DEVELOPMENT OF NEW ANALYTICAL METHODS FOR STEROID PROFILING FOR DOPING PURPOSES

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DEVELOPMENT OF NEW ANALYTICAL METHODS FOR STEROID PROFILING FOR DOPING PURPOSES

by

NORMALIZA BINTI ABDUL MANAF

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LIST OF ABBREVIATIONS

β-CD	β-cyclodextrin
µ-SPE	Micro-solid phase extraction
µ–VALLE	Micro–vortex-assisted liquid–liquid extraction
5αAdiol	5alpha-androstane-3alpha,17beta-diol
5βAdiol	5beta-androstane-3alpha,17beta-diol
A	Androsterone
AAFs	Adverse Analytical Findings
ABP	Athlete Biological Passport
CDI	Dicyclohexylmethane-4,4'-disiisocyante
CE	Collision energy
CIRs	Carbon isotope ratios
CO_2	Carbon dioxide
CSIA	Compound specific isotope analysis
d-µSPE	Dispersive-micro solid phase extraction
D_3E	Deuterated epitestosterone
D_3T	Deuterated testosterone
D_4A	Deuterated androsterone
D_4AG	Deuterated androsterone glucuronide
D_5E	Deuterated etiocholanolone
DHEA	Dehydroepiandrosterone
DHT	Dihydrotestosterone
DLLME	Dispersive liquid-liquid microextraction
dSPE	Dispersive-solid phase extraction
E	Epitestosterone

EAAS	Endogenous anabolic androgenic steroids
ERC	Endogenous reference compound
Etio	Etiocholanolone
GC	Gas chromatography
GC-C-IRMS	Gas chromatography combustion isotope ratio mass spectrometry
GC-HRMS	Gas chromatography high resolution mass spectrometry
GC-MS	Gas chromatography mass spectrometry
GC-MS/MS	Gas chromatography tandem mass spectrometry
HFB	Heptafluorobutyryl
HF-LPME	Hollow fiber-liquid phase microextraction
HIRs	Hydrogen isotope ratios
HPLC	High performance liquid chromatography
HPLC-DAD-ESI-MS	High performance liquid chromatography-diode array detector-electrospray ionization-with mass spectrometry
IOC	International Olympic Committee
IRMS	Isotope ratio mass spectrometry
LC-MS	Liquid chromatography mass spectrometry
LC-MS/MS	Liquid chromatography tandem mass spectrometry
LL	Lower limit
LLE	Liquid-liquid extraction
Log P	Logarithm of partition coefficient
LPME	Liquid phase microextraction
MDI	Methylenediphenyl diisocyanate
MP	Mid point
MSTFA	N-methyl-N-(trimethylsilyl)trifluoroacetamide
MS	Mass spectrometry
MWCNT	Multi-walled carbon nanotubes
OPP	Organophosphorus pesticides

PDB	Pee-Dee Belemnite
pK_a	p-Value of acid dissociation constant
r^2	Regression coefficient
rpm	Revolutions per minute
RT	Retention time
S/N	Signal-to-noise
SBSE	Stir bar sorptive extraction
SDME	Single drop microextraction
SPE	Solid phase extraction
SPME	Solid phase microextraction
T	Testosterone
TA	*Tongkat Ali*
TBME	Tert-butylmethyl ether
TD	Technical Document
TMS	Trimethylsilyl
TOF	Time-of-flight
UHPLC-MS	Ultra high performance liquid chromatography mass spectrometry
UL	Upper limit
UV	Ultra-violet
VALLME	Vortex-assisted liquid-liquid microextraction
V-PDB	Vienna-Pee-Dee Belemnite
V-SMOW	Vienna standard mean ocean water
WADA	World Anti-Doping Agency
$\delta^{13}C$	Detla value carbon thirteen
$\delta^{2}H$	Delta value hydrogen/deuterium
PD	Pregnandiol
11-OHA	11 hydroxyandrosterone

PERKEMBANGAN KAEDAH ANALITIKAL BAHARU UNTUK MEMPROFIL STEROID BAGI KEGUNAAN DOPING

ABSTRAK

Sebatian profil steroid iaitu testosteron (T), epitestosteron (E), androsteron (A), etiocholanolon (Etio), 5alpha-androstan-3alpha, 17beta-diol (5αAdiol), 5beta-androstan-3alpha, 17beta-diol (5βAdiol) dan nisbah T kepada E (T/E) telah ditentukan di dalam urin sukarelawan lelaki yang mengambil makanan tambahan Tongkat Ali (TA) menggunakan pengekstrakan fasa pepejal (SPE) dan fasa cecair cecair (LLE). Daripada 47 sampel, 11 sampel telah melanggar garis panduan *World Anti-Doping Agency* (WADA) dan kaedah pengesahan yang menggunakan gas kromatografi pembakaran nisbah isotop spektrometri jisim (GC-C-IRMS) menunjukkan 5 sampel adalah konsisten dengan punca eksogen ($\Delta\delta^{13}C > 3‰$). Dua kaedah pengekstrakan mikro iaitu pengekstrakan mikro cecair-cecair berbantukan vorteks (VALLME) dan pengekstrakan mikro fasa pepejal (μ-SPE) menggunakan LC-MS/MS telah dibangunkan. Turus Emas Hypersil (50 mm × 2.1 mm, 1.9 μm) dengan elusi cerunan menghasilkan pemisahan dasar untuk kesemua sebatian dalam masa 8 min. Ion yang dipantau adalah m/z 289.4> 97.3 untuk T dan E, 273.4> 255.3 untuk A dan Etio dan 275.4> 257.3 untuk 5αAdiol dan 5βAdiol menggunakan pengionan penyembur elektron dalam mod kekutuban positif. Keadaan pengekstrakan optimum untuk sampel 5 mL dalam VALLME adalah: pelarut estrak, 1-pentanol; isipadu pelarut, 150 μL; masa vorteks, 40 s; kelajuan dan masa emparan, 1000 rpm selama 1 min tanpa penambahan garam yang diperlukan dalam pengekstrakan. Keadaan pengekstrakan optimum untuk sampel 3 mL dalam μ-SPE ialah: pelarut estrak, asetonitril; isipadu pelarut, 300 μL; masa pengekstrakan, 30 min

dan masa penyerapan, 20 min untuk mengekstrak. Kaedah VALLME-LC-MS/MS dan μ-SPE-LC-MS/MS memenuhi syarat WADA dari segi kepekaan, had pengesanan, kebolehulangan, kelinearan dan lasak. Tambahan pula, teknik alternatif ini adalah mudah, pantas dan mesra alam dengan pengurangan jumlah pelarut yang ketara.

DEVELOPMENT OF NEW ANALYTICAL METHODS FOR STEROID PROFILING FOR DOPING PURPOSES

ABSTRACT

The steroid profile compounds namely testosterone (T), epitestosterone (E), androsterone (A), etiocholanolone (Etio), 5alpha-androstane-3alpha,17beta-diol (5αAdiol), 5beta-androstane-3alpha,17beta-diol (5βAdiol) and the ratio of T to E (T/E)) has been determined in urine of male volunteers who consumed *Tongkat Ali* (TA) supplement using the solid phase extraction (SPE) and liquid-liquid extraction (LLE) method. From 47 samples, 11 samples violated the World Anti-Doping Agency (WADA) guidelines and confirmation by gas chromatography combustion isotope ratio mass spectrometry (GC-C-IRMS) showed 5 samples were consistent with the exogenous origin ($\Delta\delta^{13}C > 3‰$). Two microextraction methods, i.e., vortex-assisted liquid-liquid microextraction (VALLME) and micro-solid phase extraction (µ-SPE) followed by LC-MS/MS were developed. Hypersil Gold C18 column (50 mm × 2.1 mm, 1.9 µm**)** with gradient elution resulted in baseline separation for compounds in about 8 min. The monitored ions are m/z 289.4 > 97.3 for T and E, m/z 273.4 > 255.3 for A and Etio and m/z 275.4 > 257.3 for 5αAdiol and 5βAdiol using electron spray ionization in the positive polarity mode. The optimum extraction conditions for 5 mL sample using the VALLME were: extraction solvent, 1-pentanol; volume of extractant, 150 µL; vortex time, 40 s; centrifuge speed and time, 1000 rpm for 1 min with no salt addition needed for the extraction. The optimum extraction conditions for 3 mL sample using the µ-SPE were: extraction solvent, acetonitrile; volume of extractant, 300 µL; extraction time, 30 min and desorption time, 20 min for the extraction. The VALLME-LC-MS/MS and µ-SPE-LC-MS/MS

methods meet WADA requirements in terms of sensitivity, limit of detection, reproducibility, linearity and robustness. Furthermore, these alternative techniques were simple, rapid and environmentally friendly as markedly reduced amounts of solvents were involved.

CHAPTER ONE

INTRODUCTION

1.1 Background

The use of performance-enhancing drugs or doping by professional athletes has been acknowledged as a serious problem since the 1960s. Doping is defined as the use of drugs or other substances for performance enhancement. It has become an important topic in virtually every sport (Claudia & Shane, 2014) and has been discovered in athletes of all ages and at every level of competition (Catlin and Thomas, 1996). The use of performance-enhancing drugs is prohibited in sports. Athletes who are found to have used such banned substances, either through positive drugs test or the athlete biological passport (ABP) system, will be banned for competition for a length of time which reflects the severity of the infraction.

The anabolic androgenic steroids (AAS) are a family of hormones that includes the natural male hormone testosterone (T), together with numerous closely related chemical derivatives (Kanayama et al., 2010). All AAS possess both anabolic (muscle-building) and androgenic (masculine) properties, and they affect a wide range of physiological systems. The changing trends in steroid abuse have only been preceded by the rapidly advancing analytical technologies that can cover a wider range of compounds and achieve lower limits of detection (Scarth et al., 2012). According to the regulations of the World Anti-Doping Agency (WADA), AAS are classified as prohibited substances in sports (WADA Prohibited List 2017) as

summarized in Table 1.1. This table shows that AAS are covered under section "S1. Anabolic Agents, 1. Anabolic Androgenic Steroids (AAS)" are further subdivided into "a. Exogenous AAS" refers to a substance which is not ordinarily produced by the body and "b. Endogenous AAS (EAAS)" refers to a substance which ordinarily is produced by the body naturally.

1.2 Doping Cases in Sports

The fight against doping is a top priority for the International Olympic Committee (IOC), which has established a zero-tolerance policy to combat cheating and to punish anyone responsible for using or providing doping products. The IOC's fight against doping began in earnest in the 1960s (IOC Factsheet, 2016). It is currently carried out in close cooperation with WADA which was created in 1999 in Lausanne under the initiative of the IOC and with the support and participation of inter-governmental organisations, governments, administrators and other public and private bodies involved in the fight against doping in sports (Dionne, 2005). Table 1.2 summarises doping cases carried out during the Olympic games since 1968. Overall, the percentage of doping cases reported was less than 1%. Athens 2004 was the most doped Olympics with 26 reported violations of anti-doping rules. Weightlifting is the most doped sport with 36 violations that represent 28.4% of all Olympic doping cases (Stefania and Filomena, 2014). Doping is the most serious threat to elite sports because it harms athletes' health, decrease equal opportunities for athletes and leads to unfair games (David et al., 2007).

Table 1.1: WADA Prohibited List 2017

SUBSTANCES & METHODS PROHIBITED AT ALL TIMES (IN- AND OUT-OF-COMPETITION)

PROHIBITED SUBSTANCE

S0 NON-APPROVED SUBSTANCES

Any pharmacological substance which is not addressed by any of the subsequent sections of the List and with no current approval by any governmental regulatory health authority for human therapeutic use (e.g. drugs under pre-clinical or clinical development or discontinued, designer drugs, substances approved only for veterinary use) is prohibited at all times.

S1 ANABOLIC AGENTS

Anabolic agents are prohibited.

1. ANABOLIC ANDROGENIC STEROIDS (AAS)
   a. Exogenous AAS
   b. Endogenous AAS when administered exogenously
2. OTHER ANABOLIC AGENTS

"exogenous" refers to a substance which is not ordinarily produced by the body naturally.
"endogenous" refers to a substance which is ordinarily produced by the body naturally.

S2 PEPTIDE HORMONES, GROWTH FACTORS, RELATED SUBSTANCES, AND MIMETICS

The substances and other substances with similar chemical structure or similar biological effect(s), are prohibited.

S3 BETA-2 AGONISTS

All selective and non-selective beta-2 agonists, including all optical isomers, are prohibited.

S4 HORMONE AND METABOLIC MODULATORS

S5 DIURETICS AND MASKING AGENTS

P2 BETA-BLOCKERS

PROHIBITED METHOD

M1 MANIPULATION OF BLOOD AND BLOOD COMPONENTS

M2 CHEMICAL AND PHYSICAL MANIPULATION

M3 GENE DOPING

SUBSTANCES & METHODS PROHIBITED IN-COMPETITION

S6 STIMULANTS

All stimulants, including all optical isomers, e.g. d- and l- where relevant, are prohibited.

S7 NARCOTICS

S8 CANNABINOIDS

S9 GLUCOCORTICOIDS

All glucocorticoids are prohibited when administered by oral, intravenous, intramuscular, or rectal routes.

P1 ALCOHOL

Detection will be conducted by analysis of breath and/or blood. The doping violation threshold is equivalent to a blood alcohol concentration of 0.10 g L^{-1}.

Source: WADA Prohibited List 2017

Table 1.2: Summer Olympics Doping Cases

Year	Place	No of Test	No of Doping Cases Reported	% of Doping Cases Reported
2012	London, England	5,051	9	0.18
2008	Beijing, China	4,770	25	0.52
2004	Athens, Greece	3,667	26	0.71
2000	Sydney, Australia	2,359	11	0.47
1996	Atlanta, USA	1,923	2	0.10
1992	Barcelona, Spain	1,848	5	0.27
1988	Seoul, S. Korea	1,598	10	0.63
1984	Los Angeles, USA	1,507	12	0.80
1980	Moscow, Russia	645	0	0.00
1976	Montreal, Canada	2,054	11	0.54
1972	Munich, Germany	2,079	7	0.34
1968	Mexico City, Mexico	667	1	0.15
	TOTAL	**26,900**	**119**	**0.44**

Source: IOC Factsheet – The fight against doping and promotion of athletes' health Update - January 2014 (The fight against doping and promotion of athletes' health / 21 January 2014)

Malaysia is also not free from doping cases. Many Malaysian athletes including elite athletes have been detected for doping violations. Among the well-known athletes involved in doping was Datuk Lee Chong Wei from badminton. He was sanctioned for using dexamethasone, a corticosteroid drug. This drug is usually used for allergic or inflammatory treatment. He was banned for 8 months until 2015 from any competition (Channel NewsAsia, 2015). Another elite athlete that was involved with a doping case was Sazali Samad from bodybuilding. The prohibited substance detected was steroids and he had been banned for 4 years until 2019 from any competition (FMT News, 2015). In 2016, a total of 16 athletes in Malaysia have been found positive for prohibited substances including sibutramine, diuretics and steroids (New Straits Time, 2017). Whatever reason the athletes have given, athletes need to be very cautious and take responsibility in what they are consuming, especially supplements because in some cases, the manufacturers do not provide any details of banned substance in their products. So, athletes should avoid taking

supplements unless under the supervision of qualified and knowledgeable doctors because of the associated risk of being adulterated with banned substances.

1.3 Endogenous Anabolic Androgenic Steroid (EAAS)

EAAS is a group of natural compounds that are chemically similar to T and share the same androgenic action. Administration of these substances for enhancement of athletic performance is forbidden in sports. The administration of EAAS that are capable of being physiologically produced by the human body is also prohibited in sports (Amy, 2010). As these compounds and their metabolites also occur naturally in the human body, specific methods for the detection of the exogenous administration of these steroids are required (Ghigo, 2011). For screening purposes, urinary concentrations of several endogenous steroids or metabolites is generally determined by the GC–MS method (Parr et al., 2011)

The method of steroid profiling (T/E ratio) was introduced into routine doping control by Donike et al. (1993). The ratios of these steroids have been proven to be very stable (Kerkhof et al., 2000; Donike et al., 1993). The administration of steroids such as T, its precursors are proven to alter one or more parameters of the urinary steroid profile (Kerkhof et al., 2000). The latest effective date for EAAS WADA Technical Document (TD) is TD2016EAAS. The purpose of this TD is to harmonize the approaches to the measurement and reporting of EAAS in urine, including data in support of the steroidal module of the ABP or “steroid profile”.

AAS are chemical compounds capable of enhancing the anabolic processes in humans. They affect protein metabolism by stimulating protein synthesis (anabolic effect) and inhibiting protein breakdown (anticatabolic effect) (Rooyackers and Nair, 1997). The group of AAS includes the endogenously produced T, dihydrotestosterone (DHT), T prohormones and their metabolites as well as exogenous AAS, which are synthetic derivatives of T. T is the primary male sex hormone, is both an anabolic and androgenic steroid. It is synthesized from cholesterol in the Leydig cells of the testes (Eacker et al., 2008). Small amounts of T are also secreted from the ovary and the adrenal gland. DHT is an active metabolite of T and a potent androgen in some tissues. Dehydroepiandrosterone (DHEA), androstenedione and androstenediol are steroids in the sex hormone biosynthesis pathway and are precursors in the endogenous production of T and estrogens (Michael, 2002). These steroid precursors are weak androgens secreted primarily by the adrenal glands in both sexes. They provide a pool of circulating steroids that can be converted to active androgens and estrogens in the peripheral tissues (Michael, 2002). Anabolic agents were found to be the highest performance enhancing drug (50%) responsible for the Adverse Analytical Findings (AAFs) as may be referred to the 2015 Anti-doping test findings from WADA laboratory report (Table 1.3).

Table 1.3: Summary of Substances Identified as Adverse Analytical Findings (AAFs) in Each Drug Class in Anti-Doping Administration & Management System (ADAMS) for All Sports in 2014

Substance Group	Occurrences	% of all ADAMS reported findings
S1. Anabolic Agents	1728	50%
S6. Stimulants	528	15%
S5. Diuretics and Other Masking Agents	428	12%
S9. Glucocorticosteroids	215	6%
S4. Hormone and Metabolic Modulators	152	4%
S8. Cannabinoids	127	4%
S3. Beta-2 Agonists	115	3%
S2. Peptide Hormones, Growth Factors and Related Substances	98	3%
S7. Narcotics	21	1%
P2. Beta-Blockers	19	1%
M2. Chemical and Physical Manipulation	1	0.03%
P1. Alcohol	0	0%
M1. Enhancement of Oxygen Transfer	0	0%
TOTAL	**3432**	

Source: 2015 Anti-Doping Test Findings – Laboratory Report by WADA

Steroid profiling is one of the most versatile and informative screening tools for the detection of steroid abuse in sports drug testing. The "steroid profile" in the WADA technical document is composed of T, epitestosterone (E), androsterone (A), etiocholanolone (Etio), 5alpha-androstane-3alpha,17beta-diol (5αAdiol), 5beta-androstane-3alpha,17beta-diol (5βAdiol) and the ratio of T to E (T/E) (Table 1.4). These are measured as free steroid content obtained from the free steroid fraction plus those released from the conjugated fraction following hydrolysis by glucuronidase enzymes. Other urinary steroids or ratios of steroid metabolites that could be useful in evaluating a steroid profile are A/T, A/Etio, 5αAdiol/5βAdiol and 5αAdiol/E). The confirmation criteria by WADA for steroid profile has been summarised in Table 1.5 (WADA TD2016EAAS). Any results above the criteria were categorised as "Suspicious Steroid Profile".

Table 1.4: Steroid profile compounds

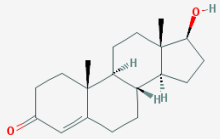
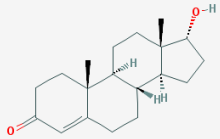
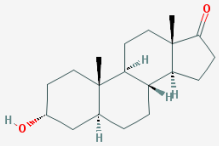
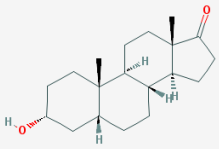
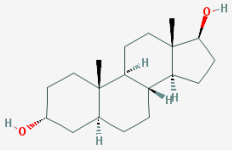
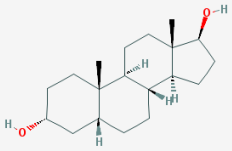
No	Name and Acronym	Structure	Mol Formula/ Mol Weight (Da)	Log P
1	Testosterone (T)		$C_{19}H_{28}O_2$ 288.424	3.34
2	Epitestosterone (E)		$C_{19}H_{28}O_2$ 288.424	3.37
3	Androsterone (A)		$C_{19}H_{30}O_2$ 290.440	3.77
4	Etiocholanolone (Etio)		$C_{19}H_{30}O_2$ 290.440	3.75
5	5alpha-androstane-3alpha,17beta-diol (5αAdiol)		$C_{19}H_{32}O_2$ 292.456	4.33
6	5beta-androstane-3alpha,17beta-diol (5βAdiol)		$C_{19}H_{32}O_2$ 292.456	3.20

Table 1.5: WADA confirmation criteria for steroid profile compounds

Compounds	Maximum Allowance by WADA*
Testosterone (T)	200 ng mL^{-1} in males or
Epitestosterone (E)	50 ng mL^{-1} in females
Androsterone (A)	10000 ng mL^{-1} combined with A/Etio
Etiocholanolone (Etio)	ratio <0.4 in males or > 4.0 in females
5αAdiol	250 ng mL^{-1}
5βAdiol	-
Ratio T to E (T/E)	< 4.0

Source: WADA TD2016EAAS - Endogenous Anabolic Androgenic Steroids Measurement and Reporting

1.4 Green Chemistry

Green chemistry is the design of chemical products and processes that reduce or eliminate the use or generation of hazardous substances. Green chemistry applies across the life cycle of a chemical product, including its design, manufacture, use, and ultimate disposal. Green chemistry is also known as sustainable chemistry (USEPA, 2017). So, in developing a method, a sustainable development or green analytical chemistry should be considered to ensure sustainability for tomorrow. Usually, the goal of green analytical chemistry is to use analytical procedures that generate less hazardous waste and that are safer to use and more benign to the environment (Keith et al., 2007). It is well known that in analytical chemistry most methods employ solvents that can harm the environment in terms of its toxicity and the volume used. So, new sustainable analytical methods are proposed that incorporate procedures that either use less hazardous chemicals or use lesser amounts of hazardous chemicals.

An important part of the green chemistry philosophy is the need to develop and adopt green analytical techniques and procedures. Analytical chemistry takes a special place in the green chemistry concept. It is aimed to detect and quantitatively determine various substances by means of methods which often use harmful reagents. As a result, the analysis itself may become a source of pollution. Analytical chemistry is considered to be a small-scale activity, but this is not always true in the case of controlling and monitoring laboratories such as anti-doping laboratories where a large number of analyses are performed. The use of instrumental methods instead of wet chemistry; the miniaturization and automation are the new trends of analytical chemistry, making this branch of chemistry more sustainable (Koel and Mihkel, 2006). The determination of a broad spectrum of analytes at low concentrations (ppb, even ppt) in samples of complex matrix composition has been facilitated by the introduction of a new generation of highly sensitive analytical devices and by the development of new sample preparation procedures. The principles of green chemistry are shown in Figure 1.1.

Most efforts in making chemical processes greener emphasize the need for using safer, less toxic, and more benign solvents, or the elimination of solvents completely, and reducing the use of reagents and auxiliaries. Other strategies include lower energy consumption through the use of milder reaction conditions (Rummi, 2017), avoiding derivatization and a preference for substrates based on renewable sources (Marek et al., 2015). In order to improve economic atom, highly selective catalytic processes should be performed instead of using additional substrates.

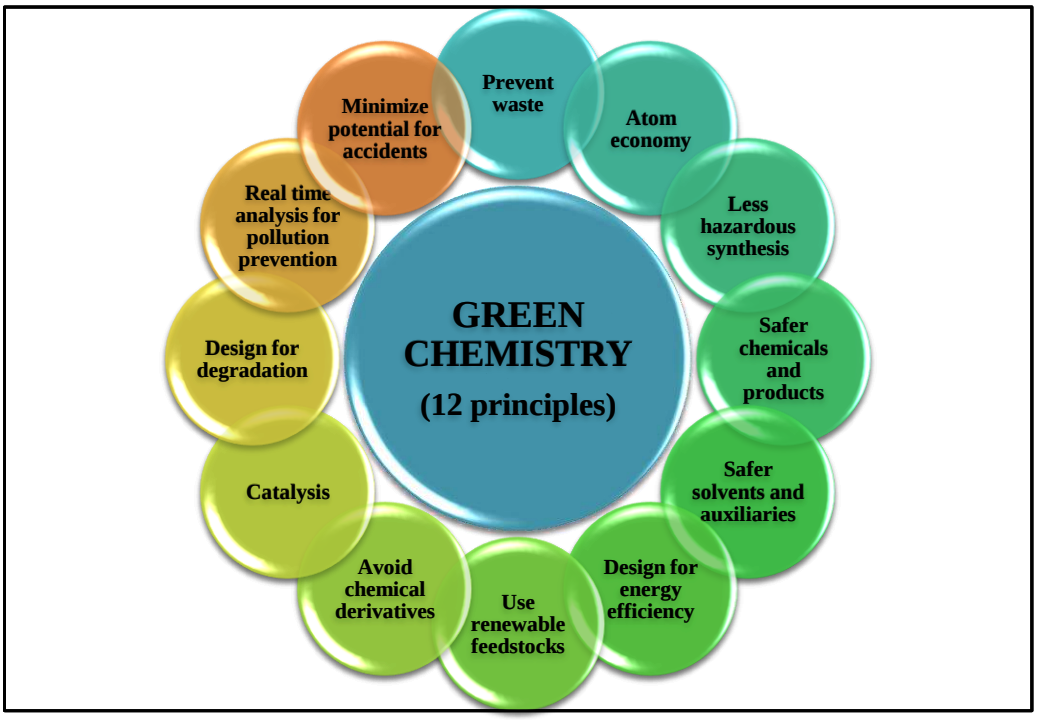


Figure 1.1: The principles of green chemistry (Anastas and Warner, 1998).

Analytical method needs solvents, reagents, energy, and it creates waste. The principles of green analytical chemistry in design of new methods includes prevention of waste (Principle 1); safer solvents and auxiliaries (Principle 5); design for energy efficiency (Principle 6); avoid chemical derivatives (Principle 8) and safer chemistry to minimize the potential of chemical accidents (Principle 12) (Agnieszka et al., 2013). The main goal is to avoid or reduce the undesirable environmental side effects of chemical analysis, while preserving the classic analytical parameters of accuracy, sensitivity, selectivity, and precision.

1.5 Sample Preparation and Analytical Method

The techniques of dope testing have improved immensely from 1972 to 2017 using improved extraction methods (Reddy et al., 2007) and sophisticated equipments (Thevis and Schanzer, 2005). Lower detection limits and better

selectivity could also be achieved with improved sample clean-up strategies in order to eliminate interferences due to the urine matrix. This approach is generally used especially in confirmatory and quantitative analysis since the procedures are often compound- or group-specific. Both isolation of analytes from the matrix and their pre-concentration are important aspects of this process. Also, it is necessary to clean up samples of difficult matrices.

Classical sample pre-treatment techniques (e.g., liquid-liquid extraction (LLE) and solid-phase extraction (SPE)) are slow and labour intensive (Gyorgy and Karoly, 2004). Often extensive amounts of hazardous organic solvents are used and sample volumes can be greater than 1 L. The main disadvantage of LLE in ultra-trace analysis is the necessity of using large amounts of very clean solvents and their subsequent evaporation is an inevitable step in obtaining significant pre-concentration. Thus, this technique is both expensive and environmentally unfriendly (Kozlowska, et al., 2003). SPE seems to be better, as smaller amounts of organic solvents are usually used. However, SPE cartridges are used once only in ultra-trace analysis; it is expensive and it also generates larger amount of waste. After use, SPE cartridges are disposed by sending to dumping grounds or, in certain cases, waste incineration plants. Both these methods are not environmentally friendly (Agnieska and Tomasz, 2011).

Immunoaffinity chromatography has been utilized to clean up urine samples for GC-MS and High Resolution Mass Spectromtry (HRMS) analysis of metabolites of stanozolol and nandrolone (Wu et al., 2012). Liquid Chromatography (LC) fractionation has been employed as an additional sample purification step in GC-

HRMS analysis of methandienone metabolites and GC tandem mass spectrometry (GC-MS/MS) and GC-MS analysis of metabolites of stanozolol, nandrolone, methyltestosterone and methandienone (Thevis et al., 2013). LLE with n-pentane and SPE with amino columns have been reported to clearly improve the quality of chromatographic signals and mass spectra in GC-MS analysis of non-polar steroids (Moon et al., 2011).

Several microextraction techniques have gained their place in modern analytical laboratories. The first solid-phase microextraction (SPME) was introduced by Arthur and Pawliszyn (1990). Since then, several other microextraction techniques have been developed such as the single-drop microextraction (SDME) (Jeannot and Cantwell, 1996; Liu and Dasgupta, 1996) and hollow-fiber liquid-phase microextraction (HF-LPME) (Pedersen and Rasmussen, 2008). Basically, microextraction offers many advantages over traditional extraction methods. Pre-concentration of analytes is normally high, sample clean-up is efficient and the number of separate stages in sample preparation is minimal (Pedersen and Rasmussen, 2008). However, LPME has not been applied to sample preparation of unconjugated AAS in urine as routine analysis.

In 2006, the dispersive liquid-liquid microextraction (DLLME) was introduced. However, the use of dispersive solvents may decrease the partitioning and the mass transfer of the analytes into the extraction solvent, thereby reducing the enrichment efficiency (Rezaee et al, 2010). The use of high density and toxic organic solvents (e.g., carbon tetrachloride, chloroform, dichloromethane) are other disadvantages of this technique (Leng et al., 2012).

Yiantzi et al. (2010) introduced another microextraction technique termed vortex-assisted liquid–liquid microextraction (VALLME) whereby dispersion of low density extraction solvent into water is obtained through vortex mixing (a mild emulsification procedure). The fine droplets could rapidly extract target analytes from water because of the shorter diffusion distance and larger interfacial area. After centrifugation, the floating extractant phase restores its initial single-drop shape. However, among the solvents (1-octanol, toluene, n-hexane, octane and cyclohexane) tested, only 1-octanol had the ability to restore its single-drop shape (Abu-Bakar et al., 2014). The rest of the tested solvents were left scattered on the surface of the aqueous samples and could not be effectively collected.

Micro-solid phase extraction (μ-SPE) is another novel alternative microextraction technique for the pre concentration analytes in complex samples. Basically, this technique involves analyte adsorption followed by solvent desorption but on a much smaller scale where the sorbent was held within a membrane envelope. The key advantages of this technique are the minimized usage of solvent as well as the simple, inexpensive and high enrichment that can be achieved. It is also suitable for extractions in complex matrices as the sample clean-up and extraction steps are carried out simultaneously (Basheer et al., 2007). Table 1.5 below shows the sample preparation technique and analytical method used for the EAAS analysis.

Table 1.6: Summary of sample preparation and analytical method for EAAS analysis.

No	Compound	Type of Sample	Sample Preparation	Instrument	References
1	T, E, A, Etio	Faeces	LLE	LC-MS	Weltring et al., (2012)
2	Steroid Profile	Urine	SPE (Oasis HLB)	UHPLC-QTOF-MS/MS	Badoud et al., (2011)
3	Steroid Profile	Urine	SPE (Detectabuse^{TM})	GC-MS	Martinez-Brito et al., (2013)
4	T	Serum	SPE (C18)	LC-MS/MS	Koren et al., (2012a)
5	Steroid Profile	Urine	LLE	GC-MS/MS	Van Eenoo et al., (2010)
6	T	Serum	LLE	LC-MS/MS	Yang, (2011)
7	T, E	Urine	SPME	LC-MS/MS	Zhan et al., (2011)
8	T	Tissue	LLE, SPE (Oasis HLB)	LC-MS/MS	Surowiec et al., (2011)
9	T, E, A, Etio	Urine	SPE (C18)	GC-MS	Ahmadkhaniha et al., (2010)
10	T, E	Saliva	DLLME	LC-MS/MS	Sobhi et al., (2014)
11	T, E	Saliva	SBSE	GC-MS	Stopforth et al., (2007)
12	T	Urine	SPME-DMF	LC-MS	Choi et al., (2016)
13	T	Urine	MIP	LC-MS/MS	Tse et al., (2010)
15	Anabolic steroids	Urine	LLE	LC-MS/MS	Deventer et al., (2006)
16	Anabolic steroids	Urine	LLE	LC-MS/MS	Mazzarino, et al., (2006)
17	Anabolic steroids	Urine	LLE	LC-MS/MS	Leinonen, et al (2002)

1.5.1 MS in Doping Analysis

In doping analysis, the detection of AAS in urine gained lots of attention with the introduction of mass spectrometric detection coupled to separating techniques like GC in the early eighties. Mass spectrometry was used for the first time in 1972 during the Munich Olympics. Since then various new chromatographic techniques such as HRMS (Atlanta Olympics Games 1996), isotope ratio mass spectrometry (IRMS) (Special Olympic Winter Games, 1998) and liquid

chromatography mass spectrometry (LC-MS) (Athens Olympic Games, 2004) have been utilised in dope testing. The WADA accredited labs around the world have their own set of testing protocols utilizing various equipments such as GC, GC-MS, HRMS, LC-MS/MS and IRMS (Mukesh, 2016).

A suitable analytical method for AAS was developed by Donike et al. (1988). Analyses were performed on a bench-top quadrupole mass spectrometry (MS) coupled with capillary GC. AAS were cleaned up on a SPE XAD-2 column, followed by hydrolysis of conjugated metabolites using β-glucuronidase enzyme and LLE with diethyl ether under alkaline conditions. Prior to GC-MS analysis in selected ion monitoring mode (SIM), steroids were derivatized with a mixture of N-methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA), trimethyliodosilane and an antioxidant to convert steroid hydroxyl and keto groups to their unique trimethylsilyl (TMS) ethers and enol ethers. Steroids excreted unconjugated in urine (free steroids) were analyzed separately by extracting urine samples directly or after SPE XAD-2 clean-up with diethyl ether at basic pH, followed by selective derivatization resulting in the formation of O-TMS derivatives. In the case of stanozolol, the heptafluorobutyryl (HFB) -O-TMS derivative (Stolman and Pranitis, 1977) was used. Modifications from the original method include SPE C18 cartridges, LLE with tertbutyl methyl ether and combination of sample pretreatment and analysis of free and conjugated steroids (Achten et al., 2001). The variation includes direct hydrolysis of urine, followed by LLE and derivatization using D_4AG and Etio for quality assurance (Adrian et al., 2011). A typical GC-MS in SIM mode takes 20-30 min and incorporates 10-15 time-programmed acquisition groups of 15-20 ions. Limits of detection (LOD) between 2-30 ng mL^{-1} can be achieved (Asakawa et al.,

1999). Negative and positive ion chemical ionization has been applied for HFB, pentafluoropropionyl, methoxy-TMS or TMS derivatives of many AAS metabolites but without significant enhancement in sensitivity (Jordi et al., 2000). Since sufficient LOD are not achieved for all steroids with the basic method, laboratories have been obliged to search complementary analytical methods to screen and confirm AAS (Reddy at al., 2009).

In the early 1990s, LC-MS were commercialised with the first application in AAS in 1996. The application of modern and powerful analytical instruments consisting of LC, sophisticated atmospheric pressure ion sources, and sensitive mass analyzers has markedly improved the quality as well as speed of doping control analyses (Thevis and Schanzer, 2005). The technique of LC–MS/MS has complemented sports drug testing strategies ever since soft ionization interfaces such as electrospray or atmospheric pressure chemical ionization (ESI or APCI, respectively) became commercially available (Lanina et al., 2007). Numerous applications have been developed that allow the determination of prohibited therapeutics that is barely detectable or undetectable with conventional GC-MS instrument. Due to the progressive nature of doping controls, the continuously changing demands originating from the dynamic pharmaceutical market, new illegal approaches that presumably increase athletic performance and modifications to the lists of prohibited compounds of regulative authorities such as WADA, numerous new applications and drug-testing strategies based on LC-MS/MS were frequently developed. With the availability of LC interfaced to an MS by sophisticated ionization techniques such as ESI and APCI, doping control analysis has many

powerful options for specific, sensitive, fast, and robust procedures (Nicoli et al., 2016).

1.5.2 Isotope Ratio Mass Spectrometry in Doping Analysis

Detection of doping with steroids that are structurally identical to those produced in the body (e.g. T, DHEA, DHT and 4-androstenedione) is a special case and a challenging task in sports drug testing, since their origin (endogenous or exogenous) is difficult to prove. Abuse of these steroids has been detected indirectly by measuring changes in absolute and relative concentrations of different endogenous steroids (steroid profile) in urine (Cawley and Flenker, 2008). The basic analysis successfully used for detection of T administration is based on the determination of the ratio to its 17α-epimer, E. At present, the cut-off level for T/E ratio is 4:1. Quantification can be carried out with the same GC-MS methods that are used for qualitative analysis of other AAS. All indirect tests rely on statistical population-based reference values and further individual investigations were often needed to exclude the possibility of an abnormal physiological or pathological condition. Doping with T and many other natural steroids can be confirmed directly by means of gas chromatography-combustion-carbon isotope ratio mass spectrometry (GC-C-IRMS) (Strahm et al., 2009).

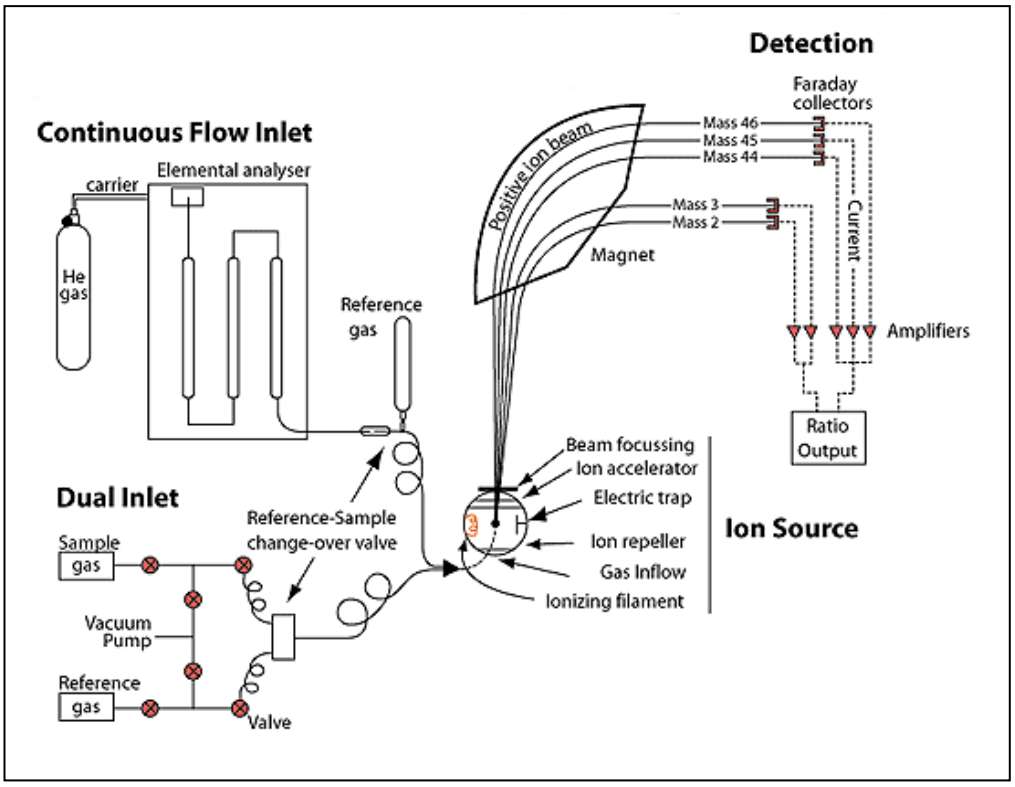


Figure 1.2: Schematic depicting of a triple-collector IRMS system to analyze the isotopic composition of CO_2. The same principle is used for analysis of the isotopes of ^{16}O and ^{1}H just with a different set-up of the collectors. (Adapted from SAHRA, 2005)

Isotope ratio mass spectrometry IRMS is a technique that gives precise and accurate measurements of the variation in the natural abundance of light stable isotopes (Sulzman, 2007). The mass spectrometric method is the most effective method for measuring isotope abundances, which is used to separate charged atoms according to their mass-to-charge ratio, denoted as m/z. IRMS instrument has two basic types which are the dual-inlet (DI-IRMS) and the continuous flow (CF-IRMS). Both of these types consist of the inlet system, ion source, mass analyzer and ion collection or detector. Figure 1.2 illustrates the difference between DI-IRMS and CF-IRMS (Sulzman, 2007).

Isotopes have the same number of protons and electrons but differing number of neutrons for an element (Sulzman, 2007). Isotopes are divided into two categories,

i.e., stable and unstable isotopes (Hoefs, 2009). Stable isotopes are those with stable energy and do not decay. Isotopes become stable when the neutrons and the protons become quite similar in numbers (Sulzman, 2007). Every element has a light isotope and one or two heavy isotopes. Examples of light isotopes are: carbon (^{12}C), nitrogen (^{14}N), oxygen (^{16}O), hydrogen (^{1}H) and sulfur (^{32}S). Examples of the corresponding heavy isotopes are ^{13}C, ^{15}N, ^{17}O, ^{18}O, ^{2}H, ^{33}S and ^{34}S (Benson et al., 2006). It is a fact that every biochemical process involves substances that contain one of these elements (C, N, H, O and S).

The δ^{13}C helped to distinguish between exogenous and endogenous steroids. It was found that, the ratio of T/E in urine can indicate synthetic steroids use (Ehleringer et al., 2007). The use of stable IRMS has shown that synthetic T has lower δ^{13}C value than the endogenous hormone (Hernandez, 2008). The natural abundance isotope ratio data are generally reported as delta values (**δ**) which are expressed in units per mil (mil=thousand) and written as ‰ (Benson et al., 2006). Delta value can be calculated and measured according to the following formula:

$$\delta = \frac{(R\ \text{Sample} - R\ \text{Standard})}{R\ \text{Standard}} \times 1000$$

R sample is the ratio of the heavy to light isotope for the sample, while *R* standard is the same ratio for the standard (Benson et al., 2006).

A positive value indicates that the sample has more heavy isotopes relative to the standard. Negative value indicates less of the heavy isotopes than the standard (Sulzman, 2007). There are many international standards that are used for isotope ratio measurement: Pee-Dee Belemnite (PDB), Atmospheric nitrogen (AIR), Vienna

standard mean ocean water (V-SMOW), Standard mean ocean chloride (SMOC) and the most important is Vienna-Pee-Dee Belemnite (V-PDB) (Benson et al., 2006). Stable isotope ratio is typically measured by a technique called isotope ratio mass spectrometry (IRMS). Table 1.6 shows the relative abundances of naturally occurring isotopes of the common elements which have been analyzed by IRMS (Benson et al., 2006).

Table 1.7: Relative abundances measurement for the isotopes of elements analyzed by IRMS (adapted from Benson et al., 2006)

Element	Isotope	Relative abundance (%)
Hydrogen (H)	^{1}H	99.984
	^{2}H	0.0156
Carbon (C)	^{12}C	98.892
	^{13}C	1.108
Nitrogen (N)	^{14}N	99.635
	^{15}N	0.365
Oxygen (O)	^{16}O	99.759
	^{17}O	0.037
	^{18}O	0.204
Sulphur (S)	^{32}S	95.020
	^{33}S	0.760
	^{34}S	4.220
	^{35}S	0.014

GC-C-IRMS is a highly specialised instrumental used to ascertain the relative ratio of light stable isotopes of carbon ($^{13}C/^{12}C$), hydrogen ($^{2}H/^{1}H$), nitrogen ($^{15}N/^{14}N$) or oxygen ($^{18}O/^{16}0$) in individual compounds separated from often complex mixtures of components. The ratio of these isotopes in natural materials varies slightly as a result of isotopic fractionation during physical, chemical and biological processes resulting, in some cases, with the relative isotopic ratio of specific compounds being highly diagnostic of key processes (Augenstein, 1999).

The technique of GC-C-IRMS is based on the compound specific isotope analysis (CSIA). Sano was the first who worked on GC-C-IRMS to measure carbon isotope ratios in 1976. From 1988, IRMS started to be commercially available (Sulzman, 2007). At the beginning only carbon was determined; then after some developments for the system, it was able to conduct analysis of nitrogen, hydrogen and oxygen isotope ratios (Benson et al., 2006). Figure 1.3 shows the schematic diagram of the basic set up of GC-C-IRMS instrument for the analysis of carbon isotope ratios (Cawley and Flenker, 2008). The sample is injected onto the GC, where it will vaporize and pass through the column by following the helium carrier gas. The different components in the sample will be separated by their affinity with the stationary phase coated onto the inner wall of the column and the carrier gas. To avoid large amounts of solvent from entering the oxidation furnace, a backflush system using helium is used directly after the GC column. To allow the eluted compounds to enter the reactor, the backflush is turned off just before the expected retention time of the compounds of interest which is then submitted to the combustion oven, which consists of oxidation and reduction furnace. To remove the water vapour which is generated during combustion, a water trap with an open split is required. All the carbon from each sample will be converted into CO_2 by the oxidation furnace, which consists of an alumina ceramic capillary tube containing Cu, Ni and Pt wire. Cu will react with high purity O_2 gas to form CuO at 650°C. Ni reacts with O_2 to form NiO at 950°C in the presence of Pt as a catalyst for this reaction. In the reduction furnace, an excess of O_2 is removed from the gas stream and nitrous oxides is reduced into N_2-N_2O. During the oxidation, H_2O will be formed and removed by a Nafion membrane, a polymeric capillary which is permeable to water. When the H_2O has been removed, the compounds enter the ConFlo IV, a gas

management device where the reference gas, CO_2, can be added. Following this, the analyte gases pass into a stream of helium into the IRMS through an open split interface (Cawley and Flenker, 2008).

In IRMS the gasses enter the ion source which is subsequently ionized by an emission of electrons under high vacuum, resulting in positively charged ions (Benson et al., 2006). These are ionized by an emission of electrons under high vacuum, creating positively charged ions. These ions will be accelerated by an electric field toward a flight tube, where the separation is performed according to the mass-to-charge ratio. These ions are accelerated to 3 kV energy and then separated according to the mass-to-charge ratio m/z. These ions are collected in an ion detector called Faraday cups (Benson et al., 2006). Faraday cups are long and narrow tubes made of metal, which prevent the ions and secondary electrons from getting out (Sulzman, 2007); this consists of three cups positioned to collect the ions of m/z 44, 45 and 46. The ion currents are sent to the data system to be reported. As an example for the analysis of CO_2, the data consists of three traces for the different isotopes and its corresponding masses are m/z 44, 45 and 46. These masses represent $^{12}C^{16}O^{16}O$, $^{13}C^{16}O^{16}O$ and $^{12}C^{16}O^{18}O$ respectively (Cawley and Flenker, 2008).

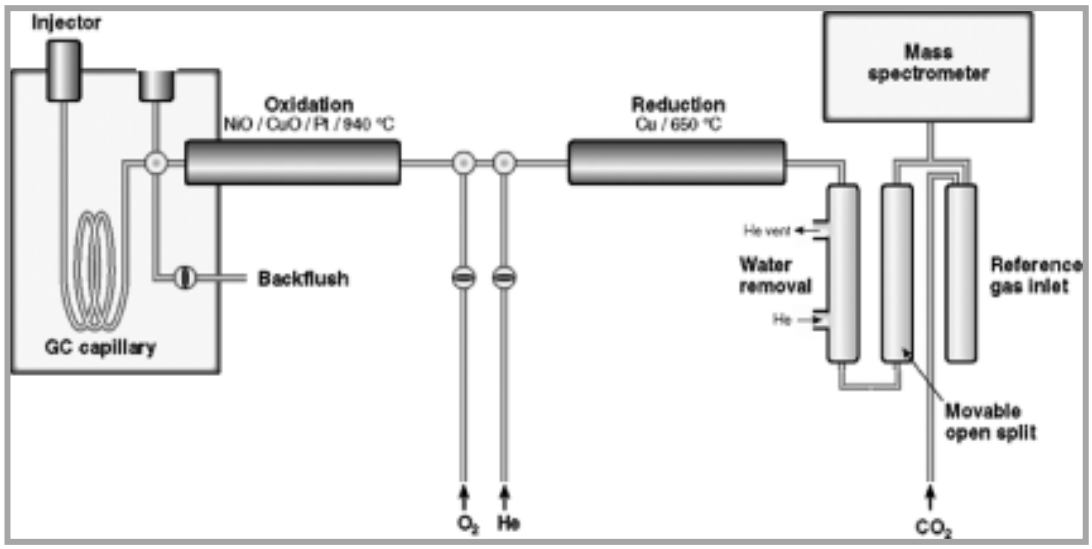


Figure 1.3: Schematic diagram of a typical GC-C-IRMS (adapted from Cawley and Flenker, 2008)

The differentiation of endogenous and exogenous steroids is important in doping analysis. The approach is based on the fact that chemically manufactured and endogenously produced steroids have small differences in their carbon isotope ($^{13}C/^{12}C$) ratio. Chemically manufactured steroids are synthesized from certain plant sterols that have low ^{13}C content, while the ^{13}C content of human body is higher and reflects the diet. In the assay, the $^{13}C/^{12}C$ ratio is determined from possibly administered steroids or their metabolites (e.g. A, Etio, 5αAdiol and 5βAdiol) and compared with the $^{13}C/^{12}C$ ratio of other endogenous steroids that are not affected by the administrated steroid (e.g. pregnandiol (PD), 11-ketoetiocholanolone (11KetoEtio)). The results will be reported as consistent with steroid administration provided that the $^{13}C/^{12}C$ value measured for the metabolite differs by three or more delta units depending on the endogenous reference compound (ERC) (WADA TD2016IRMS).