The aqueous mobile phase (A) consisted of HPLC-grade water with 0.01% TFA; the organic phase (B) was MeOH. The HPLC elution was carried out under isocratic conditions, with 55% of solution A and 45% of solution B. The flow rate was 0.9 mL/min with a 10 min run time and the UV detector was set at the wavelength of 305nm. Calibration curves were constructed by plotting peak areas against concentrations of PNP. Throughout the experimental work, data was collected and integrated using Chromeleon Software. For quantification, the peak areas were determined, and the concentrations calculated with external calibration curves. The calibration curves for PNP and PNP-G were run at the beginning of each week. The calibration curves were calculated by the least square method. Linearity was assessed by determining the coefficient of correlation (r^2) of the points of the curves. Both PNP and PNP-G showed linear response in the target concentration range of 0.4 to 500 μM (PNP) and 5 to 600 μM (PNP-G). A representative calibration curve for both PNP-G and PNP is shown in Figure VI.1.

To ensure the analytical quality of the calibration curve made at the beginning of each working week, a set of standard samples with different PNP-G and PNP concentrations were prepared and measured on each day at routine intervals.
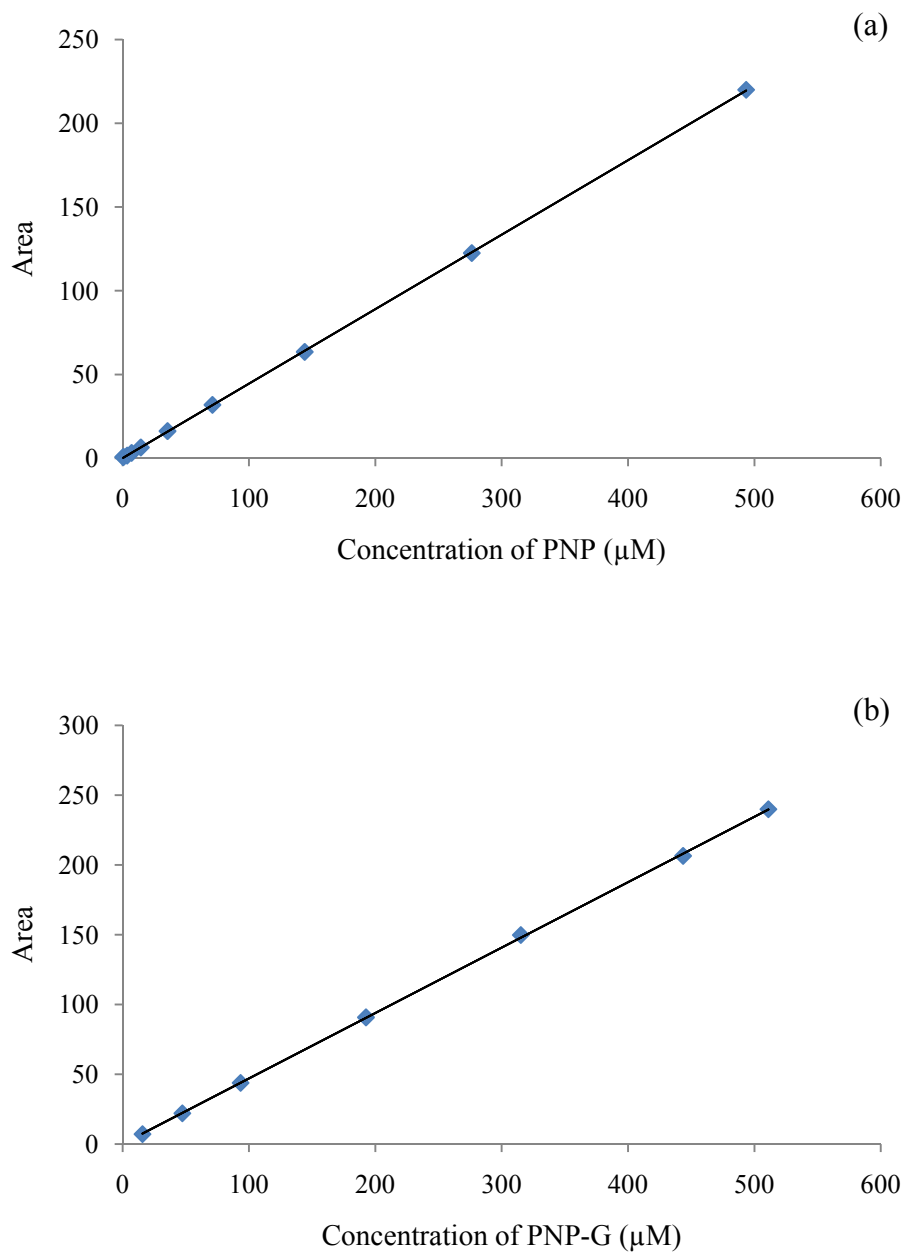
Figure VI.1 - Calibration curve obtained following HPLC-UV analysis by plotting the area of the chromatographic peak corresponding to (a) PNP and (b) PNP-G as a function of concentration. The calibration equations obtained were $y = (0.445 \pm 0.002)x + (0.1 \pm 0.3)$, $(r^2=0.9999)$, and $y= (0.469 \pm 0.006)x + (0.2 \pm 0.9)$, $(r^2=0.9998)$, for PNP and PNP-G, respectively.

VI.3.4 SAMPLE TREATMENT

Spiked sample solutions of 4-nitrophenyl-β-D-glucuronide were prepared in sodium phosphate buffer (0.1M; pH 6.8). The commercial solution of β-glucuronidase from *Escherichia coli* K12 was diluted in the same buffer (1:13). To 2mL of the spiked samples, 2 µL of the prepared enzyme solution, corresponding to 0.22µg of enzyme, was added and the enzymatic assay was carried out using two different systems as follows: a) employing conventional warming at 55ºC and b) using ultrasonic energy provided by a cup horn sonoreactor (SR) operating at 60 % of sonication amplitude. The ultrasonic amplitude was chosen based on our previous study with AAS [30]. After the hydrolysis reaction the enzyme was denatured by addiction of 10µL of TFA.

The calibration standards were prepared in 10mL volumetric flasks. In order to have the same matrix than the samples, calibration solutions were made in sodium phosphate buffer (0.1M; pH 6.8) to which was added 50µL of TFA.

VI.4 RESULTS AND DISCUSSION

The aim of this work was to compare the enzymatic activity of β-glucuronidase and the hydrolysis kinetic parameters under ultrasonic irradiation with the conventional reaction at 55ºC. For this purpose PNP-G was used as substrate. Figure VI.2 shows the hydrolytic conversion of PNP-G to PNP. It is important to stress that for all conditions assayed, three replicates were performed and that the relative standard deviation, RSD, associated was always below 9%.
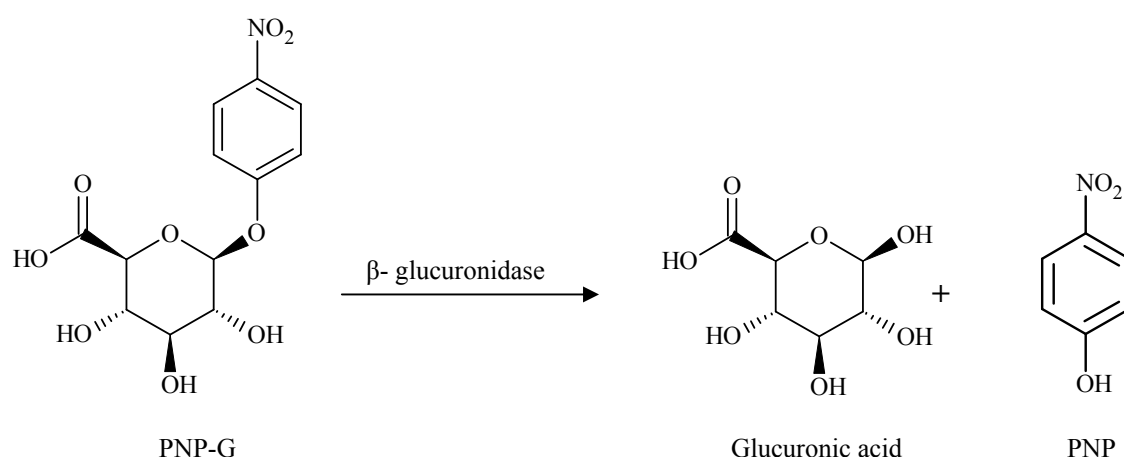

Figure VI.2 - Hydrolytic conversion of substrate PNP-G to the products PNP and D-glucuronic acid through the action of β-glucuronidase.

VI.4.1 CHROMATOGRAPHIC ASSAY TO MEASURE THE ACTIVITY OF β-GLUCURONIDASE

In order to assess the effect of ultrasonication on the kinetic of the enzymatic hydrolysis, both product formation and substrate disappearing rate were measured. Separation of the reaction mixture and detection of its components were carried out by HPLC, using UV detection at λ=305nm, which was found to be the optimal wavelength for detection of PNP-G and PNP. A representative chromatogram showing PNP-G and PNP peaks, obtained after 4 minutes of incubation with β-glucuronidase, is presented in Figure VI.3. As it may be seen, only two well resolved peaks corresponding to PNP-G and PNP are observed, making the analysis of the reaction mixture simple to achieve.

To correctly estimate the activity of β-glucuronidase under an ultrasonic field, before the enzymatic assay, the chemical stability of both reaction components was evaluated. Experiments were carried out and the concentration of both components was measured before and after ultrasonication (10 minutes at 60% of amplitude). For this study five replicates were used. The results obtained showed that both compounds remain stable after application of ultrasonic energy to the sample`s medium, exhibiting the same concentration before and after ultrasonication.
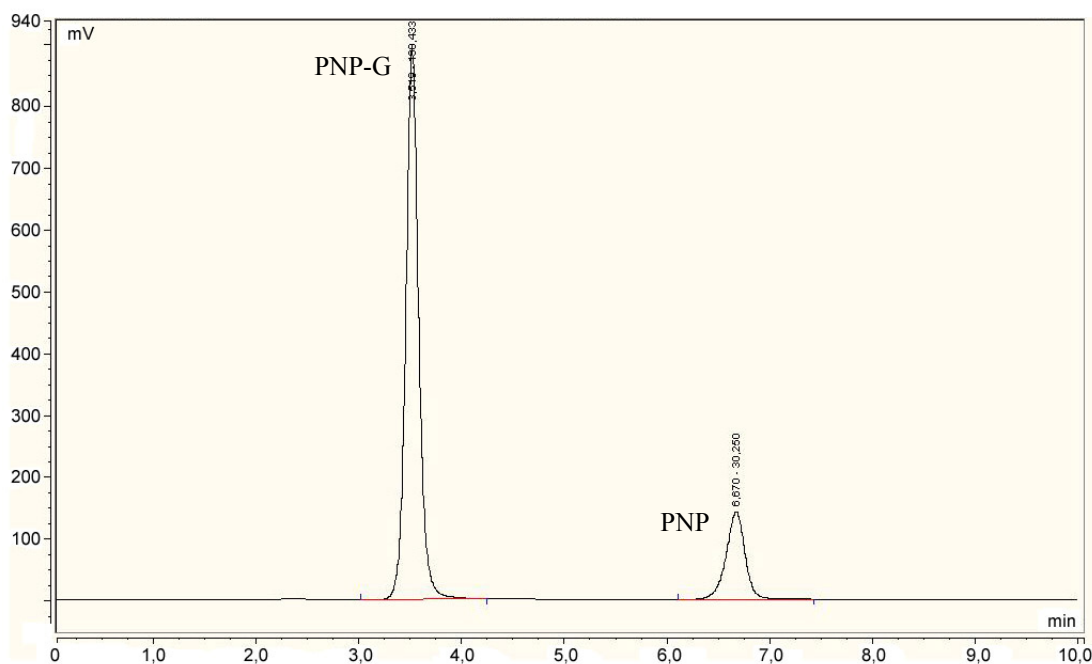

Figure VI.3 - Representative chromatogram obtained after 4 minutes of incubation of the substrate PNP-G with the enzyme β-glucuronidase. The first peak elutes at 3.51 min and corresponds to PNP-G, whereas the second peak elutes at 6.67 min and corresponds to PNP.

VI.4.2 β-GLUCURONIDASE ACTIVITY/ STABILITY UNDER ULTRASONIC IRRADIATION

The use of ultrasonic energy to enhance enzymatic reactions is still a matter of great debate within the scientific community. Recent studies focusing on the influence of ultrasonic energy in the enzyme performance have reported both enhancement and inactivation of enzyme activity [31-34]. Based on our expertise concerning the application of ultrasonic energy to enhance a variety of reactions and, particularly, enzymatic reactions, we have found that, in fact, activation and inactivation occur and are closely linked to the ultrasonic operating conditions [23, 30, 35-40]. Parameters such as ultrasonic frequency and amplitude, vial shape, time of reaction, as well as enzyme amount and temperature of the reaction bulk, deeply affect the performance of the reaction. Therefore, a consistent knowledge on the ultrasonic energy performance and ultrasonic devices´ characteristics is vital to carry out a successful experiment. For instance, when ultrasonication is applied to a liquid, a slow but constant increase in the bulk temperature occurs and, depending on the enzyme, denaturation may occur, not due to ultrasonic waves itself but to the warming generated.

To evaluate the effect of ultrasonic irradiation in both enzymatic activity and stability of β-glucuronidase, the appearance of PNP as a function of time under ultrasonic irradiation was compared with the conventional methodology of incubation at 55ºC. Experimental data was achieved using large excess of substrate; 450 μM of PNP-G and 0.22μg of β-glucuronidase. Figure VI.4 presents the appearance of PNP as a function of time and the correspondent relative enzyme activity, for both essays. The unit of the enzyme activity was considered as the enzyme activity that increases the rate of release of 1μmol of PNP per minute. As it may be seen in Figure VI.4b, the enzymatic activity under ultrasonic energy is, in the first minutes of the enzymatic reaction, higher than the one obtained under thermal incubation at 55ºC. Yet, the enzyme activity quickly decreases after 6 minutes of reaction, unlike the reaction under thermal incubation that stays almost constant along the time studied.


(a)
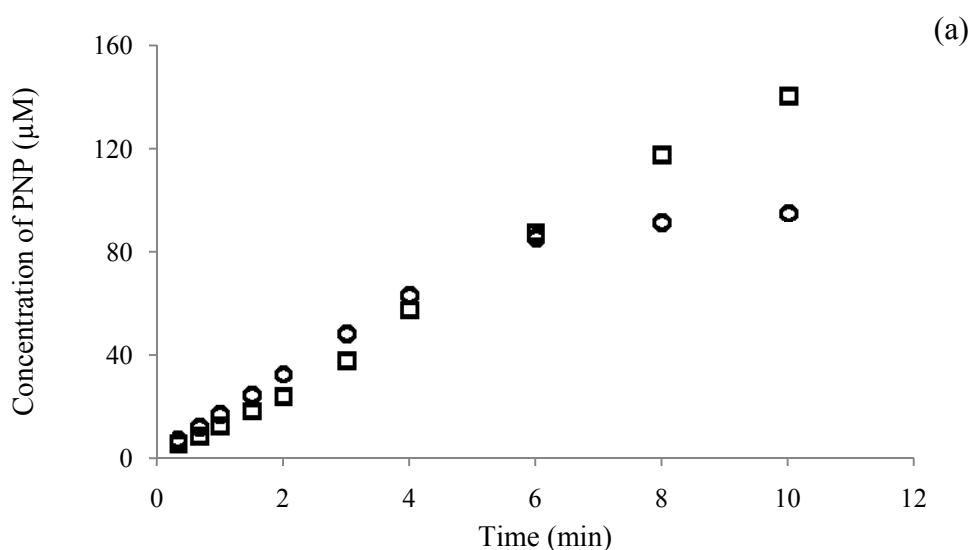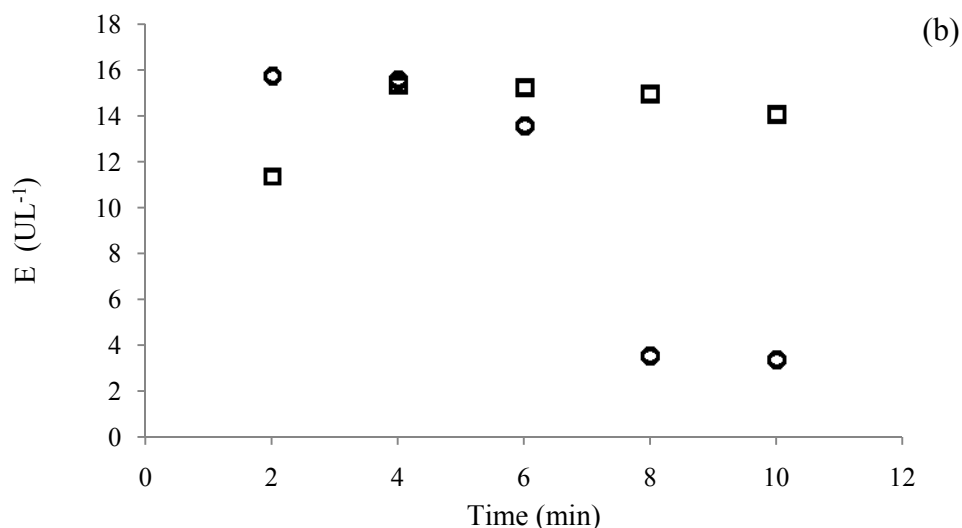
Figure VI.4 - (a) Appearance of PNP as a function of time for both incubation at 55ºC and ultrasonic irradiation essays. The experimental results were obtained using 450 μM of PNP-G. (c) Correspondent enzyme activity for both experiments. The unit of enzymatic activity was considered as the enzyme activity that increases the rate of release of 1μmol of PNP per minute. Data identification: ◻**:** 55ºC; ◯**:** Ultrasonic irradiation.

Slow inactivation of the enzyme under high ultrasonic irradiation was expected; nevertheless, when compared with our previous work, in which the enhancement of the AAS glucuronates hydrolysis by ultrasonic irradiation was studied, the enzymatic inactivation of β-glucuronidase occurred faster. The major difference between the two enzymatic assay conditions, concerns the fact that the experimental procedure developed in this study was carried out with low amount of enzyme, at large excess of substrate. Concerning our previous study on the hydrolysis of AAS, the amount of enzyme was in large excess; almost 250 fold higher than the value used in the present study [30].

To get further insight into the relation between the amount of enzyme and the enhancement of the hydrolysis reaction by ultrasonic irradiation, a set of experiments was devised in which the amount of enzyme varied whilst the concentration of substrate was maintained constant at 450 μM. Three enzyme amounts were used: 0.22μg, 0.33μg and 0.44μg. Figure VI.5 shows the relative enzyme activity for the three enzyme concentrations. As it may be seen, by increasing the amount of enzyme, the enzymatic activity is kept constant for longer reaction times. With 0.33μg of enzyme, the rate of PNP appearance increased until 8 minutes of reaction and then decreased abruptly. Likewise, by using 0.44μg of enzyme, the rate of PNP still reaches its maximum at 8 min, but at 10 min, the enzymatic activity drop is not so significant and it corresponds to 86% of the maximum activity. This result clearly indicates that the amount of enzyme is determinant under ultrasonic irradiation. In fact, at this substrate concentration value and with 0.22 μg of β-glucuronidase, the enzyme already reached its substrate saturation point (see Section VI.4.3), meaning that most active sites of the enzyme are

occupied. Therefore, the inactivation of the enzyme by ultrasonic waves has a deep effect on the product appearance rate. When the amount of enzyme is increased, the slow inactivation that occurs during ultrasonication is suppressed by the positive effect of ultrasonication on the enzyme activity.
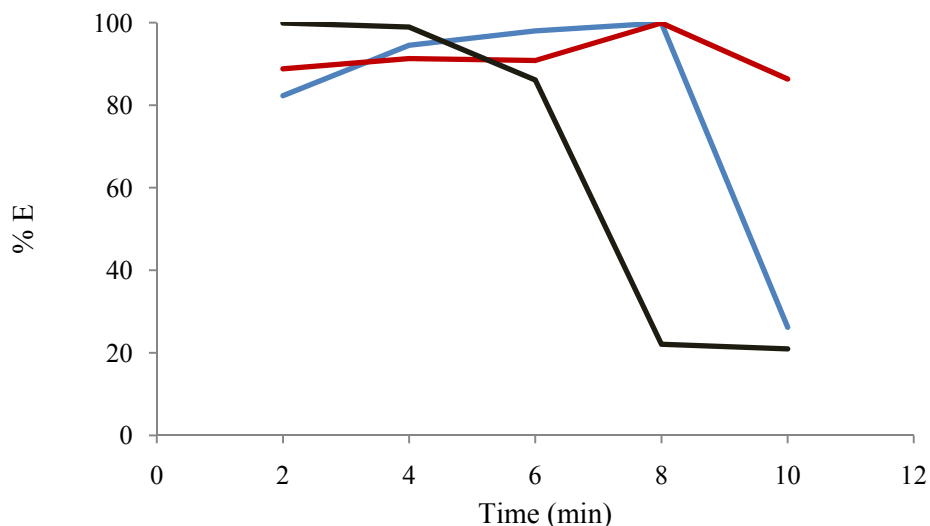
Figure VI.5 - Relative enzyme activity for the release of PNP from PNP-G. The concentration of PNP-G was fixed at 450 µM and the amount of β-glucuronidase varied from 0.22, 0.33 and 0.44 µg. Data identification: **black line:** 0.22 µg; **blue line:** 0.33 µg; **red line:** 0.44 µg.

Concerning our previous study on the hydrolysis of AAS, the amount of enzyme was in large excess; as a result, in the first ten minutes of the AAS hydrolysis reaction, the slow inactivation of the enzyme was counterbalanced by the positive ultrasonic effects on the enzyme activity. It is important to stress that, in our previous study, the hydrolysis reaction time of AAS glucuronates was greatly improved from 60 min to 10 min by using ultrasonic irradiation together with β-glucuronidase [30].

These results clearly suggest that special attention must be taken regarding the amount of enzyme, when ultrasonic waves are employed to enhance the enzymatic reaction. To diminish the effects of enzyme denaturation on the reaction rate, a slight excess of enzyme is required.

VI.4.3 β-GLUCURONIDASE KINETIC PARAMETERS

Through a brief literature search, in which we found several reports highlighting the use of ultrasound for numerous applications, it became evident that few attempts were made to understand the effect of ultrasonic irradiation on the kinetic parameters of the mentioned enzymatic reactions.

In this work, to assess the effect of ultrasonic irradiation on the reaction kinetic parameters, the enzymatic study was performed using initial rate experiments, by varying the concentration of substrate in the presence of a fixed concentration of enzyme. The initial velocity for a given concentration of PNP-G was determined by plotting the enzyme reaction time as a function of PNP concentration. Ten PNP-G concentration values were used in this study, ranging from 7.75 to 540 µM. Although the reaction time ranged from 20 seconds to 10 minutes, the initial velocities were calculated using the first 2 minutes of the reaction. Figure VI.6 presents the time courses for the appearance of PNP and the initial velocity values obtained as a function of the correspondent PNP-G concentration values, for both ultrasonic and incubation at 55ºC essays. As it was expected, the reaction velocity increased with the increase in PNP-G concentration until it reaches the substrate saturation for 0.22µg of enzyme. The enzyme substrate saturation for the ultrasonic irradiation essay occurred at a higher concentration value than for the thermal incubation essay.
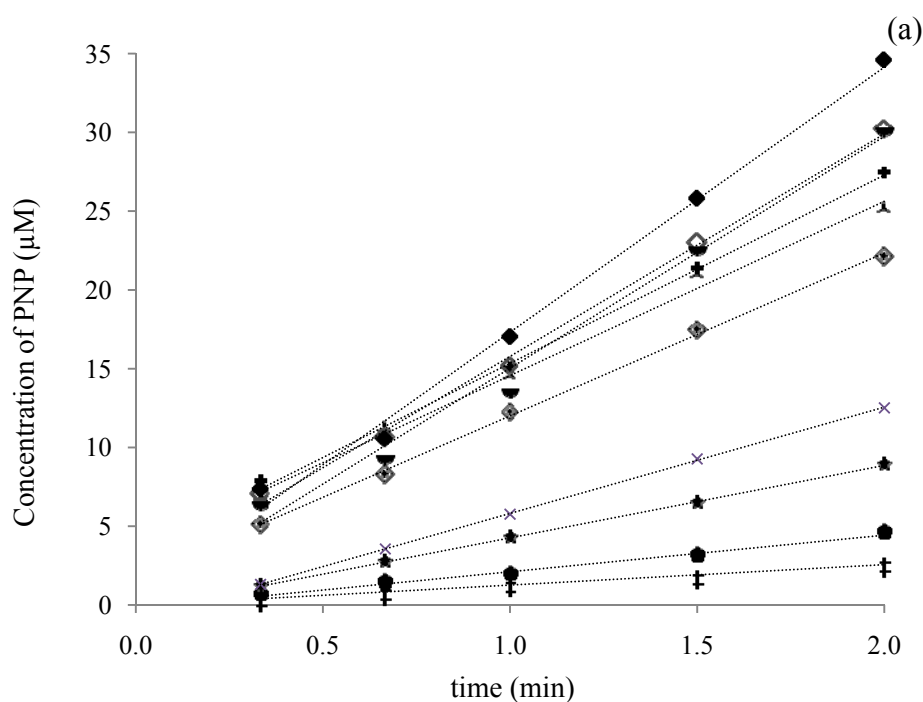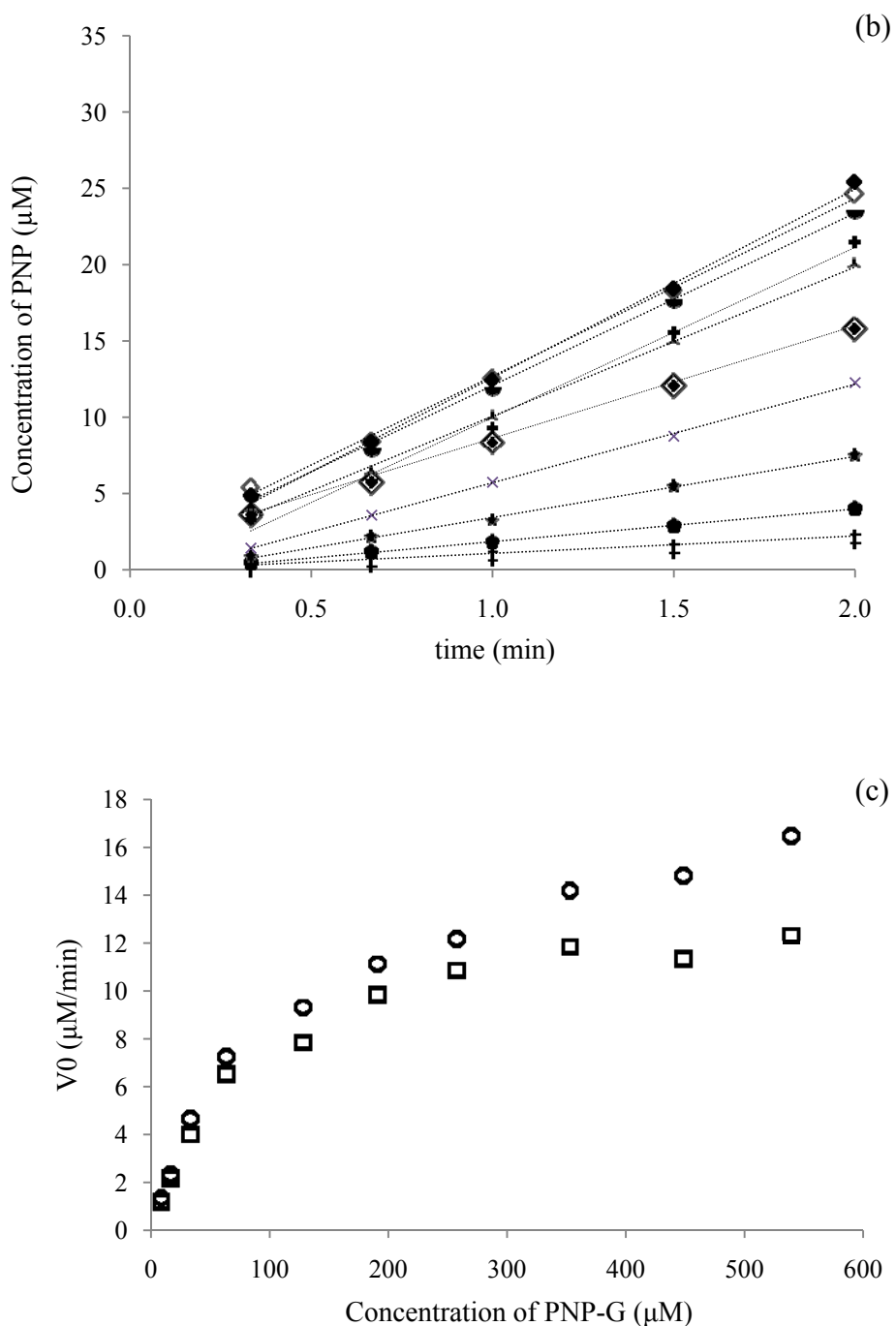
Figure VI.6 - Observed time courses for the hydrolytic conversion of PNP-G into PNP (a) at 55ºC and (b) under continuous ultrasonic irradiation, with increased PNP-G concentration. Data identification: ‡: 7.7 µM; ⬣: 15.4 µM; ★: 32.1 µM; ×: 62.9 µM; ◈: 127.3 µM; ⅄: 190.1 µM; ✚: 257.0 µM; ◇: 352.4 µM; ⬗: 448.3 µM; ◆: 539.3 µM. (c) Plot of the initial velocity values obtained as a function of the correspondent PNP-G concentration values. Data identification: ☐ **line:** 55ºC; ◯ **line:** Ultrasonic irradiation.

The kinetic parameters were determined through curve fitting, using a linear transformation of the Michaelis–Menten equation, the Lineweaver-Burk plot, in which the reciprocal of the initial rate, $1/V_0$, was considered against the reciprocal of the substrate concentration $1/[PNP-G]$ (see Figure VI.7). The data obtained was used to display the four fundamental kinetic constants for one substrate reactions: the maximal velocity of the reaction (V_{max}), the catalytic constant (k_{cat}), the Michaelis constant (K_M), and the specificity constant (k_{cat} / K_M). With the equation line of the Lineweaver-Burk plot, the value of V_{max} can be obtained from the intercept and the value of K_M/V_{max} from the slope. The catalytic constant is obtained from the total concentration of enzyme in the reaction medium, 5.01E-05 µM, and the value of the maximal velocity of the reaction. Results are shown in table VI.1. The K_M values found for both incubation at 55ºC and ultrasonic irradiation are very similar, meaning that the affinity between the enzyme and the substrate is not affected by ultrasonication. Unlike the kinetic parameter K_M, the quantity V_{max}, which reflects the limiting rate of the enzymatic reaction at substrate saturation, was positively affected by ultrasonic irradiation. Likewise, both the catalytic and specificity constants are higher under the effects of an ultrasonic field, which means that ultrasonic energy positively affects the catalytic efficiency of β-glucuronidase and, therefore, the substrate is converted into the product at an increased rate when compared with the same reaction at 55ºC.
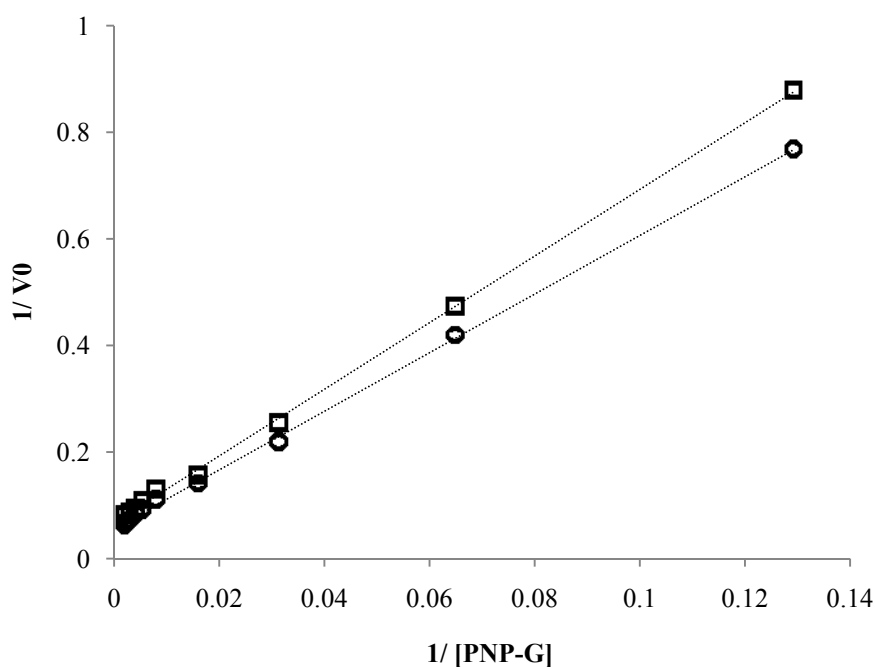
Figure VI.7 - Lineweaver-Burk plot for β-glucuronidase. Enzymatic activity was assayed with 0.22µg of enzyme at pH 6.5. The x and y axes indicate the reciprocals of the initial concentration of PNP-G and initial velocity, respectively. The value of V_{max} is given from the intercept and the value of K_M/V_{max} from the slope. Data identification: ◻ **line:** incubation at 55ºC essay; y= (6.3 ± 0.2)x + (0.067 ± 0.003), (r^2=0.9992); ◯ **line:** ultrasonic irradiation essay; y=(5.5 ± 0.1)x + (0.059 ± 0.003), (r^2=0.9979).

Given these results, we hypothesised that when ultrasonic energy is delivered into the reaction medium, it has little effect on the formation of the Enzyme-Substrate (ES) complex, since the kinetic parameters that reflect the enzyme/ substrate equilibrium, K_M, remain unaltered. Nevertheless, the ultrasonic energy must contribute to stabilise the transition state, for instance, by lowering the activation energy associated with the enzyme/ substrate transition state, since the overall rate of the reaction is affected. Moreover, the shockwaves created by cavitation bubble implosion will affect the kinetic energy of the reaction medium molecules, which probably affects the mass transfer processes between the enzyme and substrate.

Table VI.1 - Catalytic parameters of β-glucuronidase from *E.coli K12* using PNP-G as substrate.

	K_M	V_m	k_{cat}	k_{cat} / K_M
	μM	$\mu M\ min^{-1}$	min^{-1}	$\mu M^{-1}\ min^{-1}$
Incubation at 55ºC	92.5 ± 6.6	14.8 ± 0.7	295187	3192
Ultrasonic irradiation	94.8 ± 7.2	17.1 ± 0.8	340523	3593

VI.5 CONCLUSION

We have verified that ultrasonic energy enhances β-glucuronidase enzymatic kinetics by increasing both initial velocity and maximum velocity. It has been assessed that the reaction follows the Michaelis-Menten equation, remaining the respective constant unaltered. The results obtained indicate, as a first approach, that the improvement caused by ultrasonic energy is mainly due to the stabilisation of the transition state and the enhancement of mass transfer processes. It has been also proved that, at substrate excess, the positive effects of high intensity ultrasonication are quickly surpassed by the inactivation of β-glucuronidase enzyme. Hence, when ultrasonic energy is used to enhance enzymatic reactions, a concentration of enzyme superior to the one regularly used is advised.

The results obtained in this study represent an important step to understand and identify the effects of ultrasonication in the enzyme-substrate complex interaction. When compared with our previous study conducted with AAS, the effects of ultrasonication to enhance β-glucuronidase activity, using PNP-G as substrate are less accentuated. We attribute this result to the nature of substrate; the harder is the hydrolysis reaction achieved by conventional thermal incubation, more evident and advantageous is the use of high intensity ultrasonication.

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PART FOUR

GENERAL DISCUSSION AND CONCLUSIONS

CHAPTER VII

VII.1 GENERAL DISCUSSION AND CONCLUSIONS

In modern society, sports have become incredibly competitive, reaching high levels of intensity, far above the physical capacity and performance of common sportsmen. Moreover, sports "backstage" activities, such as game awards, athletes' wages and transfers, involve increasingly amounts of money. All together, this has led to an unbridled pursuit to achieve optimum physical shape.

While most athletes pursuit their athletic excellence through their natural talent, some do not respect the rules of fair competition and use all means to achieve their goals.

The use of doping substances to enhance physical performance is as old as sport itself. However, in the last decades, the development and use of new doping substances and methods increased as a consequence of anti-doping measures approved by international and national organisations, in which falls a commitment to increase the volume of research dedicated to improving and developing new detection means. WADA anti-doping laboratories play a vital role to ensure clean sport competition by leading the scientific research on this subject. Consequently, the number of analysis performed by anti-doping laboratories is ever-increasing and, therefore, the development and optimisation of new methods is demanded.

This thesis focused in the development and optimisation of both new and already implemented methodologies to improve the detection of banned substances, mainly anabolic androgenic steroids.

The new strategy for analysis of doping substances developed in this thesis was based on the MALDI ionisation technique coupled with mass spectrometry. The experimental work within this thesis allowed us to surpass the major limitation of MALDI techniques regarding the analysis of small molecules, by derivatisation of the AAS and by finding an adequate matrix, from a pool of several matrices, showing good ionisation yield and no interference in the AAS mass window. Furthermore, with this matrix, 2-(4-hydroxyphenylazo) benzoic acid, the detection limit for all the AAS studied was around 10ng/mL, which is similar to the detection limit showed by the conventional methodology, GC-MS, for the analysis of these same compounds.

In order to compete with other methodologies that are deep-rooted within anti-doping laboratories, not only the developed methodology must be easily and quickly executed, but also the data retrieved has to be easily interpreted. For this reason, and to be suitable for routine analysis of AAS, it was also developed software capable of receiving the data

generated by the device and produce reports easily interpreted by the analyst. The MLibrary software operates on excel comma-separated-values, CSV, files using the centroid mass and relative intensity data extracted from the MALDI-MS(/MS) software. The software retrieves and displays the report according to the operating mode of the MALDI. In MS mode, the software retrieves the identity of possible positives, while in MS/MS mode, the software retrieves several information, such as, the fragmentation ions that matched with the reference data, the coverage of those masses within the reference data and a percentage score for the most intense ions in both experimental and reference data. Moreover, it is also displayed a MS/MS interpretation window showing both experimental and reference spectra in opposite sides, in order to help the user to visualise the results. Additionally, some tools were added to the software taking into account future developments in this field, such as the identification of specific markers for each compound, which would simplify the data interpretation.

The major advantage of a MALDI strategy to analyse AAS, concerns the high throughput and reduce time of analysis, which is an essential requisite for anti-doping laboratories, especially, during major competitions, where the number of processed samples is very high and the results have to be quickly released. Moreover, the use of TOF analysers coupled to MALDI systems is an important feature, since it allows the analyst to identify suspicious mass ions, unlike most GC-MS systems that, to achieve low detection limits, have to work in SIM mode, recording only pre-determined ions. However, two major limitations still occur. The developed method is still confined to the analysis of exogenous AAS, whose simple presence is considered an offence to fair sport. For the analysis of endogenous compounds an additional quantification method is required. Other limitation, concerns the fact that the derivatisation procedure employed within this thesis is limited to oxosteroids. Although, these are the major class inside the AAS group, some AAS, such as Danazol, do not have a carbonyl functional group and do not react with Girard T hydrazine.

We are working towards the resolution of these limitations, in order to introduce MALDI devices in anti-doping laboratories as a valuable tool for the initial screening of these compounds in complement with chromatographic techniques for confirmatory analyses.

In parallel to the development of a MALDI-MS(/MS) strategy to improve AAS detection, the optimisation of the routine methodology used in most WADA anti-doping laboratories for the analysis of AAS by GC-MS, was also covered. The optimisation study was performed using energy delivery methods, such as ultrasonic and microwave irradiation, and covered the two keys steps of AAS sample treatment procedure, the hydrolysis of conjugated AAS with the enzyme β-glucuronidase and subsequent derivatisation with MSTFA reagent to improve AAS GC-MS characteristics.

In the initial study, ultrasonic irradiation was applied to the two critical steps of AAS sample treatment and a significant improvement in both steps was achieved when compared with the routine method that relies on thermal incubation to enhance both enzymatic and chemical reactions. The developed method reduces the total time of AAS determination from around 150 min, which is the approximate overall time spent for both routine sample treatment and subsequent GC/MS analysis, to around 85 min for most compounds studied and to 90 min if 11b-hydroxyandrosterone and 11b-hydroxyetiocholanolone are present. Regarding the enzymatic reaction, the use of ultrasonic irradiation affects mainly the reaction time, whereas for the derivatisation reaction, not only the reaction time is improved but also the reaction yield is affected by ultrasonication, since for 13 of the 35 AAS studied, the new method was able to improve the derivatisation yields obtained.

The new approach is less time-consuming, is robust, and has the same reproducibility as the traditional method.

A similar study, focusing the two critical steps of AAS sample treatment, was carried out using microwave energy. The overall result was compared with the ultrasonic irradiation methodology previously developed. For this study, not only AAS were used, but also other banned substances, such as diuretics, stimulants, β-blockers and β2-agonists, belonging to the same screening methods employed by the WADA anti-doping laboratory of Rome.

The results obtained point towards the fact that microwave energy fails in to enhance efficiently the enzymatic hydrolysis of the majority of the steroids tested, showing similar results to the routine procedure. Regarding the derivatisation reaction, this study has demonstrated that microwave irradiation improves, significantly, the derivatisation reaction time and, most important, the derivatisation yield of several compounds, which results in a great improvement of the analytical sensitivity for their detection by GC-MS.

On the overall, both ultrasonic and microwave irradiation methodologies can be successfully used to improve the routine sample treatment procedure. Regarding the ultrasonic irradiation method, it enhances both the enzymatic and chemical reactions involved and, therefore, it could be very effective as a tool to accelerate doping control tests carried out at major international events, where one of the main tasks for the analytical laboratory is to perform analyses as quickly and efficiently as possible. Concerning the microwave energy assisted method this thesis demonstrates that, although it fails to enhance the hydrolysis reaction, it improves deeply the derivatisation reaction. Even though microwave methodology is less efficient in the general sample treatment procedure, it presents higher sample throughput for the derivatisation reaction.

To better understand the effects of ultrasonic irradiation on the kinetics of β-glucuronidase enzyme, a kinetic study was performed using the compound p-nitrophenyl–β-glucuronide, which is commonly used for β-glucuronidase enzymatic essays. The results of the ultrasonic based enzymatic studies were compared with those obtained by the routine warming incubation at 55ºC. It was verified that ultrasonic energy enhances β-glucuronidase enzymatic kinetics by increasing both initial velocity and maximum velocity. It has been assessed that the reaction follows the Michaelis-Menten equation, remaining the respective constant unaltered. The results obtained indicate, as a first approach, that the improvement caused by ultrasonic energy is mainly due to the stabilisation of the transition state and the enhancement of mass transfer processes. It has been also proved that, at substrate excess, the positive effects of high intensity ultrasonication are quickly surpassed by the inactivation of β-glucuronidase enzyme. Hence, when ultrasonic energy is used to enhance enzymatic reactions, a concentration of enzyme superior to the one regularly used is advised.

The results obtained in this study represent an important step to understand the effects of ultrasonication in the enzyme-substrate complex interaction and it pretends to be a launching ramp for deeper studies on the ultrasonic effect in enzymatic reactions, particularly, β-glucuronidase hydrolysis. When compared with our previous study conducted with AAS, the effects of ultrasonication to enhance β-glucuronidase activity, using PNP-G as substrate are less accentuated. We attribute this result to the nature of the substrate: the harder is the hydrolysis reaction achieved by conventional thermal incubation, more evident and advantageous is the use of high intensity ultrasonication.

VII.2 FUTURE PROSPECTS

This thesis addresses the development of important analytical methodologies to upgrade the detection of AAS. Nevertheless, some improvements are required to incorporate the methods developed as reliable and suitable tools to be used in anti-doping laboratories. Regarding the application of MALDI-MS(/MS) to analyse AAS, experiments are currently being carried out to overcome the two major limitations reported above. Although it was not included in this thesis, the derivatisation reagent limitation related to non oxosteroids compounds will be soon overcome. Together, with Professor Carlos Lodeiro, an expertise in the fields of Photochemistry, Coordination and Bioinorganic Chemistry, we are developing a novel strategy for the fast screening of AAS, in which molecular probes are being designed and synthesized to enhance the desorption/ionization mass spectrometric properties of AAS by

acting as both derivatisation agents and MALDI matrices. With this study we pretend to produce both specific reagents for determinate functional groups as well as "universal" reagents to embrace all prohibited AAS. The active compounds should act as matrices as well as derivatisation agents, which will greatly simplify the sample treatment. Preliminary results on exosteroids are close to the ones achieved using the derivatisation reagent Girard T and matrix HABA and, therefore, are very promising.

Regarding the analysis of endogenous AAS, we are now initiating studies with deuterated compounds, in order to find a correlation factor between both endogenous and deuterated standard compounds. The software MLibrary should also include a quantification icon to help the user with this task. Moreover, studies regarding the fragmentation pattern of AAS in the MALDI system are under progress.

The use of MALDI-MS(/MS) devices in anti-doping laboratories has an incredible applicability, not only for the analysis of small molecules, such as AAS, but also for the analysis of other doping agents, such as peptide hormones that have known problems in detectability by the current means.