STRATHCLYDE INSTITUTE OF PHARMACY & BIOMEDICAL SCIENCES

TARGETED OF CANNABINOIDS IN HAIR AND ANDROGENS IN SALIVA AND UNTARGETED PROFILING OF THE EFFECTS OF EXERCISE

Mansour A M Alzahrani

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Declaration

'I declare that, except where specifically indicated, all the work presented in this report is my own and I am the sole author of all parts.'

Signed: _____

Date: _____

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List of abbreviations

Abbreviation	meaning
ACN	Acetonitrile
ATP	Adenosine triphosphate Blood
BMI	Body mass index
CBN	Cannabinol
CV-ANOVA	Cross Validated ANOVA
ESI	Electrospray ionization
EtOH	Ethanol
eV	Electron Volt
FDA	Food and drug adminstration
FMP-TS	2-Flouro-1, methyl p-toluene sulfonate
g	Gram
H2O	Distil water
HILIC	Hydrophilic Interaction Liquid Chromatography
LC-MS	Liquid chromatography-mass spectrometry
LLE	Liquid-liquid extraction
LOD	limit of detection
LOQ	limit of quantification
m/z	Mass/charge ratio
MeOH	methanol
mg	Milligram

MS/MS	Tandem Mass Spectrometry
NAD+	Nicotinamide Adenine Dinucleotide (oxidised)
NADH	Nicotinamide Adenine Dinucleotide (reduced)
ng	Nanogram
OPLS	Orthogonal Partial Least Squares
OPLS-DA	Orthogonal Partial Least Squares Discriminant Analysis
PCA	Principal Component Analysis
pg	Picogram
RP	Reversed Phase
RSD	Relative Standard Deviation
SPE	Solid-phase extraction
SIMCA	Soft-Independent Modelling of Class Analogy
SWGTOX	Scientific Working Group for Forensic Toxicology
TFA	Trifluoroacetic acid
THC	Δ9-tetrahydrocannabinol
THC-COOH	11-nor-Δ9-tetrahydrocannabinol-9-carboxylic acid
THC-COOH-glu	11-nor-Δ9-tetrahydrocannabinol-9-carboxylic acid glucuronide
V / V	volume/volume
VIP	Variable Importance in the Projection
w/v	weight/volume
µg	Microgram
µL	Microlitre

Papers published/submitted, and posters presented.

Papers

1) Benedetti, S.; Al-Tannak, N.F.; **Alzharani, M.**; Moir, H.J.; Stensel, D.J.; Thackray, A.E.; Naughton, D.P.; Dorak, M.T.; Spendiff, O.; Hill, N.; et al. Plasma Free Fatty Acids Metabolic Profile with LC-MS and Appetite-Related Hormones in South Asian and White European Men in Relation to Adiposity, Physical Activity and Cardiorespiratory Fitness: A Cross-Sectional Study. *Metabolites,* **2019**, *9*, 71.

2) **Alzahrani;** Alshuwaier; Aljaloud; Gibson; Khalaf; Alhawiti; Watson Development of a Derivatization Method for Investigating Testosterone and Dehydroepiandrosterone Using Tandem Mass Spectrometry in Saliva Samples from Young Professional Soccer Players Pre- and Post-Training. *Sci. Pharm.* **2019**, *87*, 11.

Posters

1) Alzahrani M A, Watson DG. Metabolomic Profiling of Plasma, Urine and Saliva in short Exercise at a Standardised Relative Intensity in young professional football players in Saudi Arabia, *University of Glasgow, Glasgow, Scotland, UK.* Scottish Metabolomics Network Meeting, 2nd and 3rd November 2017. (**Appendices 1 for Chapter 4).**

2) Alzahrani M A, Watson DG. Method Development and Validation of a Derivatization Method for the Analysis of Dehydroepiandrosterone (DHEA), Testosterone (T) and Epitestosterone (ET) and using mass spectrometry, *University of the Dundee, Dundee, Scotland, UK.* Scottish Student Forensic Symposium, 26th and 27th March 2018. (**Appendices 2 for Chapter 3)**.

Abstract

The thesis is made up of four chapters. The first chapter is the general introduction to prepare biological samples for analysis, including extraction methods, derivatization and LC-MS instrumentation, including separation techniques. In addition, description of metabolomics approaches which are deployed in order to determine and/or quantify any changes to the metabolites which occur within a biological system in response to different stimuli such as diet, lifestyle and physical activity.

The second chapter describes the quantification of cannabinoids in human hair through derivatization and liquid chromatography-tandem mass spectrometry. Cannabinol (CBN), Δ9-Tetrahydrocannabinol (THC) and its main metabolite 11-nor-Δ^9- Tetrahydrocannabinol carboxylic acid (THC-COOH) which are the most popular indicators of cannabis use. The use of this drug is widespread around the world and causes a serious social and health problems. In this study, a method based on solid-phase extraction (SPE) and liquid chromatography-electrospray ionization-tandem mass spectrometry (LC–ESI-MS/MS) was developed and validated. In this procedure, hair samples were extracted and derivatized with 2-fluoro-1-methyl pyridinium-p-toluenesulfonate (FMP-TS) before injecting into the LC-MS-MS. The method showed excellent linearity with a coefficient of determination (r^2) better than 0.99 for the analytes of interest. The extraction

recovery was between 81% and 105 % for all compounds. The limit of detection (LOD) and quantification (LOQ) were 2 and 20 pg/mg respectively for both CBN and THC and was 0.1 and 0.2 pg/mg for THC-COOH. Intra- and inter-assay precision were always lower than 4% and 11%, respectively, for these cannabinoids. Whereas the intra- and inter-assay bias were between (14% and -18 %) and (15 and -12 %), respectively.

Twenty-seven hair specimens from cannabis consumers were investigated using the optimized and validated method. Ultimately, CBN and THC were detected in all specimens, whereas THC-COOH was quantified in 13 specimens. Unlike CBN and THC, THC-COOH was semi-quantifiable (all values less than the limit of quantification (LOQ), but more than the limit of detection (LOD)) in 3 samples and was not detected in 11 samples. The levels of CBN, THC and THC-COOH on average were (0.022-2.562 ng/mg), (0.049-0.431 ng/mg) and (0.222-4.867 pg/mg) respectively. The median levels were (0.054 ng/mg), (0.087 ng/mg) and (0.34 pg/mg) for CBN, THC and THC-COOH respectively. Detection at least of THC-COOH metabolite in hair, especially in routine work seems to be compulsory in addition to THC and other main cannabinoids in order to distinguish between active ingestion and passive exposure.

In the last decade, high-performance liquid chromatography/tandem mass spectrometry (LC/MS/MS) combined with electrospray ionization (ESI) has been

widely used for determining low concentrations of steroids, and derivatization has often been employed to enhance detection. The third chapter describes the development of a derivatization method for quantification of testosterone and dehydroepiandrosterone in saliva samples from young professional soccer players, pre- and post-Training, using tandem mass spectrometry. In the present study, endogenous steroids were extracted using a Strata-XL polymeric reverse-phase cartridge. The isolated steroids were reacted with 2-hydrazino-1-methylpyridine (HMP). A liquid chromatography-tandem mass spectrometry (LC-MS/MS) was used in a positive mode with multiple reaction monitoring (MRM) for the quantification of testosterone (T) and its biosynthetic precursor, dehydroepiandrosterone (DHEA), in saliva samples collected from twenty young Saudi professional soccer players. The extraction recovery during the pre-treatment was >89% and gave <±20% for inter- and intra-assay precision and accuracy. The limits of quantification (LOQ) were found to be 20 pg/mL for (T and DHEA) and 50 pg/mL for Epitestosterone (EPI). The results showed no significant variation in the concentration of T between pre and post-training, whereas DHEA was significantly increased after short-term exercise. EPI could not be detected in the saliva samples.

The fourth chapter is about metabolomics profiling of plasma, urine and saliva after short-term training in young professional football players in Saudi Arabia.

Urine, plasma and saliva were collected on two days pre- and post-training. An Orbitrap Exactive mass spectrometer was used to analyse the samples. A reversed-phase (RP) column was used for the analysis of nonpolar plasma components, and a ZICpHILIC column was used for the analysis of polar metabolites in plasma, saliva and urine. There was no marked variation in the metabolite profiles between pre day1 and 2 nor between post day1 and 2 according to principal components analysis (PCA). When orthogonal partial least squares (OPLS-DA) modelling was used then the models separating pre- and post-training samples could be fitted based on the total number of significant metabolites 75, 16 and 32 for urine, plasma and saliva using hydrophilic interaction chromatography (HILIC) and 6 for plasma analysed on a reversed-phase (RP) column respectively.

Chapter 1:

General Introduction

1. General Introduction

1.1. Liquid chromatography-mass spectrometry

Liquid chromatography (LC) is used for the physical separation of components of a mixture using two phases, a mobile phase and a stationary phase. LC recently is often coupled with mass spectrometry systems. LC-MS is considered to be a potent analytical technique since the 1980s when the electrospray ion source (ESI) was developed (Fenn, Mann, Meng, Wong, & Whitehouse, 1989). In the biochemistry laboratory, this technology had been used since the mid of the 1990s (Pitt, 2009). Mass spectrometry (MS) is used for the conversion of analyte molecules to ions, resulting in the information of the target analyte ions. In the HPLC, the analytes are retained on the stationary phase for various periods according to their ability to interact with a column prior to elution by the mobile phase (Ardrey, 2003). The ionization source in MS is ionized molecules to acquire positive or negative charges allows ions to pass through the mass analyser according to their mass/charge (m/z) to reach the detector. Finally, these ions are recorded as a mass spectrum by a computer system (Ho et al., 2003).

The columns used in liquid chromatography for separation of various compounds, mass spectrometry systems and different extraction procedures used in the preparation of samples for analysis are discussed below.

1.1.1. Separation techniques

Chromatography techniques play an essential role in the analysis of target analytes in various biological samples. Chromatography is a physical technique for separation of an analyte based on the affinity between two phases, a mobile phase pressurised with a pump is carried through a stationary phase and then eluted to reach a detector (Ardrey, 2003). The elution of molecules at different times is based on the partitioning between the stationary and mobile phases.

1.1.1.1. Reversed-phase chromatography (RPC)

A reversed-phase column (RFC) is hydrophobic, where a silica gel surface is modified chemically with non-polar alkyl chains often containing 18 carbons to make it non-polar. Non-polar molecules partition into the non-polar stationary phase for a while based on the affinity between the column and mobile phase. In case of an RP column, the more hydrophobic a mobile phase, the more quickly a compound elutes from the reversed-phase column. In gradient RPC, the mobile phase starts with slightly high-water content and ends with a high organic solvent. The common RP columns are C18, C8, C4, Cyanopropyl and Phenyl, where the variation between them is in the degree of hydrophobicity ("HPLC training for the analytical chemist l CHROMacademy.com," n.d.)

1.1.1.2. Hydrophilic interaction liquid chromatography (HILIC)

HILIC is an alternative technique to RPC because it is a powerful technique for separating polar compounds which are not retained by reversed-phase columns. This technique was first reported in 1990 by Alpert (Hemstrom, Heckendorf, Jiang, Jonsson, & Appelblad, 2013). HILIC columns are often silica-based having a hydrophilic surface; among these ZIC-HILIC columns are very commonly used. In ZIC-HILIC columns a sulfobetaine zwitterionic functional group has been used to modify the silica surface thus allowing polar analytes to be retarded by partitioning in a hydrophilic environment. Apart from the partitioning of analyte between the mobile phase and water enriched layer on the stationary phase, there is also a possibility of ion exchange interactions in case of strong acids and bases. In this type of chromatography, the water at the surface of the stationary phase is behaving as a pseudo-stationary phase and separation work in a manner opposite to reversed-phase chromatography. In this mode of chromatography, the more polar the compound, the more strongly it is retained on the column or, the higher content of water in the mobile phase the more quickly it will elute the analyte (Kahsay, Song, Van Schepdael, Cabooter, & Adams, 2014).

1.1.2. Mass spectrometry systems

Mass spectrometry is an analytical tool which is used for the analysis of small organic molecules as well as large macromolecules. Since LC and the high vacuum conditions

of MS systems are incompatible, it is fundamental to form in gas phase ions (either positively, negatively or both charged) (McLafferty, 1981) prior to entry of the analyte into the mass spectrometer. These are then separated in mass analyser based on their mass to charge ratio (m/z) under a high vacuum. Therefore, the substance must evaporate or be volatilized in the mass spectrometry system. All mass spectrometers are generally composed of three essential components which include: an ionization source, mass analyser and a detector, as shown in **Figure 1-1.**

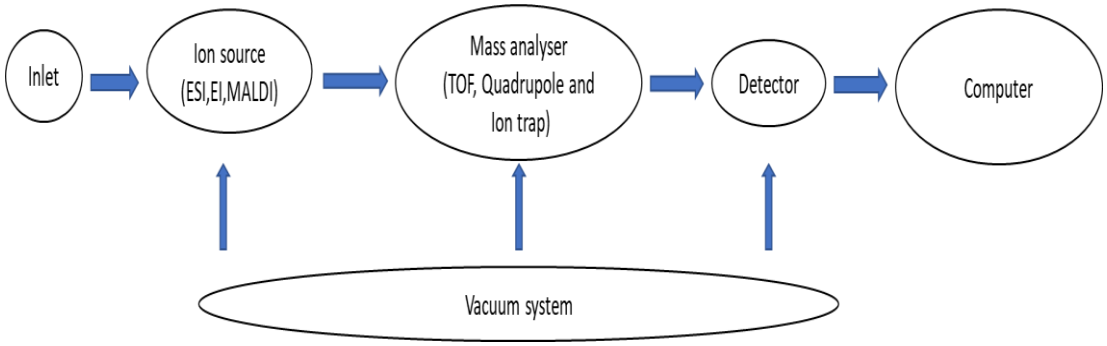


Figure 1-1. Basic components and general representation of a mass spectrometry system.

1.1.2.1. Ionization techniques

Various ionization techniques can be used in mass spectrometry for the formation of gas-phase ions such as electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI) and matrix-assisted laser desorption ionization (MALDI).

Electrospray ionization (ESI) has been the most widely used ionization technique for the last two decades because of its compatibility with HPLC. This technique has

advantages as it is considered to be a soft ionization technique since it produces little fragmentation. However, little structural information of target analytes is obtained but using tandem mass spectrometry this can be addressed (Banerjee & Mazumdar, 2012; Gaskell, 1997).

In general, the electrospray process consists of three main stages: droplet formation, droplet shrinking and gaseous ion formation. As we can see in **Figure 1-2,** the analyte in solution passes through the stainless-steel capillary tip at a specific flow rate. On the other side from the spray needle tip held at a very high voltage (2-6 Kv) at a distance of 1-3 cm, there is source-sampling cone or heated capillary, The high voltage causes the dispersion of the sample solution emerging from the tip of the needle into an aerosol of highly charged electrospray (ES) droplets due to coulombic repulsion. A coaxial flow of nebulising gas (nitrogen) that flows around the outside of capillary also supported this process to get better nebulization as well as directing the spray emerging towards the mass analyser. The drops under the repulsive forces produced by the accumulated ions elongate and form a Taylor cone which finally splits down to smaller droplets which are further reduced in size by the evaporation of solvent with the help of warm flow of drying gas passing across the front of ionization source. The drying gas usually is nitrogen. Finally, the charged sample molecules without solvent are released from the droplets as a result of an internal repulsive force, and the ions are then transferred to the high vacuum region of the mass analyser (Banerjee & Mazumdar, 2012).

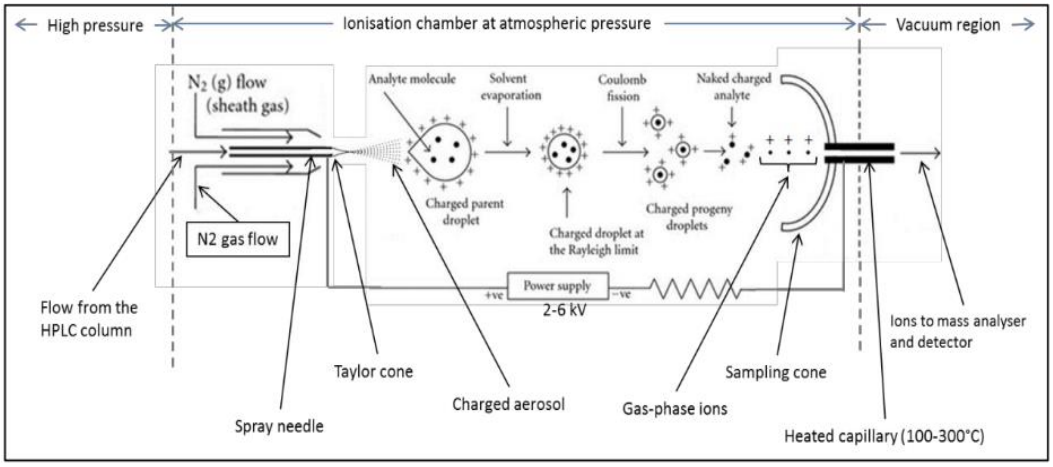


Figure 1-2. A schematic representation of the ESI-ion source (produced by Banerjee & Mazumdar, 2012; modified by Jonans Tusiimire 2016, 2016).

1.1.2.2.Mass analysers

There are many types of mass analysers such as quadrupole, ion trap, Fourier transform mass spectrometry (FTMS) and time of flight (TOF) using dynamic magnetic/electric fields for ion selection. The mass analyser used depends on the requirements for resolution, mass range, scan rate, and detection limit required for an application (Banerjee & Mazumdar, 2012).

1.1.2.2.1. Quadrupole analyser and Tandem quadrupole system

Quadrupole analysers are relatively cheap and are the most commonly used analysers in bioanalytical methods based on tandem mass spectrometry, and ions are separated according to their mass/charge ratio (Ho et al., 2003). Quadrupole analysers consist of four parallel rods which are connected in pairs having the same potential voltage but with opposite charges. In quadrupole analysers, two electric fields are applied which

are at right angles to each other for the separation of ions. These two fields are a constant direct current voltage (DC) and an alternating radio frequency (RF) voltage. The purpose of applying two electrostatic fields at right angles to each other is to produce a resonance frequency for each m/z value. Thus, the ions which have the same resonating frequency as of the quadrupole can pass through and be detected. Ions having m/z lower or higher than the resonant frequency at a particular instance will collide with the rods and will not reach the detector.

The tandem mass spectrometer uses three quadrupoles (Q1, Q2 and Q3) as mass analysers and mainly operates under the same principle as the single quadrupole mass analyser. The Q1 is a mass filter for selection of precursor (parent) ions. While Q2 controlled by only RF -performs collision induced dissociation (CID) with a gas (e.g. argon or nitrogen) producing product or daughter ions from the precursor ion. Finally, the Q3 is used for mass analysis and detection of these daughter ions see **Figure 1-3.** Multiple reaction monitoring (MRM) mode is used to allow only daughter ions from certain parent ions to be detected; using this mode results increased in the sensitivity (Johnson V., Yost A., Kelley E., & Bradford C., 1990).

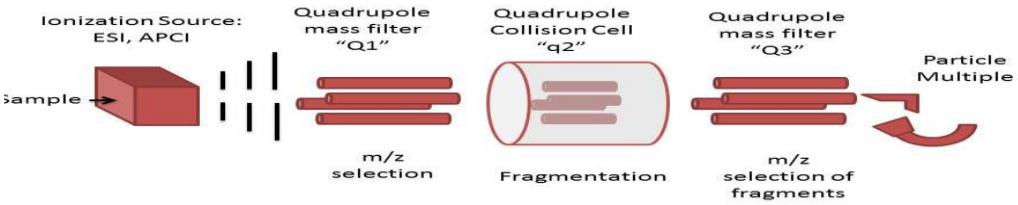


Figure 1-3. A schematic representation of a Triple Quad LC/MS system (Banerjee & Mazumdar, 2012).

1.1.2.2.2. The Orbitrap Mass Analyser

The Orbitrap is a high-resolution trap launched in 2005 which can measure the masses of ions to five decimal places or more. In the Orbitrap instrument ions from the electrospray source travel via RF-only octopoles into a linear trap the C-trap. In the C-trap, the ions are trapped and squeezed into a smaller cloud, and then ions are ejected towards the Orbitrap through the lens system by a voltage pulse across the C-trap. In the Orbitrap, an electrostatic field is used for trapping the ions. The ions are trapped so that they move around a central spindle-shaped electrode while oscillating in the Z direction. The oscillation of the ions is then detected in the form of an image current which is ultimately changed into very accurate mass information **Figure 1-4** (Scigelova, Hornshaw, Giannakopulos, & Makarov, 2011)

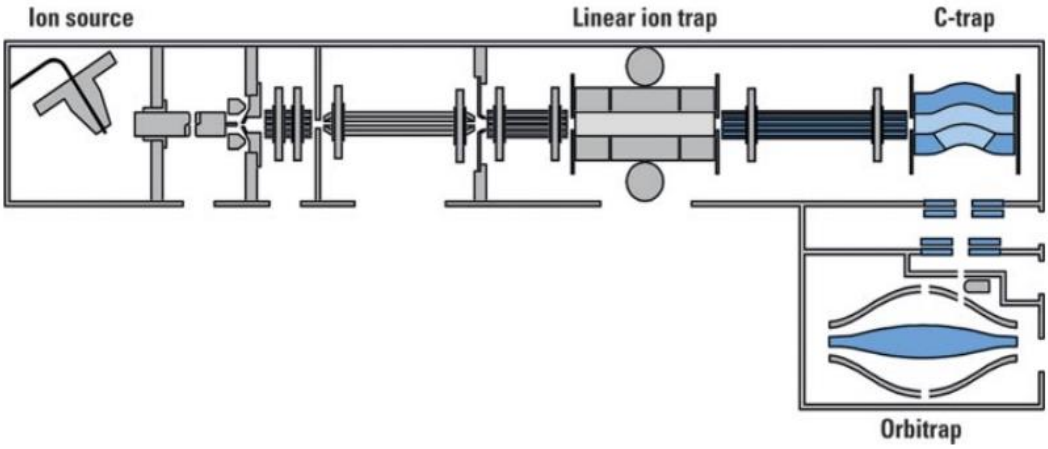


Figure 1-4 A schematic layout of the Orbitrap mass spectrometer (Scigelova et al., 2011).

1.2. Extraction methods for biological samples

1.2.1. Protein precipitation (PPT)

Removing proteins is important in order to prevent them from precipitating out on the chromatography column causing column blockage or loss of chromatographic peak as well as affecting on ionization in the electrospray ionization source. Therefore, protein precipitation is the most common and quickest method of protein removal and works for a wide range of compounds and simplest extraction way for removing proteins by adding solution or solvent to the sample to denature the proteins and precipitate them. There are several solvents used to precipitate the proteins, but results show that deproteinization caused by acetonitrile results in efficient removal of protein and good recovery (Burgess, 2009).

1.2.2. Solid-phase extraction (SPE)

Solid-phase extraction (SPE) is a technique used for the sample preparation and employs a chromatographic packing material in a cartridge to separate different components of a sample and concentrate them. Unlike protein precipitation (PPT) or Liquid-liquid extraction (LLE), SPE can take a long time as well and often requires a much higher effort toward method development, as a consequence the results are very useful providing highly selective extraction to produce very pure samples. The quantity of organic solvent used in SPE is much less than that used in LLE. A wide variety of sorbents is available for SPE, which includes reversed-phase materials, both strong and weak ion exchange materials and mixed-mode materials (Huck & Bonn, 2000).

Generally, the protocol for utilising SPE cartridge is similar for all the different types and consists of the following steps: Sample pre-treatment, Cartridge conditioning (equilibration), sample loading, sample washing and sample elution(Thurman & Mills, 1998).

Reversed-phase SPE is widely used in biological analysis. Various types of reversed-phase cartridges are based on coated silica gel or on a polymeric backbone such as C18 to C2, cyclohexyl, phenyl and cyanopropyl, where C18 functionality is used for most compounds. The mechanism of the retention is an interaction of non-polar groups of analytes with non-polar functional groups on the sorbent, via van der Waals

interactions, also known as hydrophobic interactions see **Figure 1-5 (left)** (Thurman & Mills, 1998).

When more than one type of analyte is looked for, or additional selectivity may be required, then mixed-mode SPE can prove useful. Mixed-mode SPE consists of two or more primary retention mechanisms with most common mixed-mode sorbents having hydrophobic and ion-exchange functional groups attached to the surface see **Figure 1-5 (right)**. The mechanism of the retention is both due to electrostatic attractions between charged groups as well as non-polar interactions between non-polar functional groups and hydrophobic linkers. Former interaction is considered to be the primary, whereas the latter is the secondary or weaker but still relevant (Thurman & Mills, 1998).

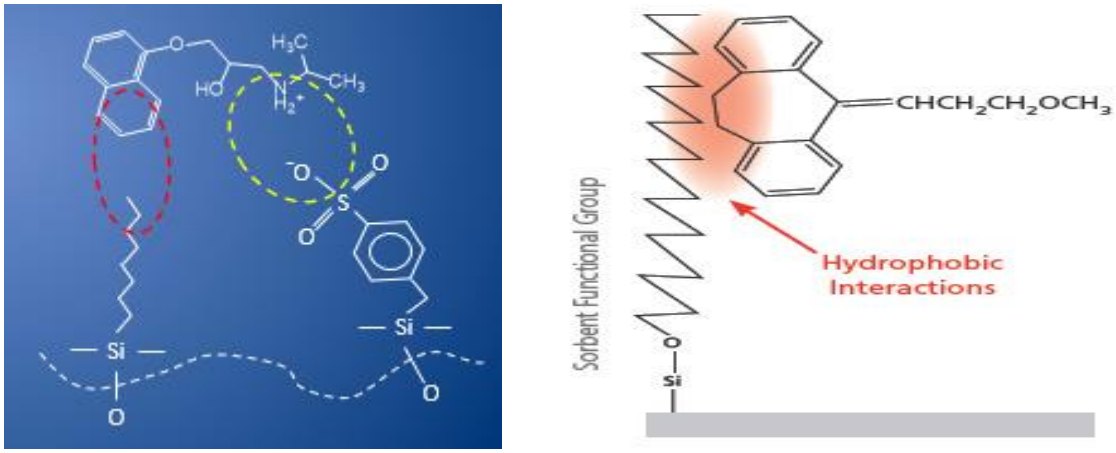


Figure 1-5. Mechanism of retention, in the mixed-mode SPE (right) (K. Zhang & Liu, 2016) and reversed-phase (left) (Henion, Brewer, & Rule, 1998).

1.3. Derivatization for mass spectrometric analysis

When some compounds with low concentration are particularly challenging to ionize in the ion source, chemical derivatizations can be used to optimize the sensitivity of detection of the analyte in ESI-MS by improving charging with suitable derivatization reagents (Higashi, Yamauchi, & Shimada, 2005; T Santa, Al-Dirbashi, & Fukushima, 2007). Derivatization is used to make compounds more ionizable in the LC/ESI-MS/MS. Moreover, upon collision-induced dissociation (CID), the target derivative analyte fragments efficiently and generates an intense product ion for the sensitive MS/MS detection (T Santa et al., 2007).

According to different reports, the benefits of chemical derivatization for LC-MS analysis are an improvement in the selectivity and separation of the target analyte, increasing the ionization efficiency in the mass spectrometry system for detection, enhancement in structure elucidation, help in isomer separation and elimination of endogenous interference (Qi et al., 2014; T Santa et al., 2007; Tomofumi Santa, 2011).

The chemical reaction of derivatization needs a functional group present on the target compound and reacting group in the derivatization reagent for the derivatization procedure to work as seen in **Figure 1-6** (Qi et al., 2014). Selection of a suitable derivatization reagent depends on the functional group of the target analyte, and the corresponding mechanism of the derivatization reactions. There are several functional

groups suitable for derivatization such as hydroxyl, carboxyl, carbonyl, amine or thiol (Tomofumi Santa, 2011).

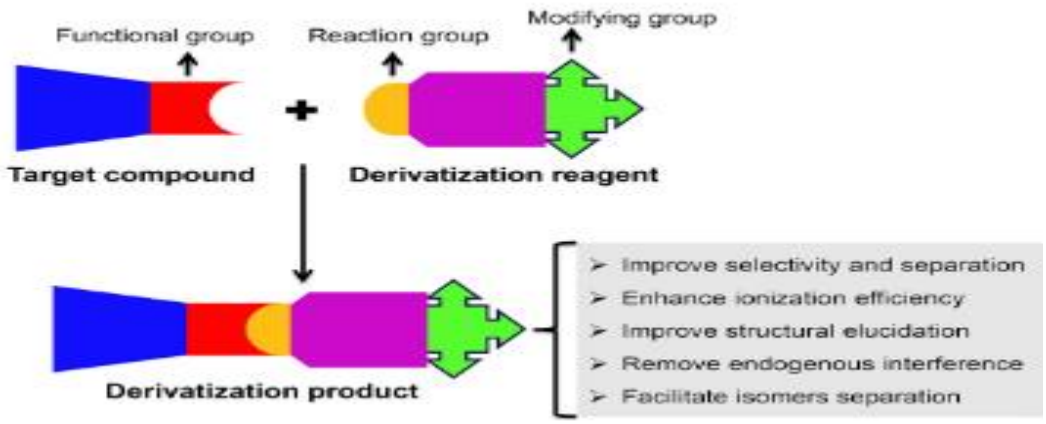


Figure 1-6. The reaction of a derivatization reagent with the functional group of a target compound. Figure reproduced from (Qi et al., 2014).

Compounds that consist of ketones and aldehydes, as well as alcohols and phenols functional group, have the poor ability for ionization in electrospray ionization (ESI). Therefore several derivatization reagents such as 2- Hydrazino-1- methylpyridine (HMP) Girard's reagent, hydroxylamine and 2, 4-Dinitrophenylhydrazine (DNPH) have been used for the derivatization of ketones and aldehydes **Figure 1-7,** and dansyl chloride and 2-fluoro-1-methyl pyridinium-p-toluenesulfonate (FMP-TS) have been used for alcohols and phenols **Figure 1-8** to introduce chargeable groups to increase the ionization (Anari et al., 2002; Higashi et al., 2005; Qi et al., 2014; Williams, Lovell, & Lynn, 2005).

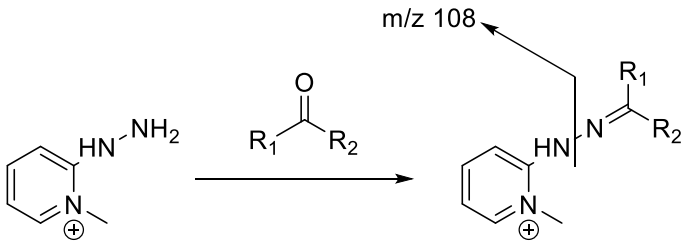


Figure 1-7. Derivatization reaction for ketones and aldehydes, and the product ion of the derivative obtained by CID using HMP derivatization reagent.

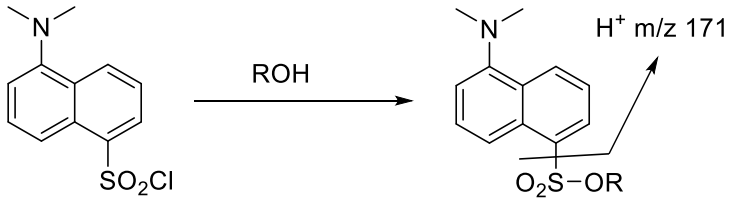


Figure 1-8. Derivatization reaction for alcohols and the product ion of the derivative obtained using dansyl chloride derivatization reagent.

Compounds containing a carboxylic acid functional group have low detection sensitivity in the mass system using negative ion mode in ESI, because of the high background noise as well as the compatibility of the mobile phase with ESI-MS for carboxylic acid detection is not always effective (T Santa et al., 2007). Therefore, derivatization of the carboxylic acid so that it carries a positive charge is a choice. Examples of such reagents include triethylamine (TEA) or dimethylaminoethyl (DMAE) ester derivatization of carboxylic acids (Johnson, 1999).

Various drawbacks related to the analysis of amines (RNH_2) on LC-MS including polarity, high water solubility and basicity along with the high matrix effects and background noise. Derivatization of amines makes these compounds more hydrophobic, resulting in improved separation and detection by LC-MS since the molecular weight is increased thus decreasing the background noise from the matrix

(T Santa et al., 2007). Examples of derivatization reagents such as 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F) (T Santa et al., 2007).

Stability seems to be a major issue in the analysis of thiols. Derivatization has been used to solve this problem by the introduction of a charged moiety leading to increased detection sensitivity in the MS system. ω-bromoacetonylquinolinium bromide, 2-Bromo-4'-chloroacetophenone, 2-Bromo-4'- bromoacetophenone, Iodoacetamide, isopropyl chloroformate and Ellman's reagents are examples for derivatization reagents used for derivativatizing thiols (Qi et al., 2014; Quirke, Berkel, & Adams, 1994).

1.4. Metabolomics

Metabolomics is the scientific term describing an optimal approach to identify and quantify the existence of the levels low molecular weight metabolites such as amino acids, amines, fatty acids, organic acids, aromatic compounds, nucleotides and steroids within biological systems. Metabolomics is derived from the word 'metabolism'; this word comes from the Greek and means 'change' (Fiehn, 2002).

The techniques utilised for metabolomics are analytical techniques which are deployed in order to determine and quantify any changes to the metabolites which occur within a biological system in response to different stimuli such as diet, lifestyle and physical activity. This approach can be classified into three broad groups, namely; targeted, semi-targeted and untargeted profiling. Targeted analysis can only be used to measure

a tiny fraction of the metabolome and can be applied quantitatively in the presence of a specific stable isotopically labelled internal standard. The calibration curve must, however, also be constructed in order to determine the absolute concentration of the target analyte. The semi-targeted is the approach between targeted and untargeted. It can measure hundreds of known metabolites and measure their approximate concentrations, whereas the global metabolomics untargeted approach can be used for measurement of many metabolites as possible in the absence of their exact concentrations (Broadhurst et al., 2018). For instance, there may be limited knowledge of the metabolite's profiles expected to be detected in a given sample such as the metabolomic differences between pre- and post-training in young professional football players in this project. As a result, the untargeted analysis produces hundreds to thousands of metabolites, where not all of them can be identified, where to identify these require a large number of standards to confirm identity, and this would be difficult since these standards may be expensive or unavailable (Broadhurst et al., 2018; Fukushima & Kusano, 2013; Patti, Yanes, & Siuzdak, 2012).

Obtaining the best analytical results in untargeted metabolomics can be achieved by applying a metabolomic approaches workflow as follows:

Firstly, data acquisition selection and preparation of biological samples, selection of analytical methods to address the various problems of data acquisition related to sampling variability or systematic drift, the signal intensity of metabolite and analytical

noise changes are addressed by using pooled quality control QC samples to correct the variation which occurs in analytical measurements (Broadhurst et al., 2018; Fukushima & Kusano, 2013; Patti et al., 2012).

Secondly, data processing, including metabolites identification, data treatment, it mainly used to extract quantified data and can be handled by analytical tools and statistical analysis software that are compatible with the instrumentation such as MZmine and MZ match (Broadhurst et al., 2018; Fukushima & Kusano, 2013; Patti et al., 2012).

Thirdly, data analysis and interpretation consist of statistical analysis and validation of biomarkers and interpretation of the results. Statistical analysis involves two approaches, which are univariate and multivariate analysis that are used for the examination of that relationship. Univariate analysis such as paired t-test (p-value) and fold changes (ratio) are dealing with each variable to find out significant metabolites. On the other hand, the multivariate analysis must be followed by some steps to determine the separation of groups based on biological components, which use an unsupervised technique, principal component analysis (PCA), and supervised techniques which include Orthogonal Partial Least Squares-Discriminant Analysis (OPLS-DA), that can be implemented using SIMCA software. Applying PCA is sometimes not enough to give a clear visualisation more than showing the overview of the dataset due to external factors such as diet or physical activity. Therefore, to

provide acceptable predictions and optimize data interpretation, then a supervised module such as OPLS-DA must be applied. OPLS-DA is the powerful technique that used to classify reliable biomarkers that have an essential association with separation between groups and are related to changes in the metabolic pathway (Broadhurst et al., 2018; Fukushima & Kusano, 2013; Patti et al., 2012).

Moreover, data validation is the next step, to the evaluation of the supervised module quality. The validity of the module assesses by a permutation test, observed versus predicted value and cross-validation ANOVA (CV-ANOVA). The purpose of previous processes is to identify the significant metabolites that use to understanding the significance of each metabolite change and the pathway envolved (Broadhurst et al., 2018; Fukushima & Kusano, 2013; Patti et al., 2012).

Chromatography method for separating endogenous compounds, combined with mass spectrometry for identification of a large number of metabolites is a powerful analytical platform for identifying biomarkers in the untargeted biochemical profiling of metabolites. In this project, an Exactive Orbitrap MS system has been used for metabolomics screening. This system has advantages over many other instruments with its high scan speed, which is ideal for fast and comprehensive metabolite profiling of tissue extracts or biofluids due to its fast polarity switching when coupled with LC separation. The instrument can provide mass resolution >100 000 and mass error <2 ppm (T. Zhang, Creek, Barrett, Blackburn, & Watson, 2012).

Chapter 2:
Quantification of cannabinoids in human hair through derivatization and liquid chromatography-tandem mass spectrometry

2. Quantification of cannabinoids in human hair through derivatization and liquid chromatography-tandem mass spectrometry

2.1. Introduction

2.1.1. Hair

Hair is a characteristic of all mammals. The hair on animals is referred to as fur. It grows from roots which begin underneath the skin and covers the mammal's body (Tobin, 2007).

Hair consists of two parts, the hair shaft which is visible above the skin and the hair follicle under the skin often called the hair root. The hair shaft is composed of the cuticle, the cortex and the medulla. The components of the follicle are surrounded by a rich blood capillary system that delivers the necessary nutrients, elements and metabolic material to the growing hair. The germination centre around the hair bulb papilla is formed by keratinocytes and melanocytes present on the basement membrane (Kučera, Byrne, Mravcová, & Lener, 1992). Melanocytes produce melanin that is released into the keratinocytes, and then the membranes of the vesicles in the keratinocytes are digested, and the melanin pigments remain (Harkey, 1993). Hair follicles are associated with three glands, the sebaceous gland, the apocrine gland, and the eccrine (sweat) gland (Harkey, 1993).

The scientific community considers the average growth rate of scalp hair for an adult male or female as one centimetre per month (1 cm/month)(Gail Audrey Ann Cooper, 2011). Hair growth starts in cells around the papilla. The epithelial cells or matrix cells are responsible for the growth of hair in a germination centre where all cells are mitotically active and integrated into the hair shaft; this cycle is considered to be the most rapid of all human tissues (Harkey, 1993). As 85% of hair follicles can be found in the vertex region of the scalp, this area was chosen as the site to obtain our test specimens. It is also considered to have the fastest hair growth rate (Harkey, 1993).

The hair shaft is made up of keratin, which is a protein. This protein plays a key role in the organisation and physical properties of hair. Hair also contains water (12 to 15 %) and traces of mineral elements (calcium, cadmium, chromium, copper, zinc, iron and silicon). Other lipids such as triglycerides, waxes, squalene, esterified cholesterol and free cholesterol come from the secretion of the sebaceous gland (Harkey, 1993).

Many factors could influence the duration of the growth cycle of hair such as ethnicity, gender, age and dietary habits and environmental alterations like day-length, disease, or cosmetic use also it is harmonized by many hormones and cytokines (Wolfram, 2003).

The incorporation of drugs into hair has been studied and reported in three ways, as shown in **Figure 2-1**. Firstly, active or passive diffusion from the bloodstream nourishing the dermal papilla. Secondly, diffusion from sweat and sebaceous glands

that release its secretions into the growing or mature hair fibre. Finally, diffusion into the mature hair fibre from smoke, vapours, powders or raw drug materials(Cone, 1996).

Lipophilicity coupled with basicity and melanin affinity are the main properties that play a role in drug incorporation into hair. Non-polar and more lipophilic parent drugs can easily pass the cell membrane from the bloodstream due to the chemical nature of the cell. Melanin pigments are reported to be the principal component for binding drugs; thus, its affinity is associated with drug basicity since it contains a negatively charged in its structure which lead to its attracting with positively charged basic drug molecules. On the other hand, many studied have reported that there is a correlation between the colour of hair and incorporation of the drug; as darker hair appears to have more binding of drugs(Borges, Wilkins, & Rollins, 2001; Nakahara & Kikura, 1996; Rollins, Wilkins, Gygi, Slawson, & Nagasawa, 1997).

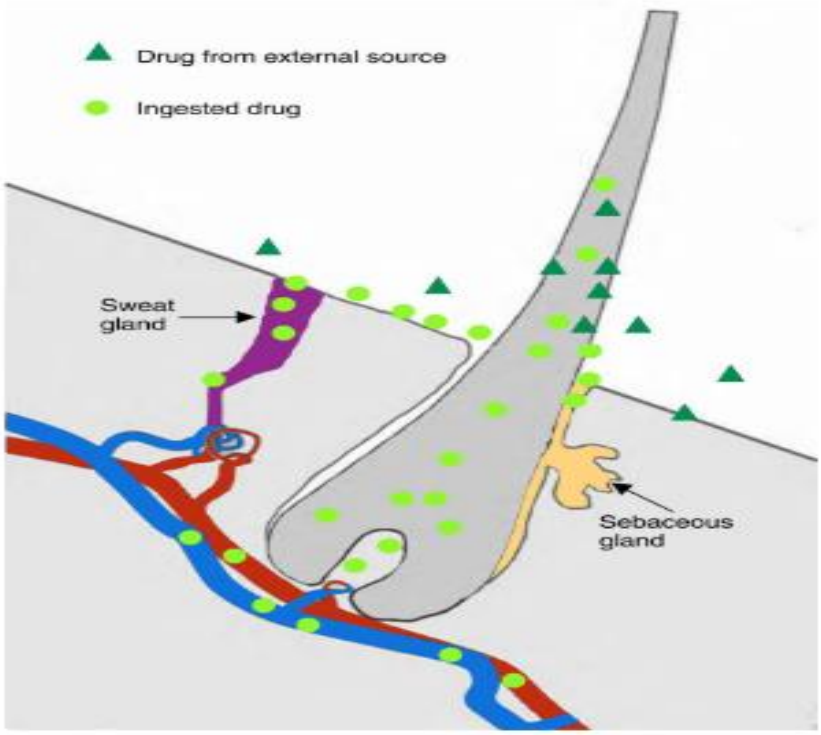


Figure 2-1. Incorporation routes of drugs into the hair. Reproduced from (Pascal. Kintz, 2006).

The analysis of hair for drugs, an exciting area for researchers, was first reported in 1858 (Sachs, 1997). In 1954 Goldblum determined barbiturates from hair (GOLDBLUM, GOLDBAUM, & PIPER, 1954). Baumgartner detected drugs of abuse in hair in 1979 (Baumgartner, Jones, Baumgartner, & Black, 1979), and then several studies were published on the detection of other drugs of abuse. Noticeably, Klug, who converted for the first time solid hair matrix into a solution using sodium hydroxide in 1980 (Sachs, 1997). Advancements in technology and innovation in the nineties have made the use of hair to detect drugs of abuse at a very low concentration widespread (P Kintz, 2006).

The Society of Hair Testing (SoHT) was established to publish recommendations and guidelines related to hair starting from sample collection to cut-offs for obtaining positive results. Using hair specimen as an alternative material for detection of drugs of abuse rather than urine or blood has benefits such as its easy and non-invasive sample collection, it provides information of long periods of drug abuse as well as being easy to store and handle hair samples. In contrast, it does not provide information of recent periods of drug use, the length of the subject's hair is not always available and external contamination usually concerns the researchers as well as a lack of understanding of drug incorporation mechanisms.

2.1.2. Cannabis

The origin of the cannabis plant seems to be the Tian Shan mountains, south of Siberia in the Altai mountains and Central Asia (Ben Amar, 2006; Cabral, 2005). It is believed that this plant has been used for 29000 years (Pringle, 1997). The therapeutic use of cannabis was banned in 1970 in the United States, once they discovered its side effects such as moral and intellectual deterioration that led to various crimes (State-By-State Medical Marijuana Laws, 2016). Soon after, most of the countries in the world, including Saudi Arabia, have prohibited cannabis use in compliance with the Convention on Psychotropic Substances Legislations instituted by the United Nations in 1971 (Ben Amar, 2006). Despite its illegality, according to the annual report of World Drug Report published by United Nations Office on Drugs and Crime (UNODC),

approximately 183 million users consumed cannabis around the world in 2014, where cannabis remains the most popular drug (UNODC The United Nations Office on Drugs and Crime, 2016).

More than 500 chemical compounds have been identified in cannabis, 100 of them are considered as cannabinoids (Ashurst et al., 2012). Gaoni et al. found that Δ9-Tetrahydrocannabinol (THC) is the primary psychoactive cannabinoid (Gaoni & Mechoulam, 1971). One of the other non-psychoactive cannabinoids is cannabinol (CBN), which was isolated the first time in 1899 by Wood (Schultz & Haffner, 1960). After smoking a joint of cannabis, the THC is metabolized in the body, mainly by the liver, to form phase I metabolites, 11-hydroxy-Δ9-tetrahydrocannabinol (THC-OH, psychotropically-active) which is further oxidized to form 11-nor-9-carboxy-Δ9-tetrahydrocannabinol (THC-COOH, psychotropically-inactive). Both metabolites can be detected in plasma (Gustafson, Moolchan, Barnes, Levine, & Huestis, 2003) and other biological specimens(Sobolesky et al., 2019).

In the last decade, detecting different drugs, including cannabinoids, in hair has become of great interest. This is because hair testing has a great potential to trace the historical pattern of someone's drug use habit, which other biological samples cannot do. Also, the hair specimen collection procedure is non-invasive, hair samples can be easily transported, and stored as well as having a low risk of adulteration (Miller, Donnelly, & Martz, 1997). For cannabinoid analysis in hair, it is crucial to target the

metabolites, THC-OH or THC-COOH, to avoid false-positive results due to environmental exposure to the cannabis smoke and also to challenge claims of passive exposure (Michael Uhl & Sachs, 2004a).

Although the determination of cannabinoids in hair has a valuable benefit, detecting them is a challenging task and needs highly sensitive analysers and robust extraction method due to their very low concentrations in hair, especially the metabolites. The Society of Hair Testing (SoHT) recommends a cut-off (0.05 ng/mg) for THC and (0.2 pg/mg) THC-COOH in hair is detected as an additional confirmatory test (Gail A A Cooper, Kronstrand, & Kintz, 2012). The steps usually required to detect these analytes in hair as described below and as summarized in **Table 2-1.**

The hair is first washed to remove any of the cannabinoids adsorbed on the hair surface, with solvent often Dichloromethane (DCM) which is a popularly used solvent(Vogliardi, Tucci, Stocchero, Ferrara, & Favretto, 2015)**.** A list of washing solvents can be found in **Table 2-1**. The washed sample is then digested, to remove the cannabinoids from the inside of the hair sample. This is usually achieved using strong alkaline hydrolysis such as sodium hydroxide (NaOH) (Vogliardi et al., 2015). The third stage of the process is extraction, liquid-liquid extraction (LLE), solid-phase extraction (SPE) or both are required for further cleaning up of the digested hair to increase the abundance of analytes in the sample as well as to remove any endogenous

interference. In general, extraction of cannabinoids from hair is carried out using both extraction approaches.

During the LLE, it is essential to adjust the pH prior to the extraction due to the variation of the chemical properties of the main cannabinoid's THC and CBN and the main metabolite THC-COOH. In some cases, LLE followed by SPE extraction is required for further cleaning up as also shown in **Table 2-1**. Unlike the previous studies where SPE was used as a supplementary extraction technique, the extraction in the current study was carried out in one single step using SPE for extraction of all analytes of interest using a Strata-X-A 33µm Polymeric Strong Anion exchange cartridge without the further step of adjusting pH as seen in **Table 2-1**.

The fourth stage of the process is a derivatization step. In order to improve detection limits, it may be necessary to derivatize cannabinoids. Various derivatization procedures for cannabinoids have been used such as methyl iodide, 2,2,3,3,3-Pentafluoro-1-propanol (PFPOH), and 1,1,1,3,3,3- Hexafluoro-2-propanol (HFIP) for esterification of carboxyl group on THC-COOH as well as trifluoroacetic anhydride (TFAA), pentafluoropropionic anhydride (PFPA), 2,2,3,3,3-Pentafluoro-1-propanol (PFPOH) and Heptafluorobutyric anhydride (HFBA) for perfluorinated of hydroxyl group, most of these reagents established for GC-MS and could also be applied to LC-MS applications (Moore, Rana, Coulter, Feyerherm, & Prest, 2006a; Thieme, Sachs, & Uhl, 2014; M. Uhl, 1997). Thieme et al., derivatized THC-COOH using methyl iodide with

acetonitrile and crystals of solid sodium carbonate. The mixed derivative led identification and quantification of THC-COOH in hair samples to below 0.1 pg/mg using LC-MS/MS due to differentiation from interfering fatty acids (Thieme et al., 2014). A 2-fluoro-1-methyl pyridinium-p-toluenesulfonate (FMP-TS) derivative was used with phenolic estrogens led to enhance the sensitivity and specificity of analysis of E2 and E1 in low abundance in plasma and serum using LC-MS/MS (Faqehi et al., 2016).

In the present work, the FMP-TS derivatizing reagent was employed to form the N-methyl pyridinium ether derivatives of the hydroxyl group of cannabinoids (CBN, THC and THC-COOH), in combination with esterification of the carboxyl group in THC-COOH using methanolic HCl to carry out the derivatisation **Figure 2-3**. These reactions yield a positively charged derivative led to the distinction between analytes, especially between THC-COOH and matrix lipids, which are uncharged and unionized, leading to increasing the sensitivity, selectivity and stability of THC-COOH.

Finally, analysis of cannabinoids in hair extracts is carried out using different techniques such as GC or LC coupled with MS/MS (Angeli, Casati, Ravelli, Minoli, & Orioli, 2018b, 2018a; Kieliba, Lerch, Andresen-Streichert, Rothschild, & Beike, 2018; Prego-Meleiro et al., 2017). Since THC-COOH is present in the hair sample in the picogram range or less, tandem MS is required to provide excellent sensitivity. Dulaurent et al., reported the determination of the main cannabinoids (THC, CBN and

CBD) and main metabolite (THC-COOH) together using LC-MS/MS for the first time with one sample preparation which utilized LLE prior to injection (Dulaurent, Gaulier, Imbert, Morla, & Lacha, 2014). In the current project, an alternative procedure using SPE and liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) for detection THC, CBN and THC-COOH in hair samples was carried out using a single sample preparation procedure and analysis method.

2.2. Aim

This project aimed to find a sensitive and selective analytical method for the quantitation of cannabinoids in hair using a 2-fluoro-1-methyl pyridinium-p-toluenesulfonate (FMP-TS) and methanolic HCl as derivatization agents. The second aim was to evaluate the developed and validated the method by applying it to authentic hair specimens obtained from known cannabis users.

Table 2-1 Summary of analysis steps for detection of cannabinoids in hair using liquid chromatography-mass spectrometry.

Substance, Date	Sample amount (mg)	Washing	Extraction procedure	pH	LLE	SPE Washing	SPE Elution	Derivatization & ion charge	Analytical techniques	LOD (pg/mg)	Ref
THC, CBN (2011)	20	Shampoo, deionized water, acetone, air-dried overnight	4mL methanol, 8h at 50 ° C + overnight at room temperature		LLE, 1mL methanol/water (1:1, v/v) filtered 0.45 PTFE syringe filter			positive ESI mode	LC–TOFMS	12.5, 5	(Domínguez-Romero, et al., 2011)
THC, CBN,CBD (2012)	50	Dichloromethane, twice	3 ml NaOH 1N at 95 ° C for 10 min		LLE, 5 ml of n-hexane/ethylacetate 90 : 10 (v/v)			positive ion mode.	LC–MS/MS	1.2,1.6,5.4	(Salomone, et al., 2012)
THC (2012)	50	2ml Dichloromethane (DCM) three times	ACN, 12h 50 ° C	9	LLE hexane/ethyl acetate SPE polymeric Reverse-phase (Strata™-X)	2ml of 5% MeOH in water and 2ml Water/MeOH/ammonium hydroxide (75/24.5/0.5, v/v)	2ml DCM/2-propanol (75/25, v/v/)		LC-ESI-MS/MS (QQQ)	50	(Lendoiro et al., 2012)
THC, THC-COOH (2013)	20	4ml MeOH	1ml NaOH, 60 ° C	7	LLE 3 ml, ethyl acetate			Negative-ESI-mode	LC-ESI-MS/MS	1 and 1	(Mercolini et al., 2013)
THCA-A, THC, CBN, CBD (2013)	50	3 times deionized water, petrol ether, methanol			2 mL methanol, 4h at room temp, shaking			Negative-ESI-mode	LC-ESI-MS/MS	20	(Roth, et al, 2013)
THC-COOH (2014)	25		2ml NaOH and 200µl ethanol at 70 ° C for 60 min	4.5	LLE, hexane/ethyl acetate (9:1) v/v	Strata™-X-C 33 µm, Polymeric Strong Cation Exchange,200µl 0.1M HCl and acetonitrile + HCl (3/7, v/v)	2ml acetonitrile + glacial acetic acid (98/2, v/v)	20 µL methyl iodide heat for 1.5h at 70 ° C. negative ESI	LC-MS/MS/MS	0.1	(Thieme et al., 2014)

THC, CBN, CBD (2014)	50	Soap, water and dichloromethane	2.0 M sodium hydroxide (100 °C, 10 min)		LLE, hexane/ethyl acetate (9:1) v/v	zircon ball mill for 20 min has been used for pulverized hair			(HPLC–MS/MS	0.25, 0.21, and 0.22	(Míguez-Framil et al., 2014)
THC, CBN, CBD THC-COOH (2014)	20	Dichloromethane, twice	1 mL of 1 M NaOH for 10 min at 100 8C	4	LLE, 7 mL of a hexane/ethyl acetate (90/10, v/v)			negative ESI mode	LC–MS/MS	50,50,50 and 0.2 respectively	(Dulaurent, et al., 2014)
THC-COOH (2014)	20	1 mL of methanol	1 mL of 1 M NaOH at 85 ◦C for 30 min	4.5	LLE, 2 mL of n-hexane:ethyl acetate (9:1) for			negative -ESI	LC–MS/MS/MS	0.05	(Park et al., 2014)
THC-COOH-Glucuronide (2015)	25	5 ml methyl alcohol and 2.5 ml diethyl ether	500 ml VMA-T M3(acidic aqueous buffer) reagent for 1 h at 100 ° C						UHPLC–MS/MS analyses	0.09	(Kenji et al , 2016)
THC-COOH (2015)			A stainless-steel bullet with 50% MeCN/water solution (200 µL)		(200 µL) of NaCl-saturated solution and MeCN (300 µL) sonicate				LC/MS/MS	0.1	(Kuwayama et al., 2015)
CBN, THC, THC-COOH (2019)	50	H_2O, Dichloromethane twice	1 mL of 1 M NaOH at 90 ◦C for 15 min	14		3 ml of H_2O & 2 ml of ACN / H2O (20:80 v/v)	3 ml of cyclohexane/ ethyl acetate/ acetic acid (80/20/5, v/v/v).	(FMP-TS) and methanolic HCl using positive ion mode.	LC/MS/MS	2,2 and 0.1	This work

2.3. Materials and methods

2.3.1. Standards, Solvents, Reagents and other Materials

The following Cerilliant certified standards were purchased from Sigma-Aldrich, (Basingstoke, UK): Δ9-Tetrahydrocannabinol (THC) at 1 mg/mL, Cannabinol (CBN) at 1 mg/mL and 11-nor-9-carboxy-Δ9-tetrahydrocannabinol (THC-COOH) at 100 µg/ml. All of these drugs were dissolved in methanol. Deuterated internal standards (ISTD); Cannabinol -*d3* (CBN-*d3)* and 11-nor-9-carboxy-Δ9-tetrahydrocannabinol- *d3* (THC-COOH-*d3*), each at 100 µg/mL, were obtained from Sigma Aldrich (Dorset, UK).

The following solvents and chemicals; HPLC grade methanol (MeOH), acetonitrile (MeCN), ethyl acetate (EtOAc), dichloromethane (DCM), also glacial acetic acid (≥99.7%) and Cyclohexane were purchased from VWR International Ltd, (Lutterworth, UK). Formic acid (≥98%) was obtained from BDH-Merck, UK. Deionized water was obtained from the in-house Millipore® system. Triethylamine (TEA; ≥ 99.5%) and 2-fluoro-1-methyl pyridinium-p-toluenesulfonate (FMP-TS) were from Sigma-Aldrich (Dorset UK).

For hair digestion, a 1 M (40 g/L) solution of sodium hydroxide was used. In addition, 1% solution methanolic HCl was prepared by adding 3ml of acetyl chloride very slowly to 100ml of MeOH.

2.3.2. Collection of hair samples

For method development, validation and QC samples, a blank matrix of hair samples was taken from friends and family members and then tested separately, found to be uncontaminated and then pooled together.

2.3.3. Pre-treatment and extraction of samples

All hair samples were washed with deionized water followed by washing twice with 5 ml dichloromethane (DCM) each, dried in the fume hood at room temperature and then cut into 3 cm pieces with pair of scissors. Approximately 50 mg of each hair sample was weighed out when possible. I ml of a 1 M sodium hydroxide (NaOH) solution was added with internal standards (400 pg and 100 ng of THC-COOH-d_3 and CBN- d_3 respectively). The mixture was incubated in a heating block at 90 °C for 15 minutes and then taken out to cool down at room temperature. The hair digest was then centrifuged for 10 minutes at high speed. Supernatants were finally ready for loading into a solid-phase extraction (SPE) cartridge.

The pH of the extract was *ca* 14. In order to study the best loading pH, the pH of the extract was adjusted to pH 8 and pH 4 by the addition of acetic acid. The adjusted solutions, along with the unadjusted extract, were then loaded onto the SPE cartridge after conditioning as described below. For the extraction, the cartridge was condition first by washing with 2 x 3 ml of methanol followed by 2 x 3ml of water. Then the samples were loaded into a Strata-X-A 33µm Polymeric Strong Anion SPE. Interferants

were washed off with 3 ml of H_2O, and the sorbent was left to dry for 2 min. A second washing step involved using 2 ml of ACN / H2O (20:80 v/v) before leaving the sorbent to dry for another 5 min. The retained analytes and deuterated internal standards were eluted under gravity with 3 ml of cyclohexane/ ethyl acetate/ acetic acid (80/20/5, v/v/v). This elution has been reported to achieve the best elution characteristics (Kieliba et al., 2018). Extracts were then evaporated at room temperature under a gentle stream of nitrogen before derivatization.

2.3.4. Derivatization of cannabinoids with FMP

The derivatization of QC and case sample extracts was carried out by adding 300 µl of methanol (MeOH) and vortexing for 10 minutes. Then, 50 µl methanolic HCl was added. The mixture was then heated up for 15 minutes at 60 °C and evaporated. The resulting residue was again reconstituted with 100 µl of fresh 2-fluoro-1-methyl pyridinium-p-toluenesulfonate (FMP-TS) solution (10 mg/ml in acetonitrile) containing 1% Triethylamine (TEA) and then heated at 40 °C for 15 minutes and evaporated to dryness under a gentle stream of nitrogen. The final residue was reconstituted in 100 µl of mobile phase (30/70 v/v) and vortexed before injecting 30 µl of the mixture.

2.3.5. Instrumentation

An Agilent LC-MS-MS triple quadruple G6430A mass spectrometer equipped with an Agilent 1200 series auto-sampler, a quaternary pump SL with a degasser, and a

thermostatic column compartment was used. Positive ion electrospray ionization (+ESI) was used, and the MS was operated in multiple reaction monitoring mode (MRM). The data were recorded using Mass Hunter software version B.06.00 (Agilent technologies).

2.3.5.1.LC-MS-MS Operating Conditions

The analytes were analysed using an ACE 3 C18-AR column (150 × 3.0 mm i.d 3µm particle size) protected by a guard column with identical packing material (20 x 2.1 mm) both obtained from Hichrom, Reading, UK. Mobile phase A consisted of 0.1% v/v formic acid in the water, and Mobile Phase B consisted of 0.1% v/v formic acid in ACN, which were used in a gradient method at the flow rate of 0.3 ml/min and pressure 400 bar. Mobile Phase B was started at 50% and remained for 3 minutes, then was increased to 90 % over 7 minutes, and then held for 3 minutes, then was decreased to its initial composition in 0.3 minutes. The system was then re-equilibrated for 6 minutes; giving a total run time of 20 minutes. 30 µl of the sample was injected for analysis. The column temperature was maintained at 30°C.

The MS parameters were as follows: the (ESI) electrospray ionization interface in positive ion mode had a needle voltage of 4 kV. The full scan range was from 75–1200 m/z. The cannabinoids and deuterated-d_3 internal standards derivatives with FMP were quantified by using an Agilent LC-MS-MS triple quadrupole G6430A mass spectrometer. The multiple reaction monitoring (MRM) conditions were used to

monitor the transitions. The infusion solutions contained a concentration of 10 µg/ml of each analyte of interest. The optimization results are listed in **table 2-2.** Additional MS parameters were optimised to achieve the best sensitivity as follows: the multiplier voltage (Delta EMV), 1000 V; gas temperature, 300 °C; gas flow, 8 L/min; nebulization pressure, 20 psi.

Table 2-2. Optimization MRM transitions of cannabinoids and deuterated-d_3 derivatives with FMP.

Analyte	MRM transition (m/z)	Fragmentation voltage (V)	CE (V)
THC-COOH-FMP	450.3 > 322.1 #	175	60
	450.3 > 382.3 *	175	45
THC-COOH-d_3-FMP	453.3 > 325.1 #	175	60
	453.3 > 385.2 *	175	45
THC-FMP	406.3 > 158.1#	200	55
	406.3 > 229.1 *	200	45
CBN-FMP	402.3 > 278.3 #	200	60
	402.3 > 179.1 *	200	45
CBN-d_3-FMP	405.3 > 281.2 #	200	40
	405.3 > 179.1 *	200	60

Note. Collision energy (CE); Voltage (V); Δ9-Tetrahydrocannabinol (THC; Cannabinol (CBN); 11-nor-9-carboxy-Δ9-tetrahydrocannabinol (THC-COOH); Cannabinol -d3 (CBN-d3) and 11-nor-9-carboxy-Δ9-tetrahydrocannabinol- d3 (THC-COOH-d3) 2-fluoro-1-methylpyr-idinium-p-toluenesulfonate (FMP-TS); quantifier (*) and qualifier (#).

2.4. Method Validation

The method was validated according to the Scientific Working Group for Forensic Toxicology (SWGTOX) guideline (the Scientific Working Group for Forensic Toxicology, 2013).

2.4.1. Extraction recovery

The extraction recovery technique was used to determine the efficiency of the current analytical method by determining the percentages of the known concentrations of analytes of interest recovered by extracting and analysing them using an optimization method (Peters, Drummer, & Musshoff, 2007).

Two sets of standards were prepared, each of which contained (0.2 and 4 ng/mg) for CBN and THC, and (1 and 12 pg/mg) for THC-COOH. One set was prepared in blank human hair, and the other set was prepared in the mobile phase. The blank hair was spiked with these two concentrations in triplicate and then extracted as described in **section 2.3.3**. The extracted samples were mixed with (100 ng and 400 pg) of CBN-d_3 and THC-COOH- d_3, respectively. The standards of the other set were directly injected after they were mixed with the same internal standards and then derivatised (i.e. the non-extracted samples). The peak area ratios of CBN, THC and COOH to internal standards were obtained for the extracted and non-extracted ratios in each concentration. The absolute recovery was determined for each analyte by dividing the average extracted ratio by that of the non-extracted ratio at the same level and then multiplying by 100.

2.4.2. Matrix effect

The matrix effect, which refers to any compound in the sample except the target analytes, which could affect the analyte response when co-eluted with them, causing

an increase in ionization efficiency (i.e., enhancement) or a decrease in ionization efficiency (i.e., suppression). The consequence is an inaccurate concentration measurement (Hall et al., 2012).

Two sets of samples were prepared in two-levels (0.2 and 4 ng/mg) for CAN, CBN-d_3 and THC, and (1 and 12 pg/mg) for COOH and THC-COOH-d_3. Set one consisted of derivative standards that were injected six times to establish a mean peak area for each concentration. Set two consist of a minimum of 10 different matrix sources. Each matrix source was extracted in duplicate, and the extract was spiked in either the low or high levels of standards and deuterated-d_3. The matrix effect was then calculated by averaging the area of each, as shown in equation 1.

$$\%ionisation\ enhancement\ or\ suppression = ((X\ Area\ of\ set2 / X\ Area\ of\ set1) - 1) * 100$$

Equation 1. Ionization suppression or enhancement percentages (Hall et al., 2012).

A negative value indicates signal suppression, whereas positive values suggest that enhancement occurred. The acceptable limits for enhancement or suppression are ± 25% (the Scientific Working Group for Forensic Toxicology, 2013).

2.4.3. Interference studies

The interferences must be examined for the non-targeted compound to evaluate the ability of the method to produce a response for only a single analyte (specificity) and to distinguish the response of single target analytes from other responses

(selectivity). Three main sources of interference were investigated. The absence of common interference was first studied for 10 hair samples obtained from donors. Secondly, examining the stable-isotope internal standards, that has been done during the calibration patch when internal standards were spiked into blank hair as well as examining the high level of the calibration curve for each analyte and spiked with blank hair without internal standard. Also, internal standards have been injected using the optimised method without extraction. Finally, solutions of reference compounds were injected using the optimised method without extraction to evaluate the endogenous compounds that might be present in the real sample at 0.02ng/ mg see **Table S2.**

2.4.4. Sensitivity: limit of detection (LOD) and limit of quantification (LOQ)

The limit of detection is defined as the lowest concentration in the sample that can be detected by the current method and can be reliably differentiated from the blank matrix at a signal to noise ratio of 3:1. Unlike the LOD, the LOQ is the lowest concentration considered to have acceptable precision at the limit of ± 20% as well as at a signal to noise ratio of >10:1. Background noise is one of the approaches in use in the SWAGTOX guideline. Therefore, a minimum of three samples per run of 50 mg of pooled hair sampled, from at least three sources, with decreasing concentrations and was analysed over three runs in duplicate. The detection and identification must be met to establish LOD and LOQ based on retention time, peak shape, mass spectral ion ratios as well as precision and accuracy for LOQ. The

calibration range for CBN and THC was 0.001, 0.002, 0.02, 0.1 and 0.2 ng/mg, whereas for THC-COOH it was 0.1, 0.2, 0.3, 0.4 and 0.5 pg/mg. The concentrations that yielded a reproducible instrument response with S/N ratio ≥ 3 and S/N ratio ≥ 10 was selected as LOD and LOQ, respectively. S/N was calculated using the instrumental software (MassHunter).

2.4.5. Linearity

The relationship between the corresponding instrument response and the concentration of the analyte is the target from the calibration curve. This correlation was assessed over five replicates of concentration. The calibration curves of the analytes of interest were built according to previous published articles that mentioned in **table 2-10**. Therefore, the calibration curve was prepared by spiking 50 mg of drug-free (blank) hair samples with 1, 10, 50, 100, 150, and 200 ng of CAN and THC; corresponding to (0.02, 0.2, 1, 2, 3 and 4 ng/mg), and 10, 50, 100, 200, 400 and 600 pg of THC-COOH; corresponding to (0.2, 1, 2, 4, 8 and12 pg/mg), plus (100 ng and 400 pg) of CBN-d_3 and THC-COOH- d_3; corresponding to 2ng/mg and 8pg/mg respectively. The calibration curves were determined by plotting the peak area ratios of CBN/ CBN- d_3, THC/ CBN- d_3 and THC-COOH/ THC-COOH-d_3 (y) against the concentrations of CBN, THC and THC-COOH (x).

2.4.6. Carryover

Carryover can be defined as the appearance of unintended analyte signal in samples transferred from a previously run positive sample. This signal will, subsequently, lead to inaccurate quantitation. As part of method validation, carryover was evaluated by injecting two blank QC samples immediately after the highest calibration level over five runs whilst establishing the calibration model.

The obtained chromatograms were then examined visually for presence of interfering signal for all analytes.

2.4.7. Bias and precision

The term bias may be described as accuracy or trueness and referred to the nearness of the mean value to the nominal concentration value of the target analytes. The mean value must be within ± 20 %. The bias of the results was determined by calculating the percentage of the expected concentrations for three replicates at spiked at three different concentrations (low, medium and high) obtained from a minimum of three sources over five different days. The bias percentage was calculated for each concentration using Equation below.

Bias (%) at a concentration $_x$ = ([$grand\ mean\ of\ calculated\ concentration_x$ − nominal $concentration_x$]/ nominal concentration x) X 100

The term precision refers to can be defined as the measure of the closeness of agreement between a series of measurements obtained from multiple samplings of

the same homogenous sample. Bias and precision tests were performed in three different concentrations (0.02, 1 and 4 ng/ mg) for CBN and THC and (1, 4 and 12 pg/mg) for THC-COOH spiked into blank hair. The samples were analysed in triplicate over five days to calculate bias as well as the precision (coefficient of variation (% CV)) of intra-day and inter-day precision.

% CV= (standard deviation (S)/mean response) X 100

2.4.8. Stability

To test the stability of derivatized analytes, extracted solutions were considered in detail, three samples for each concentration (in the low, and high concentration range) which were used previously to test precision and accuracy were injected onto the LC-MS/MS on the initial day (T0). The auto-sampler temperature stability was assessed by reinjecting these two concentrations after 12 and 24h. Long-term stability was investigated of these two extracted levels at three freeze and thaw cycles for period of 2, 6 and 9 days at 4 and -20 ° C. The average peak area ratio at each concentration was compared with that for the original vial at T0 and considered stable if the decrease in average peak area ratio of analytes was less than or equal to ± 20% (bias). These samples were analysed in triplicate.

2.5. Application to authentic cases

The current method was applied to 27 out of 28 authentic hair specimens obtained from known male cannabis users who had admitted using cannabis, and their urine samples were screened and found to be positive for cannabinoids at the time of admission to the addiction hospital (Alamal Hospital, Jeddah, Saudi Arabia). The study was approved by the National Committee of Medical and Bioethics at the Ministry of Health (MOH) in Saudi Arabia, and all participants read the information sheet and signed a consent form before participating in the study. Hair samples were collected from the posterior vertex region of the male cannabis users with no major hair treatment reported("Recommendations for hair testing in forensic cases," 2004). A 0-3 cm hair segment was cut from the proximal end of the hair sample. Each sample was labelled with a unique identifier number at the time of collection. In the laboratory, specimens were stored in a dark and dry place at room temperature until the time of analysis.

For applying the current method on these 28 hair specimens, and to check for any exogenous analytes of interest that came from exposure to cannabis smoke, which was expected to increase the level of Cannabinol (CBN) and Δ9-Tetrahydrocannabinol (THC). Sample washings using dichloromethane (DCM X 2) were examined for any exogenous analytes; using the currently developed method after allowing the washings to dry overnight. As a result, one sample was excluded (S5), since the level of CBN and THC was higher than the LOQ in the second washing. The Other 27 hair

specimens were weighed out into a 7ml screw cap tube each after being allowed to dry following the washing. Then these specimens were cut to approximately 1-2 mm pieces. Deuterated Cannabinol (CBN-d_3) and deuterated 11-nor-9-carboxy-Δ9-tetrahydrocannabinol (THC-COOH-d_3) 100 ng and 400 pg total; corresponding to 2 ng/mg and 8 pg/mg respectively, were added.

The calibration curve and quality control samples (QCs) using the standard solution were prepared and analysed before running the hair samples see **Table 2-3.** QCs were run before running the samples and after each 10 samples to ensure that the results met the acceptance criteria of method validation such as (r^2) of a calibration curve must be ≥0.99, the accuracy of each calibrator ±15% and LOQ ±20% and 67% of QCs must be ±15% of the nominal concentrations.

Finally, the concentrations were calculated using the real hair weight for each hair sample. Since the hair specimens had variable weights, the calibration curve per 50 mg was used to quantify the level of analytes of total ng of CBN and THC or/and pg of THC-COOH in hair, and then the detected concentrations were divided by the sample weight in mg to get a concentration in ng/mg or pg/mg for the analytes of interest.

Table 2-3 Unit conversion for calibration ranges of cannabinoids.

Deuterated analyte	Working solution	Volume (µl)	Total	Equivalent
CBN-d_3	2 µg/ml	50	100 ng	2 ng/mg
THC-COOH-d_3	1 ng/ml	40	400 pg	8 pg/mg

Analyte	Calibrators	Working solution	Volume (µl)	Total pg	Equivalent pg/mg	Comment
THC-COOH	Blank hair					
	Blank hair+ THC-COOH-d_3					
	Excluded from calibrator	1 ng/ml	5	5	0.1	LOD
	1		10	10	0.2	LOQ
	2		50	50	1	Low QC
	3		100	100	2	
	4	10 ng/ml	20	200	4	Medium QC
	5		40	400	8	THC-COOH-d_3
	6		60	600	12	High QC

Analyte	Calibrators	Working solution	Volume (µl)	Total ng	Equivalent ng/mg	comment
CBN & THC	Blank hair					
	Blank hair + CBN-d_3					
	Excluded from calibrator	10 ng/ml	10	0.10	0.002	LOD
	1		100	1	0.02	LOQ & QC Low
	2	1 µg/ml	10	10	0.2	
	3		50	50	1	QC Medium
	4		100	100	2	CBN-d_3
	5	10 µg/ml	15	150	3	
	6		20	200	4	QC High

Cannabinol (CBN), Δ9-Tetrahydrocannabinol (THC), 11-nor-9-carboxy-Δ9-tetrahydrocannabinol (THC-COOH), Cannabinol (CBN-d_3), deuterated 11-nor-9-carboxy-Δ9-tetrahydrocannabinol (THC-COOH-d_3), quality control (QC), limit of detection (LOD) and limit of quantification(LOQ).

2.6. Results and discussion

2.6.1. Method development

2.6.1.1. Pre-treatment and extraction of samples

The first point of sample preparation is a decontamination of hair samples to remove contaminations by exogenous substances that may lead to interference with the analytes of interest. There is no specific washing solvent or protocol, as previous studies used a different solvent such as methanol, acetone, petrol-ether, water and dichloromethane (DCM) for washing hair to detect cannabinoids (Angeli et al., 2018a; Han, Choi, Lee, Chung, & Song, 2011; Musshoff, P Junker, Lachenmeier, Kroener, & Madea, 2002; Michael Uhl & Sachs, 2004b). DCM has been chosen for this purpose with water because it does not swell the hair as methanol does and removes the surface contaminants (Vogliardi et al., 2015).

The next step is digestion of the hair for separation the cannabinoids from the solid keratinous matrix. Alkaline hydrolysis was carried out using 1ml of 1M NaOH. Many publications agreed on the use of NaOH but disagree on the method, such as incubation time and temperatures (Vogliardi et al., 2015). Based on that, several incubation times and temperatures were examined. Therefore, incubation time at two temperatures 80 and 90 °C within 10, 15, 30 and 60 minutes at each temperature were carried out on 1ng/ml triplicate of each analyte spiked into 50 mg of blank hair. Ultimately, incubation at 90 °C for 15 minutes gave the best results.

Choosing an appropriate sorbent for SPE depends on the polarity of the compounds as well as the pKa of analytes. The pH plays an essential role in the extraction of analytes. In this case, the pH of the alkaline solution used to extract all cannabinoids after adding 1M sodium hydroxide (NaOH) was 14, while the pKa value of THC-COOH is 4.2, while THC is 9.3. Based on this information, previous studies have chosen the SPE cartridge for the extraction of cannabinoids from hair samples. For instance, polymeric reverse-phase cartridge (Strata-X) has been used for detection THC, which provide strong retention of neutral, acidic, or basic analytes (Lendoiro et al., 2012), a cation exchange SPE cartridge with a hydrophobic phase column (Bond Elut Certify I and Strata X-C mixed mode) was used for extracting THC-COOH (Moore, Rana, Coulter, Feyerherm, & Prest, 2006b) and mixed-mode anion exchange cartridge (CHROMABOND HR-XA) for analysis THC,CBD,CBN, THC-OH and THC-COOH (Kieliba et al., 2018).

Four possible cartridges were tested for extraction of these analytes from the hair matrix using one sample preparation as following, polymeric reversed-phase SPE (Strata XL), polymeric Strong Cation Mixed Mode Phase (Strata X-C), polymeric weak Anion Mixed Mode Phase (Strata X-AW) and polymeric strong Anion Mixed Mode Phase (Strata X-A). They were used due to their advantages. Polymeric mixed-mode has an advantage since it includes ion exchange and non-polar interactions.

The efficiency of sorbents was evaluated for use in two ways, 1ng/ml STDs loading, and then testing different ratios of ACN/H_2O to identify the appropriate method for

washing and elution, and the second way is to add 1ng/50 mg blank hair and elute analytes with two solvents (100% ACN with 1% acetic acid or mixture of cyclohexane/ ethyl acetate/ acetic acid (80/20/5, v/v/v)). The latter elution was reported to achieve the best elution characteristic (Kieliba et al., 2018).

According to testing the recovery, polymeric reverse-phase and polymeric Strong Cation Mixed Mode Phase (Strata X-C) were excluded due to poor recovery, on the other hand, polymeric weak Anion Mixed Mode Phase (Strata X-AW) was also evaluated with different pH value of mixture (4-7), resulting in a weak extraction recovery of CBN and THC, while THC-COOH has got adequate recovery roughly 90%. Finally, a polymeric strong anion mixed-mode phase (Strata X-A) was also assessed at different pH values of the mixture before loading (4,8 and 14). Thus, optimization was done using 2 ml of ACN / H_2O (20:80 v/v) in the washing step, since it was found that using this mixture did not cause the loss of any appreciable number of analytes. As shown in **Figure 2-2**, the best elution that gave the acceptable recoveries used 3ml of the mixture (cyclohexane/ ethyl acetate/ acetic acid (80/20/5, v/v/v)) with direct loading. Acidification was used to remove the negative charge on the cannabinoids hence stop them binding to the anion exchange resin.

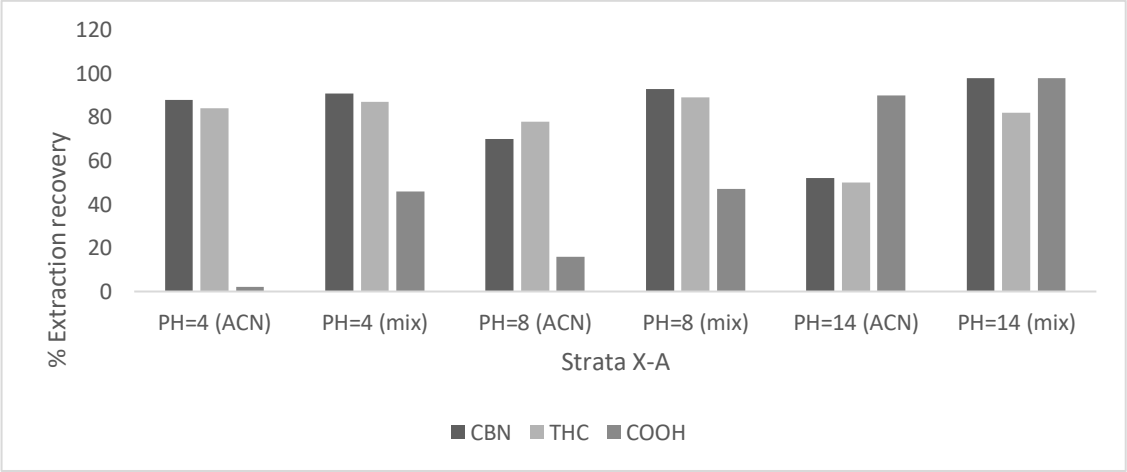


Figure 2-2 % extraction recovery of 1ng/50 mg of CBN, THC and THC-COOH spiked with blank hair using polymeric strong Anion Mixed Mode Phase (Strata X-A) with two elution solvents (100% ACN with 1% acetic acid or mixture of cyclohexane/ ethyl acetate/ acetic acid (80/20/5, v/v/v)) at three pH points of mixture before loading (4,8 and 14).

2.6.1.2.Optimization of reaction conditions in derivatization cannabinoids with FMP

First of all, FMP was used as a derivating reagent with triethylamine (TEA) as a catalyst. It has been reported that the product ions of different analytes in the present of FMP, resulting in specific fragmentation pattern (Faqehi et al., 2016; Thieme, Sachs, & Thevis, 2008). This advantage of using FMP contributed to determining these analytes at low levels in the hair sample.

A positively charge derivative resulted from reacting FMP derivatives with the phenolic hydroxyl group of the cannabinoids, as shown in **Figure 2-3**. The efficiency of the reaction was optimized by examining at different temperatures (20, 40 and 60 ° C), incubation times (15, 20, 30 and 60 minutes) and amount of FMP solution (50

and 100µl). As seen in **Figure 2-4**, it was considered that the derivatization of cannabinoids with 100 µl of (10mg/ml) FMP in the presence of 10 µl TEA was completed within 15 minutes at 40 ° C.

As THC-COOH contains carboxyl and hydroxyl groups, it was recognized that single derivatization reaction was not sufficient for detection of THC-COOH at concentration of (0.2 pg/mg) as recommended by the Society of Hair Testing (SoHT), as the lowest level was detected was (2 pg/mg) even with increasing the injection volume on the LC to 40 µl. Therefore, esterification reaction was carried out by reacting methanolic HCl with THC-COOH as shown in **Figure 2-3, A**. This esterification reaction resulted in a methyl ester $COOCH_3$, and the purpose of this modification was to enhance the stability, sensitivity and selectivity of THC-$COOCH_3$, due to increased lipophilicity and the absence of a negative charge as well as to prevent derivatization of two positions with FMP which would give a doubly charged derivative.

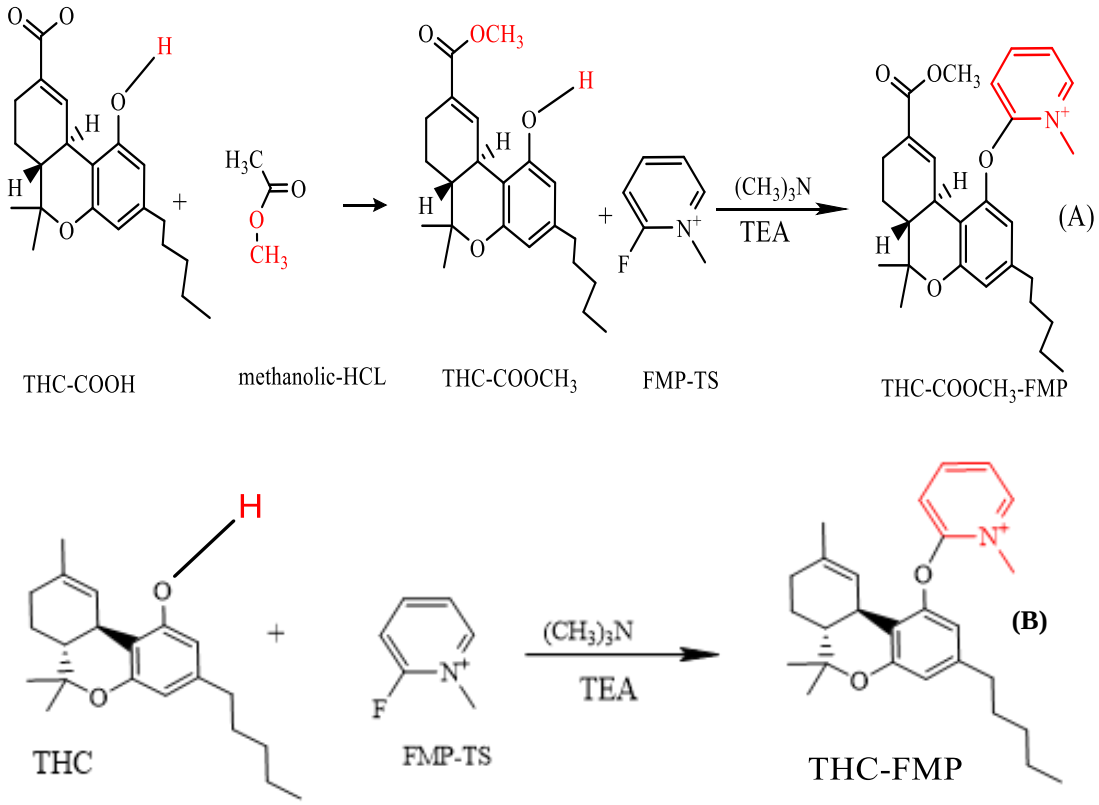


Figure 2-3. an example of derivatization of **(A)** Formation of methyl pyridinium ether derivative of phenolic THC-COOH after esterification reaction, **(B)** Formation of methyl pyridinium ether derivative of phenolic THC.

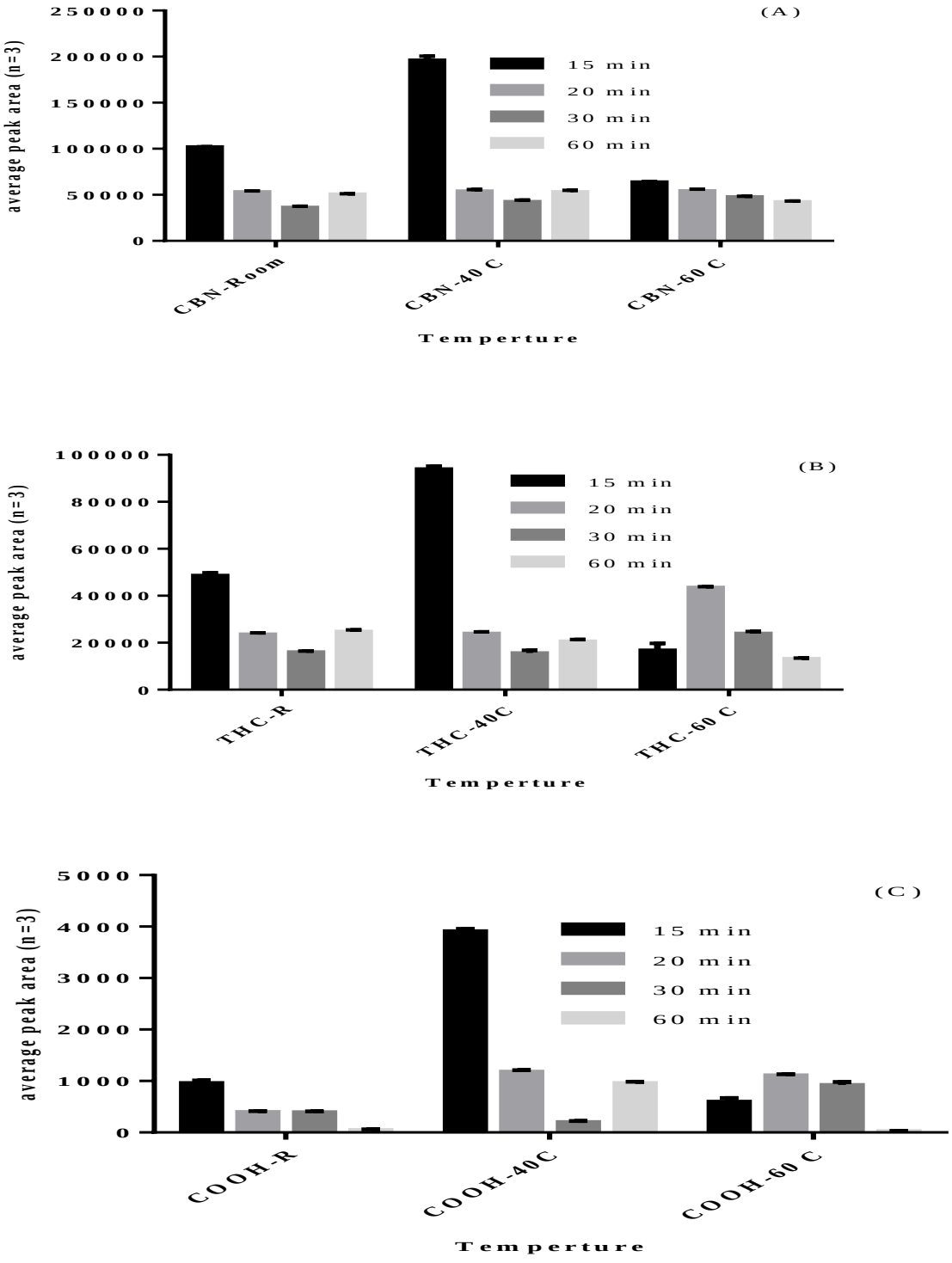


Figure 2-4. Reactivity of 100µl of FMP solution with 1ng/ml of (A) CBN, (B) THC and (C) THC-COOH at different incubation times and temperatures.

2.6.1.3.Optimization of LC conditions

The optimization of liquid chromatography (LC) parameters was achieved using an ACE 3 C18-AR column (150 × 3.0 mm i.d) protected by a guard column with identical packing material (20 x 2.1 mm). Generally, C18 column is considered to be a more popular column for many applications in forensic toxicology. The C18-AR (aromatic) has alternative selectivity to a standard C18 column, since it contains a C18 chain a phenyl functionality. Cannabinoids have aromatic functionalities in their structures; therefore, this column was chosen in the expectation it would increase the efficiency of analytes separation due to interactions with the phenyl functionality.

On the other hand, the esterification reaction of THC-COOH led to a decrease in the separation between the peak of THC-COOH and CBN from 1.5 minutes to be less than 0.5 minutes. With the introduced of MS/MS, the analytes have different precursor, and product ions, therefore, there was no interference. The separation using both isocratic and gradient systems was examined. An isocratic system with different percentages of the aqueous mobile phase was examined to check the elution time of each analyte. Since they have different molecular weights, it was expected that there would be acceptable chromatographic separation between them would be possible, as the C18-AR column has the ability to retain the drugs according to lipophilicity and aromaticity, thus the prediction separation was achieved using 60% of high organic mobile phase (ACN containing 0.1 % formic acid). The first analyte eluted was THC-

COOH after 3 minutes and then CBN and THC. All the peaks shapes and separations achieved during the chromatography were adequate.

As hair samples are complex and contain many interferants, it was decided to use a gradient system. This started with 80% of the aqueous mobile phase (H_2O containing 0.1 % formic acid) was used to start with. It was found that the run time was increased due to the equilibration time at the end of each run without any increase in the separation between THC-COOH and CBN. Different gradient methods were examined, and when the aqueous mobile phase was started from 50%, the run time was reduced to 20 minutes with the last 6 minutes for re-equilibrium. Flow rates of 150,250,350 and 400 µl/min, with oven temperatures of 20 and 30 °C and injection volume of 10, 20 and 30 µl were assessed. As a result, the flow rate of 400 µl/min, oven temperature at 30 ° C and injection volume at 30 µl/min were selected as the best LC parameters to enhance the sensitivity without affecting the quality of chromatogram. It is recommended that the injection volume does not exceed 10% of the column flow rate (R. & de Boor, 2006).

2.6.1.4.Optimization of MS conditions

Usually, direct infusion of analytes standards and internal standards into the electrospray ionization source (ESI) is used to get the appropriate precursor ions, product ions, fragmentary ions and collision energy (CE) is used. This automated protocol for optimization MRM condition was not suitable for cannabinoids even

when using (10µg/ml), where the product ions of these analytes did not appear even when using different collision energies. Therefore, the second choice was to run each analyte or internal standard (1µg/ml) dissolved in 50/50 (v/v) mobile phase, several times for each individual target as follows. **Firstly**, the actual precursor ion was identified using the scan mode. **Secondly**, different fragmentor voltages were selected to determine the optimal response of the precursor ion to identify the correct voltage of them using MS2 Selected Ion Monitoring (SIM). **Thirdly**, a product ion scan for each precursor ion, was used to identify the high abundance of product ions. Finally, using MRM acquisition mode for optimization CE (0,15,30,45 and 60 eV) for each transition, as seen in **Figure 2-5 to 2-9**. This protocol was followed for each analyte and internal standard separately, the results from which are shown in **Table 2-2**. In addition, MS parameters such as gas temperature within the range (250, 300 and 350 °C); gas flow (6, 8, 11 and 13 L/min); nebulization pressure (15, 20, 30 and 40 psi) and Delta EMV (400, 600, 800 and 1000 V) were examined to achieve the best sensitivity as follows: gas temperature, 300 °C; gas flow, 8 L/min; nebulization pressure, 20 psi; Delta EMV, 1000 V.

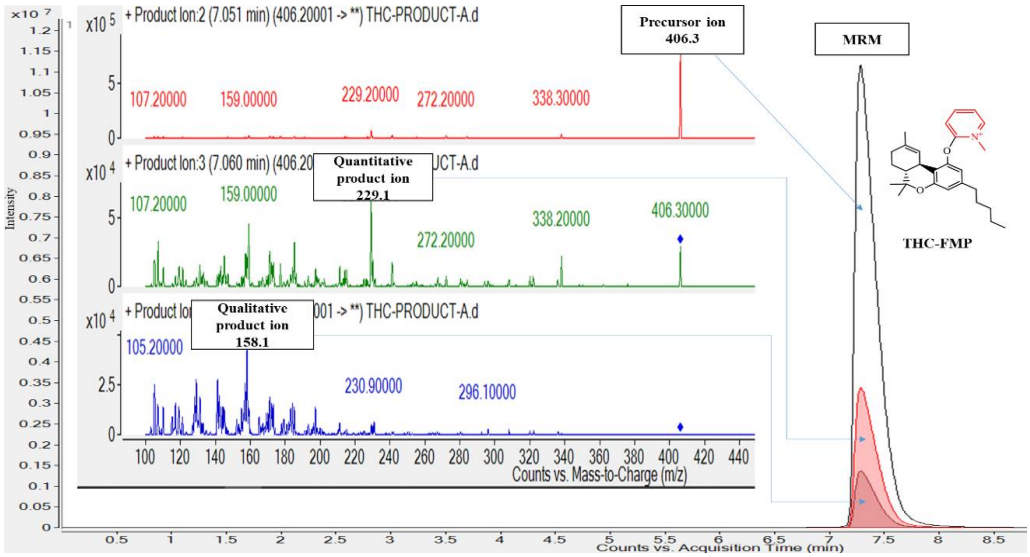


Figure 2-5. THC-FMP chemical structure as well as MRM chromatograms of precursor ion and its product ions using the appropriate collision energy of each transition; (ion transitions m/z 406.3 →229.1 and 406.3→158.1), and MS spectrums of precursor ion with different product ions using different fragmentor voltages (100,150,200 and 250).

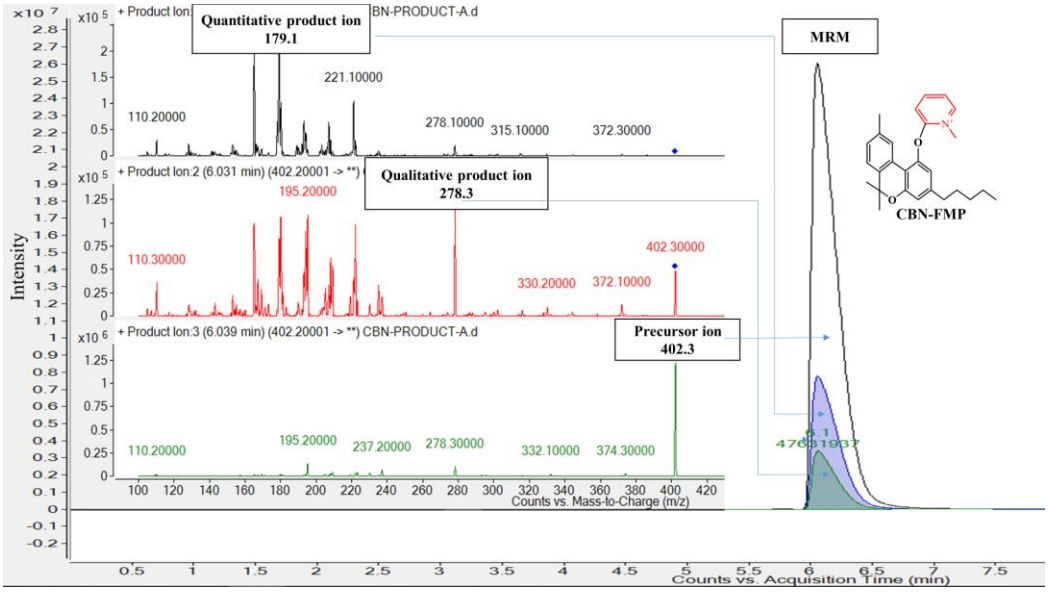


Figure 2-6. CBN-FMP chemical structure as well as MRM chromatograms of precursor ion and its product ions using the appropriate collision energy of each transition; (ion transitions m/z 402.3 →278.3 and 402.3→179.1), and MS spectrums of precursor ion with different product ions using different fragmentor voltages (100,150,200 and 250).

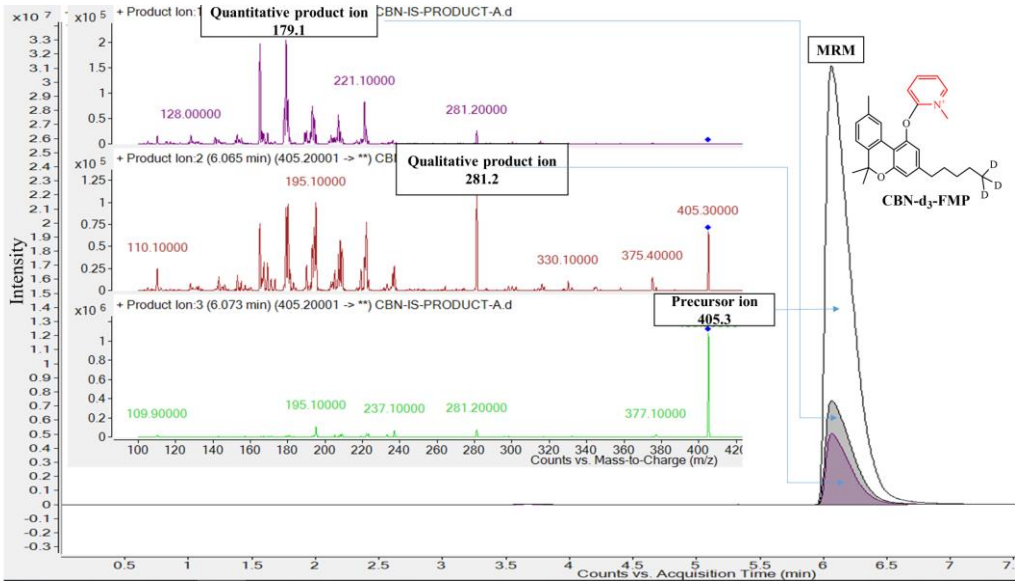


Figure 2-7. CBN-d_3-FMP chemical structure as well as MRM chromatograms of precursor ion and its product ions using the appropriate collision energy of each transition; (ion transitions m/z 405.3 →281.2 and 405.3→179.1), and MS spectrums of precursor ion with different product ions using different fragmentor voltages (100,150,200 and 250).

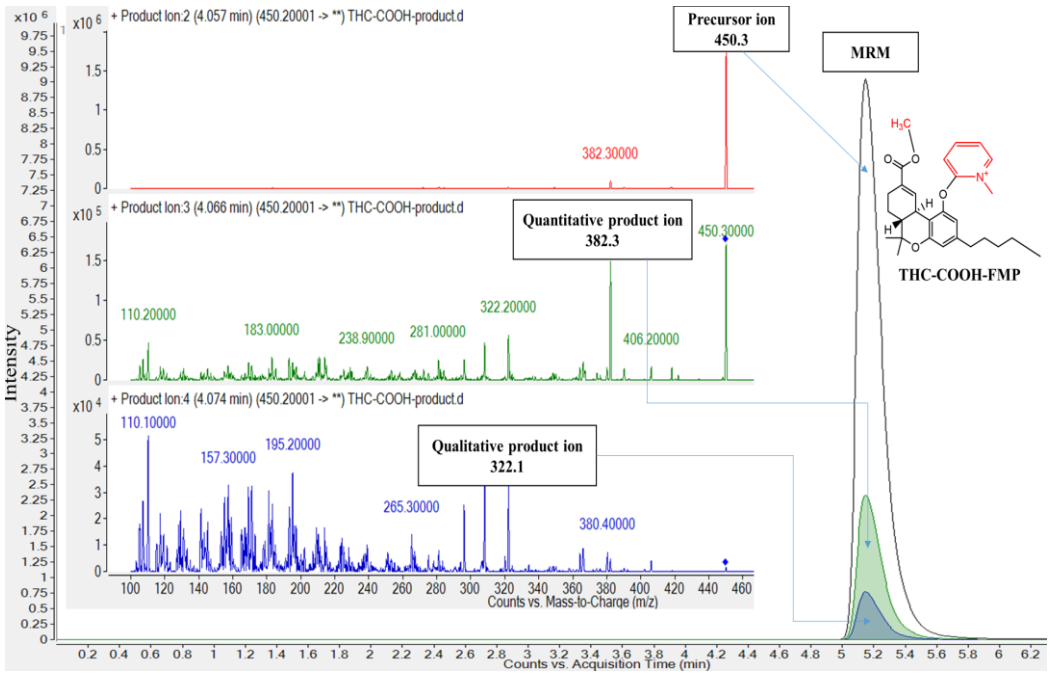


Figure 2-8. THC-COOH-FMP chemical structure as well as MRM chromatograms of precursor ion and its product ions using the appropriate collision energy of each transition; (ion transitions m/z 450.3 →382.3 and 450.3→322.1), and MS spectrums of precursor ion with different product ions using different fragmentor voltages (100,150,200 and 250).

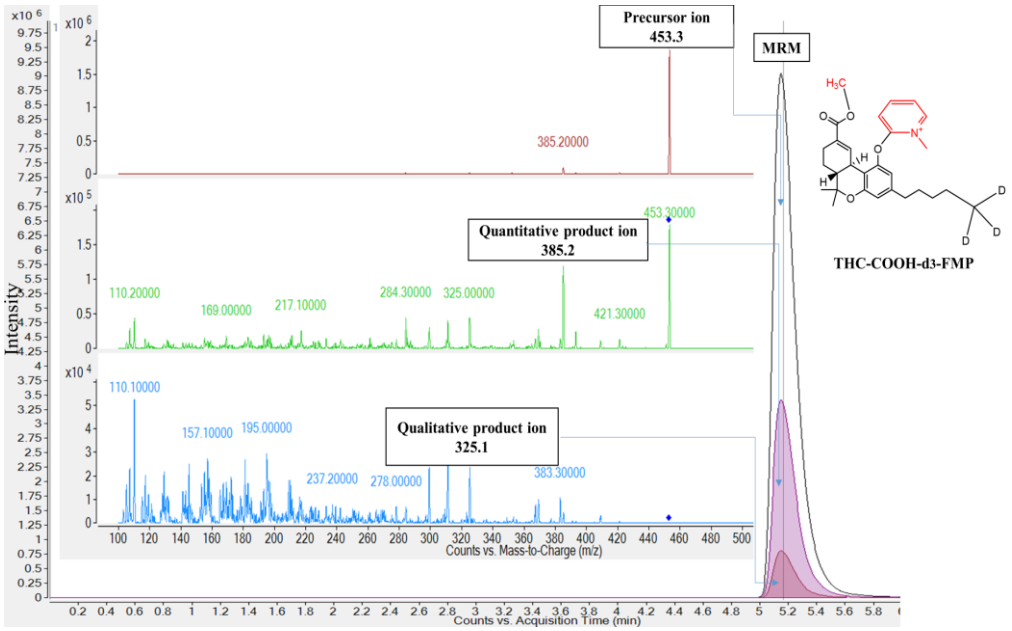


Figure 2-9. THC-COOH-d_3-FMP chemical structure as well as MRM chromatograms of precursor ion and its product ions using the appropriate collision energy of each transition; (ion transitions m/z 453.3 →385.2 and 453.3→325.1), and MS spectrums of precursor ion with different product ions using different fragmentor voltages (100,150,200 and 250).

2.6.2. Method validation

2.6.2.1.Extraction recovery

The efficiency of the extraction procedure for CBN, THC and THC-COOH using SPE was assessed by calculating the extraction recovery percentages. The extraction method chosen was able to extract more than 90% of CBN, more than 86% of THC, and more than 81% of THC-COOH at two different concentrations, as shown in **Table 2-4.**

Table 2-4 Extraction Recovery Data on cannabinoids Determined in bank hair Using LC-MS/MS

Compounds	Concentration n=3	level	Average ratio Extracted Mean± S.D	Average ratio Non-extracted Mean± S.D	Extraction recovery (%)
CBN	0.2	Low	0.13±0.012	0.145± 0.007	90.6
(ng/mg)	4	High	1.98±0.084	1.889±0.040	105.0
THC	0.2	Low	0.10±0.009	0.12± 0.014	86.0
(ng/mg)	4	High	1.76±0.118	1.781±0.124	99.0
THC-COOH	1	Low	0.28±0.049	0.311±0.012	88.8
(pg/mg)	12	High	2.50±0.103	3.091±0.110	81.0

2.6.2.2.Matrix effect

As shown in **Figure 2-10**, the matrix suppressed the response for all analytes and the deuterated-d_3 standards in the low and high concentrations, except the low level for THC-COOH-d_3 the signal was enhanced. The matrix effect was within the range of ± 25%, which was acceptable.

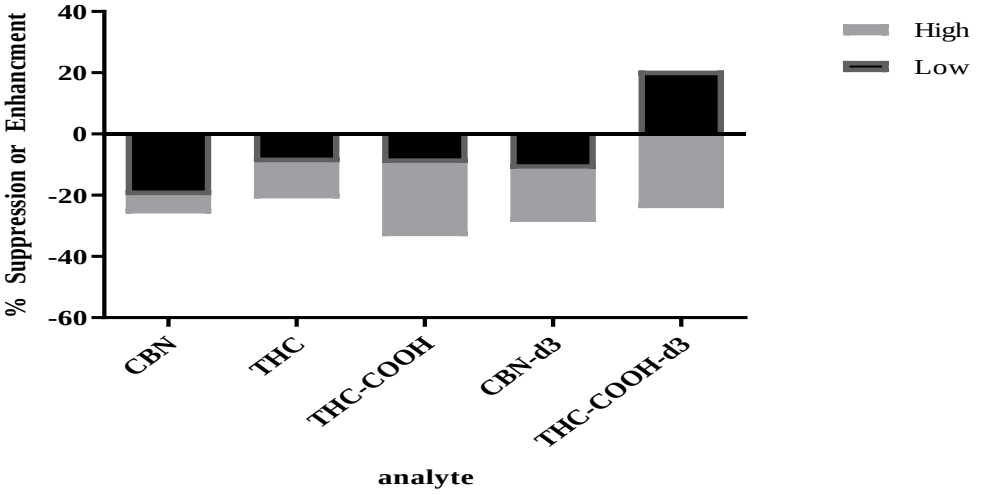


Figure 2-10. Mean matrix effect (n=6)

2.6.2.3.Interference studies

This method is specific and selective for the identification of target analytes in human hair since there was no interference between CBN and THC-COOH and their internal standards. Moreover, these analytes have good resolution and peak symmetry. Furthermore, there was no interference seen from blank hair sources nor commonly used drugs for any of the analytes or their internal standards at their retention times.

2.6.2.4.Sensitivity: limit of detection (LOD) and limit of quantification (LOQ)

LOQ and LOD of targeted analytes are summarized in **Table 2-5,** and MRM traces for LOD and LOQ are shown in **Figure 2-11**. The LOQ of THC was ≤ 0.1 ng/mg, which is the cut-off recommended by SoHT, and LOQ of THC-COOH reached the SoHT recommend level at ≤ 0.2 pg/mg. The SoHT has no recommended level for CBN in the hair (Gail A A Cooper et al., 2012). On the other hand, to the best of my knowledge, there is just one published paper which detects THC and CBN in hair samples using liquid chromatography at levels lower than 1 pg/mg where MS/MS/MS was used (Míguez-Framil et al., 2014), and one published paper reached LOD 1pg/mg for just THC (Mercolini et al., 2013). While two published papers reached the same LOD for THC-COOH as in the current case. The first work using LC-MS/MS/MS (Thieme et al., 2014) and then another paper using LC-MS/MS (Kuwayama et al., 2015), and one

published paper found < 0.05 pg/mg as the LOD using LC-MS/MS/MS (Park et al., 2014), the limits found by these three papers are summarized in **Table 2-1**.

Table 2-5 LOD and LOQ of cannabinoids in hair using LC-ESI-MS/MS achieved.

Analyte	LOD (pg/mg)	LOQ (pg/mg)
CBN	2 (0.002 ng/mg)	20 (0.02 ng/mg)
THC	2 (0.002 ng/mg)	20 (0.02 ng/mg)
THC-COOH	0.1	0.2

CBN: cannabinol; THC: Δ9-tetrahydrocannabinol; THC-COOH: 11-nor-Δ9- tetrahydrocannabinol-9-carboxylic acid.

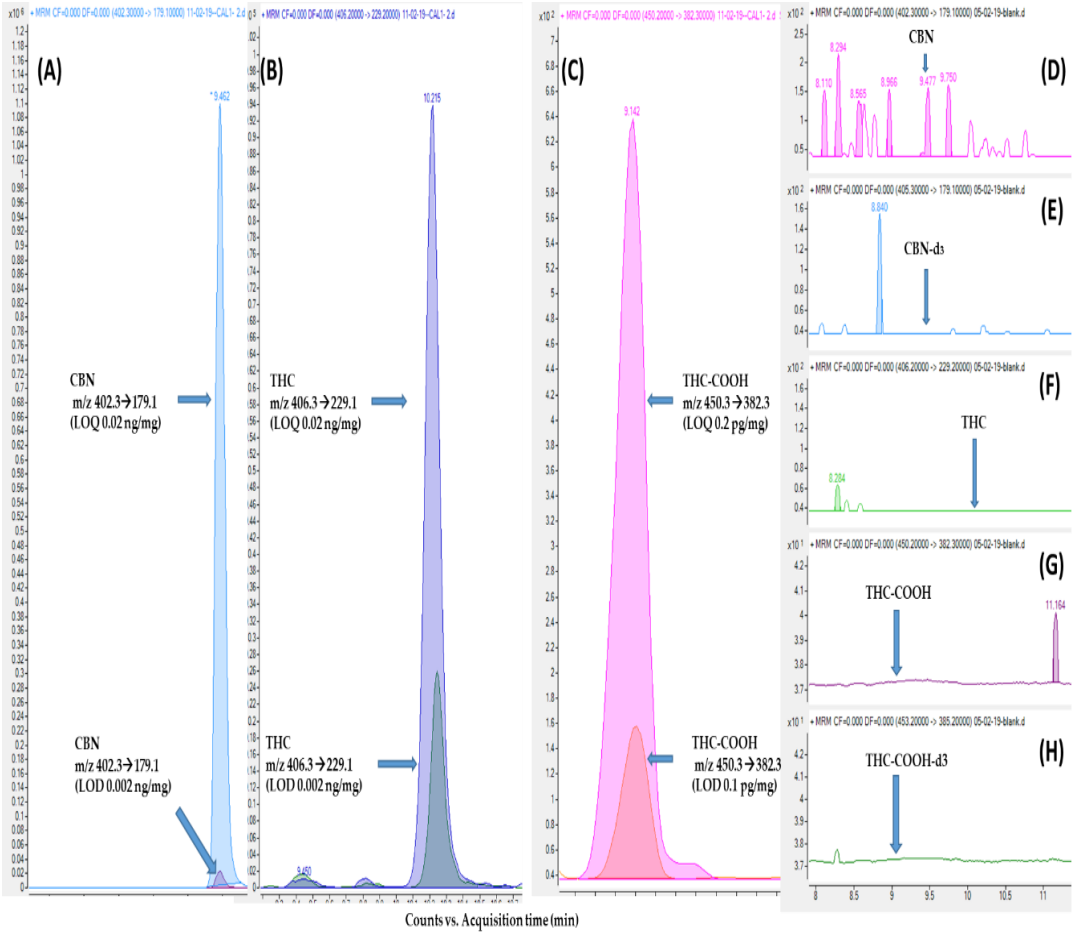


Figure 2-11. Quantifier ion chromatograms of the limit of detection (LOD) and quantification (LOQ) of **(A)** cannabinol (CBN), **(B)** Δ9-tetrahydrocannabinol (THC) and **(C)**11-nor-Δ9-tetrahydrocannabinol-9-carboxylic acid (THC-COOH). In traces **(D-H)** there are no interfering peaks appearing which could interfere either with the analytes or internal standards at the retention time in the blank hair.

2.6.2.5.Linearity and calibration of standards spiked into blank hair

The assay showed that the linearity was acceptable within the range of 0.02 - 4 ng/mg for CBN and THC, and within the range of 0.2 - 12 pg/mg for THC-COOH with correlation coefficients of ≥0.997 see **Figures 2-12, 2-13 and 2-14.**

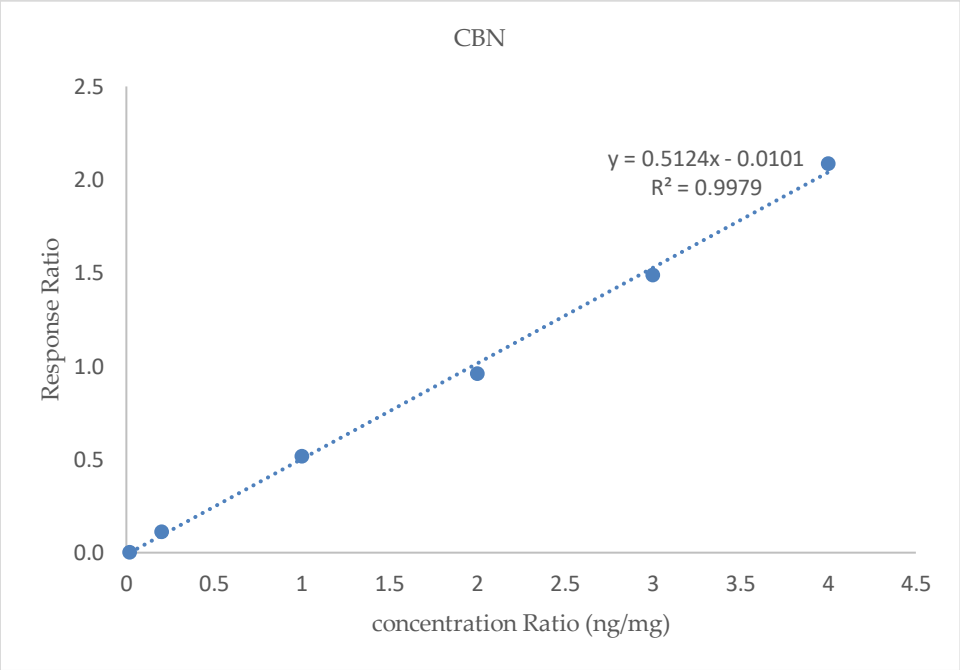


Figure 2-12. Representative calibration curves for cannabinol (CBN) plotted as the concentration per mg of hair against the response ratio.

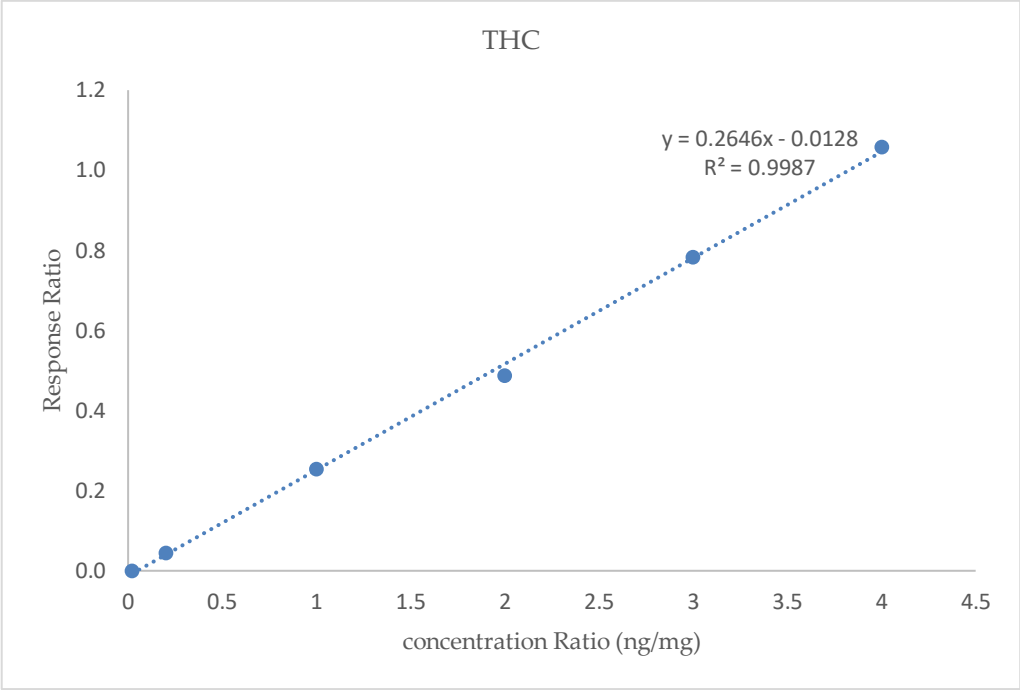


Figure 2-13. Representative calibration curves for Δ9-tetrahydrocannabinol (THC), plotted as the concentration per mg of hair ratio against the response ratio.

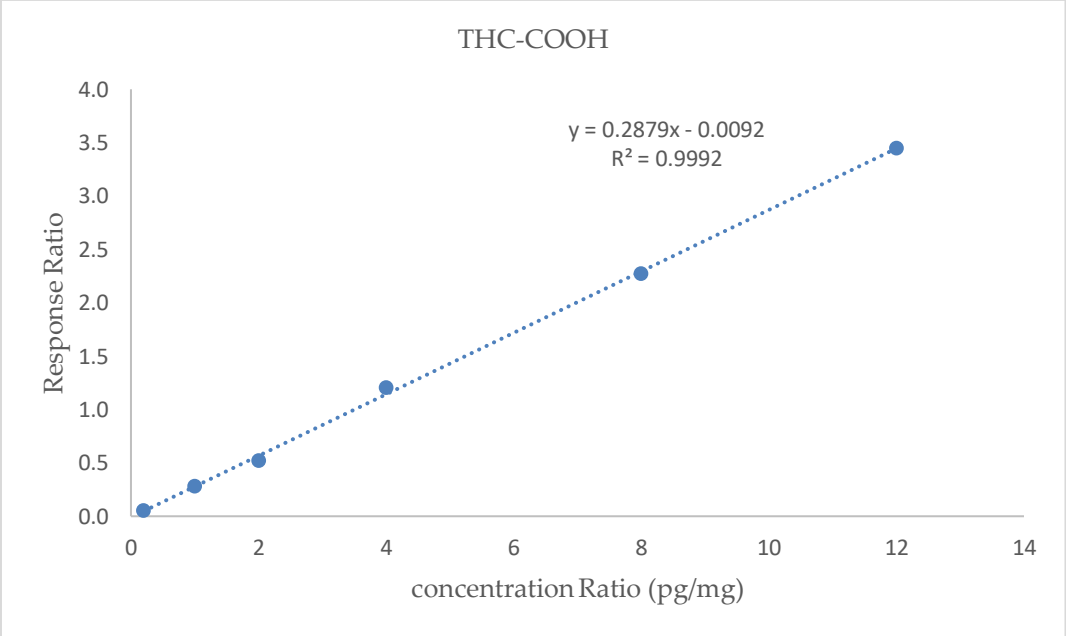


Figure 2-14. Representative calibration curves for 11-nor-Δ9- tetrahydrocannabinol-9-carboxylic acid (THC-COOH) plotted as the concentration per mg of hair ratio against the response ratio.

2.6.2.6.Carryover

No signal was observed in the blank QC samples at the retention time for THC, CBN and THC-COOH. Carryover was therefore not deemed to be a problem.

2.6.2.7.Bias and precision

The method was shown to be reproducible regarding the peak shape and retention time. The coefficient of variation (% CV) of intra- and inter-assay precision CBN, THC and THC-COOH was <11%. The bias was within the range of 82% – 115%. The results for the bias and precision of the method are shown in **Table 2-6**.

Table 2-6 Intra-and Inter-assay Precision and Accuracy Results of cannabinoids Determination in hair using LC-MS/MS (n= 5)

Analyte	Concentration	Level	Intra-day			Inter-day		
			CV[a] (%)	Mean	Bias[b] (%)	CV[a] (%)	Mean	Bias[b] (%)
CBN	0.02	Low	1.8	0.02	14.8	1.4	0.02	15.1
(ng/mg)	1	Medium	2.0	1.02	2.6	3.1	1.06	6.7
	4	High	0.3	3.76	-5.9	4.8	3.89	-2.5
THC	0.02	Low	0.6	0.01	-13.2	0.2	0.22	11.8
(ng/mg)	1	Medium	0.05	0.91	-8.1	6.6	0.92	-7.1
	4	High	0.6	3.83	-4.0	10.9	4.34	8.6
THC-COOH	1	Low	2.7	0.81	-18.3	10.9	0.87	-12.4
(pg/mg)	4	Medium	2.5	3.88	-2.7	2.2	3.79	-5.1
	12	High	3.9	11.25	-6.2	4.1	10.65	-11.2

a expressed as coefficient of variation (% CV).
b expressed as [(mean % deviation = mean calculated concentration - nominal concentration) / nominal concentration X100]

2.6.2.8.Stability

The stability of cannabinoids extracted from hair was studied by Lendoiro et al., who studied the stability of THC in an auto-sampler for 24 h and proved that there were

no significant losses (Lendoiro et al., 2012). Park et al., evaluated the stability of THC-COOH over 20 hours, resulting in a satisfactory result (Park et al., 2014). These previous studies evaluated the stability of un-derivatised cannabinoids. Thus, the current project assessed the stability of these analytes after derivatization at two concentrations of the calibration curves (low and high concentrations).

As can be seen in **Table 2-7,** the stability of extracted and derivatised CBN, THC and THC-COOH was acceptable, with the variation being < ± 20% over the storage period. CBN was observed to have less stability when it was stored in a refrigerator. THC low concentrations sample decreased in freeze-thaw (cycle 2) in both fridge and freezer. The low concentration sample of THC-COOH was observed to reach the highest acceptable percentage of bias when stored in the freezer for all cycles.

Table 2-7 Stability of extracted CBN, THC and THC-COOH.

Analyte	level	Auto-sampler		Refrigerate at 4 ° C			Freeze at -20 ° C		
		12h	24h	Cycle 1	Cycle 2	Cycle 3	Cycle 1	Cycle 2	Cycle 3
CBN	Low(0.02)	11	4	19	-4	-18	-11	15	-9
(ng/mg)	High (1)	0	0	19	-13	-16	12	4	12
THC	Low(0.02)	-6	3	-6	-18	-17	-12	-18	-14
(ng/mg)	High (1)	-1	-1	-11	-2	-8	-4	-19	-11
THC-COOH	Low (1)	17	6	-15	-4	-7	-18	-19	-19
(pg/mg)	High (12)	-8	-11	-9	4	6	-16	-1	-14

%Bias calculated as 100 × (mean−nominal)/nominal. Cycle 1 expressed of storage after 2 days, Cycle 2 expressed of storage after 6 days and Cycle 3 expressed of storage after 9 days.

2.7. Method application

The developed and validated the method for detection and quantification of cannabinoids of interest in hair was applied to 27 hair specimens taken from cannabis

users. As shown in **Table 2-9.** CBN and THC were detected in all samples, quantifier and qualifier ion chromatograms of both native cannabinoids and cannabinol-d3(CBN--d3) are illustrated in **Figure 2-15 (A-H).** While THC-COOH was detected in 13 specimens, as shown in **Figures 2-15-I**. In addition, THC-COOH gave semi-quantifiable results (all values less than the limit of quantification (LOQ), but more than the limit of detection (LOD)) in 3 samples shown in **Figure 2 15-J** and was not detected in 11 samples shown in **Figure 2 15-K.** A summary of the analytes concentration ranges, the mean and median levels were calculated using Microsoft Excel 2016 and is shown in **Table 2-8.** For CBN, THC and THC-COOH the ranges were 0.022-2.562 ng/mg, 0.049-0.431 ng/mg and 0.222-4.867 pg/mg respectively. The median values were 0.054 ng/mg, 0.087 ng/mg and 0.34 pg/mg for CBN, THC and THC-COOH respectively.

Table 2-8. Statistical results of the concentration of cannabinoids in 27 hair specimens from cannabis users.

	CBN (ng/mg)	THC (ng/mg)	THC-COOH (pg/mg)
Mean	0.221	0.143	1.130
low	0.022	0.049	0.222
Median	0.054	0.087	0.34
High	2.562	0.431	4.867
N. D	0	0	11
Trace	0	0	3

N.D; not detected. Trace; > LOD.

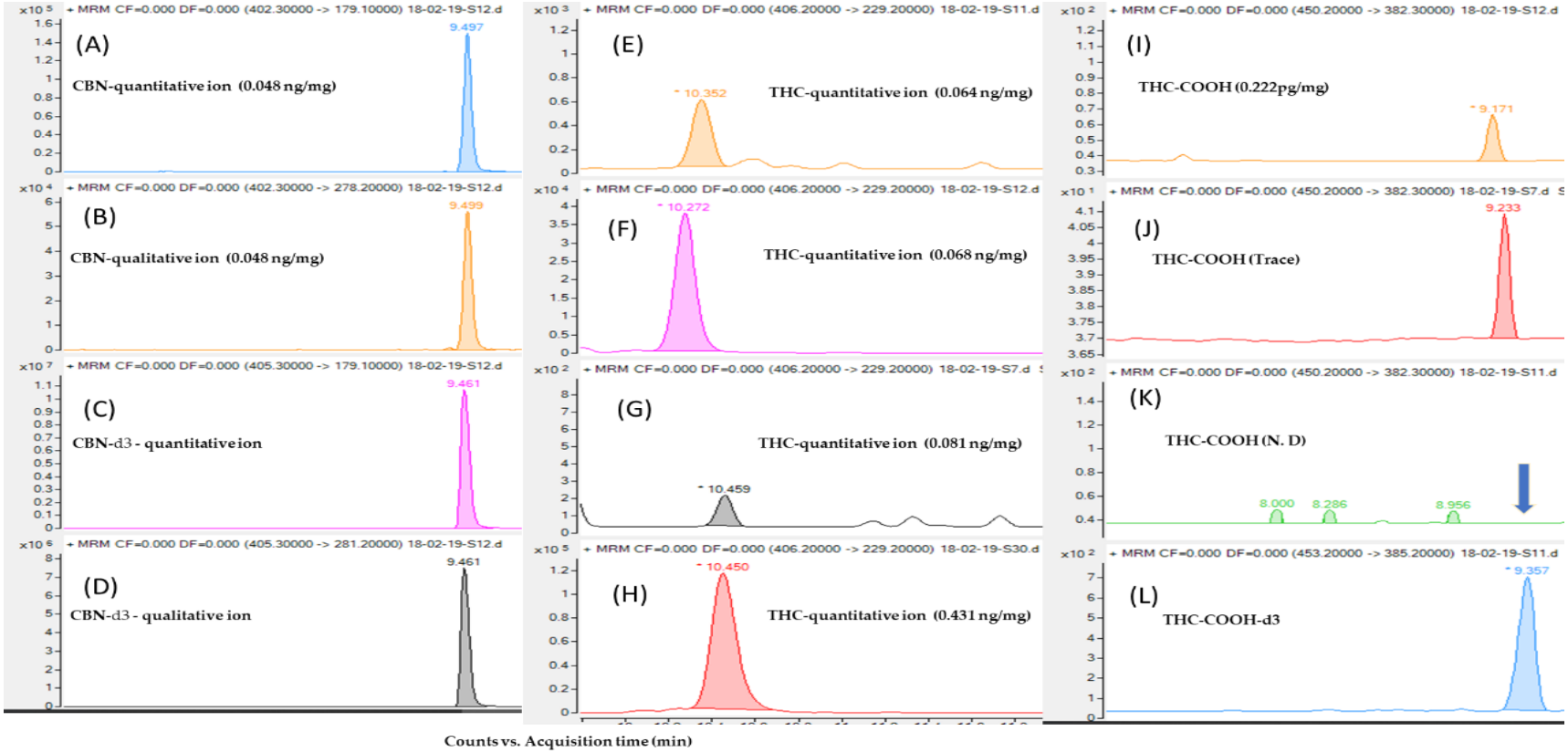


Figure 2-15. Extracted ion chromatograms of real hair specimens for **(A-D)** quantifier and qualifier ions of cannabinol (CBN) and cannabinol-d_3 (CBN--d_3) in sample (S12), **(E-H)** quantifier ions of Δ9-tetrahydrocannabinol (THC) in samples (S 11,12,7 and 30); the latter sample represent the highest concentration of THC, and **(I)** quantifier ion of 11-nor-Δ9-tetrahydrocannabinol-9-carboxylic acid (THC-COOH) in sample (S12), **(J)** quantifier ion of THC-COOH less than limit of Quantification (Trace) in sample (S7) and **(K-L)** former show not detected (N. D) any quantifier ion of THC-COOH in the specific retention time (9.34), and latter show quantifier ion of 11-nor-Δ9-tetrahydrocannabinol-9-carboxylic acid--d_3 (THC-COOH--d_3), both in sample (S11).

Table 2-9. Concentrations of cannabinoids in hair from 27 cases.

Sample ID	Weight (mg)	CBN (ng/mg)	THC (ng/mg)	THC-COOH (pg/mg)
S6	40	0.036	0.065	Trace
S7	30	0.034	0.081	Trace
S11	38	0.028	0.064	N. D
S12	46	0.048	0.068	0.222
S13	8	0.133	0.309	N. D
S14	9	0.120	0.274	N. D
S16	32	0.039	0.077	N. D
S17	48	0.117	0.087	N. D
S18	45	0.581	0.207	0.732
S19	45	0.054	0.061	1.792
S20	15	0.068	0.162	2.706
S22	45	0.075	0.109	0.339
S25	50	0.069	0.102	0.334
S27	30	0.056	0.097	N. D
S30	20	0.167	0.431	N. D
S31	15	0.109	0.171	N. D
S32	50	0.022	0.049	Trace
S34	50	0.023	0.050	0.246
S35	9	0.121	0.270	N. D
S36	46	1.281	0.420	12.128
S37	13	2.562	0.285	4.867
S39	50	0.024	0.051	0.386
S40	45	0.029	0.058	0.231
S41	50	0.033	0.054	0.241
S44	25	0.053	0.111	N. D
S47	37	0.037	0.085	N. D
S48	40	0.052	0.068	0.340

N.D; not detected. Trace; < low limit of detection (LOQ) and > limit of quantification (LOQ).

There have been various reports that detected different targeted cannabinoids in hair using LC-MS, as summarized in **Table 2-10.** The CBN, THC and THC-COOH average in hair were (0.008-0.20 ng/mg), (0.011-12.1 ng/mg) and (0.06-15.75 pg/mg) respectively. Based on these previously published ranges for the analytes, a prediction of the calibration curve range in the current project was made.

It is not unusual to detect parent cannabinoids (CBN, THC) in hair samples. Determination of THC-COOH due to the low level of this metabolite in hair is more challenging. The presence of parent cannabinoids in the hair represents exposure to

cannabis in smoke only, while quantification the metabolites such as THC-COOH in hair refers to ingestion. On the other hand, one of the advantages of the detection these analytes in hair rather than biological samples, such as blood or urine, is the length that they remain in the hair, which helps in identifying them even after a long period where each centimetre (cm) of hair indicates the data of use about a month ago (G Cooper et al., 2012; Miller et al., 1997). The hair length of the specimens provided in this project was 0-3 cm and thus referred to the consumption over three months.

Table 2-10. The range of concentrations of cannabinoids in hair determined by previous studies using LC-MS/MS.

Ref	N	CBN (ng/mg)			THC (ng/mg)			THC-COOH (pg/mg)		
		Min	Max	P	Min	Max	P	Min	Max	P
Pichini et al., 2015a	20				0.33	12.1	20	0.09	0.39	10
Dulaurent et al., 2014	1							---	3.12	1
Míguez-Framil et al., 2014	14	0.008	0.33	14	0.011	0.06	14			
Park et al., 2014	98							0.13	15.75	92
Mercolini et al., 2013	NS			NS	0.055	0.10		5	10	NS
Salomone et al., 2012	14	0.031	0.20	14	0.050	0.55	14			
Han et al., 2011	28							0.06	15.13	18
Domínguez et al., 2011	6				----	2.97	6			
Coulter et al., 2009	5				0.059	0.087	5			
Current project	27	0.022	2.562	27	0.049	0.431	27	0.222	4.867	13

A positive number (P), Number of specimens (N), not show (NS).

2.8. Conclusion

Recently many methods have been reported for detection of the main cannabinoids and their metabolites in hair using LC-MS/MS as mentioned in **Table 2-1**. In the present study, various modifications of the extraction procedures to choose the

optimal extraction conditions and sorbent were carried out. The development in sample preparation allowed a single loading of the sample with acceptable extraction recovery, and without significant issues with ion suppression using polymeric strong Anion Mixed Mode Phase (Strata X-A) for CBN, THC and THC-COOH.

The detection of THC-COOH in hair matrices requires superior sensitivity due to its extremely low concentrations in a hair sample. Employing 2-fluoro-1-methyl pyridinium-p-toluenesulfonate (FMP-TS) and methyl esterification as a new derivatization procedure of hydroxyl and carboxyl groups offered enhanced specificity and ionization for the detection of THC-COOH in hair matrices, which increased its lipophilicity and removed the negative charge on the carboxyl group.

Twenty-seven hair specimens from cannabis consumers were analysed in order to confirm the ability of the developed method to detect the target analytes in these hair specimens. Ultimately, THC-COOH was semi-quantified in 13 specimens. Unlike CBN and THC, THC-COOH non-quantifiable (all values less than the limit of quantification (LOQ), but more than the limit of detection (LOD)) in 3 samples and was not detected in 11 samples. CBN, THC and THC-COOH ranges were (0.022-2.562 ng/mg), (0.049-0.431 ng/mg) and (0.222-4.867 pg/mg) respectively. The median values were (0.054 ng/mg), (0.087 ng/mg) and (0.34 pg/mg) for CBN, THC and THC-COOH respectively. The length of the hair specimens was around 3cm. Thus the concentrations of these cannabinoids indicate cannabis has taken for three months earlier since each 1cm of hair grows per one month on average (Miller et al., 1997).

Detection of at least THC-COOH metabolite in hair, especially in routine work seems to be compulsory alongside THC and other main cannabinoids to distinguish between ingestion and passive exposure. No more comparisons were made due to the lack of information on users. There is a limited number of publications for detection of some significant metabolites in hair such as 11-Hydroxy-Δ9-tetrahydrocannabinol (11-OH-THC) and 11-nor-Δ9-tetrahydrocannabinol-9-carboxylic acid glucuronide (THC-COOH-glu) that would be an interesting subject for future work, where they are considered to prove cannabis consumption (Beasley, et al, 2016; Kieliba et al, 2019; Williams, et al, 2019; Pichini et al., 2015; Weinmann, et al, 2000). This limitation is probably due to the concentration of 11-OH-THC in hair being lower than THC-COOH metabolite, whereas, THC-COOH-glu is known to be unstable to chemical hydrolysis.

Chapter 3:

Development of a Derivatization Method for Investigating Testosterone and Dehydroepiandrosterone in Saliva Samples from Young Professional Soccer Players Pre- and Post-Training Using Tandem Mass Spectrometry

3. Development of a Derivatization Method for Investigating Testosterone and Dehydroepiandrosterone in Saliva Samples from Young Professional Soccer Players Pre- and Post-Training Using Tandem Mass Spectrometry

3.1. Introduction

3.1.1. Saliva

Saliva is an important, complex, extracellular fluid secreted by the salivary glands in the mouths of some animals, including humans that plays a key role in maintaining oral health. It is mostly made up of water (98%) and other substances (enzymes, minerals, electrolytes, white blood cells, antibacterial compounds and mucus) that help to digest food (chew, taste, and swallow), moisten the mouth and make teeth strong. There are two types of salivary glands, and they are classified according to their anatomical size; the major, (parotid gland, submandibular glands and sublingual glands), salivary glands and the minor salivary glands which are connected with ducts that open into the mouth. The former glands are responsible for the secretion of more than 95 % of the saliva volume, while the latter glands produce the rest of the oral fluids (Ghannam & Singh, 2019; Kessler & Bhatt, 2018). The glands are made up of three types of cells: acinar cells, ductal cells and myoepithelial cells and are regulated by the autonomic nervous system (Proctor, 2016).

Saliva consists of organic substances such as creatinine, fatty acids and proteins such as enzymes and amino acids as well as inorganic substances such as electrolytes, calcium and magnesium components. Some hormones can also be found in the saliva, such as in a serum, in various concentrations based on the flow status (Kessler & Bhatt, 2018).

Based on the nature of the hormones, the transfer from blood to saliva occurs, via selective transport (active or passive) and ultrafiltration. Hormones pass through the capillary wall to the interstitial fluid and penetrate the salivary glands through the cell membranes of acinus (acinar cells), or the duct cells, which then reach the duct cell cytoplasm to finally combine with the other components of saliva see **Figure 3-1.** Cortisol, progesterone and testosterone are lipid-soluble naturally occurring, non-conjugated steroidal hormones that can cross many barriers from blood to reach the saliva very fast *via* passive diffusion (Proctor, 2016; Vining, McGinley, & Symons, 1983) Since these hormones are unconjugated then they are independent of saliva flow rate which makes them appropriate for clinical use, where they provide excellent correlation with hormone concentrations in the blood (Nowak, McMillen, Redman, & Short, 1987). In contrast, lipid-insoluble or conjugated hormones such as dehydroepiandrosterone (DHEA) transfer into saliva by ultrafiltration through the tight junctions between the acinar cells, and therefore care needs to be taken to control the saliva flow rate during collection since their concentrations in saliva are highly flow-rate dependent (Vining et al., 1983).

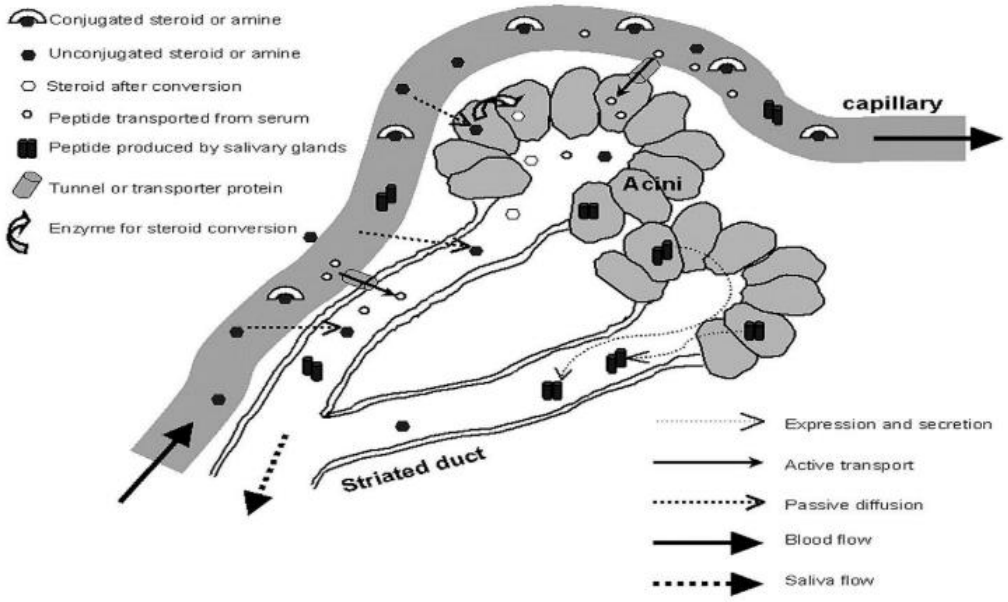


Figure 3-1. Hormones' mode of entry into saliva made by (Gröschl, 2008)

Methodological considerations for salivary diagnosis differ based on the nature and the reason for analysis of a particular hormone (Wren, Shirtcliff, & Drury, 2015). Collection of saliva requires an accurate method. Nowadays, collection of saliva can be done either under unstimulated or stimulated conditions. In the stimulating collection, the whole saliva is mainly secreted from the parotid gland during the action of chewing or by a stimulating agent such as citric acid, while in unstimulated saliva collection it is secreted from submandibular and sublingual glands these glands are responsible for secreting hormones as well. Therefore, hormones are not affected by flow rate except DHEA and other conjugated steroids due to their mode of entry

into saliva that mentioned above (Wren et al., 2015). Four protocols have been reported recently for the collection saliva. First, the draining method, which uses a funnel that is connected to a test tube with a lower lip. Secondly, passive drool or spitting which is the most common method. Thirdly, suctioning also extracts saliva from the mouth floor into a collection tube. Finally, the absorbent technique places an absorbent material, such as a cotton swab, gauze or polypropylene, in the mouth for a certain period (Celec & Ostatníková, 2012; Gröschl & Rauh, 2008; Gröschl & Rauh, 2006; Sarstedt, n.d.; Wren et al., 2015). Some researchers recommended using suction, and absorbent methods supporting the stimulation of saliva collection as well as other two collection methods recommended for the collection of unstimulated saliva. Generally, there is no gold standard for saliva collection (Wren et al., 2015).

Several factors must be avoided prior to collection of a saliva sample, since they could affect steroid hormones and influence hormone concentration, such as blood contamination which comes from brushing, bleeding gums or other dental issues, exogenous steroids (eating, drinking or smoking), dry mouth that leads to low saliva volume collection which mainly associated with elderly people, saliva flow rate, medications that contain rich amounts of steroidal hormones and sample temperature which could reduce hormones concentration especially during sample transportation.

Saliva is an alternative to serum for clinicians and researchers since it is considered to be a natural filtrate of plasma which contains only unbound free hormones. It is believed to generate a more accurate reflection of the physiologically active form of steroids. Various advantages can be provided when using saliva in comparison with other biological fluids for the analysis hormones such as it is a non-invasive, convenient, stress-free collection process, providing the ability to collect multiple samples throughout the day. On the other hand, there are various limitations related to salivary testing particular for steroids that should be addressed such as standard procedure of saliva collection, collection of sufficient volumes of saliva from some participants such as elderly people or infants could be difficult.

3.1.2. Androgens

Testosterone (T) is a primary androgen hormone and an anabolic steroid that is secreted by the testicles of males and the ovaries of females. It stimulates the development of male characteristics by binding to androgen receptors to exert its action (Mooradian & Morley, 1987). Dehydroepiandrosterone (DHEA) is the precursor of testosterone and other steroids and is produced by the adrenal glands (Granger & Zakaria, 1999). Epitestosterone (EPI) is an inactive 17 alpha-epimer of testosterone (Clark & Lobotsky, 1944). A correlation between T and DHEA in biological samples has not been confirmed. Some diseases may be related to decreased levels of DHEA in serum, such as depression, osteoporosis and metabolic syndrome (Arlt, 2004). However, it is not clear whether these diseases are the

consequence of a decline in DHEA itself, resulting from metabolic products of DHEA, such as T (Arlt, 2004; Higashi & Shimada, 2007).

Previous studies focused on the correlation between DHEA in saliva (Sal-DHEA) and serum samples. For example, in a study that was conducted in the Korean population, a radioimmunoassay (RIA) was used, demonstrating a positive correlation between the levels of DHEA in saliva and serum; additionally, there was an inverse variation in its concentration with age (*n* = 167 in men, *n* = 192 in women between 21–69 years) (Ahu & Chun, 2007). Another study investigated the correlation between T in saliva (Sal-T) and serum (Serum-T) samples, finding a positive correlation in men between the T level in saliva and serum (*n* = 104). However, there was no clear relationship of the T level in women between the saliva and serum (*n* = 91)) (Keevil et al., 2014). Unlike serum-T, Sal-T is generally referred to as a free active steroid because of its association with the level of unbound T in serum, which is less than 2% of the circulating T (Dunn et al., 1981). Using LC-MS/MS, a Japanese group compared Sal-T and Sal-DHEA levels (*n* = 114), showing a low correlation between them. A variation in the activity of one or both enzymes, 3β-hydroxysteroid dehydrogenase (HSD) and 17 β -HSD, which are responsible for the conversion of DHEA to T, may control the ratio between them (Shibayama et al., 2009).

A recent study was conducted in 2017 on a large British general population of men (*n* = 1675) aged between 18 and 69 years, and women (*n* = 2453) aged between 18 and 74 years, for the detection of Sal-T, using LC-MS/MS. The study showed an association

between increased age and a reduction in the mean Sal-T level since the annual average decline was between 1% and 1.4% in men and 1.3% and 1.5% in women (Keevil et al., 2017).

There has been a debate regarding the effects of physical activity on the level of Sal-T in sports players. Previous research showed that the effects were varied among professional players. Concentrations were increased in soccer players aged 10–16 years old by activity, whereas the level was unchanged in rugby players aged 25 years (Martyn et al., 2008; Di Luigi et al., 2006). In 2018, Australian researchers highlighted a correlation of the subsequent performance and the period of the former training load and fatigue response with the playing positions of 23 A-league football players. Regarding the impact of the training load on Sal-T, testosterone was increased for both short and long periods of training (3 to 28 days). Although the Sal-T level increased in both periods, the performance improved only for the majority of midfielders (Rowell et al., 2018a).

EPI is still under investigation regarding its metabolism and physiology. It is not produced from testosterone (Lipsett et al., 1966). To date, T and EPI can be detected only in urine. Athletes are suspected of androgen abuse if the ratio of T/EPI in urine is > 4 (WADA, 2017). Deshmukh et al. were for the first time able to determine T and EPI in human hair using LC-MS/MS. They reported that the correlation between the average T/EPI ratio was linear (Deshmukh & Naughton, 2012). A study conducted in

2014 detected EPI in rat serum, which indicated the possibility of detecting it in human saliva (Nawed IK et al., 2014).

Researchers have used several techniques to measure androgens in body fluids, hair and tissues, such as high-performance liquid chromatography (HPLC) and gas chromatography-mass spectrometry (GC-MS) (Draper & Parkinson, 1998; Shimada & Higashi, 2001). In the last decade, liquid chromatography (LC) combined with mass spectrometry (MS) was introduced in various studies, resulting in a reduced analysis time and increased sensitivity in determining low concentrations of androgens (Deshmukh et al., 2012; Gonzalo-Lumbreras & Izquierdo-Hornillos, 2003; Higashi et al., 2005; Nawed IK et al., 2014; Rauh, 2009). Only one previous study reported the detection of T and DHEA in saliva by using LC-MS/MS, as shown in **Table 3-1**.; many studies have used a radioimmunoassay.

Because some neutral steroids with low concentrations in saliva samples are particularly challenging to ionize in the ion source, chemical derivatization can be used to optimize the sensitivity for detecting analytes in saliva using ESI-MS (Büttler et al., 2016; Higashi et al., 2005; T Santa et al., 2007). In 2004, Higashi et al. reported that, when comparing the derivatization reagent 2-Hydrazino-1-methylpyridine derivative (HMP) with 2-fluoro-1-methylpyridine (FMP) and Girard's reagent P (GP), the HMP reagent yielded the best results with DHEA. The detectability of the DHEA-HMP was 1600 times underivatized DHEA in ESI (Higashi et al., 2005; Lai & Lin, 2001; Quirke & Van Berkel, 1994).

3.2. Aim

The current work follows the previous work by (Higashi et al., 2005). The first aim of this study was to develop a sample preparation method and derivatization using 2-Hydrazino-1-methylpyridine derivative (HMP) to detect testosterone, dehydroepiandrosterone and epitestosterone in saliva samples using LC-ESI-MS/MS. The secondary target was to apply the validated method to the analysis of saliva samples collected from 20 young professional soccer players pre- and post-training on two separate days.

Table 3-1 Comparison of the present method with previously reported methods of detection of T and DHEA in the saliva sample.

Author, Year (ref.)	Analyte	Instrument	Collection Method	Extraction	Derivative	Chromatography	LOQ	Case samples
Granger et al.,1999	DHEA	radioimmunoassay	directly					64 (8–11 y) 96 (12–17 y) 48 (30–45 y)
Ostatníková et al., 2002	T	radioimmunoassay	directly					77 girls & 126 boys (6–9 y)
Morley et al., 2006	T	radioimmunoassay	directly					1454 (20–89 y) male
Ahu et al. 2007	Cortisol and DHEA	radioimmunoassay	directly					359 (21–96 y) male &female
Higashi et al., 2007	DHEA	LC–ESI-MS–MS	directly	SPE Strata-X (ethyl acetate) *	(HMP) incubation (60 °C for 1 h)	ODS-H-80 column (10 µL injected)	25 pg/mL	3 (33–23 y) male 3 (22–24 y) female
Beaven et al., 2008	Cortisol and T	immunoassay	Directly					23 men rugby players
Yasuda., 2008	T	LC-MS and ELISA	directly	LLE (ethyl acetate)		CD-C18 column		51 (30–85 y) 29 (55–78 y)
Shibayamaet al.,2009	DHEA and T	LC–ESI-MS–MS	directly	SPE Strata-X (ethyl acetate) *	(HMP) incubation (60 °C for 1 h)	AYMC-Pack Pro C18 RS column (10 µL injected)	10 pg/mL	114 (21–89 y)
Cardoso et al.,2010	T	radioimmunoassay	directly					60 (20–60 y) male
Macdonald et al., 2011	T	LC-MS/MS	directly	LLE (methyl-tert-butyl ether)		C18 column	7 pg/mL	103 (16–74 y) male

Keevil et al., 2014	T	LC-MS/MS	directly	LLE (methyl-tert-butyl ether) *		C8 column (35 µL injected)	1.5 pg/mL	104 males 91 females
Maya et al., 2016	Cortisol and T	ELISA	directly					16 (22.5 ± 2.1 y) female players
Clifton et al., 2016	T	LC-MS/MS	directly	LLE (methyl-tert-butyl ether) *		C8 column (35 µL injected)	1.8 pg/mL	1599 male 2123 female (18–74 y)
Büttler et al., 2016	T	LC-MS/MS	directly	(SPE) Symbiosis online solid-phase extraction	methoxylamine hydrochloride		LOD 1.3 pg/mL	131 girls and 123 boys (8–26 y)
Keevil et al., 2017	T	LC-MS/MS	directly	LLE (methyl-tert-butyl ether) *		C8 column (35 µL injected)	1.5 pg/mL	1675 male 2453 female
Rowell et al., 2018	Cortisol and T	ELISA	directly					23 (23 ± 4.1 y) Football player
This study	DHEA and T	LC-MS/MS	Salivette Polyester	(SPE) Strata-XL polymeric reverse-phase (ACN) *	(HMP) incubation (50 °C for 30 min)	Ultracore 2.5 Superphenylhexyl column (10 µL injected)	20 pg/mL	20 (20.6± 1.4 y) Male football players

Abbreviation: (*) Elution solvent, (y) Years old. (T) Testosterone, (DHEA) Dehydroepiandrosterone, (HMP) 2-Hydrazino-1-methylpyridine, (ACN) Acetonitrile, (LOD & LOQ) lower limit of detection & quantification, (LC-ESI-MS) liquid chromatography-electrospray ionization mass spectrometry, (SPE) Solid-phase extraction and (LLE) liquid-liquid extraction

3.3. Materials and Methods

3.3.1. Chemicals and Materials

The following reagents were purchased from Sigma-Aldrich, UK: 2-fluoro-1-methyl pyridinium-p-toluenesulfonate, hydrazine hydrate, testosterone (T), dehydroepiandrosterone (DHEA), epitestosterone (EPI), deuterated testosterone (T-d_3), trifluoroacetic acid (TFA), methanol (MeOH), ethanol (EtOH). HPLC grade water, HPLC grade acetonitrile (ACN) and HPLC grade ethyl acetate were purchased from Fisher Scientific, UK. HPLC grade formic acid (98%) was obtained from BDH-Merck, UK. Individual stock solutions of T, DHEA, EPI and T-d_3 were prepared in EtOH at 10 µg/mL, each followed by dilution to prepare 1 µg/mL, 10 and 1 ng/mL of working solutions in EtOH.

3.3.2. Preparation of 2-Hydrazino-1-Methylpyridine Derivative (HMP)

HMP was synthesized according to the work of Higashi et al. (Higashi et al., 2005) as follows: hydrazine hydrate solution (80%, 132 µL) in MeCN (30 mL) was added to 2-fluoro-1-methyl pyridinium-p-toluenesulfonate (FMP-TS) (300 mg) in MeCN (6 mL) at 0 °C after stirring for 10 min at 0 °C and then maintained for 20 min at room temperature under N_2. The residue was dissolved for a second time in MeCN (2 mL) after this resulting mixture was concentrated. Following this, it was filtered, and the crude product was recrystallized (twice) from MeCN–ethyl acetate (5:1, *v*/*v*) to give

HMP (98 mg) as colourless needles (mp: 133–134 °C; ESI-MS: m/z 124.3 [M]+). Finally, HMP was stored at 4 °C; this product remained stable for at least six months.

3.3.3. Instrumentation

An Agilent liquid chromatography-mass spectrometry (LC-MS-MS) triple, quadruple G6430A mass spectrometer equipped with an Agilent 1200 series auto-sampler, a quaternary pump SL with a degasser, and a thermostatic column compartment was used for the quantitative analysis. Positive electrospray ionization (+ESI) was used, and the mass spectrometry (MS) was operated in the multiple reaction monitoring mode (MRM). The data were recorded using Mass Hunter software version B.06.00 (Agilent technologies).

3.3.3.1.Liquid Chromatographic Conditions

The analytes were separated on an ACE Ultracore 2.5 Superphenylhexyl column (150 × 3.0 mm id) with a guard column, and the temperature was maintained at 30 °C. Isocratic elution was carried out with water: acetonitrile (75:25), containing 0.01% (formic acid) FA with a flow rate of 0.4 mL/min. The injection volume and run time were 10 µL and 16 min, respectively.

3.3.3.2.Mass Spectrometric Conditions

The androgens and testosterone-d_3 (T-d_3) derivatized with 2-Hydrazino-1-Methylpyridine Derivative HMP were quantified by using an Agilent liquid

chromatography-mass spectrometry (LC-MS-MS) triple quadrupole G6430A mass spectrometer. The multiple reaction monitoring (MRM) conditions were examined by injecting infusion solutions directly into the electrospray ionization (ESI) source. The infusion solutions contained a concentration of 10 µg/mL of each analyte. The results are shown in **Table 3-2**. Additional mass spectrometry (MS) parameters were optimized to achieve the best sensitivity, as follows: Delta EMV, 800 V; gas temperature, 350 °C; gas flow, 9 L/min; nebulization pressure, 20 psi; sheath gas, 50 psi; auxiliary gas, 15 psi; capillary voltage, 5000 V.

Table 3-2 Optimization MRM transitions of androgens and testosterone -d_3 derivative with HMP.

Analyte	MRM Transition (*m/z*)	Fragmentation Voltage	CE (V)
T-HMP	394 > 108	130	40
T-d_3-HMP	397 > 108	200	50
EPI-HMP	394 > 108	210	40
DHEA-HMP	394 > 109	120	35

Collision energy (CE); Voltage (V); testosterone (T); testosterone -d_3 (T-d_3); epitestosterone (EPI); dihydroepianderston (DHEA); 2-Hydrazino-1-methylpyridine derivative (HMP); multiple reaction monitoring (MRM).

3.3.4. Experimental Design

To investigate the effect of exercise on endogenous androgens in saliva using an optimised and validated method, saliva was collected from 20 young male professional soccer players (20.6 $\pm$ 1.4 years, body mass 70.2 $\pm$ 1.6 kg, height 178 $\pm$ 2 cm and Body mass index (BMI) 20.6 $\pm$ 1.4 kg/m^2) using a Sarstedt Salivette polyester. The samples (pre & post) were collected over two days during the training sessions. No participant was injured, and no participant took any medication during the study. The participants attended the club one and a half hours before the training starts

every day. The study was approved by the Ethics Committee at King Saud University, and all players read the information sheet and signed a consent form before participating in the study.

All training sessions started at the same time each day. Two training sessions were designed for the study, beginning with a 15 min warm-up. Then, the players were divided randomly to play two small side games for 30 min, followed by a 4 min rest time. In addition, the coach set up a game for 40 min divided into two halves, followed by a 5 to 10 min cool down period. The intensity of the exercises on the first training day and the second day was designed based on the percentage of the maximum heart rate. The heart rates were 70% and 72% of the maximum on the first and second days, respectively. The mean ambient temperature was 25 ± 3 °C, and the humidity was 18 ± 4% for the first day and 26 ± 1 °C and 16 ± 2% for the second day.

3.3.5. Pre-Treatment and Extraction of Samples

The stored samples were thawed and then centrifuged at 1000× *g* for 5 min, and 0.5 mL of supernatant was taken. The saliva samples were deprotonated with 0.5 mL of acetonitrile containing 50 pg of T-d_3, vortexed for 30 s, centrifuged for 10 min and then diluted with water (1.5 mL).

For the extraction, the samples were loaded onto a Strata-XL reverse-phase cartridge, which was first prepared by washing with 1 mL of methanol (MeOH) followed by 1

mL of water (H_2O). After washing with 1 mL of H_2O followed by 1 mL of MeOH/H_2O (10:90 *v*/*v*), the retained analytes and IS were eluted under gravity with 1 mL of acetonitrile.

3.3.6. Derivatization of Androgens with 2-Hydrazino-1-Methylpyridine Derivative HMP

The derivatization of the standards (STDs) and extracts respectively, with deuterated testosterone (T-d_3) was carried out by adding 50 µL of fresh 2-Hydrazino-1-Methylpyridine Derivativing agent (HMP) solution (1 mg/mL) in methanol (EtOH) containing 25 µg of trifluoroacetic acid (TFA). The sample was then incubated at 50 °C for 30 min. The mixture was then gently evaporated under the nitrogen. The residue was reconstituted using the mobile phase (200 µL) and then vortexed. Finally, 10 µL of the mixture was injected.

3.4. Method Validation

The method was validated according to the Food and Drug Administration guidelines (FDA) (FDA & Food and Drug Administration, 2001).

3.4.1. Extraction Recovery

Two sets of standards were prepared, each of which contained 50, 200 and 400 pg, corresponding to 100, 400 and 800 pg/mL of testosterone (T), dehydroepiandrosterone (DHEA)and epitestosterone (EPI), respectively. One set was made in the pooled steroid-free saliva samples (blank), and the other set was

prepared in the mobile phase. The blank saliva samples (500 µL) were spiked with these three concentrations, respectively, in triplicate and then extracted. The saliva samples were mixed with 100 pg of deuterated testosterone (T-d_3), referring to 200 pg/mL. The standards of the other set were directly injected after they were combined with an internal standard and then derivatized (i.e., the non-extracted samples). The peak area means of T, DHEA and EPI and the (T-d_3) were obtained for the extracted and non-extracted samples in each concentration. The absolute recovery was determined for each analyte by dividing the average extracted mean by that of the non-extracted mean at the same level and then multiplying by 100 (Deshmukh et al., 2012).

3.4.2. Matrix Effect

The matrix effect refers to any compound in the sample except the analyte of interest, which could affect the analyte response when co-eluted with it, causing an increase in ionization efficiency (i.e., enhancement) or a decrease in ionization efficiency (i.e., suppression). The consequence is an inaccurate concentration measurement (Hall et al., 2012).

Two sets of samples were prepared in low (50 pg), medium (100 pg), and high (400 pg) concentrations of standards and deuterated testosterone (T-d_3), corresponding to 100, 200 and 800 pg/mL each. Set one consisted of derivative standards that were injected six times to establish a mean peak area for each concentration. Set two

involved three different pooled blank saliva samples from ten sources. Each matrix source was extracted in duplicate, and the extract was spiked in either the low, medium or high concentrations of standards and T-d_3. The matrix effect was then calculated by averaging the area of each, as shown in Equation below (Hall et al., 2012).:

$$\%ionisation\ enhancement\ or\ suppression = ((X\ Area\ of\ set2 - X\ Area\ of\ set1)/X\ Area\ of\ set1) * 100 \quad (1)$$

A negative value indicates signal suppression, whereas positive values suggested that enhancement occurred. The acceptable limits for enhancement or suppression are ± 25% (Hall et al., 2012).

3.4.3. Specificity

In the actual method, to ensure that no interference occurred between each analyte of interest by internal standard or other substances during their retention time in the saliva, three representative types of steroids, pregnenolone, dihydrotestosterone and androstenedione, were derivatized at 800 pg/mL and injected into the mass spectrometry (MS) system.

3.4.4. Linearity and Calibration Standards of the Saliva

Pooled saliva samples were prepared, and the samples were mixed for 24 h with 1 g of activated charcoal (NoritEXW, Nacalai Tesque, France) and then centrifuged at 1000 g for 20 min. The supernatant was examined to ensure that no testosterone (T),

dehydroepiandrosterone (DHEA) or epitestosterone (EPI) were detected following this treatment. Aliquots of steroid-free saliva samples (blank saliva) (0.5 mL) were spiked with standards (STDs) to prepare six different concentrations of these steroids in the range of 10–400 pg, corresponding to 20–800 pg/mL of DHEA and T; and in the range of 25–600 pg, corresponding to 50–1200 pg/mL of EPI. The level of the internal standard (T-d_3) was 100 pg/mL. After evaporation, the residue was dissolved in 200 µl of ethanol and then derivatized with the HMP reagent using the final optimised procedure described above. The calibration curves were determined by plotting the peak area ratio of T/T-d_3, DHEA/T-d_3 and EPI/T-d_3 (y) against the concentrations of T, DHEA and EPI (x, pg/mL), respectively.

3.4.5. Sensitivity: Limit of Detection (LOD) and Limit of Quantification (LOQ)

Stock solutions of testosterone (T) and dehydroepiandrosterone (DHEA) were prepared at concentrations of 10–400 pg/0.5 mL, equal to 20–800 pg/mL. Meanwhile, epitestosterone (EPI) was prepared at concentrations of 25–600 pg/0.5 mL, equal to 50–1200 pg/mL. They were then spiked into the blank saliva and analysed in triplicate following derivatization. The LOD and LOQ of T, DHEA and EPI were determined using a linear calibration curve (Shrivastava & Gupta, 2011). Three calibration curves were constructed. The LOD and LOQ were calculated according to an estimate from the standard deviation of the y-intercept (Sy) and the average slope (Am), as shown in Equation below.

$$LOD = 3 * Sy / Am$$
$$LOQ = 10* Sy / Am \quad (2)$$

3.4.6. Accuracy and Precision

The accuracy of the results was determined by calculating the percentage recovery of five determinations at three different concentrations obtained from five sources in a linearity range of 50, 400 and 800 pg/mL. The concentrations were calculated from the corresponding regression equations.

Precision determinations were performed for three different concentrations of pure standards of steroids (50, 400 and 800 pg/mL for testosterone (T), dehydroepiandrosterone (DHEA) and epitestosterone (EPI), respectively) spiked into the saliva. They were analysed in triplicate in a single day and on five following days to calculate the intra-day (i.e., repeatability) and inter-day precision (i.e., intermediate precision).

3.4.7. Stability

Two terms were investigated to study the stability of the analytes. In the short-term study, 0.5 mL of the blank saliva samples was spiked with low and high concentrations of the analytes (50 and 400 pg) and deuterated testosterone (T-d_3) (50 pg), corresponding to 100 and 800 pg/mL each, as well as 100 pg/mL for the T-d_3, before being stored at different temperatures (4, −20 °C and in the auto-sampler) and then

analysed after thawing and extraction. Regarding the freeze-thaw stability, two cycles in the short-term study were assessed in triplicate after 24 h and 48 h. In addition, the low and high concentrations of the analytes (100 and 800 pg/mL) and T-d_3 (100 pg/mL) were stored at −20 °C for six weeks and then analysed after more than three freeze-thaw cycles. In the long-term evaluation, 24 random samples were chosen from the overall samples, analysed and then stored at −80 °C for six weeks, and then rerun. The first run of each concentration in the short or long-term study was considered a time-zero response. Thus, the stability was satisfactory if the response was within 15% of the time zero response.

3.5. Results and Discussion

3.5.1. Method development

3.5.1.1.Optimization of Reaction Conditions in Derivatization Steroids with HMP

Two isomeric (E &Z) peaks were formed, as a result of reacting the 2-Hydrazino-1-Methylpyridine (HMP) derivatization reagent with testosterone (T) and epitestosterone (EPI0. In contrast, dehydroepiandrosterone (DHEA-HMP) formed just one peak (possibly due to a steric hindrance from the methyl group at the 18 position in the steroid nucleus inhibiting the formation of the syn isomer).

It was found that the evaporation step was crucial since only 2% of the steroid was derivatized without evaporation, while 99% was derivatized when the solvent was

evaporated with nitrogen gas. Shou et al. (Shou & Naidong, 2005) justified this increase, reporting that when using trifluoroacetic acid (TFA) as a catalyst, it has the power to form ion pairs, which leads to the prevention and loss of the electrospray ionization (ESI) signal. To overcome this problem, nitrogen evaporation was used to dry the sample, which led to improved results.

The reaction times of 30 min and 1, 2, 4 and 24 h were used with two temperatures (room temperature and 50 °C). The results are shown in **Figure 3-2** and indicate that the highest reaction efficiency between the androgens and HMP occurred after 30 min of incubation at both room temperature and 50 °C. However, when the androgens were heated at 50 °C, the derivatization was complete within 30 min (99.8%). Therefore, it was concluded that heating at 50 °C for 30 min yielded the best result.

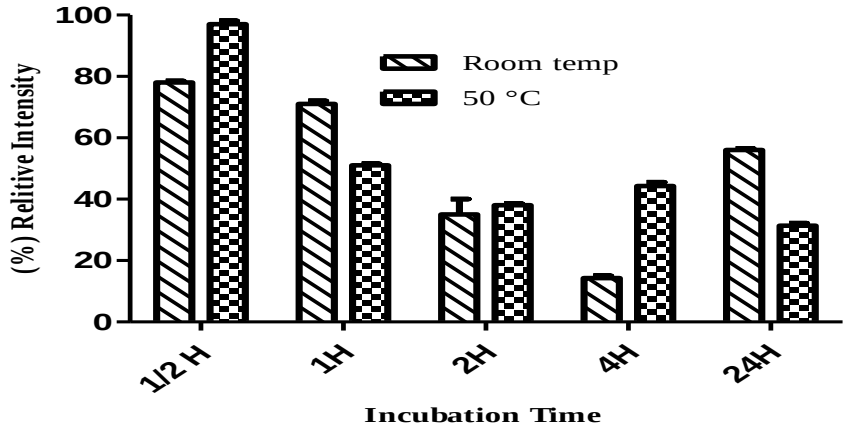


Figure 3-2 The yield of the HMP derivative of testosterone depending on reaction temperature and time.

3.5.1.2.Optimization of LC Conditions

Three columns were examined to determine their efficiency in separating the steroids. This testing involved an ACE 3 C18 column (150 × 3.0 mm i.d.; Hichrom, Reading, UK), an ACE 5 C18-AR column (150 × 4.6 mm, 5 µm Hichrom) and an ACE Ultracore 2.5 Superphenylhexyl (150 × 3.0 mm id.; Hichrom). The C18 column did not provide any acceptable separation between the steroid derivatives. The C18-AR gave only a poor separation between the dehydroepiandrosterone (DHEA-HMP) peak and the first peak of testosterone (T-HMP), which led to co-elution. Because it was not possible to achieve an acceptable separation between the first peak of T-HMP and DHEA-HMP by using the C18-AR column, an ACE Ultracore 2.5 Superphenylhexyl column (150 × 3.0 mm id) was used and produced an adequate separation of the steroids. All the analyte peaks appeared within the range of 5.6 to 15 min (**Figure 3-3),** using the conditions described in **Section 3.3.3.1**; on the other hand, because of the presence of twin peaks for T-HMP and EPI-HMP**,** and since the second eluting isomers gave higher abundances than the first, they were used for the quantification.

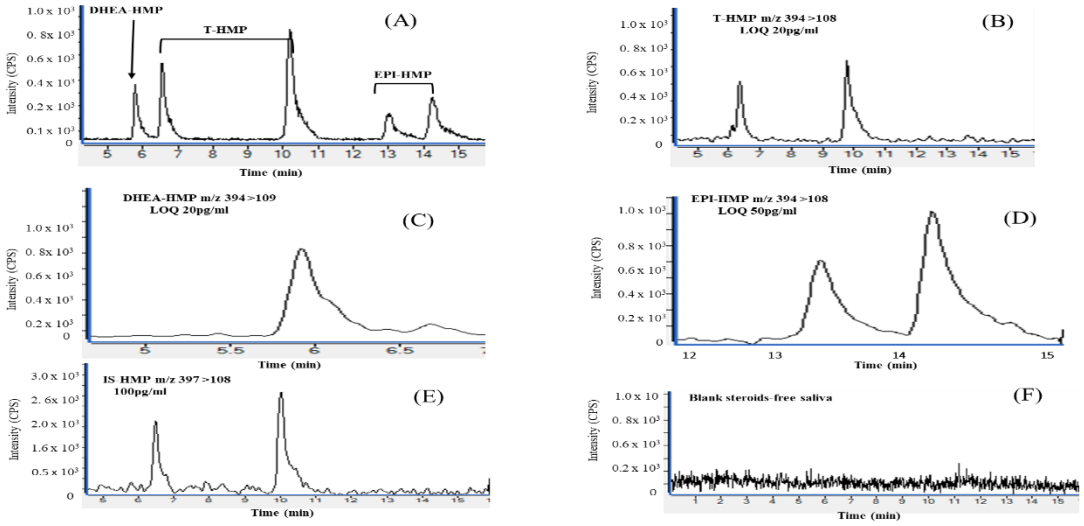


Figure 3-3 Chromatograms of HMP derivatives of testosterone (T-HMP), dehydroepiandrosterone (DHEA-HMP), epitestosterone (EPI-HMP) and Testosterone-d3 HMP (T-d3-HMP) in healthy human saliva spiked after treatment with activated charcoal: (A) chromatographic resolution of derivative testosterone (T), dehydroepiandrosterone (DHEA) and epitestosterone (EPI), (400 pg/mL) spiked in steroid-free saliva. (B) T-HMP multiple reaction monitoring (MRM) illustrating low limit of quantification (LOQ) m/z 394 > 108, (20 pg/mL); (C) DHEA-HMP limit of quantification (LOQ) m/z 394 > 109, (20 pg/mL); (D) EPI-HMP limit of quantification (LOQ) m/z 394 > 108, (50 pg/mL); (E) Testosterone-d3 HMP m/z 397> 108, (100 pg/mL); (F) Blank: saliva treated with activated charcoal, before being extracted and derivatized. The liquid chromatography-mass spectrometry (LC-MS/MS) conditions are described in section 2.3.2. Counts per second (cps).

3.5.1.3.Optimization of MS Conditions

The parameters of the mass spectrometer (MS) operating conditions are provided in **Table 3-3.** The optimal MS parameters were achieved by the direct infusion of the derivatives of the steroids into the electrospray ionization (ESI) source in the positive mode. The infusion solution consisted of 50/50 (*v*/*v*) of aqueous and organic mobile phases at a concentration of 10 µg/mL for all the analytes. The optimal MS transitions had only one product ion with the chosen collision energy, as follows: m/z 394 > m/z 108; *m*/*z* 394 > *m*/*z* 109; *m*/*z* 394 > *m*/*z* 108; and *m*/*z* 397 > *m*/*z* 108, for testosterone

(T-HMP), dehydroepiandrosterone (DHEA-HMP), epitestosterone (EPI-HMP) and deuterated testosterone (T-d_3-HMP), respectively, as described in **Section 3.3.3.2 (Table 3-2).** The product ion is formed by the cleavage of the N-N bond of the hydrazine, as shown in **Figure 3-4**; the derivatization of the T and EPI with HMP results in hydrazones with just a fragmentation ion $[M]^+$, whereas DHEA-HMP gave a fragmentation ion with $[M+1]^+$.

Table 3-3 Summary of the optimum conditions for ion source parameters

Parameter	Ranges	The Optimum Conditions
Delta	400–600–800	800
Gas temperature	300–325–350 °C	350 °C
Gas flow	8–9–10–11 l/min	9 L/min
Nebulization pressure	15–20–25–30 psi	20 psi

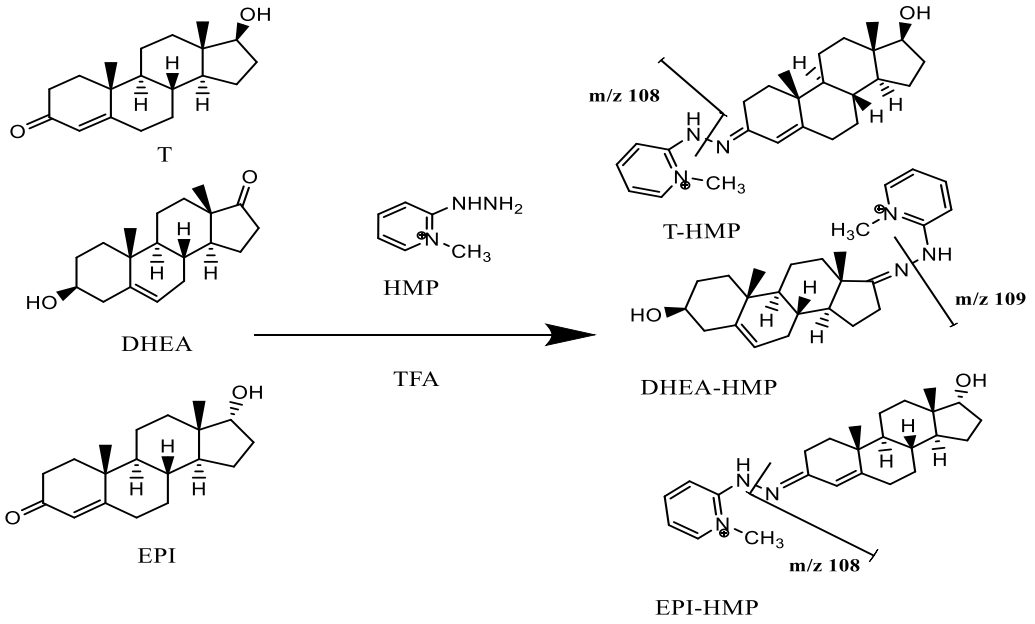


Figure 3-4 The scheme shows the derivatization of testosterone (T), dehydroepiandrosterone (DHEA) and epitestosterone (EPI) with 2-hydrazino-1-methylpyridine (HMP) in the presence of Trifluoracetic acid (TFA).

3.5.1.4.Collection of Saliva and Pre-Treatment

There are many ways to collect saliva; the most popular method is by passive drool or spitting, as mentioned in **Table 3-1**. Saliva collection can be stimulated by chewing gum or citric acid, which could cause a reduction of the analyte concentration (Choo & Huestis, 2004). Also, the collection of Saliva testosterone (Sal-T) and other steroids, using different devices such as Salivette® (Sarstedt) with cotton, polyester and polyethylene, were evaluated. The polyester and polyethylene Salivette have been given weak recovery for androgen. On the other hand, Salivette® cotton is not recommended (Gröschl et al., 2008; Gröschl & Rauh, 2006). Polyester Salivette was used for the collection of saliva samples in this project. Therefore, the evaluation of recovery from this device has been made and was compared with the direct collection by collecting a sample from a participant 5 times for both collection types following the collection protocol. Recoveries were compared and calculated by using an independent t-test. The results for the recovery of Sal-T using the Salivette device in comparison to the direct collection was 86%, and the *p*-value was 0.1, whereas the saliva dehydroepiandrosterone (Sal-DHEA) recovery was 83.5% and the *p*-value was 0.2.

3.5.1.5.Solid-phase Extraction Cartridge Selection

Two kinds of cartridges (C18-E and Strata-XL polymeric reverse-phase) were examined. After suitable sorbents for the analytes were chosen, the 10-bottle

optimization SPE extraction approach, which is recommended by the Agilent company, was applied (Agilent technologies, n.d.). The optimization was started using a procedure where 11 cartridges from each sorbent were conditioned with 1 mL methanol, followed by 1 mL of water (H_2O). Then, 100 µl of ethanol containing 100 pg of standards (STDs) were added to 1 mL H_2O and loaded on 10 cartridges of each sorbent. Only 1 mL H_2O without any STDs was loaded on the blank number 11 for each sorbent. **Figure 3-5, C** shows that 10% to 20% of methanol (MeOH) and 80% to 90% of H_2O could be used in the washing step because of the low quantity of steroids that was lost from both cartridges.

As shown in **Figure 3-5 A, B,** 1 mL of acetonitrile (ACN) yielded the highest extraction recovery for all the steroids. Overall the Strata-XL cartridge gave a slightly better performance. Therefore, the final extraction procedure used to extract the steroids from the saliva samples was as follows: the sample was loaded onto the Strata-XL cartridge, which was prewashed with 1 mL of MeOH followed by 1 mL of H_2O. After washing with 1 mL H_2O followed by MeOH: H_2O (10:90 v/v), the target analytes were eluted with 1 mL of ACN.

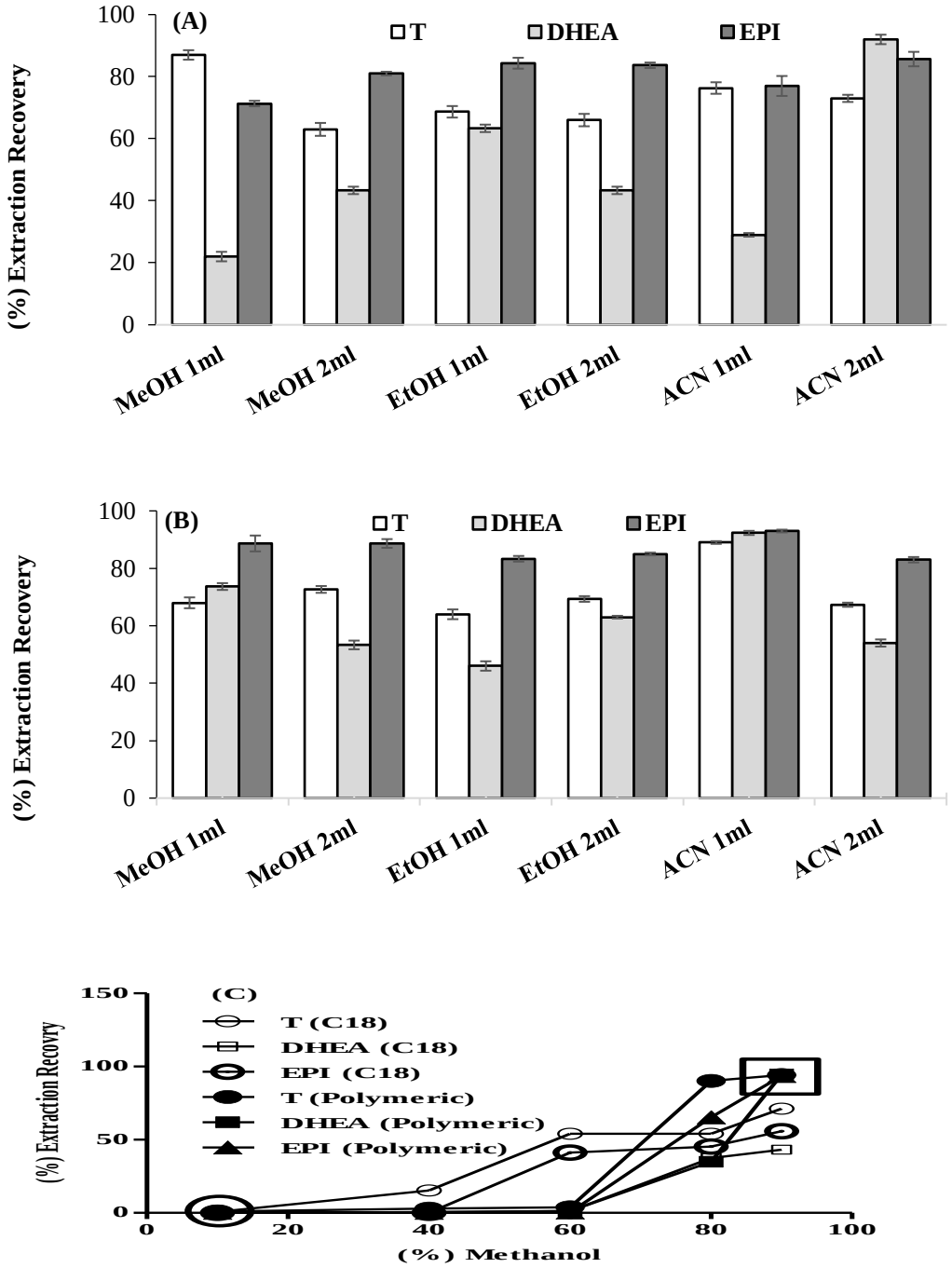


Figure 3-5 Development of an SPE method for androgens using two sorbents of SPE cartridges: (A) effect of elution different solvents and volumes using C18 cartridge; (B) effect of elution with different solvent mixtures and volumes using the polymeric cartridge; (C) effect of different % of methanol in the washing step (n=3).

3.5.2. Method Validation

3.5.2.1.Extraction Recovery from Spiked Human Saliva

The efficiency of the extraction procedure of testosterone (T), dehydroepiandrosterone (DHEA) and epitestosterone (EPI) using solid-phase extraction cartridge (SPE) was assessed by calculating the extraction recovery percentages. The extraction method was able to extract more than 93% of T, more than 86% of DHEA, and more than 96% of EPI from the three different concentrations, as shown in **Table 3-4.**

Table 3-4 Extraction Recovery Data on Androgens Determined in Saliva Using LC-MS/MS

Compounds	Concentration (pg/mL)	Level	Average Ratio		Extraction recovery
			Extracted	Non-extracted	
	n = 3		Mean ± S.D. *	Mean± S.D. *	(%)
T	100	Low	7037 ± 416	6650 ± 197	105.8
	400	Medium	21,797 ± 766	21,590 ± 1569	101.0
	800	High	56,725 ± 2196	63,539 ± 5556	89.3
DHEA	100	Low	5072 ± 207	4880 ± 532	103.9
	400	Medium	21,760 ± 1214	23,187 ± 1181	93.8
	800	High	48,749 ± 1297	52,424 ± 4102	93.0
EPI	100	Low	3853 ± 233	3828 ± 261	100.7
	400	Medium	12,629 ± 745	12,420 ± 119	101.7
	800	High	23,231 ± 1872	23,424 ± 2244	99.2
$T\text{-}d_3$	200	IS	7727 ± 882	8720 ± 883	112.8

(*) Expressed as standard deviation (S.D.); testosterone(T), dehydroepiandrosterone, (DHEA) and epitestosterone (EPI)

3.5.2.2.Matrix Effects

As shown in **Figure 3-6,** the matrix slightly enhanced the response for all steroids and the deuterated testosterone (T-d_3) in the low and medium concentrations, whereas at the highest level the signal was slightly suppressed. The matrix effect was within the range of ± 25%, which was acceptable.

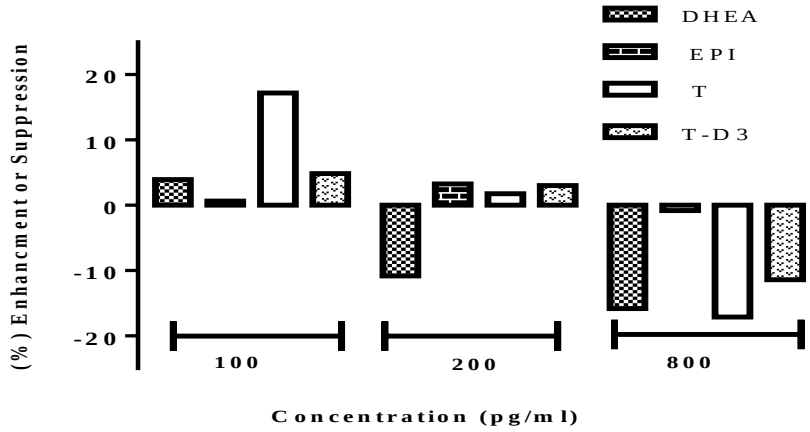


Figure 3-6 Mean matrix effect (n = 3).

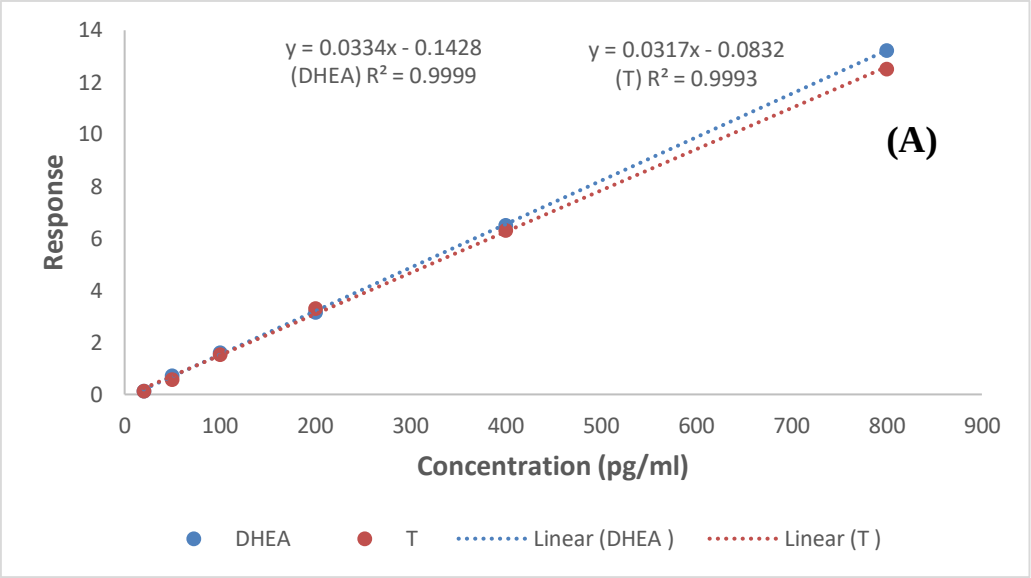
3.5.2.3.Specificity

The specificity was determined using the liquid chromatography-mass spectrometry (LC-MS-MS) conditions described in **Section 3.3.3**. Three steroids, pregnenolone, dihydrotestosterone and androstenedione, were investigated to determine the most productive ion, and the retention time was as follows: pregnenolone (PREG) m/z 422.5 [M]+, m/z 108.0 and retention time 7.3 and 11.5; 5α dihydrotestosterone (DHT) m/z 396.2 [M]+, m/z 108.0 and retention time 7.5 and 11.8; and androstenedione

(AD) m/z,497.4 [M−1]+, m/z 108.0 and retention time 7.3 and 11.5 min, all of which had twin peaks. All these steroids were not detected by the multiple reaction monitoring (MRM) conditions used for testosterone (T), dehydroepiandrosterone (DHEA) and epitestosterone (EPI), that were derivatized by 2-Hydrazino-1-Methylpyridine Derivatiing agent (HMP) when 800 pg/mL of the potentially interfering steroids were injected.

3.5.2.4.Linearity and Calibration of Standards Spiked into Saliva

The assay showed that the linearity was acceptable within the range of 10–400 pg corresponding respectively to 20–800 pg/mL for testosterone (T) and dehydroepiandrosterone (DHEA); and within the range of 25–600 pg corresponding respectively to 50–1200 pg/mL for epitestosterone (EPI), with correlation coefficients of ≥ 0.995 (**Figure 3-7**). In addition, reliable resolution of the peaks and acceptable peak shapes were achieved for T, DHEA, EPI and deuterated testosterone (T-d_3). The ratios obtained for DHEA and T against the (T-d_3) were roughly equal; therefore, some points in the calibration curve appear as one point.



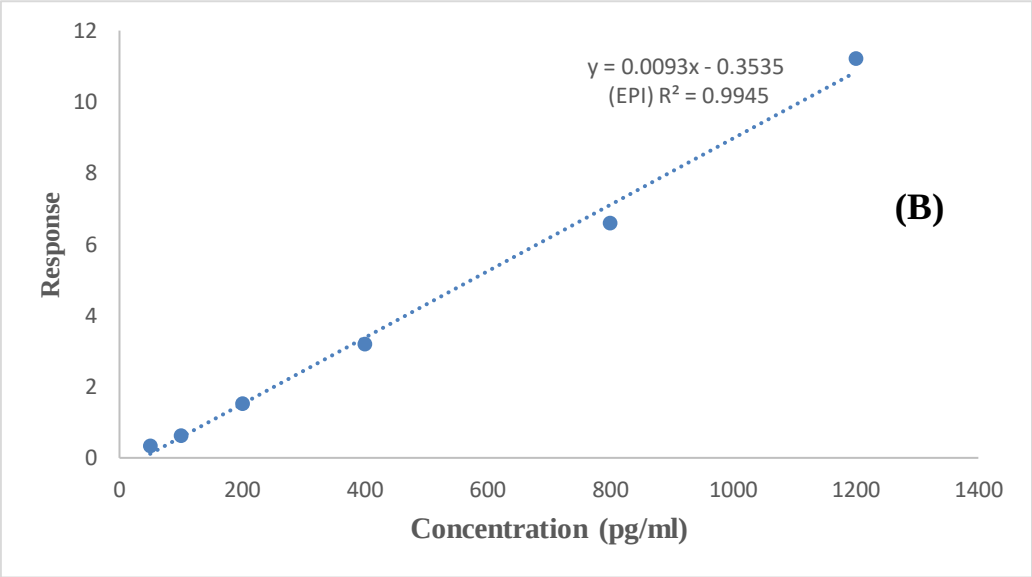


Figure 3-7 Calibration curve for (**A**): (T) testosterone (orange colour), (DHEA) Dehydroepiandrosterone (blue colour) in the range of 20-800 pg/ml each, and (**B**): (EPI) Epitestosterone in the range of 50-1200 pg/ml.

3.5.2.5.Sensitivity: Limit of Detection (LOD) and Limit of Quantification (LOQ)

As summarized in **Table 3-5,** LOQ and LOD were calculated for testosterone (T) and dehydroepiandrosterone (DHEA) at concentrations of 20–800 pg/mL, and epitestosterone (EPI) at concentrations of 50–1200 pg/mL. The LOQ was 20 pg/mL for T and DHEA and 50 pg/mL for EPI. However, the LODs for T, DHEA and EPI were 4, 6 and 14 pg/mL, respectively.

Table 3-5 Calibration Curve Parameters (*n* = 3).

Analyte	LOD pg/mL	LOQ pg/mL	Slope			Intercept		r^2
			Mean	S.D.	RSD	Mean	S.D.	Mean
T	4	20	0.0337	0.000954	2.83068	0.280067	0.016757	0.9978
DHEA	6	20	0.034333	0.000551	1.60414	0.224933	0.023755	0.9996
EPI	14	50	0.0186	0.000173	0.93121	0.326333	0.041834	0.995

3.5.2.6.Accuracy and Precision

The method was shown to be reproducible regarding the peak shape and retention time. The relative standard deviation (RSD%) of the intra- and inter-assay precision for testosterone (T), dehydroepiandrosterone (DHEA) and epitestosterone (EPI) was < 11%. The accuracy was within the range of 93–114%. The results for the accuracy and precision of the method are shown in **Table 3 6.**

Table 3-6 Intra-and Inter-Assay Precision and Accuracy Results of Androgen Determination in Saliva Using LC-MS/MS (*n* = 5).

Analyte	Concentration (pg/mL)	Level	Intra-day			Inter-day		
			RSD[a] (%)	Mean (pg/mL)	Accuracy[b]	RSD[a] (%)	Mean (pg/mL)	Accuracy[b]
T	50	Low	6.8	46.8	93.5	9.9	47.0	93.9
	400	Medium	5.9	401	100.5	4.1	409.8	102.4
	800	High	3.0	816	102.0	5.5	760.4	95.1
DHEA	50	Low	4.1	51.2	102.4	8.6	57.2	114.5
	400	Medium	1.8	405.0	102.5	10.8	398.4	99.6
	800	High	2.3	795.8	99.5	7.4	784.8	98.1
EPI	50	Low	8.9	49.8	99.6	9.9	47.0	93.9
	400	Medium	3.4	427.8	106.9	4.1	409.8	102.4
	800	High	4.7	731.4	91.4	5.5	760.4	95.0

[a] expressed as the relative standard deviation (RSD). [b] expressed as [mean % deviation = mean calculated concentration/ nominal concentration X100].

3.5.2.7.Stability

Table 3-7 shows that after two cycles of freezing/ thawing at two different temperatures and on auto-sampler, the stability was found to be satisfactory for the analytes (testosterone (T), dehydroepiandrosterone (DHEA) and epitestosterone (EPI)) during the short-term tests (24 and 48 h). However, there were slight decreases from zero-response in the auto-sampler. In contrast, the degradation of the analytes in low and high concentrations of 100 and 800 pg/mL, respectively, and the deuterated testosterone (T-d_3) in a concentration of 100 pg/mL, was >50% when they were stored for six weeks at −20 °C after freezing/thawing for a third cycle. The stability of the derivative steroids was acceptable after 24 samples that were reanalysed after being stored at −80 °C for six weeks. When the samples were rerun, the percentages compared with the original response were 95% and 97% for testosterone (T) and dehydroepiandrosterone (DHEA), respectively.

Table 3-7 Stability of T, DHEA and EPI in the short term.

Treatment		T [a]		DHEA [a]		EPI [a]	
		100	**800**	**100**	**800**	**100**	**800**
Freezing/ thawing, 1 cycle	−20 °C	100.1 ± 9.1	98.0 ±1.7	100.1 ± 0.1	97.1 ± 1.8	98.0 ± 0.5	97.2 ± 5.7
	4 °C	98.1 ± 3.9	97.2 ±0.6	96.4 ± 0.6	96.7 ± 1.6	94.8 ± 2.3	95.2 ± 5.1
	Auto-sampler	97.0 ± 2.2	96.9 ±3.2	95.1 ± 1.0	95.7 ± 1.4	95.7 ± 0.8	92.5 ± 3.3
Freezing/ thawing, 2 cycles	−20 °C	97.2 ± 4.0	98.7 ±0.0	99.8 ± 2.3	96.9 ± 2.3	94.4 ± 3.1	95.7 ± 2.2
	4 °C	98.1 ± 2.6	95.7 ±3.3	96.7 ± 5.7	97.3 ± 4.9	92.8 ± 2.7	93.5 ± 0.1
	Auto-sampler	93.8 ± 1.6	94.6 ±6.4	95.5 ± 1.8	92.3 ± 1.0	94.8 ± 5.5	91.4 ± 6.5

[a] expressed as mean% ± S.D. of triplicate runs.

3.6. Method Application

Endogenous androgens in saliva samples were determined in twenty professional soccer players. All players attended the training sessions during the mid-season; the players train 90 min a day over five days a week, and they play one match per week. The samples were collected over two days before and after the training sessions.

The protocol of the injection sample in liquid chromatography-mass spectrometry (LC-MS-MS) was as follows: four samples belonging to each player, pre- and post-exercise, were injected sequentially on subsequent days. Seven pooled samples were prepared randomly from overall samples to examine technical variations. The results showed that the average of testosterone (T) and dehydroepiandrosterone (DHEA) levels in the pooled samples were 226.2 pg/mL ± 5.7% and 40.7 pg/mL ± 8.7%, respectively.

A paired t-test was used for the statistical comparison of the variables (T and DHEA) both pre- and post-exercise. The post-exercise DHEA levels increased significantly

(mean = 397.45 pg/mL) by about 105 pg/mL (*p*-value = 0.008) compared to the pre-exercise level mean = 292.50 pg/mL. However, there was no significant change in the T levels between the post-exercise mean = 84.35 pg/mL and pre-exercise mean = 84.85 pg/mL. We compared the mean level of T in pre and post-exercise in the current study with a Japanese study (Shibayama et al., 2009). They mentioned that between 20 to 30 years, for healthy men, the mean level of saliva testosterone (Sal-T) was high in the morning (rough mean= 55.0 pg/mL) while in the current study 84.35 pg/mL was obtained. The collection saliva in the current study was in the afternoon time and was also from very fit subjects. The greater T level in the current study could be due to the long term impact of physical activity, which is in agreement with other studies (Cormack & Cormie, 2008; Rowell et al., 2018b). In the present study, T levels do not reveal any significant differences based on player positions **(Figure 3-8, A),** and that may be because training sessions were considered as having a moderate intensity based on the percentage of the heart rate of the maximum heart beats. On the other hand, in Australian football players, they found that the T levels increased in all positions, but the performance was increased just in midfielder players, who have a higher training load than other positions (Rowell et al., 2018b).

ANOVA was used to compare the differences in DHEA for three positions (defender, midfielder and attacker). The results showed no specific differences in any of the positions between pre- and post-exercise. The Body mass index (BMI) and age were

not compared because of small variations in both ranges. The comparison was performed using a one-way ANOVA **(Figure 3-8, B).**

Previous studies have found that the highest levels of DHEA and T occurred in the early morning, which then decreased gradually during the day (Shibayama et al., 2009). The current results indicated that brief, intense physical activity could significantly increase DHEA, which could lead to improved well-being and protection from many diseases (Arlt, 2004).

To study the correlation between the level of saliva dehydroepiandrosterone (Sal-DHEA) and –T (*n* = 20), a Pearson correlation was calculated, and the result **(Figure 3-9 A, B)** indicated that there was no correlation between them in pre or post-exercise since the Pearson correlation R^2 and *p*-values were 0.014, 0.0002 and 0.935 for pre, and 0.407, 0.166 and 0.075 for post-training. T can be formed from DHEA via androstenedione or androst-5-ene-3β, 17β-diol, and it might be that the build-up of DHEA leads to increased testosterone after a longer period of post-training. Epitestosterone was not detected in any of the collected samples.

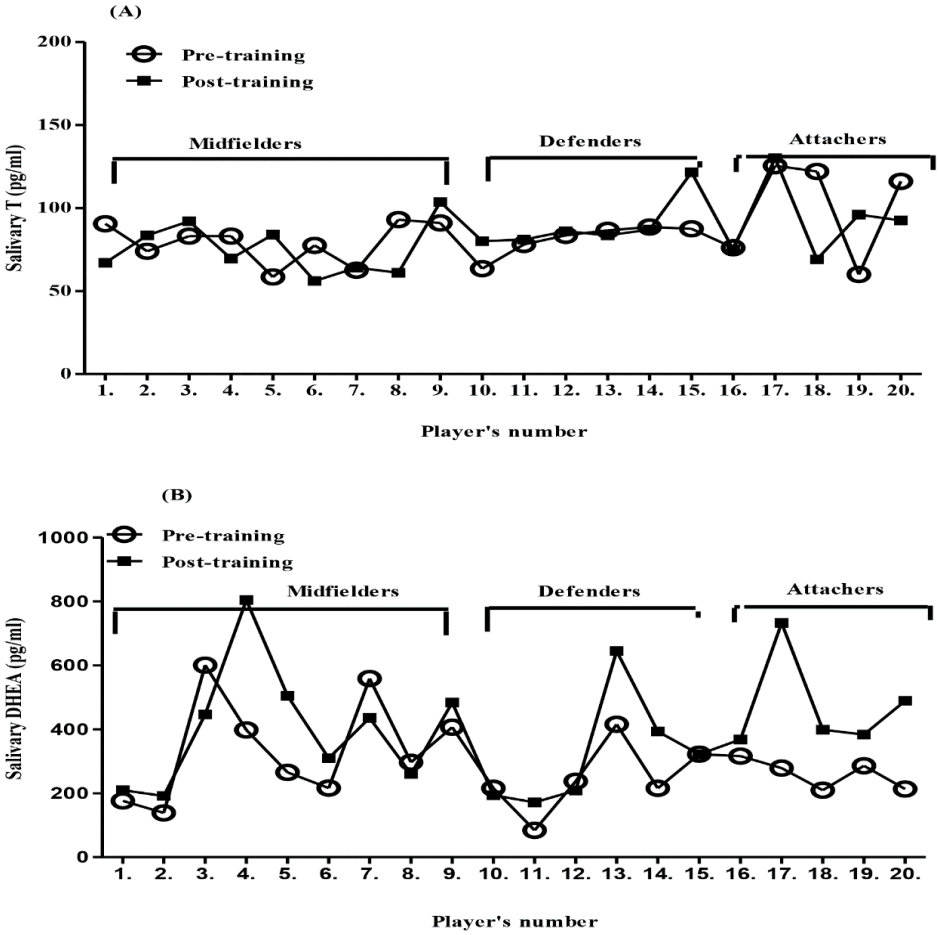


Figure 3-8 Pre- and post-exercise rhythms of (A) Sal-T levels and (B) Sal-DHEA levels in 20 young professional soccer players in three different positions.

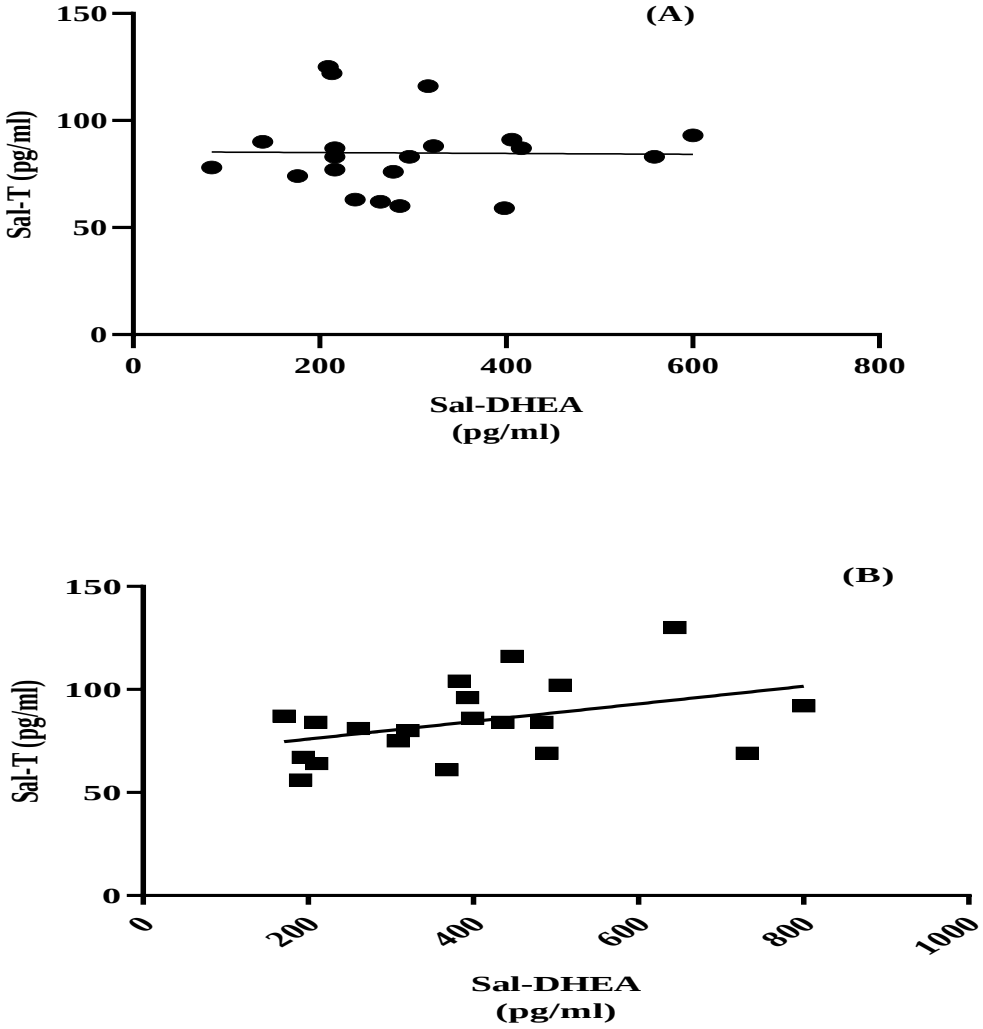


Figure 3-9 Correlation between the concentrations of Sal-DHEA and Sal-T (n=20) were (A) pre-training and (B) post-training.

3.7. Conclusion

The development of a derivatization method for endogenous androgens in a saliva sample followed by analysis by using liquid chromatography-mass spectrometry (LC-MS-MS) was successfully achieved. The 2-Hydrazino-1-Methylpyridine Derivative (HMP) reagent was chosen for the derivatization, and the reaction conditions were optimized. The optimization of the derivatization involved several steps, including the ratio temperature and incubation time. The acceptable separation between these analytes was achieved by using an ACE Ultracore 2.5 Superphenylhexyl column.

A rapid procedure for the extraction of the steroids from the saliva samples was developed by using a polymeric reverse solid-phase extraction cartridge (SPE). Testosterone (T) and dehydroepiandrosterone (DHEA) were extracted from the saliva, and an LC-ESI-MS/MS method for detecting the steroids in the saliva sample was developed. The process was validated according to FDA guidelines and had acceptable specificity, sensitivity, predictability, repeatability and accuracy.

This method was applied to the analysis of saliva samples collected from football players pre-and post-training over two days. The concentrations T and DHEA in the saliva samples were determined, whereas epitestosterone (EPI) was not detected. The levels of saliva testosterone (Sal-T) detected in the pre-and post-exercise samples showed no significant difference in Sal-T before and after training. However, the saliva dehydroepiandrosterone (Sal-DHEA) level registered a considerable difference.

The correlation between the level of Sal-T and Sal-DHEA in the pre-exercise or the post-exercise samples was not high, suggesting no change in the activity for the enzymes responsible for producing T from DHEA. However, the number of subjects was relatively small, and it might be that a different outcome would be achieved with larger sample size. Since the analytical method worked effectively and was shown to be stable, it would be possible to use the method to analyse samples from different cohorts of subjects undergoing different types of physical activity, e.g., ultramarathon runners.

Chapter 4:

Metabolomics Profiling of Plasma, Urine and Saliva After Short-term Training in Young Professional Football Players in Saudi Arabia

4. Metabolomics Profiling of Plasma, Urine and Saliva After Short-term Training in Young Professional Football Players in Saudi Arabia

4.1. Introduction

Physical activity plays a key role in the maintenance of general health and wellbeing as well as keeping the body fit. However, besides the health benefits, exercise helps in the prevention of many diseases without the need for medical intervention and can contribute as an alternative treatment for people who suffer from diseases such as type 2 diabetes (Cecchini et al., 2010; Sarris & Berk, 2014). In recent times, diseases among humans such as obesity, cardiovascular disease and depression may occur as a result of bad habits or personal choices, including a lack of exercise (Sarris et al., 2014).

On the other hand, some people do exercise regularly or daily, such as professional soccer players. These players run various distances during daily practice or full football matches, resulting in a variation in the intensity of activity between high moderate and low intensity, which in turn places varying demands on different metabolic pathways including the energy delivery pathways (Drust & Cable, 2000). Several personal factors can affect the variation of the intensity of physical activity, such as lifestyle, gender and age (Baker & Tang, 2010; Hunter& Fauth, 2011; Knechtle & Kohler, 2009; Lepers & Cattagni, 2012; Sparling & Snow, 1998).

Depending on the type of physical activity, aerobic or anaerobic metabolism is used as the primary means of providing ATP for muscle contraction, therefore changes in the duration and intensity of exercise lead to changes in the metabolic pathways required to produce energy(Berg & Stryer, 2015; Drust et al., 2000).

Using the maximum heart rate ($\%HR_{max}$) measurement can provide extremely useful information to help optimize the training intensity, and would be useful in determining the impact of using this measure on metabolism (Karvonen & Vuorimaa, 1988).

Various metabolomics studies have been conducted to study the changes to the metabolic profile due to exercise by analyzing urine or plasma samples. These studies have also identified significant changes in metabolites after long-term physical activity in colder weather (Alshehri et al., 2018; Burleigh et al., 2016; Huffman et al., 2014). To the best of the knowledge, just one metabolomics study has evaluated the effects of short-term exercise on the metabolic variations from samples of human saliva utilising NMR spectroscopy as the analysis platform (Paci et al., 2013). The significant metabolites which are reported to be affected by exercise across several studies include purine metabolism and fatty acid metabolism, particularly the formation of acylcarnitine (Fiehn et al., 2016; Huffman et al., 2014). The purine metabolite hypoxanthine has been proposed as a marker of fitness since in trained individual's purine conservation is much more

efficient, and thus hypoxanthine levels measured in physiological fluids post-exercise do not rise as much (ZIielinski & Rychlewski, 2011).

4.2. Aim

This study aimed to highlight the impact of controlled short physical activity on young professional soccer players by analysing the metabolomic profiles from samples of their urine, plasma and saliva.

4.3. Materials and Methods

4.3.1. Chemicals and Solvents

HPLC grade acetonitrile (ACN) was purchased from Fisher Scientific, UK. HPLC grade water was produced by a Direct-Q 3 Ultrapure Water System from Millipore, UK. AnalaR-grade formic acid (98%) was obtained from BDH-Merck (Poole, UK). Ammonium carbonate was purchased from Sigma-Aldrich (Poole, UK). Authentic stock standards were prepared as stated previously in the literature and diluted four times with ACN before LC-MS analysis, then distributed into seven different standard solutions. (R. Zhang et al., 2014).$^{13}C_2$ glycine was purchased from (Sigma-Aldrich, Poole, UK). A mixture of fatty acid standards was prepared from a mix of 37 fatty acid methyl ester standards supplied by Sigma Aldrich (Supelco 37 component FAME Mix) by hydrolysis with 1 M methanolic KOH.

4.3.2. Participants

Twenty-six professional soccer players (age 20.6 $\pm$ 1.4 y, body mass 70.2 $\pm$ 1.6 kg, height 178 $\pm$ 2 cm and BMI 22.2 $\pm$ 0.5 kg/m^2 participated in the study. All players attended the training sessions during the mid-season in February 2018. Players train for five days, 90 min per day and play one match per week. The two days on which the collection of samples occurred was during the training sessions. No participants were injured, and no participants took any medication during the study. The participants attended the club one and a half hours before the commencement training starts every day. The Ethics Committee at King Saud University approved the study, and all players read the information sheet and signed a consent form before participating in the study. Seven of players are members of the National team under 23 years of ages.

Unfortunately, not all 32 participants contributed samples in all cases. Plasma and urine samples were collected from 20 soccer players out of the 32 players. While 18 participants out of 20; who donated urine and plasma samples, also donated saliva samples and 8 soccer players only donated saliva samples to make a total of 26 saliva samples from the soccer players out of the 32 soccer players who contributed samples for the study.

4.3.3. Experimental Design

The collection of plasma, urine and saliva samples from young Saudi professional soccer players was conducted in Riyadh, Saudi Arabia by King Saud University, College of Sports Science and Physical Activity, exercise physiology department who had ethical approval. Samples were stored at -80 °C in the laboratory of the exercise physiology department. Samples were sent on dry ice and took two days to arrive for analysis at the University of Strathclyde, UK.

The collection of samples was done on two consecutive days, pre and post-training sessions. The total number of samples were, 80 plasma and urine samples each, as well as 104 samples of saliva. The training sessions took roughly two hours. All training sessions were started at the same time each day. Two training sessions were designed for the study, which began with a 15 minutes warm-up. The players were then divided randomly into groups to play two small side games for 30 minutes, followed by a 4 minutes rest period. The coach also set up a game for 40 minutes divided into two halves, followed by a 5 to 10 minutes cool down. The intensity of the training on the first and the second training day was designed based on the percentage of maximum heart rate. The heart rate averages were 70 % and 72 % of the maximum on the first and second days respectively. The mean ambient temperature was 25 ± 3 ^{o}C, and humidity was 18 ± 4 % for the first day and 26 ± 1 °C and 16 ± 2% for the second day.

Individual containers in sealed coded bags were given to the players for the pre- and post-training samples. Before starting the collection of samples, the BMIs of all participants was determined pre and post-training session on each day.

On the days of collection, the methods used for collecting samples were explained to the participants. As a part of the collection procedure, volunteers placed their labelled samples directly into the fridge until the end of the training sessions. Subsequently, all samples were placed in environmental storage and transported immediately to the laboratory at the College of Sports Sciences and Physical Activity for proper storage.

4.3.4. Samples collection

For the collection of urine samples, soccer players were required to clean their hands and penis first, as well as collect midstream urine during a full flow of urine to avoid any contamination. Samples were collected in a universal 30 ml container and placed in a small sealed plastic labelled bag and left it in the fridge. Finally, to empty their bladder during per training sample collection. Individual urine samples were transferred in aliquots of 3 ml into Eppendorf tubes before being stored at -80 ^{o} C.

For the collection of plasma samples, whole blood samples were collected from veins into a 10 ml EDTA tube that contained an anticoagulant (BD Vacutainer Systems, Plymouth, UK). All samples were placed on ice and delivered immediately

to the laboratory. All blood tubes were centrifuged immediately at 1500 × g for 15 min at 4 ^{o}C. Aliquots of 3 ml of the plasma samples were then transferred into Eppendorf tubes before being stored at -80 ^{o}C.

For the collection of saliva samples, a Sarstedt Salivette polyester tube was used. The sampling procedure was carried out as follows: after hand washing, the wad is taken into the mouth, and left for 2 minutes and then returned to tube. The volunteers were asked not to brush their teeth within 1 hour of collecting the samples. They are also required not to have any food or drink within 30 minutes of taking a sample to avoid any contamination or interference matrix. All samples were placed on ice and delivered immediately to the laboratory. According to the manufacturer's instructions, samples were centrifuged at 1000 × g for 20 min at 4 ^{o}C; then the inlay was removed from tubes and samples were then stored at -80 °C.

4.3.5. Samples preparation

Samples stored at -80 °C were allowed to thaw and equilibrate to room temperature for 1-2 hours before further use. Metabolites were extracted by transferring 200 µL of samples to an Eppendorf tube followed by the addition of 800 µL of acetonitrile (ACN) containing 5 µg/mL of $^{13}C_2$ glycine as an internal standard to ensure retention time stability and then vortexed. The samples were then centrifuged at 8000 revolutions per minute for 10 minutes. The supernatant

was then collected into an HPLC vial as a final solution ready for LC-MS analysis. Samples were organised into batches corresponding to the player donor and each group injected together as follows B = pre-day 1, R = post-day 1, G = pre-day 2 and K = post-day 2, for example, B1, R1, G1 and K1, were samples collected from player 1 in pre- and post-training for the two days of training.

The accuracy, as well as reproducibility of the analytical method, were measured by regularly injecting authentic standard metabolite mixtures and quality control (QC) samples throughout the runs. The analytical standards were prepared by adding 10 µg/mL as the final concentration of each metabolite standard plus $^{13}C_2$ glycine, into the seven different standard solutions(R. Zhang et al., 2014). The pooled quality control (QC) samples were prepared by pipetting 10 µL from random groups and then mixing them. A mixture of fatty acid standards was prepared from a mix of 37 fatty acid methyl ester standards supplied by Sigma Aldrich (Supelco 37 component FAME Mix) by hydrolysis with 1 M methanolic KOH followed by extraction into hexane.

4.3.6. LC-MS Condition

An Accela HPLC system interfaced to an Exactive Orbitrap mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) was used for the liquid chromatographic separations. A ZIC-pHILIC (150 _ 4.6 mm, 5 µm) and an ACE C4 (150 _ 3.0 mm, 3 µm) HPLC column supplied by HiChrom (Reading, UK) were used

for the analysis. The former column was used for all biological samples, while the latter was used for just the plasma samples. Samples were run on the LC-MS under the following conditions: the ZIC-pHILIC mobile phase consisted of 20 mM ammonium carbonate in HPLC-grade water (A) and acetonitrile (B); the solvent gradient used was 80% B (0 min), 20% (30 min), 8% (31–36 min), and 80% (37–45 min) at a flow rate of 0.3 mL/min. For the ACE C4 column, the mobile phase was 1 mM acetic acid in water (A) and 1 mM acetic acid in acetonitrile (B). The solvent gradient used was 40% B (0 min), 100% (30–36 min) and 40% (37–41 min) at a flow rate of 0.4 mL/min. The nitrogen sheath and auxiliary gas flow rates were maintained at 50 and 17 arbitrary units. The electrospray ionization (ESI) interface was employed in positive/negative dual polarity mode, with a spray voltage of 4.5 kV for positive mode and 4.0 kV for negative mode, while the ion transfer capillary temperature was set at 275 °C. The full scan data were obtained in the mass-to-charge ratio (m/z) range between 75 and 1200 amu for both ionization modes. The data was collected and processed using Xcalibur 2.1.0 software (Thermo Fisher Scientific, Bremen, Germany).

4.3.7. LC-MS Data Processing with m/z mine and Statistical analysis

The results processing was done using m/z mine 2.14 for raw LC-MS files (Pluskal & Orešič, 2010). The Metlin database, the Lipid Maps and Human Metabolome Data Base HMDB web (http://www.hmdb.ca/) were used to prepare an in house

metabolite database to investigate metabolites and identify the accurate masses. All reported metabolites were within 3 ppm of their exact masses.

Univariate and Multivariate data analysis were employed. The former analysis was achieved using Microsoft Excel 2016 and paired t-tests between pre and post samples and differences were considered significant at $p < 0.05$. Whereas, the latter analysis was performed using the SIMCA-P software v.14.0 (Umetrics, Umea, Sweden), which included analysis (PCA-X) and (OPLS-DA).

4.4. Results

4.4.1. Metabolic Profiling

Metabolomics profiling of samples was carried out using LC-MS. Extraction of data to get the output for the metabolites according to either their exact mass (with <3 ppm deviation) or their exact mass plus retention time matching to a standard. A sample with pooled quality control (QC) samples were injected, and the QC was taken place after every 12 samples to monitor any instrumental effects over time. A clustering of QC samples that refer to the stability of the instrument throughout the run was observed following principal component analysis (PCA). QC samples were not in the centre of the plot due to the collection being random, as seen in **Figure 4-S1**. Otherwise, the four analysis carried out of the different sample types and the indicated clustering of the pooled samples indicated excellent instrument stability.

The polar metabolites were obtained by analysis of all biological samples using the ZIC-pHILIC column, while non-polar metabolites were derived by analysis of the plasma samples using a reverse-phase (RP) column (ACE C4 column).

Metabolites with relative standard deviation (RSD) values >20% within the (QC) pooled samples were excluded from the analysis. In addition, the inclusion and exclusion of metabolites were done according to various values variables, which have been summarised in **Table 4-1**.

Table 4-1. filtration processes using Microsoft Excel and SIMCA-P version 14.0

Filtration	Plasma (C4) metabolites	Plasma (HILIC) metabolites	Urine (HILIC) metabolites	Saliva (HILIC) metabolites
Total number of features, data extracted using m/z Mine 2.14	838	887	9403	9402
Unknown features which were excluded	720	587	7346	7176
Identified Metabolites with RSD > 20% which were excluded	34	300	2057	1714
Metabolites with P value < 0.05 were included	20	211	126	331
Biomarkers were only reported as significantly influencing the models	6	75	16	32

Regarding the metabolites analysed on the ZIC-pHILIC column, **Figures 4-1, 2 and 3** show an unsupervised classification method using PCA plots for plasma, urine and saliva samples to discover whether or not there were significant variations between pre- and post-exercise groups based on 211, 126 and 331 metabolites respectively. There is a reasonable degree of separation for plasma metabolites

profiles pre and post-exercise (**Figure4-1**) using the PCA on both days. **Figure 4-2** shows the PCA plots for the urine samples pre- and post-exercise, and in this case, the separation is more marked than for the plasma samples. The PCA plot for the saliva samples produces a less clear-cut separation between pre- and post-exercise samples in **Figure 4-3**. PCA plots for the plasma metabolites analysed on an ACE C4 column produced a less clear separation between pre- and post-exercise samples, as shown in **Figure 4-4.**

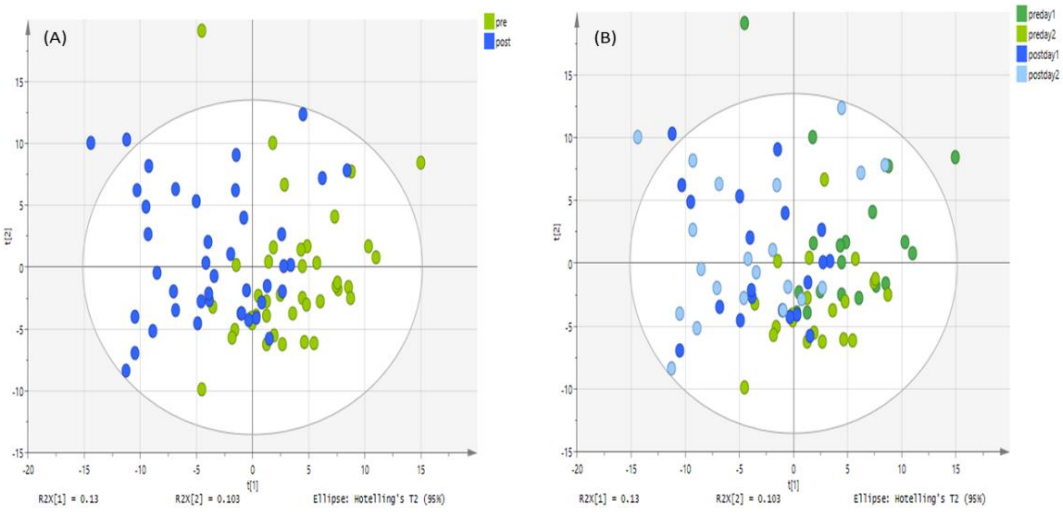


Figure 4-1 PCA score plot showing groups of plasma samples based on 211 metabolites analysed on a ZIC-pHILIC column. The data was Pareto scaled. **(A)** green is control; pre-training for both day1 and 2 respectively (n=40), and blue is treated; post-training for both day1 and 2 respectively (n=40), **(B)** green and light green are control; pre-training for both day1 and 2 respectively, (n=20 of each day) and blue and light blue are treated; post-training for both day1 and 2 respectively (n=20 of each day).

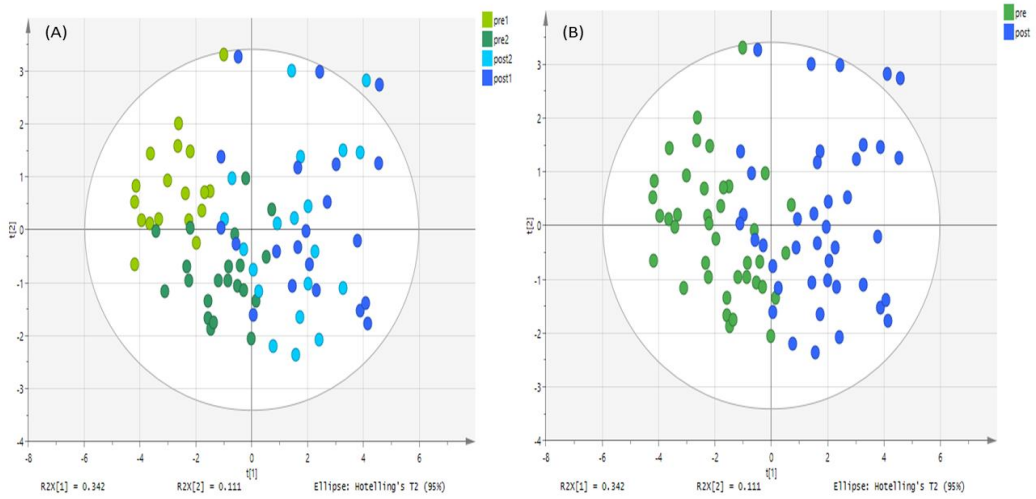


Figure 4-3 PCA score plot showing groups of urine samples based on 126 metabolites analysed on a ZIC-pHILIC column. The data was Pareto scaled. **(A)** green and light green are controls; pre-training for both day1 and 2 respectively (n=20 of each day), and blue and light blue are treated; post-training for both day1 and 2 respectively (n=20 of each day), **(B)** green is control; pre-training for both day1 and 2 respectively, (n=40), and blue is treated; post-training for both day1 and 2 respectively (n=40).

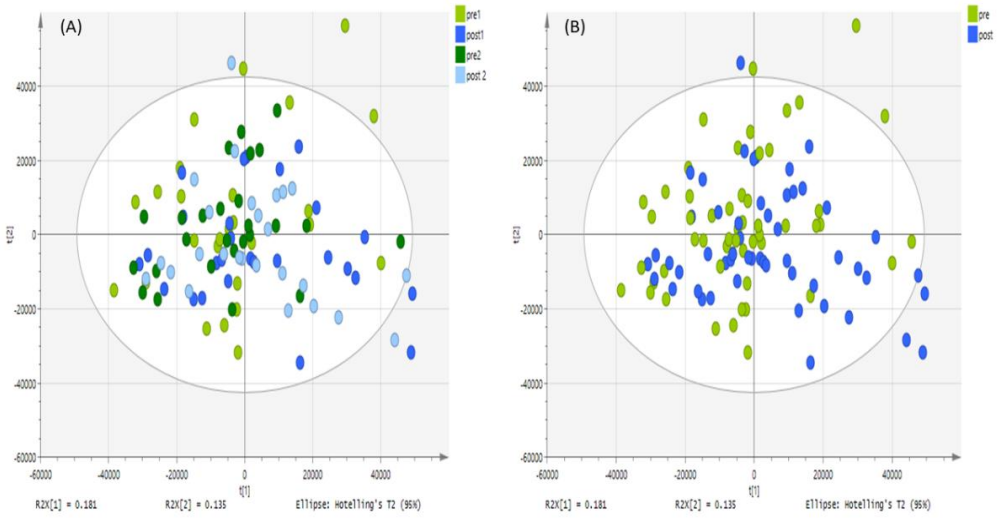


Figure 4-2 The PCA score plot showing groups of saliva samples based on 331 putative metabolites analysed on a ZIC-pHILIC column. The data was Pareto scaled. **(A)** green and light green are controls; pre-training for both day1 and 2 respectively (n= 26 of each day), and blue and light blue are treated; post-training for both day1 and 2 respectively (n=26 of each day), **(B)** green is control; pre-training for both day1 and 2 respectively (n= 52) and blue is treated; post-training for both day1 and 2 respectively (n=52).

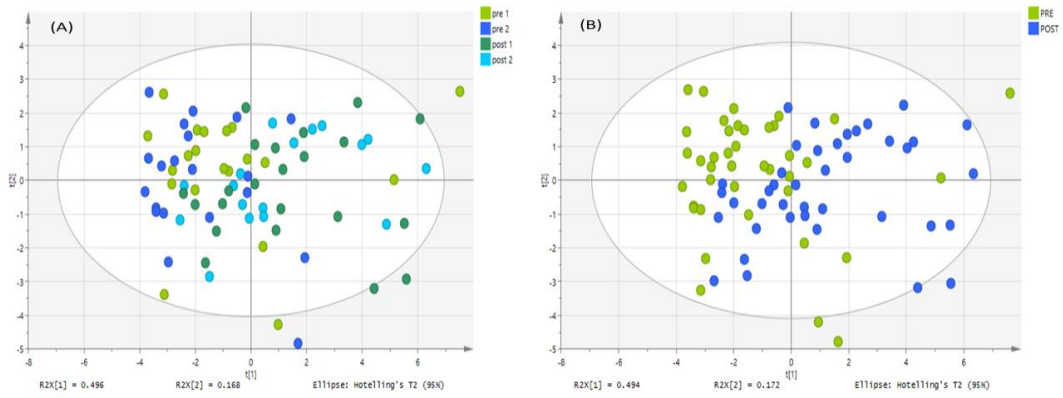


Figure 4-4 PCA score plot showing groups of plasma samples based on 20 metabolites analysed on an ACE C4 column. The data was Pareto scaled. **(A)** green and blue are control; pre-training for both day1 and 2 respectively (n= 20 of each day), and light green and light blue are treated; post-training for both day1 and 2 respectively (n= 20 of each day), **(B)** green is control; pre-training for both day1 and 2 respectively (n= 40) and blue is treated; post-training for both day1 and 2 respectively (n= 40).

When both groups were specified, and orthogonal partial least squares discriminant analysis (OPLS-DA) was used, there was a clear separation between the pre- and post-samples in all cases (**Figure 4-5)**. Moreover, models were built based on the readings of 75, 16 and 32 significant putative polar metabolites of plasma, urine and saliva analysed on a ZIC-pHILIC column, and 6 significant putative non-polar metabolites of plasma analysed on an ACE C4 column, obtained based on P-Value < 0.05, 95% confidence interval and VIP predictive/orthogonal ratio ≥ 1 as shown in **Table 4-2 for day 1, and Table 4-S1.** On the other hand, the plots clearly show significant separation between the two groups with P CV-ANOVA = 7.81E-29, 1.17E-027 and 3.11E-020 of plasma, urine, saliva analysed on a ZIC-pHILIC column respectively, where P CV-ANOVA = 7.74E-012 of plasma analysed on an ACE C4 column. The validity of these supervised modules was

assessed based on the permutations test plot and cross-validation. In the permutation test plots, there were R2 and Q2 parameters obtained from the original model that compared to newly permuted R2 and Q2, and to confirm the validity using this test, The new parameters generated from this permutation should all be lower in value than the original values as well as the regression line of the predictive model should cross the horizontal zero line see **Figures 4-S2, S4, S6 and S8.** The Observed vs Predicted plot of all models were plotted are shown in **Figures 4-S3, S5, S7 and S9.** These plots display the observed versus the predicted value of the selected Y- variable. Also, the regression line R2 is close to one, which indicates an excellent and valid model. However, the regression line in the plots were R2 = 0.95, 0.95 and 0.85 for plasma, urine and saliva respectively, analysed on a ZIC-pHILIC column, and R2 = 0. 77 for plasma analysed on an ACE C4 column, indicating the validity of the cross-validation of the model.

In **Table 4-2,** the univariate comparisons of the changes in the metabolites from pre- and post-exercise for day one samples are shown, while those for day two are shown **Table 4-S1**. Hypoxanthine is the only significantly polar metabolite, which was found to change in all cases (plasma, urine and saliva). Otherwise, several significant differences in metabolites were obtained from plasma, the levels of polar and non-polar (lipophilic) metabolites of plasma analysed on both columns were increased in the majority of them. Moreover, the levels of polar metabolites obtained from urine and saliva were also found to be slightly increased in many

cases compared to the pre-training levels. Many of the metabolites matched the retention times of the analytical standards.

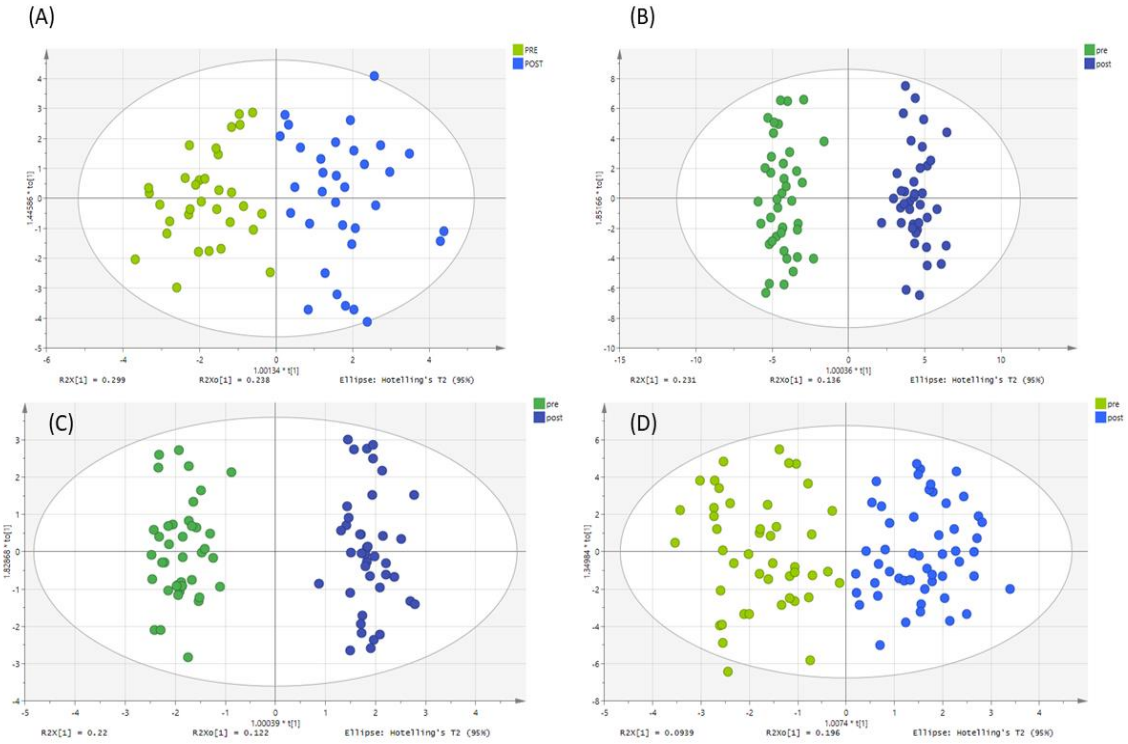


Figure 4-5 OPLS-DA score plots the samples shown above according to their classification coloured based on their group (green) represents the pre-training samples while (blue) represents the post-training. Models fitted based on: **(A)** 20 putative metabolites in plasma samples, analysed on an ACE C4 column **(B)** 211 putative metabolites in plasma samples, analysed on a ZIC-pHILIC column, **(C)** 126 putative metabolites in urine samples, analysed on a ZIC-pHILIC column, **(D)** 331 putative metabolites in saliva samples, analysed on a ZIC-pHILIC column, the data was Pareto scaled and log 2 transformed.

Table 4-2 All the metabolites affected significantly by the short-term training trial in day 1 (p-value < 0.05) or fold change >2 or <0.5. * Matches retention time of standard. ‡ Data from runs on ACE C4 column, otherwise run on the pHILIC column.

Mode	m/z	RT(min)	Putative Metabolite	Urine		Plasma		Saliva	
				Ratio	p-Value	Ratio	p-Value	Ratio	p-Value
			Purine metabolism						
+	136.061	8.7	Adenine			1.457	0.182	1.334	0.002
+	137.046	9.7	Hypoxanthine *	3.453	<0.001	1.735	0.002	1.796	0.003
-	151.026	11.8	Xanthine			2.027	<0.001		
-	167.021	13.3	Urate			0.766	0.009		
+	252.108	7.7	Deoxyadenosine					1.406	0.002
+	253.093	8.3	Deoxyinosine	1.042	0.024			1.713	0.037
-	283.069	13.0	Xanthosine *					0.136	0.001
			Arginine and proline metabolism						
-	104.071	13.9	4-Aminobutanoate *	1.394	0.004			0.661	0.019
-	112.052	9.4	Creatinine			1.278	<0.001		
-	130.051	9.3	L-Glutamate 5-semialdehyde	0.776	0.020				
+	131.118	21.2	N-Acetylputrescine					1.313	0.017
+	133.097	23.5	Ornithine *			1.318	0.001	1.353	0.053
+	146.092	14.9	4-Guanidinobutanoate			1.603	0.014	0.585	0.017
+	148.060	15.1	Glutamate *			2.014	<0.001		
+	173.104	26.1	arginine *			1.521	0.009	1.577	0.013
-	176.103	15.9	L-Citrulline *			1.456	<0.001		
-	188.057	9.0	N-Acetyl-L-glutamate			1.748	<0.001		
+	217.129	14.6	N-acetylarginine			1.929	<0.001		
+	247.140	13.9	N-(Carboxyethyl) arginine			3.494	<0.001		

Mode	m/z	RT(min)	Putative Metabolite	Urine		Plasma		Saliva	
				Ratio	p-Value	Ratio	p-Value	Ratio	p-Value
+	298.096	6.4	5-Methylthioadenosine *			2.395	<0.001		
			Pyrimidine metabolism						
+	112.051	8.8	Cytosine			1.234	0.002	1.793	0.003
+	127.050	13.2	Thymine			1.365	0.004	1.824	0.023
+	129.066	14.6	5,6-Dihydrothymine	1.511	0.006				
+	243.097	12.4	Thymidine					2.287	0.002
			Carnitine metabolism						
+	204.123	10.4	Acetylcarnitine *			2.411	<0.001		
+	218.138	9.1	Propionylcarnitine	0.652	0.046				
+	230.138	9.0	Butenylcarnitine	0.924	0.047				
+	244.154	7.6	Tiglylcarnitine			1.549	0.012		
+	248.149	10.8	Hydroxybutyrylcarnitine			2.399	<0.001		
	274.201	6.3	Heptanoylcarnitine			1.884	0.036		
+	276.144	12.8	Glutarylcarnitine			1.812	<0.001		
+	290.159	12.2	Methylglutarylcarnitine			1.578	0.004		
+	302.232	5.1	dimethylheptanoylcarnitine			1.929	0.054		
+	312.217	5.1	Decadienoylcarnitine			1.855	0.013		
+	330.227	5.1	Keto-decanoylcarnitine			1.536	0.031		
+	330.263	4.7	dimethylnonanoylcarnitine	0.954	0.058			1.298	0.002
+	384.270	4.8	Hydroxytetradecadiencarnitine			1.411	0.041		
+	386.290	4.7	Hydroxytetradecenoylcarnitine			1.505	0.045		

Mode	m/z	RT(min)	Putative Metabolite	Urine		Plasma		Saliva	
				Ratio	p-Value	Ratio	p-Value	Ratio	p-Value
			TCA cycle						
-	115.004	13.5	Fumarate *			1.741	<0.001		
-	117.019	9.2	Succinate*			1.235	0.024		
-	133.014	16.9	Malate *			3.226	<0.001		
-	145.014	15.9	2-Oxoglutarate *			1.954	<0.001		
-	173.009	19.0	cis-Aconitate *			3.632	<0.001		
-	191.020	19.2	Citrate *			2.010	<0.001		
			Pentose phosphate pathway						
-	133.051	9.3	Deoxyribose			1.33	0.008		
-	149.046	11.9	Ribose *			1.545	<0.001		
-	151.061	12.9	Xylitol or isomer *			1.328	0.008		
-	193.036	16.8	Glucuronate or isomer *			1.896	<0.001		
-	195.051	15.0	Gluconic acid *			2.852	0.002		
-	229.011	15.7	Ribose 5-phosphate *					0.145	0.051
			Glycine, serine and threonine metabolism						
+	76.076	10.5	Aminopropan-2-ol			2.002	0.018		
+	104.107	20.9	Choline *			0.853	0.056		
-	105.019	12.6	Glycerate			1.665	0.001		
+	118.061	15.8	Guanidinoacetate	1.604	0.028				
+	120.065	11.3	Threonine *			1.586	0.015		

Mode	m/z	RT(min)	Putative Metabolite	Urine		Plasma		Saliva	
				Ratio	p-Value	Ratio	p-Value	Ratio	p-Value
			Lysine metabolism						
-	126.056	7.2	2,3,4,5-Tetrahydropyridine-2-carboxylate	1.154	0.021	1.614	0.001		
+	146.081	14.6	hexanoic acid			1.364	0.048	0.232	0.024
+	146.117	12.9	Trimethylammoniobutanoate			1.237	0.001		
+	147.113	23.6	L-lysine *			1.611	0.02		
+	160.097	6.9	Acetamidopentanoate	1.151	0.028				
+	162.112	13.3	L-Carnitine *			3.264	0.011		
	189.159	22.1	N6,N6,N6-Trimethyl-L-lysine			1.859	<0.001		
+	204.086	14.2	N2-Acetyl-L-aminoadipate			1.532	<0.001		
+	205.118	10.3	N6-Acetyl-N6-hydroxy-L-lysine			2.175	<0.001		
+	219.133	14.4	Carboxyethyllysine					1.465	0.017
			Histidine metabolism						
+	137.036	7.6	Urocanate *			1.733	<0.001		
+	141.066	9.7	Methylimidazoleacetic acid			1.632	0.010		
+	156.076	15.4	L-Histidine	1.138	<0.001				
+	157.061	11.6	Imidazolone-5-propanoate			1.286	0.059	1.446	0.01
+	170.092	12.8	methylhistidine					0.629	0.029
			Tryptophan metabolism						
+	118.065	10.1	Indole *			1.506	0.028	1.596	0.003
+	154.050	6.8	3-Hydroxyanthranilate	1.199	0.025				
+	161.107	10.1	Tryptamine					1.912	0.003
+	177.102	13.4	Serotonin					1.362	0.056

Mode	m/z	RT(min)	Putative Metabolite	Urine		Plasma		Saliva	
				Ratio	p-Value	Ratio	p-Value	Ratio	p-Value
-	204.067	8.1	Indolelactate			1.394	<0.001	1.352	0.058
+	205.097	11.4	L-Tryptophan *			1.163	0.036		
-	219.077	8.9	5-Hydroxy-L-tryptophan *					2.043	<0.001
			Tyrosine metabolism						
+	138.091	5.7	Tyramine *					1.212	0.006
-	179.035	8.3	Hydroxyphenylpyruvate			1.606	<0.001		
-	181.051	9.5	Hydroxyphenyllactate			1.782	<0.001		
+	182.081	12.3	L-Tyrosine *	0.802	0.056			1.333	0.055
			Valine, leucine and isoleucine degradation						
-	115.04	4.8	3-Methyl-2-oxobutanoic acid			0.752	0.002		
-	129.056	4.3	Methyl-oxopentanoic acid			0.761	<0.001		
+	132.101	10.8	L-Leucine					1.669	<0.001
			Phenylalanine metabolism						
+	122.096	4.7	Phenethylamine					1.322	0.004
+	123.044	13.3	Benzoate					1.396	0.047
+	136.075	13.3	Phenylacetamide					1.387	0.021
+	149.059	10.1	Cinnamate					1.585	0.003
-	166.086	10.1	Phenylalanine *					1.606	0.004
			Methionine metabolism						
+	150.058	11.3	L-Methionine *	1.404	0.053				
+	166.053	13.4	Methionine S-oxide			1.628	<0.001		
-	176.039	7.2	N-Formyl-L-methionine			1.241	0.004		

Mode	m/z	RT(min)	Putative Metabolite	Urine		Plasma		Saliva	
				Ratio	p-Value	Ratio	p-Value	Ratio	p-Value
+	192.105	4.9	trihomomethionine			1.408	0.010		
			Alanine and aspartate metabolism						
-	88.04	14.6	L-Alanine *			1.439	0.002		
-	225.099	11.7	Carnosine *					2.051	0.007
			Fatty acids and metabolites [C4]						
-	131.071	2.0	Hydroxyhexanoic acid*‡			1.526	<0.001		
-	227.202	15.9	Tetradecanoic acid ‡			1.498	0.015		
-	241.218	17.4	pentadecanoic acid * ‡			1.208	0.035		
-	253.218	16.8	Hexadecenoic acid ‡			1.374	0.047		
-	255.233	18.8	Hexadecanoic acid isomer ‡			1.233	0.054		
-	281.249	19.5	octadecenoic acid ‡			1.366	0.032		
			Miscellaneous						
-	147.03	15.8	(R)-2-Hydroxyglutarate			0.674	0.006		
+	241.031	16.5	L-Cystine *	1.041	0.041				
-	259.022	15.8	D-Glucose 1-phosphate *			1.304	<0.001		
+	345.139	12.3	Melibiitol			0.337	0.003		
-	448.307	5.2	Glycodeoxycholate			0.196	<0.001		
-	464.302	5.5	Glycocholate *			1.278	<0.001		

4.5. Discussion

The primary aim of this study was to identify the effect of short-term physical activity of professional soccer players on the change in the pattern of their untargeted metabolic response by comparing post- with pre-training exercise metabolite levels.

The intensity of the physical activity has been reported to play a prominent role in the changes observed in some metabolites which have been investigated in many studies when two cohorts were compared, e.g., pre- and post-training (Alshehri et al., 2018; Burleigh et al., 2016; Daskalaki & Watson, 2015; Huffman et al., 2014). In the current study, the heart rate level was used to indicate the effect of physical activity on metabolism since the body uses different substrates to produce energy according to the intensity of exercise and heart rate measurement. The heart rates of the players were 70 % and 72 % of the maximum on the first and second training days respectively. These percentages indicate that the training could be described as moderate-intensity exercise according to the participant's ages, which may explain the metabolites ratios after-training (Karvonen & Vuorimaa, 1988). In general, in metabolomics exercise studies, two measurements have usually been used, the maximum heart rate (%HR_{max}) and oxygen uptake (VO_{2max}). The last measurement can be favourable for high-intensity exercise because the oxygen uptake under anaerobic respiration or maximum load increases more than %HR_{max} measurement (Karvonen & Vuorimaa, 1988).

Adenosine triphosphate (ATP) is the primary source of energy in the body, which is used by cells for such movements or biosynthesis (Berg et al., 2015),therefore, it is essential first to understand the process for generating ATP to produce energy in the body within the rest and active situations, and this understanding is leading us to realise the interaction of the effects on metabolism according to the intensity of exercise.

ATP function is to allow muscle contraction and fuel active transport pumps (Knowles, 1980). The last phosphate in the triphosphate structure is an unstable bond, which can be broken through a hydrolysis reaction; when H_2O is introduced with the assistance of the ATPase enzyme to form adenosine diphosphate (ADP). ATP is then converted to ADP with the release of energy; this energy is utilised by the cell for functions such as synthesis or movement. Therefore, because cells need ATP always for energy, ATP must be formed from ADP by creatine kinase to form creatine and ATP, and vice versa. ADP has converted to ATP again via an endothermic process that requires energy; that comes from cellular respiration (aerobic), this cycle is called the ATP cycle (Berg et al., 2015). Consequently, when muscles are being active or contract through exercise, the production of ATP occurs in three steps depending on the intensity of exercise. First, creatine phosphate transfers its phosphate group back to ADP to form creatine and ATP as a high-energy phosphate, which then can be used to create muscle contraction, this ATP cycle runs out very quickly and is not sufficient for a long duration of exercise (Berg et al., 2015).

Secondly, since creatine phosphate is depleted quickly within a short period, the muscles then turn to the glycolysis process as a second source for producing ATP. Glucose is broken down to generate ATP but not as quickly as creatine phosphate generate it due to the length of the process of glycolysis. Glucose can diffuse straight into the muscle from the blood or can be formed from glycogen. Glycogen is a polysaccharide, which is stored in the muscle tissue after conversion from glucose. Glycogen must first be converted to glucose by the glycogenolysis process (Berg et al., 2015). The glucose resulting from both processes undergoes glycolysis, which is a series of reactions that ultimately produces two ATP molecules and pyruvate. Pyruvate or pyruvic acid can be used for two things. It can be broken down into lactic acid by reaction with the reduced form of nicotinamide adenine dinucleotide (NADH), catalysed by lactate dehydrogenase. Therefore, pyruvate gains electrons to become lactate and NADH is oxidised to form NAD+. The NAD+ molecule allows glycolysis to keep going. This process is called anaerobic respiration. This process, not efficient because it only makes 2ATP molecules per glucose molecule which is ultimately going to be depleted when the intensity of exercise is high for a short time (Berg et al., 2015).

The third source of generating ATP; that is good for long-duration exercise, is called aerobic cellular respiration. This occurs following the formation of pyruvate if oxygen is available. The oxygen is used to break down glucose or other nutrients to produce carbon dioxide (CO_2), H_2O and ATP. There are three inputs for aerobic respiration, which are pyruvate, fatty acids and amino acids (Berg et al., 2015). First, glucose is

broken down by glycolysis to produce pyruvate, which is first used for aerobic respiration. Second, when glucose is depleted, then the body turns to broken down fat to convert it into fatty acids and glycerol to participate in the aerobic process. Finally, when protein is high or fat and glucose stores are deficient, then protein can be broken down to amino acids to be used in this process (Berg et al., 2015). This catabolism occurs within the mitochondria, which is the powerhouse of the muscle cell in the presence of oxygen from either haemoglobin in the blood or oxygen attached to myoglobin in the muscles.

NADH produced from pyruvate, by β-oxidation of fatty acids or oxidation of amino acids can enter aerobic respiration in a two-step process in the mitochondria (Berg et al., 2015). In the Krebs or citric acid cycle (TCA), pyruvate can be used to produce NADH. The NADH produced enters the electron transport system of the terminal respiratory chain and is used to produce ATP with the protons from NADH, in the end, being transferred to molecular oxygen. The outcome again is the formation of CO_2, H_2O and ATP. This process is efficient because it makes 36 ATP molecules per glucose molecule and 100 molecules ATP per fatty acid (Berg et al., 2015).

On the other hand, this process needs a steady supply of oxygen, which makes it much slower (Berg et al., 2015) than glycolysis. This process can be approached in terms of stress since aerobic exercise burns fat at low-intensity exercise and stress, while high-intensity anaerobic exercise burns sugar as the primary fuel. The body

needs to control this high stress by releasing a stress hormone called cortisol to raise the blood sugar level, and as a consequence, produces lactic acid (Berg et al., 2015).

By looking at the training design in this project and the significantly changed metabolites that are summarised in **tables 4-1 and 4 S-1**, and compared that with previous studies, then the output of metabolomics changes could be modified according to the style of exercise (Alshehri et al., 2018; Burleigh et al., 2016; Daskalaki et al., 2015).In the current case, it was found that the majority of the polar and nonpolar metabolites were increased slightly after training due to the type of exercise.

Purine metabolites are considered a leading indicator of the effects of exercise, specifically hypoxanthine (Daskalaki et al., 2015). Activation of this pathway requires high physical activity. Hypoxanthine was increased by more than half in plasma and saliva and more than three times in urine, as well as the product compound of hypoxanthine, which is xanthine Figure **4-64-7**, whose concentration doubled and was observed just in plasma. The possible justification is that hypoxanthine is converted to xanthine by xanthine dehydrogenase; that is present in liver and the intestine, where hypoxanthine flows out of the bloodstream and saliva and is therefore present highly in the urine (Kaya et al., 2006). Elevation of hypoxanthine post-exercise samples was observed in previous studies and is increased in high-intensity training as well as being higher in urine than in plasma (Alshehri et al., 2018; Burleigh et al., 2016). Adenine was found to be increased in plasma and saliva. However, since it is

re-absorbed by the kidney, this may explain its absence in urine, (Berg, Jeremy et al., 2015).

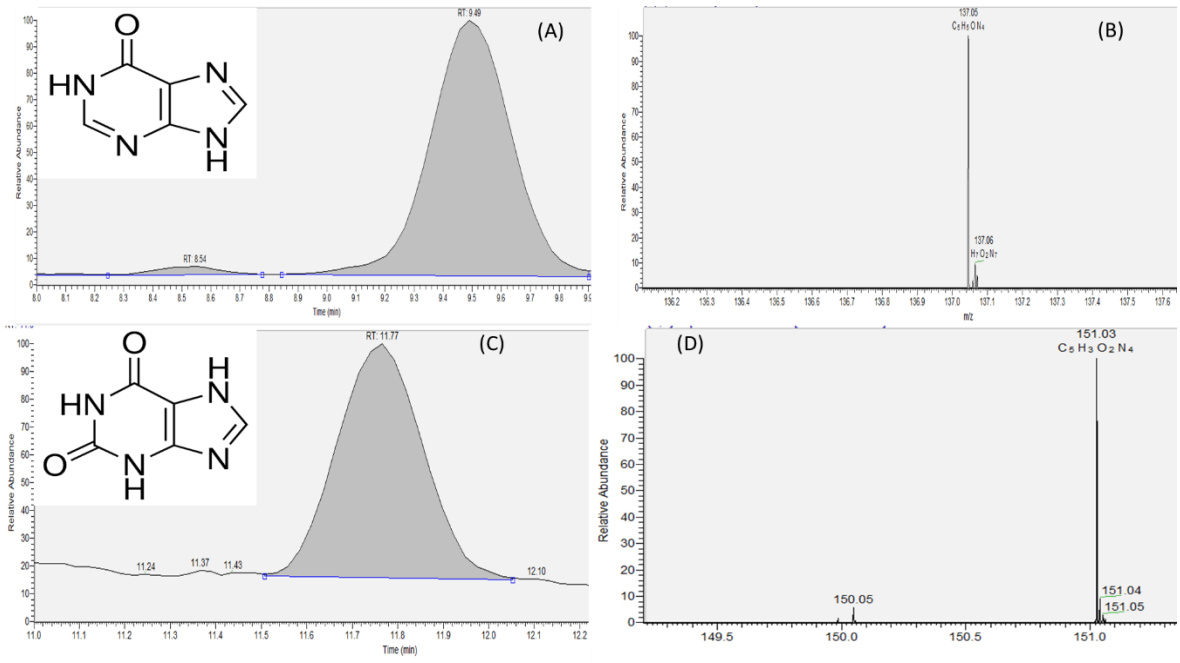


Figure 4-6. Chromatograms with structures (A and C) and spectrums (B and D) of hypoxanthine in the saliva sample, and xanthine in a plasma sample post-exercise, respectively.

The metabolic pathway of arginine-proline appears to have high numbers of significant metabolites in plasma samples, and it was found that ornithine and arginine metabolites were changed significantly in plasma and saliva samples. Also, glutamate was increased and doubled in the plasma samples. It has been found that glutamate can be an essential source for cell energy metabolism, where it is involved in brain energy metabolism and neuronal functions and survival (Hertz & Dienel,

2002). N-(Carboxyethyl) arginine; which is formed from arginine, was found to be increased three-fold after training. Also, arginine increased by one half, which can serve as a precursor of various amino acid substances such as glutamine, citrulline, proline and creatine, which are shown to change with exercise (Jr, 2006; McConell, 2007). The conversion arginine to citrulline, which is elevated in plasma following exercise, produces nitric oxide which produces vasodilation.

The carnitine pathway was affected after training and produced the highest number of significant metabolites in plasma. This impact of exercise on the carnitine metabolites could be due to mitochondrial fatty acid oxidation since acetylcarnitine accumulation has been identified to be high in plasma following exercise, which indicates an increased demand for stored energy (Fiehn et al., 2016). Acylcarnitines could also play an essential role in the regulation exertion when interacting with the neurons regulating muscle activity (Fiehn et al., 2016).

TCA and fatty acid metabolites showed higher responses in samples post-training.; the change was observed only in the plasma samples. Once fatty acid metabolites were increased, due to hydrolysis of triglycerides to fatty acids and glycerol. The fatty acids can be broken down during the beta-oxidation process to acetyl-CoA, which used in the citric acid cycle (TCA). Citrate cycle metabolites have been observed as the changed metabolites. It has been proposed that increased fat oxidation could lead to a reduced risk of cardiovascular disease (CVD) (Ginsburg et al., 1996). It has been reported that increases in the concentration of malate lead to increased rates

of oxidation of succinate and citrate and it seems to be the indicate an increased rate of oxidation of fatty acids (Bobyleva-Guarriero & Lardy, 1986). In the present work, malate was increased significantly in plasma; three-fold post-training in comparison to pre-training, which led to an increase in the succinate and citrate rates.

4.6. Conclusions

The significant increase in metabolites involved in fatty acid oxidation was observed post-exercise. Also, purine metabolites were increased slightly, especially hypoxanthine and its product xanthine. Although there are many metabolites, which were affected by this type of exercise, the majority of metabolites were increased. However, the increases in the metabolites were not very high in comparison to other studies that it might be due to the short duration and calibrated intensity of the exercise in combination with the fitness of the participants. This is the first study using an untargeted metabolomics approach of plasma, urine and saliva samples to determine the metabolic profile in response to short-term training in young professional football players.

5. General conclusion

The thesis covers three projects, the details of which are described here briefly as:

The first chapter is made up of the general introduction, which includes a brief introduction to the instruments and the sample preparation methods used for the project.

The second chapter of the thesis describes the quantification of cannabinoids from samples of human hair utilising a derivatization protocol and liquid chromatography-tandem mass spectrometry. The development of a derivatization method for detection cannabinoids; CBN, THC and THC-COOH, from hair samples. It includes the optimization of the protocol utilised for the preparation samples using SPE; modification the structure of cannabinoids CBN, THC and THC-COOH using the derivatizing agent (FMP-TS) as well as esterification of the carboxyl group of THC-COOH to enhance the stability, sensitivity and selectivity, in order to reach the level (0.2 pg/mg) that is recommended by SoHT. These cannabinoid derivatives were separated and identified efficiently based on multiple reaction monitoring (MRM) transitions using a triple quadrupole mass spectrometer. Once the method was optimized to detect these cannabinoids from hair as well as achieve the recommended cut-off of SoHT as follows: (0.05 ng/mg) for THC and (0.2 pg/mg) for THC-COOH in hair, the method was then validated according to the SWGTOX guideline (the Scientific Working Group for Forensic Toxicology, 2013). Finally, the ability of the method to detect the target analytes was examined by analysing 27 hair

specimens from cannabis users. THC-COOH being quantified for 13 specimens. Unlike CBN and THC, the levels of THC-COOH were semi-quantifiable (all values less than the limit of quantification (LOQ), but more than the limit of detection (LOD)) for 3 samples and THC-COOH was not detected at all for 11 samples.

The challenge in this project was deciding to use one step for extraction of CBN, THC and THC-COOH. In 2014, Dulaurent et al. reported the determination of the main cannabinoids (THC, CBN and CBD) and main metabolite (THC-COOH) in the hair together using LC-MS/MS for the first time (Dulaurent & Lachâtre, 2014). On the other hand, FMP-TS is believed to be a derivatization reagent that can dramatically improve the detection sensitivity of CBN, THC and THC-COOH. Thus, a method was developed based hydrolysis of hair with 1M NaOH followed by a one-step SPE extraction. The method achieved the target sensitivity proposed by SoHT for the main cannabinoids. It was then applied to their analysis in hair samples. A limitation of the study was the small number of hair samples, insufficient quantities of hair samples were collected and lack of information about their history. Addressing these limitations could be helpful to study the correlation between concentrations of the drugs in hair and weekly use score or among daily and non-daily users.

The third chapter is focused on the detection of testosterone (T) and dehydroepiandrosterone (DHEA) from a saliva sample. HMP was used as a derivatization reagent, increasing the sensitivity of the quantification of T and DHEA using liquid chromatography-mass spectrometry (LC-MS-MS) with an ACE Ultracore

2.5 Superphenylhexyl column. The development method included the extraction using a polymeric reverse solid-phase extraction cartridge (SPE) and an LC-ESI-MS/MS method for detecting the steroids in the saliva samples. The process was validated according to FDA guidelines and gave acceptable specificity, sensitivity, predictability, repeatability and accuracy. EPI was included in method development and validated as well. Finally, this procedure was applied to the analysis of saliva samples collected from football players pre-and post-training over two days. As a result, the concentrations T and DHEA in the saliva samples were determined, whereas epitestosterone (EPI) was not detected.

The levels of saliva testosterone (Sal-T) detected in the pre-and post-exercise samples showed no significant difference in Sal-T before and after training. However, the saliva dehydroepiandrosterone (Sal-DHEA) level registered a considerable difference. The correlation between the level of Sal-T and Sal-DHEA in the pre-exercise or the post-exercise samples was not high, suggesting no change in activity for the enzymes responsible for producing T from DHEA.

However, the main issue here was that the number of subjects was relatively small, and a different outcome might be achieved with a larger sample size.

The fourth chapter studied the impact of physical activity in response to short-term training in young professional football players on the change in metabolic profile. Plasma, urine and saliva samples were analysed for their metabolomic profiles to determine the metabolic changes due to short-term exercise in order to gain some

insight into how metabolism is adapted for short-term intense exercise performance. Using a statistical approach (multi- and univariate analysis) is the main benefit of getting reliable biomarker selection.

6. Future work

Studying the correlation between the concentration of the cannabinoids in head hair and body hair (pubic, axillary and beard hair) would be an interesting subject for future work. In addition, detection of 11-Hydroxy-Δ9-tetrahydrocannabinol (11-OH-THC) and 11-nor-Δ9-tetrahydrocannabinol-9-carboxylic acid glucuronide (THC-COOH-glu) in hair would also be an interesting subject for future work, where they are considered to prove cannabis consumption.

For the third chapter, since the analytical method works effectively and is stable, it would be possible to use the method to analyse samples from different cohorts of subjects undergoing different types of physical activity, e.g., ultramarathon runners.

For final project, confirmation of these untargeted metabolites needs standards, in addition to the 200 standards run with the samples. Standards are not always available and using MS^2 fragmentation can help to confirm identity in the absence of standards.

References

Agilent technologies. (n.d.). TAKE CONTROL OF YOUR SAMPLES. Retrieved November 7, 2017, from https://www.agilent.com/cs/library/posters/public/5991-7020EN.pdf

Ahu, R. S., Lee, Y. J., Choi, J. Y., Kwon, H. B., & Chun, S. Il. (2007). Salivary cortisol and DHEA levels in the Korean population: Age-related differences, diurnal rhythm, and correlations with serum levels. *Yonsei Medical Journal*, Vol. 48, pp. 379–388. https://doi.org/10.3349/ymj.2007.48.3.379

Alshehri, A., Spendiff, O., Muggeridge, D., Boyd, M., Howe, C., Watson, D., … Moir, H. (2018). Untargeted Metabolomics Profiling of an 80.5 km Simulated Treadmill Ultramarathon. *Metabolites*, *8*(1), 14. https://doi.org/10.3390/metabo8010014

Anari, M. R., Bakhtiar, R., Zhu, B., Huskey, S., Franklin, R. B., & Evans, D. C. (2002). Derivatization of ethinylestradiol with dansyl chloride to enhance electrospray ionization: Application in trace analysis of ethinylestradiol in rhesus monkey plasma. *Analytical Chemistry*, *74*(16), 4136–4144. https://doi.org/10.1021/ac025712h

Angeli, I., Casati, S., Ravelli, A., Minoli, M., & Orioli, M. (2018a). A novel single-step GC–MS/MS method for cannabinoids and 11-OH-THC metabolite analysis in hair. *Journal of Pharmaceutical and Biomedical Analysis*, *155*, 1–6. https://doi.org/10.1016/j.jpba.2018.03.031

Angeli, I., Casati, S., Ravelli, A., Minoli, M., & Orioli, M. (2018b). Journal of Pharmaceutical and Biomedical Analysis Short communication A novel single-step GC – MS / MS method for cannabinoids and 11-OH-THC metabolite analysis in hair. *Journal of Pharmaceutical and Biomedical Analysis*, *155*, 1–6. Retrieved from https://ac.els-cdn.com/S0731708518303194/1-s2.0-S0731708518303194-main.pdf?_tid=fa091bb3-0d31-4186-a47a-ab657ceb6211&acdnat=1538333080_ad840d891b1e59ee3980ac2aef19fbe3

Ardrey, R. E. (2003). *Liquid chromatography-mass spectrometry : an introduction*. Retrieved from https://www.wiley.com/en-us/Liquid+Chromatography+Mass+Spectrometry%3A+An+Introduction-p-9780471498018

Arlt, W. (2004). Dehydroepiandrosterone and ageing. *Best Practice & Research Clinical Endocrinology & Metabolism*, *18*(3), 363–380. https://doi.org/10.1016/J.BEEM.2004.02.006

Ashurst, P. R., Bohlmann, F., Farkas, L., Gaoni, Y., Kling, H., Mechoulam, R., … Gaoni, Y. (2012). Recent Advances in the Chemistry of Hashish. In *Fortschritte der Chemie Organischer Naturstoffe / Progress in the Chemistry of Organic Natural Products / Progrès dans la Chimie des Substances Organiques Naturelles* (pp. 175–213). https://doi.org/10.1007/978-3-7091-8164-5_6

Baker, A. B., & Tang, Y. Q. (2010). Aging performance for masters records in athletics, swimming, rowing, cycling, triathlon, and weightlifting. *Experimental Aging Research*, *36*(4), 453–477. https://doi.org/10.1080/0361073X.2010.507433

Banerjee, S., & Mazumdar, S. (2012). Electrospray Ionization Mass Spectrometry: A Technique to Access the Information beyond the Molecular Weight of the Analyte. *International Journal of Analytical Chemistry*, *2012*, 1–40. https://doi.org/10.1155/2012/282574

Baumgartner, A. M., Jones, P. F., Baumgartner, W. A., & Black, C. T. (1979). Radioimmunoassay of Hair for Determining Opiate-Abuse Histories. *In Vitro Nucler Medicine*, 748–753. Retrieved from https://europepmc.org/abstract/med/541713

Beasley, E., Francese, S., & Bassindale, T. (2016). Detection and Mapping of Cannabinoids in Single Hair Samples through Rapid Derivatization and Matrix-Assisted Laser Desorption Ionization Mass Spectrometry. *Analytical Chemistry*, *88*(20), 10328–10334. https://doi.org/10.1021/acs.analchem.6b03551

Ben Amar, M. (2006). Cannabinoids in medicine: A review of their therapeutic potential. *Journal of Ethnopharmacology*, *105*(1–2), 1–25. https://doi.org/10.1016/j.jep.2006.02.001

Berg, Jeremy, M., Tymoczko, J. L., Gatto, G. J., & Stryer, L. (2015). Biochemistry 8th Edition. In *Biochemistry*. https://doi.org/10.1007/978-3-8274-2989-6

Bobyleva-Guarriero, V., Wehbie, R. S., & Lardy, H. A. (1986). The role of malate in hormone-induced enhancement of mitochondrial respiration. *Archives of Biochemistry and Biophysics*, *245*(2), 477–482. https://doi.org/10.1016/0003-9861(86)90240-7

Borges, C. R., Wilkins, D. G., & Rollins, D. E. (2001). Amphetamine and N-acetylamphetamine incorporation into hair: An investigation of the potential role of drug basicity in hair color bias. *Journal of Analytical Toxicology*, *25*(4), 221–227. https://doi.org/10.1093/jat/25.4.221

Broadhurst, D., Goodacre, R., Reinke, S. N., Kuligowski, J., Wilson, I. D., Lewis, M. R., & Dunn, W. B. (2018). Guidelines and considerations for the use of system suitability and quality control samples in mass spectrometry assays applied in untargeted clinical metabolomic studies. *Metabolomics*, Vol. 14, p. 72. https://doi.org/10.1007/s11306-018-1367-3

Burgess, R. R. (2009). Chapter 20 Protein Precipitation Techniques. In *Methods in Enzymology* (Vol. 463, pp. 331–342). https://doi.org/10.1016/S0076-6879(09)63020-2

Burleigh, M., Easton, C., Muhsen Ali, A., Zhang, T., Daskalaki, E., & Watson, D. (2016). Metabolomic Profiling of Submaximal Exercise at a Standardised Relative Intensity in Healthy Adults. *Metabolites*, *6*(1), 9. https://doi.org/10.3390/metabo6010009

Büttler, R. M., Peper, J. S., Crone, E. A., Lentjes, E. G. W., Blankenstein, M. A., & Heijboer, A. C. (2016). Reference values for salivary testosterone in adolescent boys and girls determined using Isotope-Dilution Liquid-Chromatography Tandem Mass Spectrometry (ID-LC-MS/MS). *Clinica Chimica Acta*, *456*, 15–18. https://doi.org/10.1016/j.cca.2016.02.015

C. MARTYN BEAVEN, 1, NICHOLAS D. GILL, 1 AND CHRISTIAN J. COOK 2, & 1SchoolofSport. (2008). Salivary testosterone and cortisol responses following four resistance training protocols in professional rugby players. *Journal of Strength and Conditioning Research*, *22*(2), 426–432.

Cabral, G. A. (2005). Marijuana and Cannabinoids. In *Journal of Cannabis Therapeutics* (Vol. 1). https://doi.org/10.1300/j175v01n03_06

Cecchini, M., Sassi, F., Lauer, J. A., Lee BHSci, Y. Y., Guajardo-Barron, V., & Chisholm, D. (2010). Tackling of unhealthy diets, physical inactivity, and obesity: health effects and cost-effectiveness. *The Lancet*, *376*(376), 1775–1784. https://doi.org/10.1016/S0140-6736(10)61891-0

Celec, P., & Ostatníková, D. (2012). Saliva collection devices affect sex steroid concentrations.

Clinica Chimica Acta, Vol. 413, pp. 1625–1628. https://doi.org/10.1016/j.cca.2012.04.035

Choo, R. E., & Huestis, M. A. (2004). Oral fluid as a diagnostic tool. *Clinical Chemistry and Laboratory Medicine*, *42*(11), 1273–1287. https://doi.org/10.1515/CCLM.2004.248

Clark, L. C., Kochakian, C. D., & Lobotsky, J. (1944). *THE IN VITRO METABOLISM OF Al-ANDROSTENEDIONE;3,17 TO TESTOSTERONE, &TESTOSTERONE, AND SEVERAL UNIDENTIFIED STEROIDS**. Retrieved from http://www.jbc.org/content/171/2/493.full.pdf?sid=94eaaa35-be64-400a-b464-e0cbbef77644

Cone, E. J. (1996). Mechanisms of drug incorporation into hair. *Therapeutic Drug Monitoring*, *18*(4), 438–443. https://doi.org/10.1097/00007691-199608000-00022

Cooper, Gail A A, Kronstrand, R., & Kintz, P. (2012). Society of Hair Testing guidelines for drug testing in hair. *Forensic Science International*, Vol. 218, pp. 20–24. https://doi.org/10.1016/j.forsciint.2011.10.024

Cooper, Gail Audrey Ann. (2011, November). Hair testing is taking root. *Annals of Clinical Biochemistry*, Vol. 48, pp. 516–530. https://doi.org/10.1258/acb.2011.011112

Cormack, S. J., Newton, R. U., McGuigan, M. R., & Cormie, P. (2008). Neuromuscular and endocrine responses of elite players during an Australian rules football season. *International Journal of Sports Physiology and Performance*, *3*(4), 439–453. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/19223670

Daskalaki, E., Easton, C., & G. Watson, D. (2015). The Application of Metabolomic Profiling to the Effects of Physical Activity. *Current Metabolomics*, Vol. 2, pp. 233–263. https://doi.org/10.2174/2213235x03666150211000831

Deshmukh, N. I. K., Barker, J., Petroczi, A., & Naughton, D. P. (2012). Detection of testosterone and epitestosterone in human hair using liquid chromatography-tandem mass spectrometry. *Journal of Pharmaceutical and Biomedical Analysis*, *67–68*, 154–158. https://doi.org/10.1016/j.jpba.2012.04.011

Deshmukh, N. I. K. K., Barker, J., Petroczi, A., & Naughton, D. P. (2012). Detection of testosterone and epitestosterone in human hair using liquid chromatography-tandem mass spectrometry. *Journal of Pharmaceutical and Biomedical Analysis*, *67–68*, 154–

158. https://doi.org/10.1016/j.jpba.2012.04.011

Di Luigi, L., Baldari, C., Gallotta, M., Perroni, F., Romanelli, F., Lenzi, A., & Guidetti, L. (2006). Salivary Steroids at Rest and After a Training Load in Young Male Athletes: Relationship with Chronological Age and Pubertal Development. *International Journal of Sports Medicine*, *27*(9), 709–717. https://doi.org/10.1055/s-2005-872931

Draper, A. J., Madan, A., Smith, K., & Parkinson, A. (1998). Development of a non-high pressure liquid chromatography assay to determine testosterone hydroxylase (CYP3A) activity in human liver microsomes. *Drug Metabolism and Disposition: The Biological Fate of Chemicals*, *26*(4), 299–304. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9531515

Drust, B., Reilly, T., & Cable, N. T. (2000). Physiological responses to laboratory-based soccer-specific intermittent and continuous exercise. *Journal of Sports Sciences*, *18*(11), 885–892. https://doi.org/10.1080/026404100750017814

Dulaurent, S., Gaulier, J. M., Imbert, L., Morla, A., & Lacha, G. (2014). cannabinol and 11-nor-D 9 -tetrahydrocannabinol-9-carboxylic acid in hair using liquid chromatography – tandem mass spectrometry. *Forensic Science International Jou*, *236*, 151–156.

Dulaurent, S., Gaulier, J. M., Imbert, L., Morla, A., & Lachâtre, G. (2014). Simultaneous determination of δ9-tetrahydrocannabinol, cannabidiol, cannabinol and 11-nor-δ9-tetrahydrocannabinol-9-carboxylic acid in hair using liquid chromatography-tandem mass spectrometry. *Forensic Science International*, *236*, 151–156. https://doi.org/10.1016/j.forsciint.2014.01.004

DUNN, J. F., NISULA, B. C., & RODBARD, D. (1981). Transport of Steroid Hormones: Binding of 21 Endogenous Steroids to Both Testosterone-Binding Globulin and Corticosteroid-Binding Globulin in Human Plasma. *The Journal of Clinical Endocrinology & Metabolism*, *53*(1), 58–68. https://doi.org/10.1210/jcem-53-1-58

Faqehi, A. M. M., Cobice, D. F., Naredo, G., Mak, T. C. S., Upreti, R., Gibb, F. W., … Andrew, R. (2016). Derivatization of estrogens enhances specificity and sensitivity of analysis of human plasma and serum by liquid chromatography tandem mass spectrometry. *Talanta*, *151*, 148–156. https://doi.org/10.1016/j.talanta.2015.12.062

FDA, F. and D. A., & Food and Drug Administration. (2001). Guidance for Industry:

Bioanalytical method validation. In *U.S. Department of Health and Human Services*. https://doi.org/http://www.labcompliance.de/documents/FDA/FDA-Others/Laboratory/f-507-bioanalytical-4252fnl.pdf

Fenn, J. B., Mann, M., Meng, C. K., Wong, S. F., & Whitehouse, C. M. (1989). Electrospray ionization for mass spectrometry of large biomolecules. *Science (New York, N.Y.)*, *246*(4926), 64–71. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/2675315

Fiehn, O. (2002). Metabolomics--the link between genotypes and phenotypes. *Plant Molecular Biology*, *48*(1–2), 155–171. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11860207

Fiehn, O., Burnett, D. J., Hunter, G. R., Hughen, R. W., Adams, S. H., Zhang, J., … Newman, J. W. (2016). Acylcarnitines as markers of exercise-associated fuel partitioning, xenometabolism, and potential signals to muscle afferent neurons. *Experimental Physiology*, *102*(1), 48–69. https://doi.org/10.1113/ep086019

Fukushima, A., & Kusano, M. (2013). Recent Progress in the Development of Metabolome Databases for Plant Systems Biology. *Frontiers in Plant Science*, *4*, 73. https://doi.org/10.3389/fpls.2013.00073

Gaoni, Y., & Mechoulam, R. (1971). The isolation and structure of delta-1-tetrahydrocannabinol and other neutral cannabinoids from hashish. *Journal of the American Chemical Society*, *93*(1), 217–224. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/5538858

Gaskell, S. J. (1997). Electrospray: Principles and practice. *Journal of Mass Spectrometry*, *32*(7), 677–688. https://doi.org/10.1002/(SICI)1096-9888(199707)32:7<677::AID-JMS536>3.0.CO;2-G

Ghannam, M. G., & Singh, P. (2019). Anatomy, Head and Neck, Salivary Glands. In *StatPearls*. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/30855909

Ginsburg, G. S., Agil, A., O'Toole, M., Rimm, E., Douglas, P. S., & Rifai, N. (1996). Effects of a single bout of ultraendurance exercise on lipid levels and susceptibility of lipids to peroxidation in triathletes. *JAMA*, *276*(3), 221–225. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/8667567

GOLDBLUM, R. W., GOLDBAUM, L. R., & PIPER, W. N. (1954). Barbiturate concentrations in

the skin and hair of guinea pigs. *The Journal of Investigative Dermatology*, *22*(2), 121–128. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/13130894

Gonzalo-Lumbreras, R., Pimentel-Trapero, D., & Izquierdo-Hornillos, R. (2003). Development and method validation for testosterone and epitestosterone in human urine samples by liquid chromatography applications. *Journal of Chromatographic Science*, *41*(5), 261–265. https://doi.org/NO_DOI

Granger, D. A., Schwartz, E. B., Booth, A., Curran, M., & Zakaria, D. (1999). Assessing dehydroepiandrosterone in saliva: a simple radioimmunoassay for use in studies of children, adolescents and adults. *Psychoneuroendocrinology*, *24*(5), 567–579. https://doi.org/10.1016/S0306-4530(99)00013-X

Gröschl, M. (2008). Current status of salivary hormone analysis. *Clinical Chemistry*, *54*(11), 1759–1769. https://doi.org/10.1373/clinchem.2008.108910

Gröschl, M., Köhler, H., Topf, H. G., Rupprecht, T., & Rauh, M. (2008). Evaluation of saliva collection devices for the analysis of steroids, peptides and therapeutic drugs. *Journal of Pharmaceutical and Biomedical Analysis*, *47*(3), 478–486. https://doi.org/10.1016/j.jpba.2008.01.033

Gröschl, M., & Rauh, M. (2006). Influence of commercial collection devices for saliva on the reliability of salivary steroids analysis. *Steroids*, *71*(13–14), 1097–1100. https://doi.org/10.1016/j.steroids.2006.09.007

Gustafson, R. A., Moolchan, E. T., Barnes, A., Levine, B., & Huestis, M. A. (2003). Validated method for the simultaneous determination of 9-tetrahydrocannabinol (THC), 11-hydroxy-THC and 11-nor-9-carboxy-THC in human plasma using solid phase extraction and gas chromatography-mass spectrometry with positive chemical ionization. *Journal of Chromatography B*, *798*, 145–154. https://doi.org/10.1016/j.jchromb.2003.09.022

Hall, T. G., Smukste, I., Bresciano, K. R., Wang, Y., McKearn, D., & Savage, R. E. (2012). Identifying and Overcoming Matrix Effects in Drug Discovery and Development. *Intech Europe*, *18*, 389–420. Retrieved from http://cdn.intechopen.com/pdfs/29012/InTech-Identifying_and_overcoming_matrix_effects_in_drug_discovery_and_development.pdf

Han, E., Choi, H., Lee, S., Chung, H., & Song, J. M. (2011). A study on the concentrations of 11-

nor-D 9-tetrahydrocannabinol-9-carboxylic acid (THCCOOH) in hair root and whole hair. *Forensic Science International Jou*, *210*, 201–205. https://doi.org/10.1016/j.forsciint.2011.03.017

Harkey, M. R. (1993). Anatomy and physiology of hair. *Forensic Science International*, *63*(1–3), 9–18. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/8138238

Hemstrom, P., Heckendorf, A., Jiang, W., Jonsson, T., & Appelblad, P. (2013). Controlling Selectivity on Zwitterionic HILIC Columns by Adjusting pH and Buffer Strength. *Chromatography Today*, (May/June), 28–30.

Henion, J., Brewer, E., & Rule, G. (1998). Sample preparation for LC/MS/MS: analyzing biological and environmental samples. *Analytical Chemistry*, *70*(19), 650A-656A. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9784742

Hertz, L., & Dienel, G. A. (2002). Energy metabolism in the brain. *International Review of Neurobiology*, *51*, 1–102. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/12420357

Higashi, T., Shibayama, Y., & Shimada, K. (2007). Determination of salivary dehydroepiandrosterone using liquid chromatography-tandem mass spectrometry combined with charged derivatization. *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences*, *846*(1–2), 195–201. https://doi.org/10.1016/j.jchromb.2006.08.050

Higashi, T., Yamauchi, A., & Shimada, K. (2005). 2-Hydrazino-1-methylpyridine: a highly sensitive derivatization reagent for oxosteroids in liquid chromatography–electrospray ionization-mass spectrometry. *Journal of Chromatography B*, *825*(2), 214–222. https://doi.org/10.1016/j.jchromb.2004.12.007

Ho, C. S., Lam, C. W. K., Chan, M. H. M., Cheung, R. C. K., Law, L. K., Lit, L. C. W., … Tai, H. L. (2003). Electrospray ionisation mass spectrometry: principles and clinical applications. *The Clinical Biochemist. Reviews*, *24*(1), 3–12. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/18568044

HPLC training for the analytical chemist l CHROMacademy.com. (n.d.). Retrieved April 28, 2019, from https://www.chromacademy.com/hplc-training.html

Huck, C. W., & Bonn, G. K. (2000, July 14). Recent developments in polymer-based sorbents

for solid-phase extraction. *Journal of Chromatography A*, Vol. 885, pp. 51–72. https://doi.org/10.1016/S0021-9673(00)00333-2

Huffman, K. M., Koves, T. R., Hubal, M. J., Abouassi, H., Beri, N., Bateman, L. A., … Kraus, W. E. (2014). Metabolite signatures of exercise training in human skeletal muscle relate to mitochondrial remodelling and cardiometabolic fitness. *Diabetologia*, *57*(11), 2282–2295. https://doi.org/10.1007/s00125-014-3343-4

Hunter, S. K., Stevens, A. A., Magennis, K., Skelton, K. W., & Fauth, M. (2011). Is there a sex difference in the age of elite marathon runners? *Medicine and Science in Sports and Exercise*, *43*(4), 656–664. https://doi.org/10.1249/MSS.0b013e3181fb4e00

Johnson, D. W. (1999). Dimethylaminoethyl esters for trace, rapid analysis of fatty acids by electrospray tandem mass spectrometry. *Rapid Communications in Mass Spectrometry*, *13*(23), 2388–2393. https://doi.org/10.1002/(SICI)1097-0231(19991215)13:23<2388::AID-RCM803>3.0.CO;2-X

Johnson V., J., Yost A., R., Kelley E., P., & Bradford C., D. (1990). Tandem-in-space and tandem-in-time mass spectrometry: triple quadrupoles and quadrupole ion traps. *Analytical Chemistry*, *62*(20), 2162. https://doi.org/10.1021/ac00219a003

Jonans Tusiimire 2016. (2016). *A Study of the Biological Activity of Bee Venom and its Fractions With Regard to Cosmetic Science and Immunology A Thesis Submitted in Fulfilment of the Requirements for the Degree of Doctor of Philosophy in the Strathclyde Institute of Pharmacy and Biome*.

Jr, S. M. M. (2006). Arginine: beyond protein 1– 4. *American Journal of Clinical Nutrition*, *83*(2), 508–512. https://doi.org/10.1093/ajcn/83.2.508S

Kahsay, G., Song, H., Van Schepdael, A., Cabooter, D., & Adams, E. (2014). Hydrophilic interaction chromatography (HILIC) in the analysis of antibiotics. *Journal of Pharmaceutical and Biomedical Analysis*, *87*(April), 142–154. https://doi.org/10.1016/j.jpba.2013.04.015

Karvonen, J., & Vuorimaa, T. (1988). Heart Rate and Exercise Intensity During Sports Activities: Practical Application. *Sports Medicine: An International Journal of Applied Medicine and Science in Sport and Exercise*, *5*(5), 303–311. https://doi.org/10.2165/00007256-198805050-00002

Kaya, M., Moriwaki, Y., Ka, T., Inokuchi, T., Yamamoto, A., Takahashi, S., … Yamamoto, T. (2006). Plasma concentrations and urinary excretion of purine bases (uric acid, hypoxanthine, and xanthine) and oxypurinol after rigorous exercise. *Metabolism: Clinical and Experimental*, Vol. 55, pp. 103–107. https://doi.org/10.1016/j.metabol.2005.07.013

Keevil, B. G. B. G. B. G. B. G., MacDonald, P. R., Macdowall, W., Lee, D. M. M. D. D. M. M., Wu, F. C. W. F. C. F. F. C. W., Durdiaková, J., … Symons, R. G. (2014). Salivary testosterone measurement by liquid chromatography tandem mass spectrometry in adult males and females. *Annals of Clinical Biochemistry*, *51*(3), 368–378. https://doi.org/10.1177/0004563213506412

Keevil, B. G., Clifton, S., Tanton, C., Macdowall, W., Copas, A. J., Lee, D., … Wu, F. C. W. (2017). Distribution of Salivary Testosterone in Men and Women in a British General Population-Based Sample: The Third National Survey of Sexual Attitudes and Lifestyles (Natsal-3). *Journal of the Endocrine Society*, *1*(1), 14–25. https://doi.org/10.1210/js.2016-1029

Kessler, A., & Bhatt, A. (2018). Review of the Major and Minor Salivary Glands, Part 2: Neoplasms and Tumor-like Lesions. *Journal of Clinical Imaging Science*, *8*(1), 48. https://doi.org/10.4103/jcis.jcis_46_18

Kieliba, T., Lerch, O., Andresen-Streichert, H., Rothschild, M. A., & Beike, J. (2018). Simultaneous quantification of THC-COOH, OH-THC, and further cannabinoids in human hair by gas chromatography–tandem mass spectrometry with electron ionization applying automated sample preparation. *Drug Testing and Analysis*, (August 2018), 267–278. https://doi.org/10.1002/dta.2490

Kieliba, T., Lerch, O., Andresen-Streichert, H., Rothschild, M. A., & Beike, J. (2019). Simultaneous quantification of THC-COOH, OH-THC, and further cannabinoids in human hair by gas chromatography–tandem mass spectrometry with electron ionization applying automated sample preparation. *Drug Testing and Analysis*, *11*(2), 267–278. https://doi.org/10.1002/dta.2490

Kintz, P. (2006). *Analytical and Practical Aspects of Drug Testing in Hair* (Vol. 15). https://doi.org/10.1201/9781420006193

Kintz, Pascal. (2006). *Analytical and Practical Aspects of Drug Testing in Hair* (Vol. 15). https://doi.org/10.1201/9781420006193

Knechtle, B., Wirth, A., Knechtle, P., Zimmermann, K., & Kohler, G. (2009). Personal best marathon performance is associated with performance in a 24-h run and not anthropometry or training volume. *British Journal of Sports Medicine*, *43*(11), 836–839. https://doi.org/10.1136/bjsm.2007.045716

Knowles, J. R. (1980). Enzyme-Catalyzed Phosphoryl Transfer Reactions. *Annual Review of Biochemistry*, *49*(1), 877–919. https://doi.org/10.1146/annurev.bi.49.070180.004305

Kučera, J., Byrne, A. R., Mravcová, A., & Lener, J. (1992). Vanadium levels in hair and blood of normal and exposed persons. *Science of the Total Environment, The*, *115*(3), 191–205. https://doi.org/10.1016/0048-9697(92)90329-Q

Kuwayama, K., Miyaguchi, H., Yamamuro, T., Tsujikawa, K., Kanamori, T., Iwata, Y. T., & Inoue, H. (2015). Micro-pulverized extraction pretreatment for highly sensitive analysis of 11-nor-9-carboxy-Δ9-tetrahydrocannabinol in hair by liquid chromatography/tandem mass spectrometry. *Rapid Communications in Mass Spectrometry*, *29*(22), 2158–2166. https://doi.org/10.1002/rcm.7363

Lai, C.-C., Tsai, C.-H., Tsai, F.-J., Lee, C.-C., & Lin, W.-D. (2001). Rapid monitoring assay of congenital adrenal hyperplasia with microbore high-performance liquid chromatography/electrospray ionization tandem mass spectrometry from dried blood spots. *Rapid Communications in Mass Spectrometry*, *15*(22), 2145–2151. https://doi.org/10.1002/rcm.493

Lendoiro, E., Quintela, Ó., de Castro, A., Cruz, A., López-Rivadulla, M., & Concheiro, M. (2012). Target screening and confirmation of 35 licit and illicit drugs and metabolites in hair by LC-MSMS. *Forensic Science International*, Vol. 217, pp. 207–215. https://doi.org/10.1016/j.forsciint.2011.11.006

Lepers, R., & Cattagni, T. (2012). Do older athletes reach limits in their performance during marathon running? *Age*, *34*(3), 773–781. https://doi.org/10.1007/s11357-011-9271-z

Lipsett, M. B., Sarfaty, G. A., Wilson, H., Wayne Bardin, C., & Fishman, L. M. (1966). Metabolism of Testosterone and Related Steroids in Metastatic Interstitial Cell Carcinoma of the. *Journal of Clinical Investigation*, *45*(11). Retrieved from

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC292854/pdf/jcinvest00269-0062.pdf

McConell, G. K. (2007). Effects of L-arginine supplementation on exercise metabolism. *Current Opinion in Clinical Nutrition and Metabolic Care*, *10*(1), 46–51. https://doi.org/10.1097/MCO.0b013e32801162fa

McLafferty, F. W. (1981). Tandem mass spectrometry. *Science (New York, N.Y.)*, *214*(4518), 280–287. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/7280693

Mercolini, L., Mandrioli, R., Protti, M., Conti, M., Serpelloni, G., & Raggi, M. A. (2013). Monitoring of chronic Cannabis abuse: An LC-MS/MS method for hair analysis. *Journal of Pharmaceutical and Biomedical Analysis*, *76*, 119–125. https://doi.org/10.1016/j.jpba.2012.12.015

Míguez-Framil, M., Cocho, J. Á., Tabernero, M. J., Bermejo, A. M., Moreda-Piñeiro, A., & Bermejo-Barrera, P. (2014). An improved method for the determination of {increment}9-tetrahydrocannabinol, cannabinol and cannabidiol in hair by liquid chromatography-tandem mass spectrometry. *Microchemical Journal*, *117*, 7–17. https://doi.org/10.1016/j.microc.2014.05.015

Miller, M. L., Donnelly, B., & Martz, R. M. (1997). The forensic application of testing hair for drugs of abuse. *NIDA Research Monograph*, *167*, 146–160. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9243561

Mooradian, A. D., MORLEY, J. E., Korenman, S. G., & A. D. Mooradian, J. E. Morley, S. G. K. (1987). Biological actions of androgens. *Endocrine Reviews*, *8*(1), 1–28. https://doi.org/10.1210/edrv-8-1-1

Moore, C., Rana, S., Coulter, C., Feyerherm, F., & Prest, H. (2006a). Application of two-dimensional gas chromatography with electron capture chemical ionization mass spectrometry to the detection of 11-nor- Δ9-tetrahydrocannabinol-9-carboxylic acid (THC-COOH) in hair. *Journal of Analytical Toxicology*, *30*(3), 171–177. https://doi.org/10.1093/jat/30.3.171

Moore, C., Rana, S., Coulter, C., Feyerherm, F., & Prest, H. (2006b). Application of two-dimensional gas chromatography with electron capture chemical ionization mass spectrometry to the detection of 11-nor- Δ9-tetrahydrocannabinol-9-carboxylic acid (THC-COOH) in hair. *Journal of Analytical Toxicology*, *30*(3), 171–177.

https://doi.org/10.1093/jat/30.3.171

Musshoff, F., P Junker, H., Lachenmeier, D., Kroener, L., & Madea, B. (2002). Fully Automated Determination of Cannabinoids in Hair Samples using Headspace Solid-Phase Microextraction and Gas Chromatography-Mass Spectrometry. In *Journal of analytical toxicology* (Vol. 26). https://doi.org/10.1093/jat/26.8.554

Nakahara, Y., & Kikura, R. (1996). Hair analysis for drugs of abuse. XIII. Effect of structural factors on incorporation of drugs into hair: The incorporation rates of amphetamine analogs. *Archives of Toxicology*, *70*(12), 841–849. https://doi.org/10.1007/s002040050348

Nawed IK, C. J., Deshmukh, N. I., Shah, I., Zachár, G., Székely, A. D., Petroczi, A., & Naughton, D. P. (2014). LC-MS/MS-Based Assay for Free and Deconjugated Testosterone and Epitestosterone in Rat Urine and Serum. *Journal of Analytical & Bioanalytical Techniques*, *s5*(01). https://doi.org/10.4172/2155-9872.S5-006

Nowak, R., McMillen, I. C., Redman, J., & Short, R. V. (1987). The correlation between serum and salivary melatonin concentrations and urinary 6-hydroxymelatonin sulphate excretion rates: two non-invasive techniques for monitoring human circadian rhythmicity. *Clinical Endocrinology*, *27*(4), 445–452. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/3436070

Paci, M., Dinallo, V., D'Ottavio, S., Barbato, G., Bernardini, S., & Santone, C. (2013). Saliva metabolomics by NMR for the evaluation of sport performance. *Journal of Pharmaceutical and Biomedical Analysis*, Vol. 88, pp. 441–446. https://doi.org/10.1016/j.jpba.2013.09.021

Park, M., Kim, J., Park, Y., In, S., Kim, E., & Park, Y. (2014). Quantitative determination of 11-nor-9-carboxy-tetrahydrocannabinol in hair by column switching LC-ESI-MS 3. *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences*, *947–948*, 179–185. https://doi.org/10.1016/j.jchromb.2013.12.036

Patti, G. J., Yanes, O., & Siuzdak, G. (2012, April 22). Innovation: Metabolomics: the apogee of the omics trilogy. *Nature Reviews Molecular Cell Biology*, Vol. 13, pp. 263–269. https://doi.org/10.1038/nrm3314

Paul, R., Williams, R., Hodson, V., & Peake, C. (2019). Detection of cannabinoids in hair after

cosmetic application of hemp oil. *Scientific Reports*, *9*(1), 2582. https://doi.org/10.1038/s41598-019-39609-0

Peters, F. T., Drummer, O. H., & Musshoff, F. (2007). Validation of new methods. *Forensic Science International*. https://doi.org/10.1016/j.forsciint.2006.05.021

Pichini, S., Marchei, E., Martello, S., Gottardi, M., Pellegrini, M., Svaizer, F., … Pacifici, R. (2015). Identification and quantification of 11-nor-δ9-tetrahydrocannabinol-9-carboxylic acid glucuronide (THC-COOH-glu) in hair by ultra-performance liquid chromatography tandem mass spectrometry as a potential hair biomarker of cannabis use. *Forensic Science International*, *249*, 47–51. https://doi.org/10.1016/j.forsciint.2015.01.011

Pitt, J. J. (2009). Principles and applications of liquid chromatography-mass spectrometry in clinical biochemistry. *The Clinical Biochemist. Reviews*, *30*(1), 19–34. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/19224008

Pluskal, T., Castillo, S., Villar-Briones, A., & Orešič, M. (2010). MZmine 2: Modular framework for processing, visualizing, and analyzing mass spectrometry-based molecular profile data. *BMC Bioinformatics*, *11*(1), 395. https://doi.org/10.1186/1471-2105-11-395

Prego-Meleiro, P., Lendoiro, E., Concheiro, M., Cruz, A., López-Rivadulla, M., & de Castro, A. (2017). Development and validation of a liquid chromatography tandem mass spectrometry method for the determination of cannabinoids and phase I and II metabolites in meconium. *Journal of Chromatography A*, *1497*, 118–126. https://doi.org/10.1016/j.chroma.2017.03.066

Pringle, H. (1997, August 29). Ice age communities may be earliest known net hunters. *Science*, Vol. 277, pp. 1203–1204. https://doi.org/10.1126/science.277.5330.1203

Proctor, G. B. (2016, February). The physiology of salivary secretion. *Periodontology 2000*, Vol. 70, pp. 11–25. https://doi.org/10.1111/prd.12116

Qi, B. L., Liu, P., Wang, Q. Y., Cai, W. J., Yuan, B. F., & Feng, Y. Q. (2014, July). Derivatization for liquid chromatography-mass spectrometry. *TrAC - Trends in Analytical Chemistry*, Vol. 59, pp. 121–132. https://doi.org/10.1016/j.trac.2014.03.013

Quirke, J. M. E., Adams, C. L., & Van Berkel, G. J. (1994). Chemical Derivatization for Electrospray Ionization Mass Spectrometry. 1. Alkyl Halides, Alcohols, Phenols, Thiols,

and Amines. *Analytical Chemistry*, *66*(8), 1302–1315. https://doi.org/10.1021/ac00080a016

Quirke, J. M. E., Berkel, G. J. V., & Adams, C. L. (1994). Chemical Derivatization for Electrospray Ionization Mass Spectrometry. 1. Alkyl Halides, Alcohols, Phenols, Thiols, and Amines. *Analytical Chemistry*, *66*(8), 1302–1315. https://doi.org/10.1021/ac00080a016

R., J., & de Boor, C. (2006). A Practical Guide to Splines. *Mathematics of Computation*, *34*(149), 325. https://doi.org/10.2307/2006241

Rauh, M. (2009). Steroid measurement with LC-MS/MS in pediatric endocrinology. *Molecular and Cellular Endocrinology*, *301*(1–2), 272–281. https://doi.org/10.1016/j.mce.2008.10.007

Recommendations for hair testing in forensic cases. (2004). *Forensic Science International*, *145*(2–3), 83–84. https://doi.org/10.1016/j.forsciint.2004.04.022

Rollins, D. E., Wilkins, D. G., Gygi, S. P., Slawson, M. H., & Nagasawa, P. R. (1997). Testing for Drugs of Abuse in Hair - Experimental Observations and Indications for Future Research. *Forensic Sci Rev*, *9*(1), 23–36. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/26270864

Rowell, A. E., Aughey, R. J., Hopkins, W. G., Esmaeili, A., Lazarus, B. H., & Cormack, S. J. (2018a). Effects of training and competition load on neuromuscular recovery, testosterone, cortisol, and match performance during a season of professional football. *Frontiers in Physiology*, *9*(JUN), 668. https://doi.org/10.3389/fphys.2018.00668

Rowell, A. E., Aughey, R. J., Hopkins, W. G., Esmaeili, A., Lazarus, B. H., & Cormack, S. J. (2018b). Effects of training and competition load on neuromuscular recovery, testosterone, cortisol, and match performance during a season of professional football. *Frontiers in Physiology*, *9*(JUN), 668. https://doi.org/10.3389/fphys.2018.00668

Sachs, H. (1997). History of hair analysis. *Forensic Science International*, *84*(1–3), 7–16. https://doi.org/10.1016/S0379-0738(96)02043-9

Santa, T, Al-Dirbashi, O. Y., & Fukushima, T. (2007). Derivatization reagents in liquid chromatography/electrospray ionization tandem mass spectrometry for biomedical analysis. *Drug Discov Ther*, *1*(2), 108–118. https://doi.org/10.1002/bmc

Santa, Tomofumi. (2011). Derivatization reagents in liquid chromatography/electrospray ionization tandem mass spectrometry. *Biomedical Chromatography*, *25*(1), 1–10. https://doi.org/10.1002/bmc.1548

Sarris, J., O'Neil, A., Coulson, C. E., Schweitzer, I., & Berk, M. (2014, December 10). Lifestyle medicine for depression. *BMC Psychiatry*, Vol. 14, p. 107. https://doi.org/10.1186/1471-244X-14-107

Sarstedt. (n.d.). *saliva collection for diagnostics and monitoring*.

Schultz, O. E., & Haffner, G. (1960). [On the question of the biosynthesis of cannabinol. Part III]. *Archiv Der Pharmazie Und Berichte Der Deutschen Pharmazeutischen Gesellschaft*, *293*(65), 1–8. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/14443791

Scigelova, M., Hornshaw, M., Giannakopulos, A., & Makarov, A. (2011). Fourier transform mass spectrometry. *Molecular & Cellular Proteomics : MCP*, *10*(7), M111.009431. https://doi.org/10.1074/mcp.M111.009431

Shibayama, Y., Higashi, T., Shimada, K., Odani, A., Mizokami, A., Konaka, H., … Namiki, M. (2009). Simultaneous determination of salivary testosterone and dehydroepiandrosterone using LC-MS/MS: Method development and evaluation of applicability for diagnosis and medication for late-onset hypogonadism. *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences*, *877*(25), 2615–2623. https://doi.org/10.1016/j.jchromb.2008.10.051

Shimada, K., Mitamura, K., & Higashi, T. (2001). Gas chromatography and high-performance liquid chromatography of natural steroids. *Journal of Chromatography A*, *935*(1), 141–172. https://doi.org/10.1016/S0021-9673(01)00943-8

Shrivastava, A., & Gupta, V. (2011). Methods for the determination of limit of detection and limit of quantitation of the analytical methods. *Chronicles of Young Scientists*, *2*(1), 21. https://doi.org/10.4103/2229-5186.79345

Sobolesky, P. M., Smith, B. E., Hubbard, J. A., Stone, J., Marcotte, T. D., Grelotti, D. J., … Fitzgerald, R. L. (2019). Validation of a liquid chromatography-tandem mass spectrometry method for analyzing cannabinoids in oral fluid. *Clinica Chimica Acta*, *491*, 30–38. https://doi.org/10.1016/j.cca.2019.01.002

Sparling, P. B., O'Donnell, E. M., & Snow, T. K. (1998). The gender difference in distance

running performance has plateaued: an analysis of world rankings from 1980 to 1996. *Medicine and Science in Sports and Exercise*, *30*(12), 1725–1729. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9861606

State-By-State Medical Marijuana Laws. (2016). https://doi.org/10.1201/9781315367583-15

the Scientific Working Group for Forensic Toxicology. (2013). Scientific Working Group for Forensic Toxicology (SWGTOX) Standard Practices for Method Validation in Forensic Toxicology. *Journal of Analytical Toxicology*, *37*(7), 452–474. https://doi.org/10.1093/jat/bkt054

Thieme, D., Sachs, H., & Thevis, M. (2008). Formation of the N-methylpyridinium derivative to improve the detection of buprenorphine by liquid chromatography-mass spectrometry. *Journal of Mass Spectrometry*, *43*(7), 974–979. https://doi.org/10.1002/jms.1433

Thieme, D., Sachs, H., & Uhl, M. (2014). Proof of cannabis administration by sensitive detection of 11-nor-Delta(9)-tetrahydrocannabinol-9-carboxylic acid in hair using selective methylation and application of liquid chromatography- tandem and multistage mass spectrometry. *Drug Testing and Analysis*, *6*(1–2), 112–118. https://doi.org/10.1002/dta.1565

Thurman, E. M. (Earl M., & Mills, M. S. (1998). *Solid-phase extraction : principles and practice*. Wiley.

Tobin, D. J. (2007). Biology of Hair. The Biogenesis and Growth of Human Hair. In D. J. Tobin (Ed.), *Hair in Toxicology* (pp. 1–33). https://doi.org/10.1039/9781847552518-00001

Uhl, M. (1997). Determination of drugs in hair using GC/MS/MS. *Forensic Science International*, *84*(1–3), 281–294. https://doi.org/10.1016/S0379-0738(96)02072-5

Uhl, Michael, & Sachs, H. (2004a). Cannabinoids in hair: Strategy to prove marijuana/hashish consumption. *Forensic Science International*, *145*(2–3), 143–147. https://doi.org/10.1016/j.forsciint.2004.04.029

Uhl, Michael, & Sachs, H. (2004b). Cannabinoids in hair: Strategy to prove marijuana/hashish consumption. In *Forensic science international* (Vol. 145). https://doi.org/10.1016/j.forsciint.2004.04.029

UNODC The United Nations Office on Drugs and Crime. (2016). *CHAPTER I Cannabis 43*. Retrieved from www.infodrogas.gub.uy.

Vining, R. F., McGinley, R. A., & Symons, R. G. (1983). Hormones in saliva: Mode of entry and consequent implications for clinical interpretation. *Clinical Chemistry*, *29*(10), 1752–1756. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/6225566

Vogliardi, S., Tucci, M., Stocchero, G., Ferrara, S. D., & Favretto, D. (2015). Sample preparation methods for determination of drugs of abuse in hair samples: A review. *Analytica Chimica Acta*, *857*, 1–27. https://doi.org/10.1016/j.aca.2014.06.053

WADA. (2017). Prohibited List | World Anti-Doping Agency. Retrieved from https://www.wada-ama.org/en/resources/science-medicine/prohibited-list

Weinmann, W., Vogt, S., Goerke, R., Müller, C., & Bromberger, A. (2000). Simultaneous determination of THC-COOH and THC-COOH-glucuronide in urine samples by LC/MS/MS. *Forensic Science International*, *113*(1–3), 381–387. https://doi.org/10.1016/S0379-0738(00)00210-3

Williams, T. I., Lovell, M. A., & Lynn, B. C. (2005). Analysis of derivatized biogenic aldehydes by LC tandem mass spectrometry. *Analytical Chemistry*, *77*(10), 3383–3389. https://doi.org/10.1021/ac048265+

Wolfram, L. J. (2003). Human hair: A unique physicochemical composite. *Journal of the American Academy of Dermatology*, *48*(6 SUPPL.), S106–S114. https://doi.org/10.1067/mjd.2003.276

Wren, M. E., Shirtcliff, E. A., & Drury, S. S. (2015). Not All Biofluids Are Created Equal: Chewing over Salivary Diagnostics and the Epigenome. *Clinical Therapeutics*, *37*(3), 529–539. https://doi.org/10.1016/j.clinthera.2015.02.022

Zhang, K., & Liu, X. (2016). Reprint of "Mixed-mode chromatography in pharmaceutical and biopharmaceutical applications." *Journal of Pharmaceutical and Biomedical Analysis*, *130*, 19–34. https://doi.org/10.1016/j.jpba.2016.09.013

Zhang, R., Watson, D. G., Wang, L., Westrop, G. D., Coombs, G. H., & Zhang, T. (2014). Evaluation of mobile phase characteristics on three zwitterionic columns in hydrophilic interaction liquid chromatography mode for liquid chromatography-high resolution mass spectrometry based untargeted metabolite profiling of Leishmania parasites.

Journal of Chromatography A, *1362*, 168–179. https://doi.org/10.1016/j.chroma.2014.08.039

Zhang, T., Creek, D. J., Barrett, M. P., Blackburn, G., & Watson, D. G. (2012). Evaluation of coupling reversed phase, aqueous normal phase, and hydrophilic interaction liquid chromatography with orbitrap mass spectrometry for metabolomic studies of human urine. *Analytical Chemistry*, *84*(4), 1994–2001. https://doi.org/10.1021/ac2030738

Zlielinski, J., Kusy, K., & Rychlewski, T. (2011). Effect of Training Load Structure on Purine Metabolism in Middle-Distance Runners. *Medicine & Science in Sports & Exercise*, *43*(9), 1798–1807. https://doi.org/10.1249/mss.0b013e318215d10b

Appendices

Appendices 1 for Chapter 2: Ethical approval from Saudi MOH

وزارة الصحة
Ministry of Health

المملكة العربية السعودية
Kingdom of Saudi Arabia
مديرية الشؤون الصحية بمحافظة جدة
Directorate of Health Affairs - Jeddah
(٢٠٢/٢٧٥)

To Whom it may concern:

DR. Farouq Faisal AL-Zahrani

DR. Ahmed Ibrahim AL-Asmari

Ethical Application Ref:

47/302/26771

1st august

2018

I am pleased to confirm that hair samples have been collected from Alamal Hospital, Jeddah, Saudi Arabia, and it has been granted a research ethical approval letter (mentioned above). In addition, the approval letter was submitted for hair testing for drugs of abuse. Therefore. DR. Dave Watson and Mr Mansour Alzahrani have the right to apply more analysis on the collected samples mentioned above for scientific purpose only.

For further request, please contact us at AlAL-Asmari2@moh.gov.sa
Sincerely,

Dr Ahmed Al-Asmari
Phd, MSc
Director of Jeddah Poison Control & Forensic Medical Chemistry Center
Consultant Forensic Toxicologist
Ministry of Health
Jeddah, Kingdom of Saudi Arabia
P. O. Box 6470 Jeddah 21442
Tel: 00966126374900-ext-170
Fax: 00966126374900-ext-100
Mobile: 00966599155725
e-mail: AlAL-Asmari2@moh.gov.sa
e-mail: pcc-jeddah@moh.gov.sa

وزارة الصحة
Ministry of Health
مركز مراقبة السموم والكيمياء الطبية الشرعية بمحافظة جدة

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الرقم : / /٤٧/ج التاريخ : / / ١٤هـ المشفوعات : .....

هاتف : ٠٢-٦٩٧٠٠٠٦ / ٠٢-٦٨٣١٣٧٧ فاكس : ٠٢-٦٦٢٢٩٦١ ص.ب : ١٢٤٩٣ جدة : ٢١١٧٦
Tel.: 02-6831377. 02-6970006 Fax : 02-6622961 P.O. Box : 12493 Jeddah 21176
موقع إلكتروني : www.mohj.gov.sa

Appendices 2 for Chapter 2: Drugs of abuse and prescribed drugs tested for interference study.

Drug groups	Drug lists
Drugs of abuse/ Abused prescription drugs	Amphetamine-type stimulants: amphetamine, methamphetamine, 3,4-methylenedioxy-methamphetamine (MDMA), 3,4-methylenedioxy-amphetamine (MDA), 3,4-methylenedioxy –N-ethyl-amphetamine (MDEA) Opioid drugs: 6-monoacetylmorphine (6-AM), morphine, codeine, dihydrocodeine (DHC), methadone, 2-ethylidene-1,5-dimethyl-3,3-diphenyl pyrrolidine (EDDP), buprenorphine, norbuprenorphine, tramadol, propoxyphene Cocaine and its metabolites: cocaine, benzoylecgonine, ecgonine methyl ester (EME), cocaethylene Cannabis and its Hallucinogenic drug: ketamine
Sedative-hypnotic drugs	Benzodiazepines: diazepam, desmethyldiazepem (nordiazepam), oxazepam, temazepam, lorazepam, clonazepam, chlordiazepoxide, alprazolam, phenazepam, etizolam Non-benzodiazepine hypnotic drugs (Z-drugs): zolpidem, zopiclone
Antidepressant drugs	amitriptyline, clomipramine, desipramine, fluoxetine, norfluoxetine, paroxetine, duloxetine, mirtazapine, venlafaxine, trazodone, citalopram, propranolol, lamotrigine (adjunctive treatment for bipolar disorder, depressive episode)
Antipsychotic drugs	chlorpromazine, haloperidol, clozapine, olanzapine, risperidone, quetiapine, valproic acid (adjunctive treatment for schizophrenia

Appendices 3 for Chapter 3: Ethical approval from Saudi KSU

جامعة الملك سعود (034)
هاتف 51 31 806 11 966+
فاكس 70 33 806 11 966+

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To Whom It May to Concern

Mr Ghareeb Alshuwaier
King Saud University
College of Sport Sciences and Physical Activity
Exercise Physiology Department
Ethical Application Ref:
4/67/352673

13th MAY 2017

I am pleased to confirm that Mr Ghareeb Alshuwaier from Exercise Physiology Department in the College of Sport Sciences and Physical Activity at King Saud University, and he has been granted a research ethical approval letter (mentioned above). In addition, the approval letter was submitted for his studies as part of his PhD thesis and was mentioned that blood samples, urine and saliva and all collected data for the approved researcher can be used in further studies in future. Therefore, Mr Alshuwaier, Dr Dave Watson and Mr Mansour Alzahrani have the right to apply more analysis on the collected samples mentioned above for scientific purpose only. Mr Ghareeb Alshuwaier has been given this letter at his request.

For further request please contact us via Khaljaloud@ksu.edu.sa

Sincerely,

Khalid Saad Aljaloud MSc,PhD.
Vice Dean for Graduate Studies and Research

٦/٣/٣٥٣٣١٥ الرقم
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المرفقات

Appendices 4 for Chapter 3 (poster): Method Development and Validation of a Derivatization Method for the Analysis of Dehydroepiandrosterone (DHEA), Testosterone (T) and Epitestosterone (ET) and using mass spectrometry

Method Development and Validation of a Derivatisation Method for the Analysis of Dehydroepiandrosterone (DHEA), Testosterone (T) and Epitestosterone (ET) and using mass spectrometry

M Alzahrani, D.G.Watson

Pharmacy and Biomedical Science, Strathclyde University, Glasgow, UK

Introduction

Various analytical techniques have been used for analysis of androgen steroids by using different instruments and applying these methods on different biological samples. This project has been focusing on the method development for preparation sample, derivatisation and optimisation the detection of the instruments and validation the method as well. Therefore, the optimization of derivatisation method using derivative reagents HMP was reached. Otherwise, the chromatographic acceptable separation of these steroids was achieved using HMP reagent, ACE Ultracore 2.5 Superphenylhexyl column (150 × 3 mm id) and finally identified by Agilent 6410 triple quadrupole (QQQ) tandem mass spectrometers.

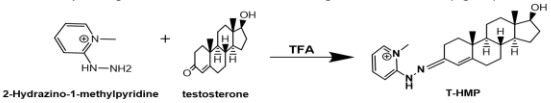
Since steroids are only present in small amounts in saliva, the current work focused on developing a reliable sample preparation and extraction procedure to isolate steroids. Non-polar SPE is extraction process when molecules with non-polar function group are extracted from a polar matrix such as steroids from biological fluids. Optimisation the extraction method was employed using polymeric reveres phase cartridge. Validation was carried out according to the guideline of Food and Drug Administration (FDA).

Detection steroids in the body are not easy mission due to its low concentration in the body. Therefore, this report has been concentrated on:
1- Development of derivatisation method to the enhancement of detection and structure evaluation of Testosterone(T), Dehydroepiandrosterone (DHEA) and Epitestosterone *(EPI)*, using chemical derivatizations (2-Hydrazino-1-methylpyridine (HMP)), using LC-MS analyses.
2- Optimise the extraction method for the three steroids in saliva samples, and Validate the method using the guideline of Food and Drug Administration (FDA).

Optimization of reaction conditions in derivatization steroids with HMP

2-Hydrazino-1-methylpyridine (HMP) was advancing as derivatization reagent technique to enhance the detection sensitivity, to detect compounds in low concentration, by making them more ionisable in the LC-MS system. Many protocols have been developed to get the best method to detect these steroids with HMP derivative (Figure 1).

Due to the importance of heat in chemical reactions, the reaction has proceeded at different incubation times. The percentage of chemical derivatisation was examining at each incubation time (Figure 2).



2-Hydrazino-1-methylpyridine testosterone T-HMP

Figure 1. Derivatization of T with HMP

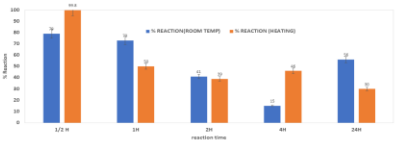


Figure 2 HMP's reactivity with testosterone in different times and ways of incubation.

Optimisation of chromatographic separation

Some different styles of columns were tried first to exam their efficiency in separating the different steroids. This testing involved an ACE 3 C18 column (150 × 3.0mm i.d.; Hichrom, Reading, UK.), an ACE 5 C18-AR column (150 × 4.6mm, 5µm Hichrom, Reading, UK) and ACE Ultracore 2.5 Superphenylhexyl (150 × 3 mm id).

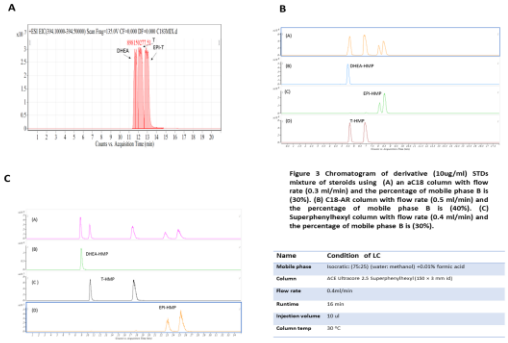
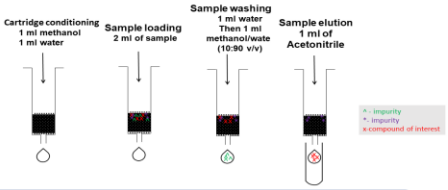


Figure 3 Chromatogram of derivative (10ug/ml) STDs mixture of steroids using (A) an aC18 column with flow rate (0.3 ml/min) and the percentage of mobile phase B is (30%), (B) C18-AR column with flow rate (0.5 ml/min) and the percentage of mobile phase B is (40%). (C) Superphenylhexyl column with flow rate (0.4 ml/min) and the percentage of mobile phase B is (30%).

Name	Condition of LC
Mobile phase	Isocratic: (75:25) (water: methanol) +0.01% formic acid
Column	ACE Ultracore 2.5 Superphenylhexyl (150 × 3 mm id)
Flow rate	0.4ml/min
Runtime	16 min
Injection volume	10 µl
Column temp	30 °C

Extraction

Sample pre-treatment: deprotonate sample with 0.5 ml Acetonitrile and then diluted with 1.5 ml water.



Method validation

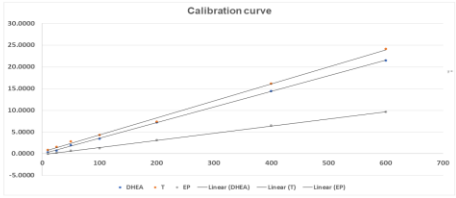


Figure 4 linearity for DHEA, testosterone and epitestosterone from range 10-600 pg/ml using LC-ESI-MS/MS.

Parameter	DHEA	Testosterone	Epitestosterone
	Human saliva	Human saliva	Human saliva
Range pg/ml	10-600	10-600	10-600
Regression Equation	y = 0.0392x + 0.4441	y = 0.036x - 0.0296	y = 0.017x - 0.1844
Correlation coefficient (r)	0.9978	0.9997	0.9984
Selectivity[a]	95.03±1.23	90.12 ± 2.3	97.3 ± 0.8
LOQ (pg/ml)	25	10	50
LOD (pg/mL)	8	3	16
Intra assay precision[b]	2.4	2.6	0.6
Inter assay precision[c]	0.7	2.5	0.2
Accuracy[d]	92.9%	90.5%	94.9%

Table 1. Validation parameters of the proposed method

1- LC-ESI-MS/MS method has benn validated for the determination of these three steroids with good linearity, accuracy, and precision.
2- the effect of matrix was examined for these three steroids and D3-T as well. The percentage was less than -25% which could be acceptable effect.

Conclusions

The main goal was achieved is derivative these steroids using HMP derivative reagent.
In addition, The separation of DHEA, T and ET was gained using ACE Ultracore 2.5 Superphenylhexyl column (150 × 3 mm id) column.
Moreover, by demonstrating the LC-ESI-MS/MS method for detecting these steroids in the saliva sample.
The method was validated according to FDA guideline with acceptable specificity, sensitivity, predictable, repeatable and accurate.
Fast extraction procedure for extraction these steroids from saliva sample partially achieved. Where DHEA and T were extracted but ET seemed to be not existed or need method very low sensitive that has LOQ within fg/ml.
For more forth work, this method going to apply on saliva sample to study the correlation between DHEA and T. this saliva sample collected from more than 20 players pre and post-training within two days.

References

Higashi, T., Yamauchi, A. and Shimada, K. (2005b) '2-Hydrazino-1-methylpyridine: a highly sensitive derivatization reagent for oxosteroids in liquid chromatography–electrospray ionization-mass spectrometry', *Journal of Chromatography B*, 825(2), pp. 214–222. doi: 10.1016/j.jchromb.2004.12.007.

Appendices 5 for Chapter 3 (paper): Method Development and Validation of a Derivatization Method for the Analysis of Dehydroepiandrosterone (DHEA), Testosterone (T) and Epitestosterone (ET) and using mass spectrometry

Scientia Pharmaceutica

Article

Development of a Derivatization Method for Investigating Testosterone and Dehydroepiandrosterone Using Tandem Mass Spectrometry in Saliva Samples from Young Professional Soccer Players Pre- and Post-Training

Mansour A. Alzahrani [1,*], Ghareeb O. Alshuwaier [2], Khalid S. Aljaloud [2], Colin Gibson [3], Abedawn Khalaf [3], Aliyah S. Alhawiti [1] and David G. Watson [1,*]

[1] Strathclyde Institute of Pharmacy and Biomedical Sciences, University of Strathclyde, The John Arbuthnott Building, 161 Cathedral Street, Glasgow G4 0RE, UK; Aliyah.alhawiti@strath.ac.uk
[2] College of Sports Sciences and Physical Activity, King Saud University, Building 69, Code 11441, Riyadh P.O. Box 1949, Saudi Arabia; galshuwaier@KSA.EDU.SA (G.O.A.); Khaljaloud@KSA.EDU.SA (K.S.A.)
[3] Department of Pure and Applied Chemistry, University of Strathclyde, 295 Cathedral Street, Glasgow G1 1XL, UK; clgibson@virginmedia.com (C.G.); abedawnkhalaf@yahoo.co.uk (A.K.)
* Correspondence: mansour.alzahrani@strath.ac.uk (M.A.A.); D.G.Watson@strath.ac.uk (D.G.W.)

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Abstract: In the last decade, high-performance liquid chromatography/tandem mass spectrometry (LC/MS/MS) combined with electrospray ionization (ESI) has been widely used for determining low concentrations of steroids, and derivatization has often been employed to enhance detection. In the present study, endogenous steroids were extracted using a Strata-XL polymeric reverse phase cartridge. The isolated steroids were reacted with 2-hydrazino-1-methylpyridine (HMP) at 50 °C for 30 min. A liquid chromatography-tandem mass spectrometry (LC-MS/MS) was used in a positive mode with multiple reaction monitoring (MRM) for the quantification of testosterone (T) and its precursor, dehydroepiandrosterone (DHEA), in saliva samples collected from twenty young Saudi professional soccer players. The analytes were separated on an ACE Ultracore 2.5 Superphenylhexyl column (150 × 3.0 mm id). The extraction recovery during the pre-treatment was >89% and gave <±20% for inter- and intra-assay precision and accuracy. The limits of quantification (LOQ) were found to be 20 pg/mL for (T and DHEA) and 50 pg/mL for Epitestosterone (EPI). The results showed no significant variation in the concentration of T between pre and post training, whereas DHEA was significantly increased after short-term exercise. These results could indicate that there is no correlation between T and its precursor DHEA level following short term physical activity. EPI concentrations could not be detected with a LOQ of 50 pg/mL in the saliva samples.

Keywords: testosterone; dehydroepiandrosterone; epitestosterone; 2-hydrazino-1-methylpyridine; liquid chromatography; mass spectrometry

1. Introduction

Testosterone (T) is a primary androgen hormone and an anabolic steroid that is secreted by the testicles of males and the ovaries of females. It stimulates the development of male characteristics by binding to androgen receptors to exert its action [1]. Dehydroepiandrosterone (DHEA) is the precursor of testosterone and other steroids and is produced by the adrenal glands [2]. Epitestosterone (EPI) is an inactive 17 alpha-epimer of testosterone [3]. A correlation between T and DHEA in biological samples has not been confirmed. Some diseases may be related to decreased levels of DHEA in serum,

Appendices 6 for Chapter 3 (paper): Plasma Free Fatty Acids Metabolic Profile with LC-MS and Appetite-Related Hormones in South Asian and White European Men in Relation to Adiposity, Physical Activity and Cardiorespiratory Fitness: A Cross-Sectional Study

Article

Plasma Free Fatty Acids Metabolic Profile with LC-MS and Appetite-Related Hormones in South Asian and White European Men in Relation to Adiposity, Physical Activity and Cardiorespiratory Fitness: A Cross-Sectional Study

Simone Benedetti [1], Naser F. Al-Tannak [2], Mansour Alzharani [3], Hannah J. Moir [1], David J. Stensel [4,5], Alice E. Thackray [4,5], Declan P. Naughton [1], Mehmet T. Dorak [1], Owen Spendiff [1], Natasha Hill [1], David G. Watson [3,*] and Judith Allgrove [1,*]

1 Applied & Human Sciences, School of Life Sciences, Pharmacy & Chemistry, Kingston University London, Kingston upon Thames KT1 2EE, UK; S.Benedetti@kingston.ac.uk (S.B.); H.Moir@kingston.ac.uk (H.J.M.); D.Naughton@kingston.ac.uk (D.P.N.); M.Dorak@kingston.ac.uk (M.T.D.); O.Spendiff@kingston.ac.uk (O.S.); N.Hill@kingston.ac.uk (N.H.)
2 Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Kuwait University, P.O. Box 23924, Safat, Kuwait City 13110, Kuwait; dr_altannak@HSC.EDU.KW
3 Strathclyde Institute of Pharmacy and Biomedical Sciences, University of Strathclyde, The John Arbuthnott Building, 161 Cathedral Street, Glasgow G4 0RE, UK; mansour.alzahrani@strath.ac.uk
4 National Centre for Sport and Exercise Medicine, School of Sport, Exercise and Health Sciences, Loughborough University, Loughborough LE11 3TU, UK; D.J.Stensel@lboro.ac.uk (D.J.S.); A.E.Thackray@lboro.ac.uk (A.E.T.)
5 University Hospitals of Leicester NHS Trust, Infirmary Square, Leicester LE1 5WW, UK
* Correspondence: d.g.watson@strath.ac.uk (D.G.W.); J.Allgrove@kingston.ac.uk (J.A.); Tel.: +44-141-54-82-651 (D.G.W.); +44-208-41-77-360 (J.A.)

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Abstract: South Asians have a greater cardiovascular disease (CVD) and type 2 diabetes (T2D) risk than white Europeans, but the mechanisms are poorly understood. This study examined ethnic differences in free fatty acids (FFAs) metabolic profile (assessed using liquid chromatography-mass spectrometry), appetite-related hormones and traditional CVD and T2D risk markers in blood samples collected from 16 South Asian and 16 white European men and explored associations with body composition, objectively-measured physical activity and cardiorespiratory fitness. South Asians exhibited higher concentrations of five FFAs (laurate, myristate, palmitate, linolenic, linoleate; $p \leq 0.040$), lower acylated ghrelin (ES = 1.00, $p = 0.008$) and higher leptin (ES = 1.11, $p = 0.004$) than white Europeans; total peptide YY was similar between groups ($p = 0.381$). South Asians exhibited elevated fasting insulin, C-reactive protein, interleukin-6, triacylglycerol and ratio of total cholesterol to high-density lipoprotein cholesterol (HDL-C) and lower fasting HDL-C (all ES ≥ 0.74, $p \leq 0.053$). Controlling for body fat percentage (assessed using air displacement plethysmography) attenuated these differences. Despite similar habitual moderate-to-vigorous physical activity (ES = 0.18, $p = 0.675$), $\dot{V}O_{2max}$ was lower in South Asians (ES = 1.36, $p = 0.001$). Circulating FFAs in South Asians were positively correlated with body fat percentage ($r^2 = 0.92$), body mass ($r^2 = 0.86$) and AUC glucose ($r^2 = 0.89$) whereas in white Europeans FFAs were negatively correlated with total step counts ($r^2 = 0.96$). In conclusion, South Asians exhibited a different FFA profile, lower ghrelin, higher leptin, impaired CVD and T2D risk markers and lower cardiorespiratory fitness than white Europeans.

Keywords: metabolomics; free fatty acids; cardiovascular disease; Type 2 diabetes; South Asian; physical activity; cardiorespiratory fitness; inflammation; appetite hormones; metabolic markers

Appendices 7 for Chapter 4: (poster) Metabolomic Profiling of Plasma, Urine and Saliva in short Exercise at a Standardised Relative Intensity in young professional football players in Saudi Arabia

Metabolomic Profiling of Plasma, Urine and Saliva in short Exercise at a Standardised Relative Intensity in young professional football players in Saudi Arabia

*Mansour Alzahrani [1], Ghareeb O Alshuwaier [2], Khalid S Aljaloud [2], and David G. Watson[1]**

[1] School of Psychological & Health Sciences, Room 538, Graham Hills Building, University of Strathclyde, Glasgow, G1 1QE, U.K,

[2] College of Sport Sciences and Physical Activity, King Saud University, Building 69, Code 11441, P.O. Box 1949, Riyadh KSA.

Correspondence: D.G.Watson@strath.ac.uk

Background

Regular physical exercise attenuates disease risk, improves wellbeing and prolongs life expectancy [1]. Understanding of the metabolomics effects of physical exercise and how they correlate with physical performance could give insights into comprehensive and individualised healthcare plans to promote wellbeing.

Aims

To determine the metabolic changes due to extreme exercise in order gain some insight into how metabolism is adapted for endurance performance.

Materials & Methods

- **Participants**: Plasma, Urine and Saliva samples collected from more than 21young Saudi soccer players that conducted in Riyadh by King Saud University, College of Sports Science and Physical Activity, exercise physiology department with a mean (±SD): age 20.6±1.4 years; body mass 70.2±1.6 kg, and BMI 20.6±1.4 kg/m^2.
- **Time points:** samples collected pre and post- training over two days during the training sessions.
- **Sample prep:** Test and pooled samples extracted with acetonitrile containing 2 ^{13}C glycine as internal standard for retention time locking; centrifugation to obtain supernatant.
- **Standards:** Authentic standards from Sigma-Aldrich (Dorset, UK) spiked with 2 ^{13}C glycine internal standard.
- **LC-MS:** Accela HPLC + Exactive Orbitrap MS from Thermo Fisher (Bremen, Germany); columns: ZIC-pHILIC (150 × 4.6 mm, 5 µm) & ACE C4 (150 × 3.0 mm, 3 µm) both from HiChrom Reading UK; MP: 20mM $(NH_4)_2CO_3$ in water and acetonitrile (HILIC) and 1mM acetic acid in water and 1mM acetic acid in acetonitrile (Reversed phase).

Results

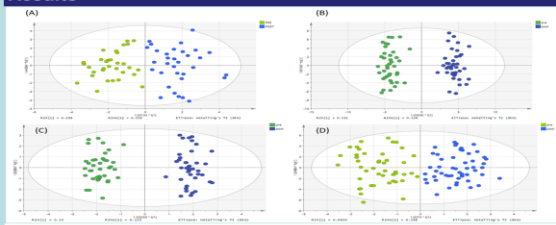

Fig 1. OPLS-DA score plots the samples according to their classification coloured based on their group (green) represent the pre-training samples while (blue) represent the post training. Model fitted based on(A) 18 putative metabolites of plasma samples, analysed on an ACE C4 column (B) 93 putative metabolites of plasma samples, analysed on a ZIC-pHILIC column, (C) 55 putative metabolites of urine samples, analysed on a ZIC-pHILIC column, (D) 67 putative metabolites of saliva samples, analysed on a ZIC-pHILIC column, the data was Pareto scaled and log 2.

Observation 1: Exercise induced a large number of metabolic changes with many amino acids slightly increasing in abundance also there were almost (½ fold) increases in the levels of many carnitines, fatty acids, carbohydrate and nucleotide.

Metabolites	Effect of physical activity	Probable Interpretation
Amino acids	Slightly decreased in urine and increase in plasma and saliva	Protein synthesis
carnitines	Increased in plasma and slightly decrease in urine	Muscle metabolism
fatty acids	Increased in plasma using C4 column	Muscle metabolism
Carbohydrate	Increase in plasma	Produce energy
Purine, pyrimidine	Slightly increased in plasma and saliva and slightly decrease in urine	Purine conservation

Table 1: Summary of the major metabolic changes induced by the short training event. Clearest differences observed was in plasma in amino acids, acylcarnitines, and fatty acids.

Conclusions

- The amino acids used in protein biosynthesis might be due to an increase in protein biosynthesis during exercise [2].
- The marked elevations in acylcarnitine levels suggest that these might provide a source of energy ready for export to mitochondria in muscle cells and they may also regulate exertion via action on neurons [3].
- The main fuel for moderate intensity exercise are carbohydrate and fatty acid that converted to produce ATP .
- Thus the pattern of fatty acids and carnitines observed in the current study points strongly towards a large increase in peroxisomal metabolism providing acetyl carnitine as a fuel [4].

Acknowledgements

- Appreciation goes to the Saudi Cultural Bureau for financial support towards Mansour ALzahrani's PhD studies which enabled him to undertake this research.

References

1. Daskalaki, E. *et al*. The Application of Metabolomic Profiling to the Effects of Physical Activity. *Curr. Metabolomics* 2015, 2, 233–263.
2. Walker, D.K. *et al*. Exercise, amino acids and aging in the control of human muscle protein synthesis. *Medicine and Science in Sports and Exercise* 2011, 43, 2249.
3. Zhang, J. *et al*. Acylcarnitines as markers of exercise-associated fuel partitioning, xenometabolism, and potential signals to muscle afferent neurons. *Experimental Physiology* 2017, 102, 48-69.
4. Wanders, R.J.; Waterham, H.R. Biochemistry of mammalian peroxisomes revisited. *Annu. Rev. Biochem*. 2006, 75, 295-332..

Appendices 8 for Chapter 4: PCA scores plots for QC (pooled) extract samples

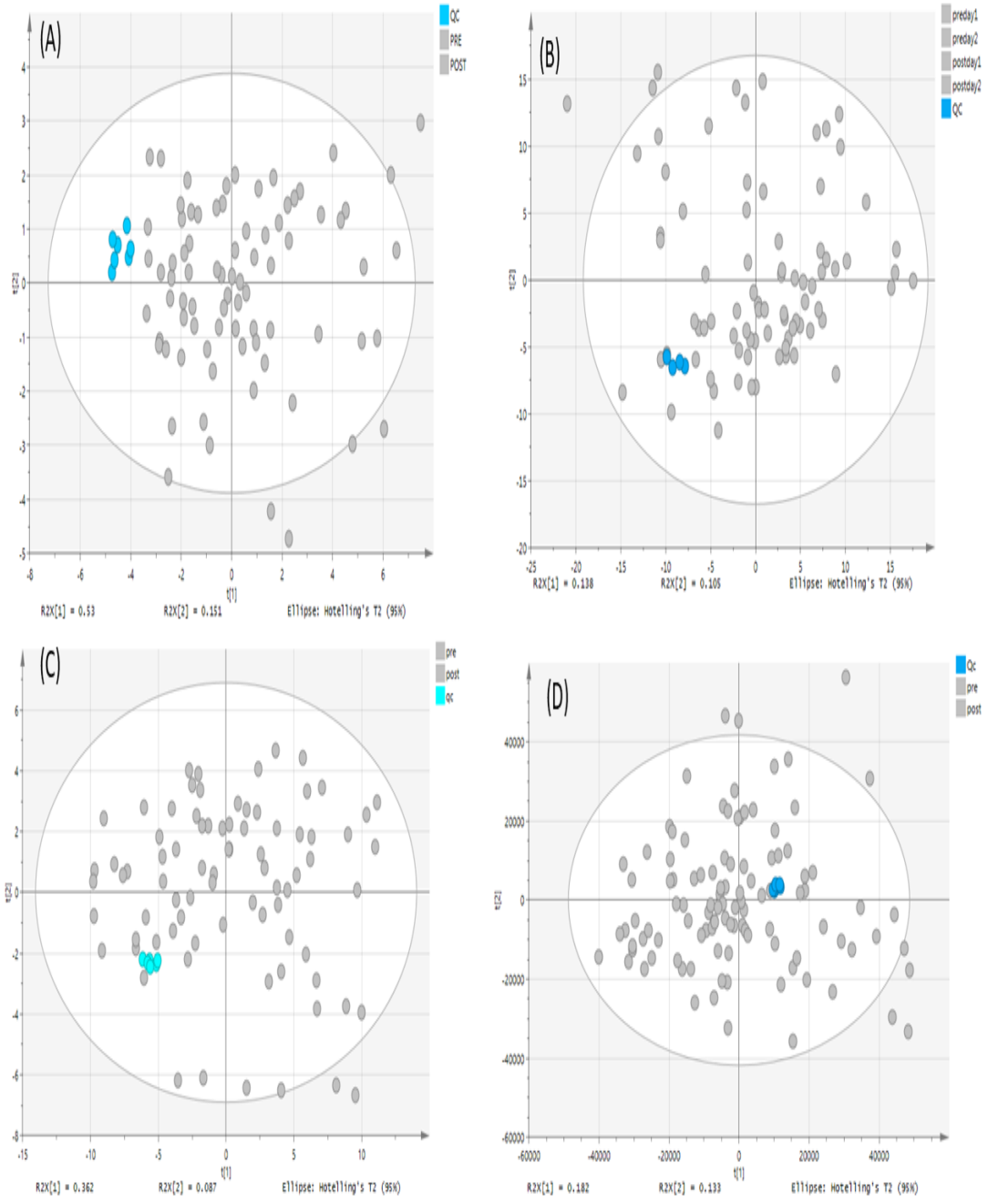

Figure 4-S1: PCA scores plots for QC (pooled) extract samples of **(A)** plasma (n=7), analysed on an ACE C4 column. **(B)** plasma (n=6), analysed on a ZIC-pHILIC column. **(C)** urine (n=5), analysed on a ZIC-pHILIC column. **(D)** Saliva (n=6), analysed on a ZIC-pHILIC column. The plots show the clustering of pooled samples (QC) compared to the rest of the samples (grey-No class), the data was Pareto scaled.

Appendices 9 for Chapter 4: validation of all samples and significant metabolites of day 2

1- PLASMA analysed on a C4 column

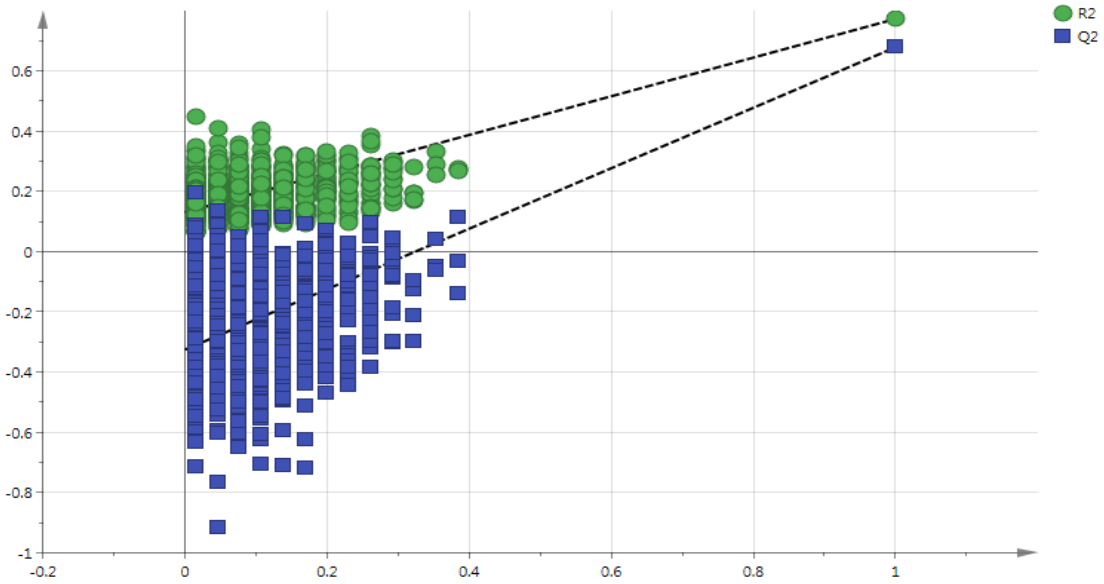

figure 4-S2 Permutations test. The plot shows, for a selected Y-variables, on the vertical axis the values of R2 and Q2 for the original model (far to the right) and of the Y-permuted models further to the left. The horizontal axis shows the correlation between the permuted Y-vectors and the original Y-vector for the selected Y. The original Y has the correlation 1.0.

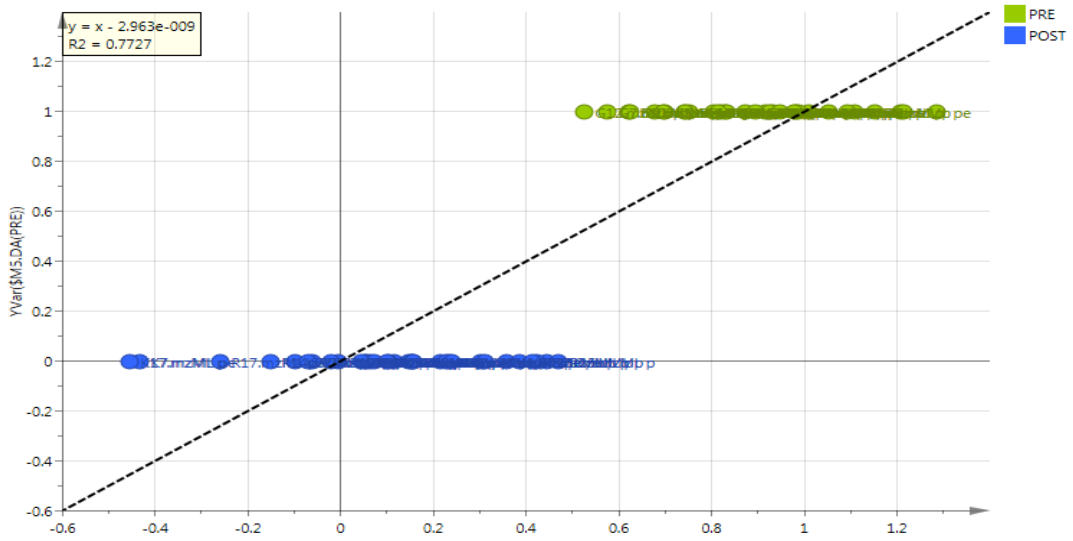

figure 4-S3 The plot displays the observed (y-axis) versus predicted (x-axis) values of the selected Y-variable of the model. The R2 of the regression line indicates the goodness of Fit = 0.77.

1- PLASMA analysed on a ZIC-pHILIC column

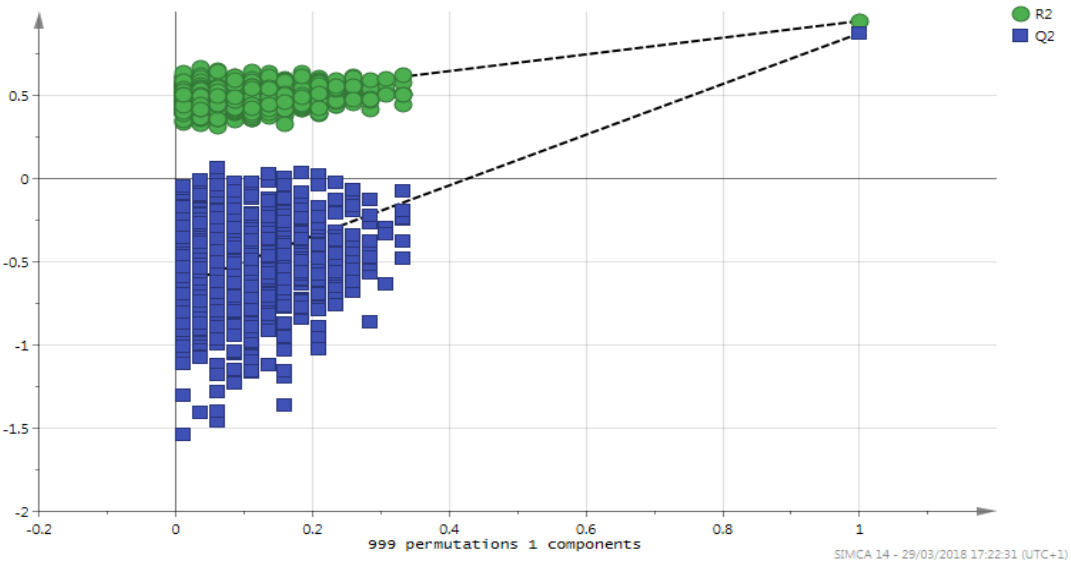

figure 4-S4 Permutations test. The plot shows, for a selected Y-variables, on the vertical axis the values of R2 and Q2 for the original model (far to the right) and of the Y-permuted models further to the left. The horizontal axis shows the correlation between the permuted Y-vectors and the original Y-vector for the selected Y. The original Y has the correlation 1.0

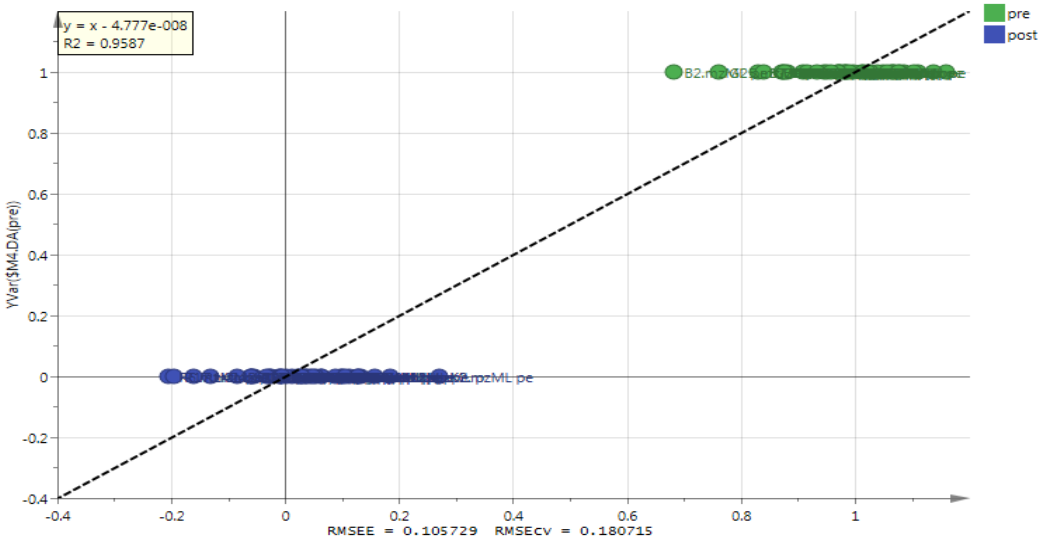

figure 4-S5 The plot displays the observed (y-axis) versus predicted (x-axis) values of the selected Y-variable of the model. The R_2 of the regression line indicates the goodness of Fit = 0.95.

2- Urine analysed on a ZIC-pHILIC column

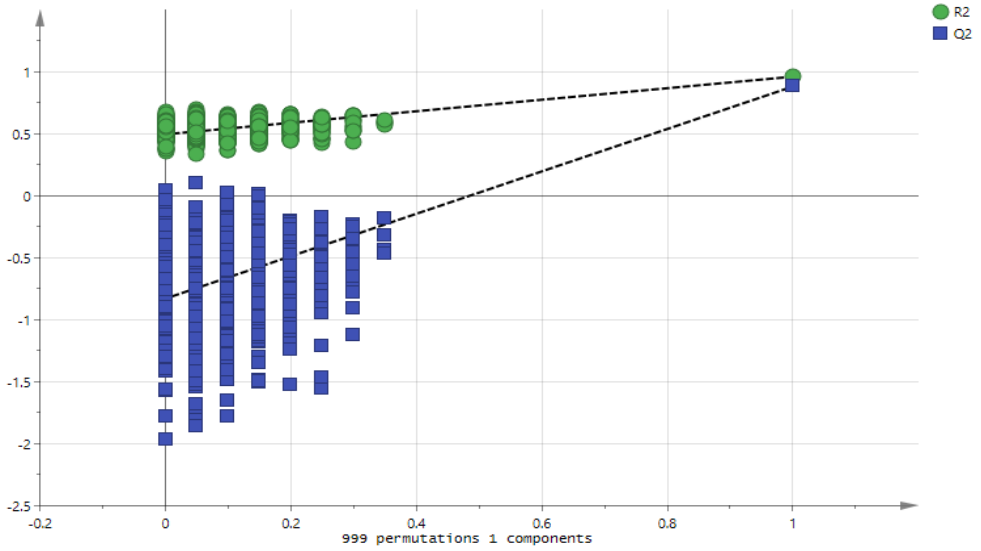

figure 4-S6 Permutations test. The plot shows, for a selected Y-variables, on the vertical axis the values of R2 and Q2 for the original model (far to the right) and of the Y-permuted models further to the left. The horizontal axis shows the correlation between the permuted Y-vectors and the original Y-vector for the selected Y. The original Y has the correlation 1.0

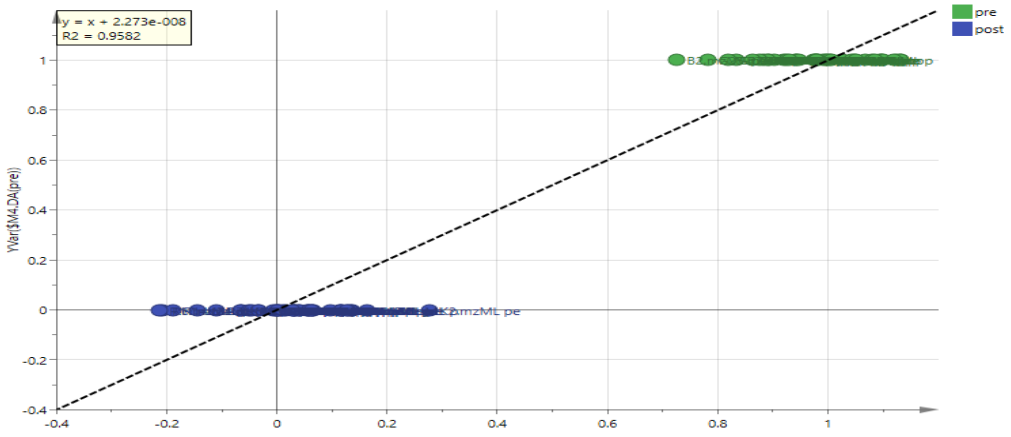

figure 4-S7 The plot displays the observed (y-axis) versus predicted (x-axis) values of the selected Y-variable of the model. The R_2 of the regression line indicates the goodness of Fit = 0.95.

3- Saliva analysed on a ZIC-pHILIC column

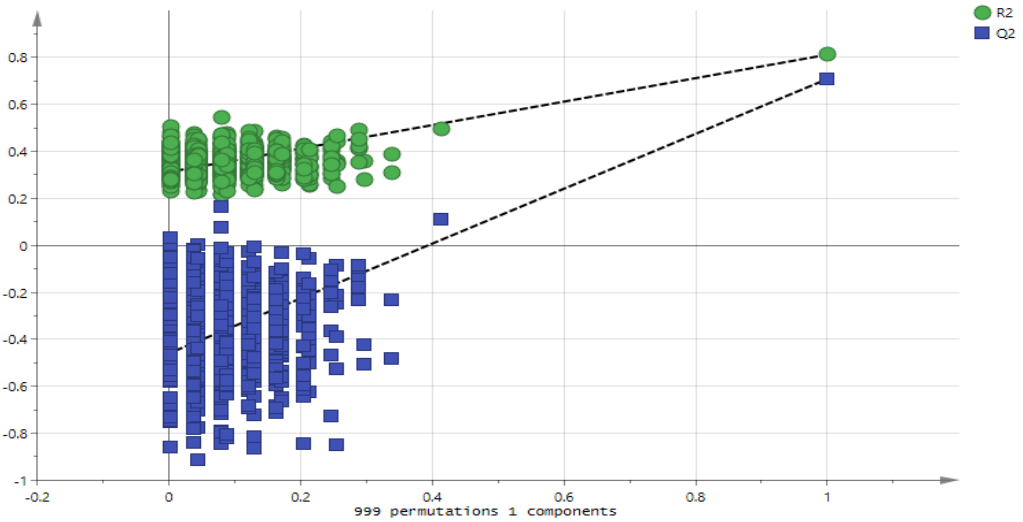

figure 4-S 8 Permutations test. The plot shows, for a selected Y-variables, on the vertical axis the values of R2 and Q2 for the original model (far to the right) and of the Y-permuted models further to the left. The horizontal axis shows the correlation between the permuted Y-vectors and the original Y-vector for the selected Y. The original Y has the correlation 1.0

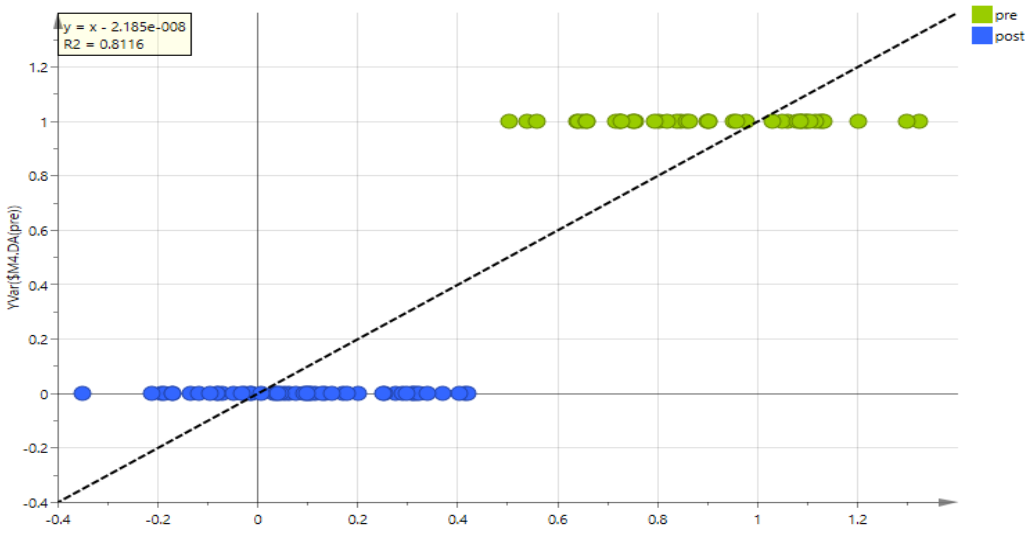

figure 4-S9 The plot displays the observed (y-axis) versus predicted (x-axis) values of the selected Y-variable of the model. The R2 of the regression line indicates the goodness of Fit = 0.81,.

Table 4-S 1 All the metabolites affected significantly by the short-term training trial in the day 2 (p-value < 0.05) or fold change >2 or <0.5. * Matches retention time of standard. ‡ Data from runs on ACE C4 column, otherwise run on the pHILIC column.

Mode	m/z	RT(min)	Putative Metabolite	Urine		Plasma		Saliva	
				Ratio	p-Value	Ratio	p-Value	Ratio	p-Value
		Purine metabolism							
+	136.061	8.7	Adenine			1.354	0.015	1.314	0.032
+	137.046	9.7	Hypoxanthine *	0.514	0.001	0.758	0.046		
-	151.026	11.8	Xanthine			1.376	<0.001		
+	252.108	7.7	Deoxyadenosine					1.311	0.021
+	253.093	8.3	Deoxyinosine	1.042	0.024			1.534	0.048
	298.096	6.5	methylthioadenosine			1.481	0.003		
		Arginine and proline metabolism							
-	104.071	13.9	4-Aminobutanoate *	1.335	0.059	0.832	0.025		
-	112.052	9.4	Creatinine			1.176	<0.001		
+	133.097	23.5	Ornithine *			1.415	<0.001	1.956	<0.001
+	146.092	14.9	4-Guanidinobutanoate					0.446	0.008
+	146.046	15.1	Glutamate *			1.259	0.015		
-	176.103	15.9	L-Citrulline *			1.292	0.002		
+	247.14	13.9	N-(Carboxyethyl) arginine			1.635	0.001		
		Pyrimidine metabolism							
+	112.051	8.8	Cytosine			1.134	0.039	1.406	0.02
-	113.036	7.2	Dihydrouracil			0.841	0.013		
+	127.050	13.2	Thymine			1.211	0.006	2.161	<0.001
+	243.097	12.4	Thymidine					1.384	0.01

Mode	m/z	RT(min)	Putative Metabolite	Urine		Plasma		Saliva	
				Ratio	p-Value	Ratio	p-Value	Ratio	p-Value
		Carnitine metabolism							
+	204.123	10.4	Acetylcarnitine *			2.269	<0.001		
+	218.138	9.1	Propionylcarnitine	1.078	0.026				
+	248.149	10.8	Hydroxybutyrylcarnitine			2.209	<0.001		
+	274.201	6.3	Heptanoylcarnitine			1.692	0.003		
+	302.232	5.1	dimethylheptanoylcarnitine			1.988	0.001		
+	312.217	5.1	Decadienoylcarnitine			1.101	0.032		
+	314.232	4.9	Decenoylcarnitine *			1.632	0.001		
+	330.227	5.1	Keto-decanoylcarnitine			1.597	<0.001		
+	342.264	9.1	Dodecenoylcarnitine *			1.875	0.001		
+	360.274	5.1	2-Hydroxylauroylcarnitine			1.594	0.006		
+	384.270	4.8	Hydroxytetradecadiencarnitine			1.658	<0.001		
+	386.290	4.7	Hydroxytetradecenoylcarnitine			1.700	0.001		
		Citrate cycle (TCA cycle)/acyllyadenineis							
-	115.004	13.5	Fumarate *			1.178	0.037		
-	145.014	15.9	2-Oxoglutarate *			1.164	0.048		
		Pentose phosphate pathway							
-	151.061	12.9	Xylitol or isomer *			1.153	0.048		
-	193.036	16.8	Glucuronate or isomer *			1.253	0.011		
		Glycine, serine, uratCystiene and threonine metabolism							
+	103.039	12.9	2-Oxobutanoate			0.796	0.007		
-	106.050	15.9	Serine *			0.872	0.031		

Mode	m/z	RT(min)	Putative Metabolite	Urine		Plasma		Saliva	
				Ratio	p-Value	Ratio	p-Value	Ratio	p-Value
-	116.035	10.5	L-2-Amino-3-oxobutanoic acid			1.665	0.001		
+	120.065	11.3	Threonine *			1.384	0.018		
		Lysine metabolism							
-	126.056	7.2	2,3,4,5-Tetrahydropyridine-2-carboxylate			1.271	0.034		
+	146.081	14.6	hexanoic acid					0.465	0.017
+	162.112	13.3	L-Carnitine *			2.745	<0.001		
+	204.086	14.2	N2-Acetyl-L-aminoadipate			1.438	<0.001		
+	205.118	10.3	N6-Acetyl-N6-hydroxy-L-lysine			1.239	0.044		
+	219.133	14.4	Carboxyethyllysine			1.557	0.026	1.711	0.005
		Histidine metabolism							
+	138.043	8.8	Urocanate *			1.425	0.008		
+	141.066	9.7	Methylimidazoleacetic acid			1.430	0.020		
+	156.076	15.4	L-Histidine	1.646	0.046				
+	170.092	12.8	methylhistidine					0.520	0.011
		Tryptophan metabolism							
+	118.065	10.1	Indole *					1.503	0.008
+	161.107	10.1	Tryptamine					2.021	0.002
+	177.102	13.4	Serotonin					1.580	0.002
+	192.065	10.4	Hydroxyindoleacetate			0.876	0.049		
-	204.067	8.1	Indolelactate					1.551	0.001
-	209.092	10.4	Kynurenine *			0.863	0.049		
-	219.077	8.9	5-Hydroxy-L-tryptophan *					2.158	<0.001

Mode	m/z	RT(min)	Putative Metabolite	Urine		Plasma		Saliva	
				Ratio	p-Value	Ratio	p-Value	Ratio	p-Value
		Tyrosine metabolism							
-	179.035	8.3	Hydroxyphenylpyruvate			1.424	0.002		
-	181.051	9.5	Hydroxyphenyllactate			1.361	0.005		
+	182.081	12.3	L-Tyrosine *					1.449	0.002
		Valine, leucine and isoleucine degradation							
-	115.040	4.8	3-Methyl-2-oxobutanoic acid			0.765	0.005		
-	129.056	4.3	Methyl-oxopentanoic acid			0.718	0.001		
		Phenylalanine metabolism							
+	122.096	4.7	Phenethylamine						
+	123.044	13.3	Benzoate					1.545	0.002
+	136.075	13.3	Phenylacetamide					1.501	0.002
+	149.059	10.1	Cinnamate					1.394	0.020
-	166.086	10.1	Phenylalanine *					1.537	0.004
		Methionine metabolism							
+	178.089	5.3	dihomomethionine			1.385	0.001		
		Alanine and aspartate metabolism							
+	161.092	11.2	D-Alanyl-D-alanine			0.851	0.050		
-	225.099	11.7	Carnosine *					2.570	0.001
		Fatty acids and metabolites [C4]							
-	131.071	2.0	Hydroxyhexanoic acid* ‡			1.213	0.038		
-	227.202	15.9	Tetradecanoic acid ‡			1.763	0.008		
-	241.218	17.4	pentadecanoic acid * ‡			1.243	0.004		

Mode	m/z	RT(min)	Putative Metabolite	Urine		Plasma		Saliva	
				Ratio	p-Value	Ratio	p-Value	Ratio	p-Value
-	253.218	16.8	Hexadecenoic acid ‡			2.158	0.002		
-	255.233	18.8	Hexadecanoic acid isomer ‡			1.465	0.001		
-	281.249	19.5	octadecenoic acid ‡			1.992	0.001		
		Miscellaneous							
+	160.108	14.9	Guanidinovaleric acid			0.649	0.002		
-	259.022	15.8	D-Glucose 1-phosphate *			1.41	0.002		
+	345.139	12.3	Melibiitol			1.304	<0.001		
-	464.302	5.5	Glycocholate *			0.337	0.003		