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Grau de Química

Treball Final de Grau

Preparation of reference materials for anti-doping control: study of the lyophilisation conditions.

Preparación de materiales de referencia en el control anti-dopaje: estudio de las condiciones de liofilización.

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Sorprendernos por algo es el primer paso de la mente hacia el descubrimiento.

Louis Pasteur

Me gustaría agradecer primero de todo a Rosa Ventura, Rosa Bergés y Àngels Sahuquillo por el apoyo dado para realizar este proyecto. A Sonia por enseñarme con tanta paciencia. A mis compañeros de despacho Georgina, Argi, Ali, Àlex, Sergi, Aleix y Eli y a todos mis compañeros de IMIM porque sin ellos este proyecto no habría sido igual. A mi pareja, familia y amigos por apoyarme incondicionalmente durante estos meses.

REPORT

CONTENTS

1. SUMMARY

Urine reference materials used in interlaboratory comparison must be homogeneous and stable over time. The lyophilisation process is one of the methods applied when preparing urine reference materials for ensuring stability during distribution, transport and storage. Urine samples of 11- nor- Δ9- tetrahydrocannabinol- 9- carboxylic acid (THC-COOH), Benzoylecgonine (BE), Ethyl glucuronide/Ethyl sulphate (EtG/EtS) and ethanol (EtOH) were prepared from blank urine previously filtered. Samples were fortified with the adequate standard solutions and they were divided in aliquots. Aliquots stored at 4 ºC and lyophilized aliquots were used to evaluate the homogeneity of the samples. Others were frozen at -20 ºC and they were used as reference aliquots.

In the first analysis, all prepared samples, except EtOH sample, were considered homogeneous. Concentration differences of THC-COOH and BE between lyophilized aliquots and reference aliquots were minimum (lower than 5 %). However, differences higher than 8 % were observed for EtG/EtS. If these differences were maintained constant in subsequence quantifications, they could be attributed to the evaporation of the analytes during the lyophilisation process. Results obtained for EtOH sample show a completely loss of the analyte due to its high volatility.

Keywords: Drugs of abuse, lyophilized urine, doping, GC/MS, LC/MS-MS

2. RESUMEN

En los ejercicios interlaboratorio deben utilizarse materiales de referencia que sean homogéneos y estables a lo largo del tiempo. El proceso de liofilización es uno de los métodos utilizados para preparar materiales de referencia de orina y asegurar su estabilidad durante su distribución, transporte y almacenaje. Se prepararon muestras de orina conteniendo: ácido 11-nor- Δ9- tetrahidrocannabinol- 9- carboxilico (THC-COOH), Benzoilecgonina (BE), Etil glucurónido/Etil sulfato (EtG/EtS) y Etanol (EtOH), a partir de un blanco de orina previamente filtrado. Las muestras se fortificaron con las soluciones estándar adecuadas y se dividieron en alícuotas. Las alícuotas almacenadas a 4 ºC y las liofilizadas se utilizaron para evaluar la homogeneidad de las muestras. Otras se congelaron a -20 ºC y se utilizaron como alícuotas de referencia.

En el primer análisis se observó que para los analitos estudiados, excepto para el EtOH, las muestras eran homogéneas. Los diferencias en concentración de THC-COOH y BE de las alícuotas liofilizadas respecto las de referencia fueron mínimas (menores al 5 %). En cambio, para EtG/EtS se observaron diferencias superiores al 8 %. Si estas diferencias se mantuvieran constantes en cuantificaciones posteriores, éstas se podrían atribuir a la evaporación de los analitos durante el proceso de liofilización. Los resultados obtenidos para la muestra de EtOH revelan una completa pérdida del analito durante la liofilización debido a la gran volatilidad de éste.

Palabras clave: Drogas de abuso, orina liofilizada, dopaje, CG/EM, CL/EM-EM

3. INTRODUCTION

Nowadays, to work as a laboratory of anti-doping is essential to be accredited by ISO 17025 and by WADA (World Anti-Doping Agency)[1].Currently, there are 32 laboratories around the world accredited by WADA to conduct human doping control sample analyses. While most WADA-accredited laboratories can also perform blood analyses in support of the haematological module of the Athlete Biological Passport (ABP), there are currently an additional 5 approved laboratories (non WADA-accredited laboratories) that are also permitted to conduct such analyses[1]. In Spain, there are two accredited laboratories, Madrid Anti-Doping Laboratory and Fundació Institut del Mar d'Investigacions Mèdiques (IMIM)[2] where this project has been performed.

Participation in interlaboratory comparisons and the regular use of reference material are two of the requirements of testing laboratories to assure the quality of analytical results according to international quality standards such as ISO 17025. It is important to verify homogeneity and drug stability from production until end-use to be sure that differences in results between laboratories are not related to drug instability or the lack of sample homogeneity.

3.1. HISTORY OF ANTI-DOPING

The word *doping* is probably derived from the Dutch word *dop*, the name of an alcoholic beverage made of grape skins used by Zulu warriors in order to enhance their prowess in battle. The term became current around the 20th century. The practice of enhancing performance through foreign substance or other artificial means, however, is as old as competitive sport itself.

In 1928 the IAAF (International Association of Athletics Federations) became the first International Sport Federation (IF) to ban doping, the use of stimulating substances. Many other International Sport Federations followed suit, but restrictions remained ineffective as no tests were performed.

The death of Knud Enemark Jensen (Danish cyclist) during competition at the Olympic Games in Rome in 1960 increased the pressure for sports authorities to introduce drug test, due to the autopsy revealed traces of amphetamine.

In 1966 *Union Cycliste Internationale* (UCI) and *Fédération Internationales de Football Association* (FIFA) were among the first IFs to introduce doping tests in their respective World Champions. In the next year the International Olympic Committee (IOC) instituted its Medical Commission and set up its first list of prohibited substances[1].

3.1.1. Doping

Doping in general is defined as the use of substances or forbidden methods in the sport. However, since the introduction of the international code of WADA, the concept is applied as the occurrence of one or more of the anti-doping rule violations.

WADA defines doping as the fact of violate any of these anti-doping rules[3]:

1. Presence of a prohibited substance or its metabolites or markers in an athlete's sample.
2. Use or attempted use by an athlete of a prohibited substance or a prohibited method.
3. Evading, refusing or failing to submit sample collection.
4. Whereabouts failures.
5. Tampering or attempted tampering with any part of doping control.
6. Possession of a prohibited substance or a prohibited method.
7. Trafficking or attempted trafficking in any prohibited substance or prohibited method.
8. Administration or attempt administration to any athlete in-competition of any prohibited substance or prohibited method, or administration or attempted administration to any athlete out-of-competition of any prohibited substance or any prohibited method that is prohibited out-of-competition.
9. Complicity.
10. Prohibited association.

Doping substances can be classified in different groups; some of them are listed below (see **Table 1**):

Steroids	Substances that influencing the production of amino acids, contribute to increase muscle mass, strength and aggressiveness.
Stimulants	Substances are used to be more awake and to delay the fatigue.
Narcotic analgesic	Substances that relieve pain and get a calming effect.
Beta blockers	Substances that decrease the heartbeat.
Diuretics	Substances used to lose weight quickly.

Table 1. Different groups of substances.

3.2. WORLD ANTI-DOPING AGENCY

The WADA was established in 1999 as an international independent agency composed and funded equally by the sport movement and governments of the world. Its key activities include scientific research, education, development of anti-doping capacities, and monitoring of the World Anti Doping Code- the document harmonizing anti-doping policies in all sports and all countries[1].

WADA works towards a vision of a world where all athletes compete in a doping-free sporting environment.

The First World Conference on Doping in Sport held, in Lausanne, on 1999, produced the Lausanne Declaration on Doping in Sport. This document provided the basis for the creation of an independent international anti-doping agency to be operational for the Games of the XXVII Olympiad in Sydney in 2000.

Pursuant to the terms of the Lausanne Declaration, the World Anti-Doping Agency(WADA) was established on November, 1999, in Lausanne to promote and coordinate the fight against doping in sport internationally[1].

WADA publishes the prohibited list every year, where the prohibited substances in- and out-of-competition and in particular sports are listed along with the prohibited methods too. The 2015 prohibited list[4] of substances is shown below in a summary table (see **Table 2**).

Substances prohibited in- and out-of-competition
Anabolic agents
Peptide hormones, growth factors, related substances and mimetics
Beta-2 agonists
Hormone and metabolic modulators
Diuretics and masking agents
Substances prohibited in-competition
Stimulants
Narcotics
Cannabinoids
Glucocorticoids
Substances prohibited in particular sports
Alcohol
Beta-blockers

Table 2. The 2015 list of prohibited substances.

3.3. THE ANALYTICAL TASK OF AN ANTI-DOPING LABORATORY

IMIM laboratory receives and analyzes different types of matrixes. The most common are urine and blood, from both humans and horses.

The laboratory is usually receiving two individual subsamples of each analysis to be performed, which are independently sealed (A and B). The authorized personal carry out the reception of the samples and assigned the intern code of the laboratory to all them.

If the sample is an athlete's sample, the subsample A is kept sealed in a cold chamber. The sample B is also kept sealed, in a freezer as potential counter-analysis sample.

As soon as possible, the subsample A is inspected. The internal code and the internal information are revised, and some visual characteristics of the sample are registered. Then the subsample is divided in aliquots. Every aliquot will be subjected to a screening analysis of some family or families of substances.

If the result is negative for all the screening procedures, the report of results will be emitted. If the sample is presumably positive to some substances, a new aliquot of the subsample A will be distributed to carry out a specific confirmation procedure. The person responsible has to decide if these results are definitive or if it is necessary to apply new confirmation procedures.

When the results are considered definitive, the report of results is sent to the appropriate sports authority which can request the analysis of the subsample B (counter-analysis). **Figure 1** is summarising the process.

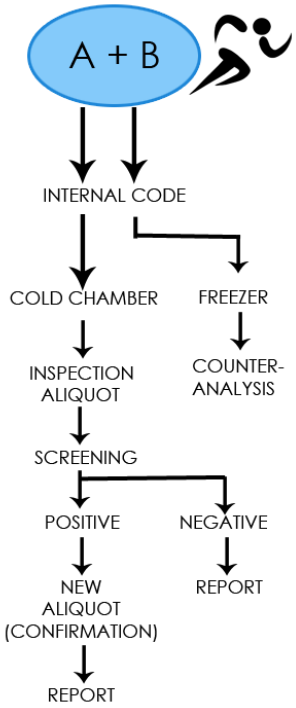


Figure 1. Scheme summarising the process of the reception of samples

The participation in interlaboratory comparisons and the regular use of reference material are two of the requirements of testing laboratories to assure the quality of analytical results according to international quality standards such as ISO 17025. IMIM is coordinator of interlaboratory comparison. IMIM prepare reference materials and it has a control of the quality of the analytical results of the laboratory as every anti-doping laboratory. In some exercise of interlaboratory comparison some problems of instability were detected by IMIM.

During the transport, the reception and the analysis of samples can pass a large period of time. Some irregularities can occur and affect samples. To minimize this and to have control of what can happen to samples during this process, some studies have been carried out for some laboratories to ascertain the stability of various samples in different condition[5]–[14]. Some conditions as for example the storage temperature, the type of containers and studies that simulate the situation of the transport and distribution of the sample have been described in the

literature. For example, the sample is stored at -20ºC, and then is 3 days at room temperature, and then is frozen again.

All these studies are so important in anti-doping control mainly because the presence of prohibited substances in these matrices may involve legal consequences for individuals. In anti-doping control it is also important to ensure that analyte instability will not affect the analytical results during the retesting period[14].

3.4. REFERENCE MATERIALS

3.4.1. Introduction

The growing importance of chemical measurements in modern society has rendered urgently the development of measures to improve the quality of analytical results and to guarantee their quality to the end users.

The objective of the chemicals analysis is to provide information on the composition of materials. If neither losses of information nor increase of the content are achieved, the result can be compared to other results obtained with the same guarantee of quality.

In all analyses, the analyst has to demonstrate the quality of his result and that it leads to reliable- traceable-results. The only way to achieve this demonstration is to compare the result to the value assigned to a known material. Such a material is called a “Reference Material” (RM).

Reference Materials allow the comparison of a real sample to a known material for the identification and the quantification of the substance of interest. Reference materials are used in interlaboratory studies (such as proficiency testing schemes, PTS) or statistical control schemes (control charts).

A reference material, according to the guide ISO 30:2015, is a material sufficiently homogeneous and stable with respect to one or more specified properties, which has been established to be fit for its intended use in a measurement process.

When the property values are certified by a procedure that establishes its traceability to an accurate value with its uncertainty, it becomes a Certified Reference Material (CRM). The use of certified reference material is the easiest way to demonstrate accuracy and to verify the performance of the laboratory at any desired moment.

A certified reference material, according to the guide ISO 30:2015, is a reference material characterized by a metrologically valid procedure for one or more specified properties, accompanied by a reference material certificate that provides the value of the specified property, its associated uncertainty, and a statement of metrological traceability.

So that a material was considered as a certified reference material, it has to accomplish some properties[15]. The most important are:

- Traceability
- Homogeneity
- Stability
- Similarity with the real samples
- Uncertainty

Besides the requirement to use Certified Reference Materials (CRMs) to verify the accuracy of their measurements and validate their methods, accredited laboratories have to monitor the long-term performance of their analytical methods by using non-certified Reference Materials, that is Quality Control Materials (QCMs) .

3.4.2. Methods and tools for reference materials production[16]

For liquid reference materials the handling during production may be subjected to specific problems, such as contamination, evaporation, or leakage. Particles suspended in the liquid will increase the inhomogeneity, and filtration should be applied.

Some issues that are important to produce a reference material are listed below:

- Contamination control: the purpose of any material is that the compounds of interest, the analytes, are present in about the same concentration range as in the sample that is analyzed in a routine operation. In reference material production, the procedures should be established to prevent or at least minimize contamination.
- Bulk sampling: the mass of the bulk material depends on the required number of units, losses during processing and losses during bottling.
- Pretreatment for the handling of liquids:
    - Pretreatment: it is important to have controlled the temperature to minimize degradation of organic or organo-metallic compounds. Oxidation of the matrix or analyte can create a problem, forcing operation and storage to be

carried out under a protective atmosphere when necessary. For similar reasons the liquids should be kept out of direct sun light, minimizing photo-oxidation.

- Filtration: Suspended particles most probably have typical different contents of analyte, and as a consequence they are a serious source of inhomogeneity. Therefore, when liquid contain particulate matter they are best filtered before homogenization.
- Homogenisation: Mechanical stirring is a first option. Mixing time should be the main parameter. Purging by nitrogen or argon can be also a way of homogenization.
- Containers: liquid reference materials can be contained in bottles or vials. They can be made of glass, quartz or artificial material. Various types of stoppers are available. The most common types are made of rubber with or without a thin Teflon layer.
- Bottling of liquids: The first litres from the system are discarded, as they are used to clean the pump and tubing system.
- Labelling: the text on the label of reference material should indicate the name of the product, a reference material name or number and an individual sample identification number. It should give information on the composition and purpose.
- Transport and storage: Depending on the expected stability of the material or the analyte, the reference material has to be stored under the appropriate conditions. For example, temperature, humidity and light should be controlled, if necessary.

3.4.3. Testing[16]

In order to assess the main properties of a candidate reference material, there are different properties to be tested. The most important are explained below.

3.4.3.1. Homogeneity testing

After the bottling has been completed, the units should be tested for the homogeneous distribution of the analytes, also called the specific homogeneity, both for between units and

within units. For reference materials that are produced to be used in total, so called “single shot”, only the between-unit homogeneity is tested.

Nearly every material is at least to some extent inhomogeneous. It is therefore necessary to check (using a reasonable number of samples) if the differences between units stay within acceptable limits. The method must be selected for its repeatability (precision), not its trueness (accuracy, absence of bias). If it is possible, all samples are to be measured on the same day, with the same instrument, by the same operator, against the same calibrants, that is under repeatability conditions.

If the material is not prepared in portions for single use but in larger units from which test portions are to be taken, it is necessary to check if all sub-samples from one unit are identical in terms of the properties of interest.

To evaluate the homogeneity, an F-test comparing variances of between and within units (random error) is performed.

$F_{CALC.} > 1$, $F_{CALC.} = \frac{S^2 STUDIED\ FACTOR}{S^2 RANDOM\ ERROR}$

The material will be considered homogeneous when $F_{CALC.} < F_{CRIT.}$ .Where $F_{CRIT.}$ is a tabulated value.

Another criterion that can be applied to evaluate the homogeneity is the precision Model of Horwitz. This model indicates that the standard deviation of a model varies with the concentration according to the following equation:

$$RSD = 2^{(1-0,5 \cdot logc)}$$

$s_{HORWITZ}$ is obtained applying the relative standard deviation (RSD) obtained with the average value of the concentration.

$$s_{HORWITZ} = (RSD * 2 * \bar{C})/100$$

s_{SAMPLE} is obtained applying the following equations using the values obtained in the ANOVA test:

$$s^2_{SAMPLE} = \frac{s^2 BETWEEN - S^2 WITHIN}{Nº\ REPLICATED}$$

$$s_{SAMPLE} = \sqrt{S^2_{SAMPLE}}$$

Finally, the material will be considered homogeneous when $S_{SAMPLE} / S_{HORWITZ}$ < 0,3 according to *The international harmonized protocol for the proficiency testing of analytical chemistry laboratories*. This condition is called "sufficient homogeneity".

3.4.3.2. Stability testing

The most common approach to stability testing is to expose the reference material to conditions that are likely to lead the degradation or transformation of the analyte.

For stability testing, a sufficiently large selection of bottles is stored under conditions that are expected to accelerate any process of modification or degradation. Assuming now the deteriorating cause is the temperature (as in most cases), samples are stored in parallel at:

- The normal storage temperature
- An elevated temperature (to speed up deterioration processes, and to mimic transport conditions that are less well under control)
- At a temperature where no changes are expected, the so called "safe temperature"

Stability is evaluated by analyzing samples, stored at different condition, as a function of time. The time steps will highly depend on the intended use. Independent analyses are carried out in triplicate for each storage time and temperature.

The measurement method to be applied for the purpose of stability testing should be one with the best available long-term reproducibility. For example, all aliquots of the sample are stored at different conditions of temperature as 4 ºC, -20 ºC. For the t= 3 month result, sample stored at 20 ºC and sample stored at 4 ºC are analyzed. For the t=6 months the same routine is performed. This routine can be performed at 0, 3, 6, 12, 24 and 36 months for example. This method can be applied for different conditions of samples as for example samples stored at 4 ºC, lyophilized samples stored at 4 ºC, samples stored at different temperatures (stored 2 months at 4 ºC, then stored 3 days at room temperature...).

Samples stored at -20ºC act as reference for the samples stored at different temperatures.

The evaluation makes use of the ratio, which is based on the value of 3 determinations made after storage at elevated temperature, divided by the mean value for 3 measurements of samples stored at the safe temperature.

If some changes occur at the safe temperature, the material is not be considered stable. On the other hand, if small changes occur at refrigerator temperatures and significant changes at ambient temperature, it is certain that the reference material will need storage in a freezer, and probably require cooled transport. Finally, if no changes occur at any of these conditions, the material will be considered stable during the self-life of the material.

Some literature about the preparation of reference materials of lyophilized urine samples have been consulted[14], [17], [18]. In these literatures studies of stability during a large period of time, and studies of homogeneity were performed to the preparation of the different reference materials. Conditions of lyophilisation used in literature [14] and [17] were similar in the pressure used and in the temperature reached by the apparatus.

Literature about protocols for stability and homogeneity studies of drugs for its application to doping control has been consulted[13]. In this literature, different protocols were performed which were protocols to study the adsorption of analytes on sterilizing filters, protocols for homogeneity testing and protocols for stability testing. Long-term stability and short-term stability was evaluated in liquid urine stored at different temperatures. Homogeneity was tested with a minimum of 5 aliquots.

3.5. LYOPHILISATION

Freeze-drying, also referred to as lyophilisation, is the process of removing water from a sample by sublimation and desorption[14]. That is to say that lyophilisation is a dehydration process of samples at low pressure, vacuum, and moderated temperature. In this process, the evaporation of water is produced by the sublimation of the ice. For this reason, the samples have must be solidified during the drying[19].

This procedure involves a minimum alteration of the molecular structure of the sample so urine should not be affected by changes in temperature and should ensure its stability[14]. The samples keep all their original characteristics.

The process of lyophilisation is divided in two stages, freezing and drying. The freezing of the samples is the most important step because it may interferes in the appearance and the properties of the final product.

If the freezing is slow, water will create big ice crystals. These crystals will produce open structures after the drying. On the other hand, if the freezing is quickly, water will create small crystals. These crystals will make difficult the escape of water vapour[20].

The principal methods to perform freezing are:

- Refrigeration by direct contact of the sample with a surface or environment cooled.
- Rotational refrigeration in a bath.
    - Spin freezer
    - Shell freezing

The size of the crystals during the solidification will define the appearance and a lot of properties of the final product. Products solidified quickly will have a lightly colouring compared with a product solidified slowly. Also, some properties as concentration and viscosity of the product will influence in the entire process.

Sometimes the cooled product presents a deceptive appearance looking like the solidification is completely, but when it is subjected at the vacuum, it foam.

During drying, sublimation is an important step. Sublimation depends of some factors as for example the thickness of the cooled product, thermal conductivity, the composition of the product, etc.

Sublimation is the physic process where the substance goes from solid to vapour. For the production of this process is important to control the pressure and the temperature.

In the technique of lyophilisation, the drying is divided in two steps:

- Primary dry: ice is sublimated.
- Secondary dry: residual moisture is removed from the sample.

At the beginning of the drying, the sublimation succeeds at the surface of the cooled sample, and then continues along the depth of the sample. The vapours will have to migrate through the dry product.

At secondary step, water confined by absorption is removed. It is possible, if necessary, to accelerate the process, heating the trays of the instrument.

3.6. ANALYTES

In this project different analytes will be analysed. Briefly, some information about every analyte will be explained below.

Both, studies of homogeneity and quantification of analytes of the samples, used quantification procedures that include hydrolysis, extraction solid-liquid, analysis by gas chromatography coupled with mass spectrometry (GC/MS) and analysis by liquid chromatography coupled with tandem mass spectrometry (LC/MS-MS).

The structures of the studied analytes are shown in **Figure 2**.

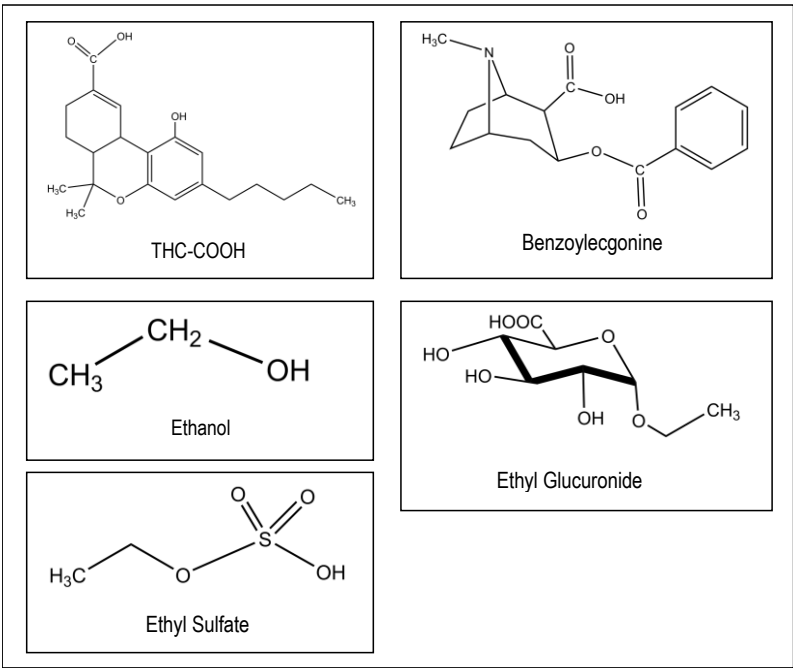


Figure 2. Structure of the studied analytes.

3.6.1. Ethanol, Ethyl glucuronide and Ethyl sulphate

Briefly, ethanol is a volatile, flammable, colourless liquid with a strong chemical odour. Its structural formula is CH_3CH_2OH.

Alcohol is a central nervous system depressant that slows down the actions of brain and body. The purpose of alcohol abuse is the reduction of anxiety, reduction of tremor and relaxation. Consequently alcohol is abused in sports needing high concentration[21].

Ethanol is an exogenous substance mentioned in the Prohibited list of WADA from 2015. Alcohol is prohibited in-competition only, in the following sports: air sports (FAI), archery (WA), automobile (FIA), motorcycling (FIM) and powerboarting (UIM)[4].

On one hand, ethyl glucuronide (EtG) is a direct metabolite of ethanol. Its presence in urine may be used to detect recent ethanol ingestion, even after ethanol is no longer measurable. The presence of this metabolite in urine is an indicator that alcohol was ingested.

On the other hand, in addition to EtG some scientific studies have identified ethyl sulphate (EtS) as a second specific metabolite or biomarker of ethanol.

The detection of both metabolites offers greater sensitivity and accuracy for determination of recent alcohol ingestion, than by detection of either biomarker alone.

The presence of EtG and EtS in urine indicates that ethanol was ingested within the previous 3 to 4 days, or approximately 80 hours after alcohol has been ingested.

3.6.2. Benzoylecgonine

Benzoylecgonine (BE) is the main metabolite of cocaine. When a person takes cocaine (chemically benzoylmethylecgonine), it gets metabolized in the liver to form benzoylecgonine. The metabolite is then excreted out of the body via urine[22].

Cocaine is a substance mentioned in the Prohibited list of WADA from 2015. Cocaine is prohibited in-competition. It is a non- specific stimulant.

3.6.3. 11- nor- Δ^9- tetrahydrocannabinol- 9- carboxylic acid

Cannabis continues to be the most widely used illicit drug in many countries around the world. Δ^9- tetrahydrocannabinol (THC), the major psychoactive component of cannabis, is rapidly metabolized to the inactive metabolite THC-COOH, conjugated with glucuronic acid, and excreted in urine[23].

THC is a substance mentioned in the Prohibited list of WADA from 2015. Cannabinoids are prohibited in-competition.

4. OBJECTIVES

The main objective of this project is try to solve some problems detected in various fresh urine samples used for the interlaboratory comparison by IMIM. Degradations in reference materials of 11- nor- Δ^9- tetrahydrocannabinol- 9- carboxylic acid (THC-COOH) and Benzoylecgonine (BE) prepared by IMIM (coordinator of interlaboratory comparison) were detected in passed assays. These samples were analyzed approximately 2 months after their production and these degradations were observed. For this reason reference materials of 11-nor- Δ^9- tetrahydrocannabinol- 9- carboxylic acid (THCCOOH), benzoylecgonine (BE), ethanol (EtOH) and ethyl glucuronide (EtG) and ethyl sulphate (EtS) will be prepared to study the conditions of lyophilisation as a solution for extending the shelf life and ensuring the stability of the drug and realise if during the process some loss of any of the analytes could exists.

5. EXPERIMENTAL SECTION

5.1. APPARATUS AND INSTRUMENTS

- Gas chromatography coupled with mass spectrometry: an Agilent 6890N series GC system equipped with a quadrupole MS (Agilent 5973N mass selective detector) and autosampler (Agilent 7683 series injector).
- Liquid chromatography coupled with tandem mass spectrometry: an Acquity UPLC system (Waters Corporation) equipped with a triple quadrupole (XEVO TQMS) mass spectrometer equipped with an orthogonal Z-spray-electrospray ionization source (ESI) (all from Waters-Corporation, Milford, MA, USA).
- Lyophilizer: Model lioalfa-6 (Telstar)
- Evaporator: Turbovap LV Evaporator (Zymark)
- Ph meter: Model GLP21 (Crison)
- Refractometer: Palette Digital refractometer (ATAGO)
- Analytical balance: Model XS205D4 (Mettler Toledo)
- Vacuum oven: Gallenkamp
- Dry bath: Multiplaces (Selecta)

5.2. STANDARD SOLUTIONS

The standard solution of EtG 1 mg/mL, EtS 1 mg/mL and the mixed standard solution of Ethyl glucuronide-d5 10 µg/mL and ethyl sulphate-d5 10 µg/mL were prepared using methanol as a solvent.

The standard solution of BE 1 mg/mL was prepared in water. The standard solution of benzoylecgonine-d3 10 µg/mL was prepared using methanol as a solvent.

The standard solution of THC-COOH 100 µg/mL and the standard solution of THC-COOH-d3 1 µg/mL were prepared using methanol as a solvent.

5.3. PRELIMINARY TESTS FOR ESTABLISHING LYOPHILISING CONDITIONS

Different tests were performed to decide the most suitable lyophilisation conditions and the best container and volume of urine to lyophilize the samples without losses of sample. Three different containers were used during the tests (see **Figure 3**).

(a)

Topaz bottle with a capacity of **10 mL**

⌀ 20 mm

(b)

Topaz bottle with a capacity of **30 mL**

⌀ 30 mm

(c)

Transparent bottle with a capacity of **50 mL**

⌀ 20 mm

Figure 3. Different containers used during the tests. The symbol ⌀ is referred to the diameter of the top of the bottle.

Five tests were carried out.

In the first test, three different bottles were filled with water and with blank urine. Bottles with a capacity of 30 mL and 50 mL were filled with 20 mL. Bottles with a capacity of 10 mL were filled with 5 mL. The purpose of this test was to check if the freeze- drier worked properly with this type of matrix as only solid samples were used beforehand.

In second test, bottles were filled with different volumes of blank urine and Milli Q water. Bottles with a capacity of 30 mL and 50 mL were filled with 5 mL, 10 mL, 15 mL and 20 mL. Bottles with a capacity of 10 mL were filled with 2 mL and 5 mL. The purpose of this test was to check the maximum volume advisable to fill the bottles and to assure no losses of sample during the process.

In third test, bottles were filled with treated blank urine and untreated blank urine. Bottles with a capacity of 30 mL were filled with 5 mL and 10 mL. Bottles with a capacity of 10 mL were filled with 2 mL and 5 mL. Also a tray of the instrument was filled with 80 mL of treated blank urine. The purpose of this test was to check if any substance in the urine could influence in the correct lyophilisation of the samples.

In fourth test, bottles were filled with three different dilutions of blank urine with water. The dilutions were 50 %, 25 % and 12,5 %. Bottles with a capacity of 30 mL and 50 mL were filled with 5 mL and 10 mL. Bottles with a capacity of 10 mL were filled with 1 mL, 2 mL and 5 mL. The purpose of this test was to check if the dilution factor of the blank urine could have an effect on the lyophilisation process.

Finally, in fifth test, bottles were filled with two dilutions of blank urine with water. The dilution were 25 % and 12,5 %. Bottles with a capacity of 50 mL were filled with 15 mL, 20 mL and 25 mL. The purpose of this test was to check if higher dilution factors allowed working with higher volumes without losses.

Different combinations of bottling tests are listed in **Table 3**.

Test	Type of bottle	Volume and matrix	Nº bottles tested
1	Topaz bottle (10 mL) ⌀≈ 20 mm	5 mL Milli Q water	5
		5 mL blank urine	5
	Topaz bottle (30 mL) ⌀≈ 30 mm	20 mL Milli Q water	5
		20 mL blank urine	5
	Transparent bottle (50 mL) ⌀≈ 20 mm	20 mL Milli Q water	5
		20 mL blank urine	5
2	Topaz bottle (10 mL) ⌀≈ 20 mm	2 mL Milli Q water	1
		2 mL blank urine	2
		5 mL Milli Q water	1
		5 mL blank urine	2
	Topaz bottle (30 mL) ⌀≈ 30 mm	5 mL blank urine	2
		10 mL blank urine	2
		15 mL Milli Q water	1
		15 mL blank urine	2
		20 mL Milli Q water	1
		20 mL blank urine	2
	Transparent bottle (50 mL) ⌀≈ 20 mm	5 mL blank urine	2
		10 mL blank urine	2
		15 mL Milli Q water	1
		15 mL blank urine	2
		20 mL Milli Q water	1
		20 mL blank urine	2

Table 3. Summary table of preliminary tests for establishing lyophilising conditions.

Test	Type of bottle	Volume and matrix	Nº bottles tested
3	Topaz bottle (10 mL) ⌀≈ 20 mm	2 mL blank urine	2
		2 mL untreated blank urine	2
		5 mL blank urine	2
		5 mL untreated blank urine	2
	Topaz bottle (30 mL) ⌀≈ 30 mm	5 mL blank urine	2
		5 mL untreated blank urine	2
		10 mL blank urine	2
		10 mL untreated blank urine	2
	Tray	80 mL blank urine	1
4	*These combinations with blank urine diluted 50%, 25% and 12,5%*		
	Topaz bottle (10 mL) ⌀≈ 20 mm	1 mL blank urine	2
		2 mL blank urine	2
		5 mL blank urine	2
	Topaz bottle (30 mL) ⌀≈ 30 mm	5 mL blank urine	2
		10 mL blank urine	2
	Transparent bottle (50 mL) ⌀≈ 20 mm	5 mL blank urine	2
		10 mL blank urine	2
5	*These combinations with blank urine diluted 25% and 12,5%*		
	Transparent bottle (50 mL) ⌀≈ 20 mm	15 mL blank urine	2
		20 mL blank urine	2
		25 mL blank urine	2

Table 3 (cont.). Summary table of preliminary tests for establishing lyophilising conditions.

5.4. ANALYTICAL METHOD

A scheme summarising all the steps performed during the analytical method is shown in **Figure 4.**

5.4.1. Preparation of samples for study of lyophilisation conditions

5.4.1.1. Preparation of blank urine

Two litres of blank urine collected from a healthy volunteer were used to prepare the samples. Blank urine was analyzed to verify the absence of interfering compounds. If any prohibited substance was detected, the blank urine will be rejected.

Urine was stabilized with sodium azide 0,1 % w/v. A volume of urine was adjusted to pH 5,50 with HCl to prepare the samples of ethanol, ethyl glucuronide, ethyl sulphate and benzoylecgonine. Some studies[12][9] demonstrate that BE is more stable at pH 5. However, studies demonstrate that THC-COOH is more stable at pH 7.

On the other hand a separate volume was adjusted to pH 6,96 to prepare the sample of THCCOOH. This volume of urine was adjusted with KOH 6M from blank urine with pH 5,50.

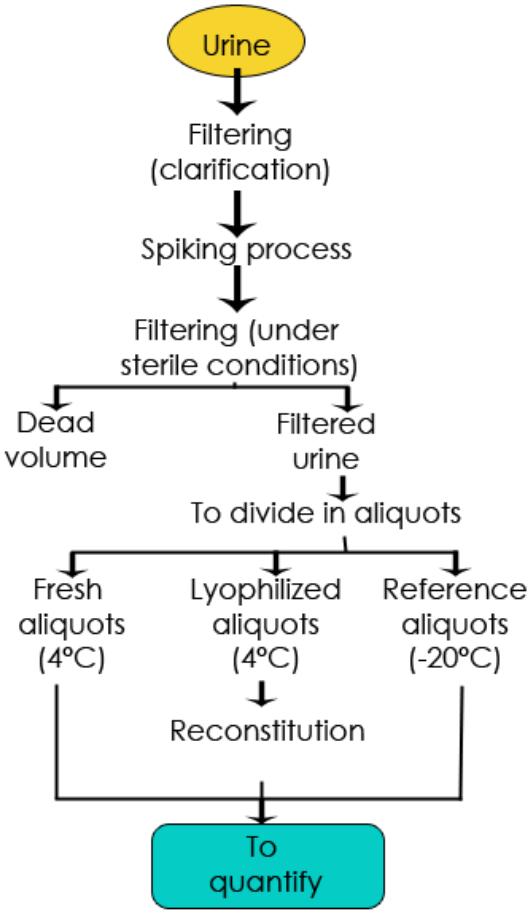


Figure 4. Scheme summarising the process of the analytical method.

Then the urine was clarified by filtration using three different filters (one cellulose reinforced disc membrane filter and two glass fiber filters of different pore size). Firsts 200 mL were rejected (see **Figure 5**).

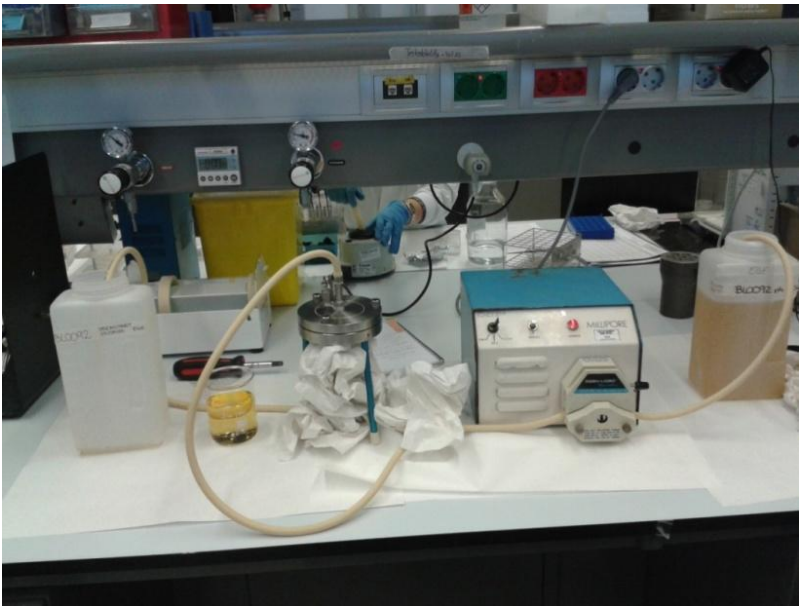


Figure 5. Filtration of blank urine.

5.4.1.2. Preparation of urine samples

Once blank urine was filtered, bulk urine sample were diluted down to 12,5 % with double deionised water and all the samples were spiked with the adequate volumes of the standard solution of the drug to result in the target concentration listed in **Table 4** by using an automatic pipette. These target concentrations were chosen to prepare positive samples taking into account the limit concentration imposed by WADA. Spiking volumes of standard solutions were from 500 µL to 2,5 mL according to the concentration of stock standard solution (see **Table 4**).

The adequate volume of the standard solution of THC-COOH was necessary to evaporate in an evaporator at 17 ºC and with a nitrogen pressure minor of 10 psi due to the standard solution was prepared with methanol as a solvent. Then, it was reconstituted with blank urine until the entire product was pulled.

The measured values for specific gravity and pH of the samples are listed in **Table 5**.

Sample	Compound	Target concentration [mg/L]	Volume of sample [mL]	Concentration of standard solutions	Spiked volume [µL]
Sample 1	THC-COOH	0,3	800	100 µg/mL	2400
Sample 2	BE	1,5	800	1 mg/mL	1500
Sample 3	EtOH	4000	100	-	505
Sample 4	EtG/EtS	10	100	1 mg/mL	1000

Table 4. Composition of the sample, target concentrations, total volume of the sample, concentration of standard solutions and spiked volumes.

Sample	Compound	pH	Specific gravity
Sample 1	THC-COOH	6,96	1,0014
Sample 2	BE	5,50	1,0014
Sample 3	EtOH	5,50	1,0022
Sample 4	EtG/EtS	5,50	1,0024

Table 5. pH values and specific gravity of the samples.

5.4.1.3. Preparation of aliquots

First of all, the laminar flow cabinet was prepared. All the needed material was introduced in the cabinet: samples, the pump, sterile bottles, sterile stoppers, a Bunsen burner, two beakers, a pair of sterile gloves, an sterile filter, a pipette, a stand and another pipette to shake the sample during the process.

The laminar flow cabinet was closed and the Ultraviolet (UV) light was switched on for at least 30 minutes. Then, the light and flow of the cabined were switched on, and the UV was turned off.

The assembly was prepared and the sterile filter was connected to the pump. Sample 1 and 2 were shaking and samples were completely filtered under sterile conditions to an intermediate bulk (beaker) after discarding 150 mL (dead volume).

After all the sample was completely filtered, 15 mL of the sample were pipette in transparent bottles (see **Figure 3c**). A total of 36 bottles were prepared.

Just after filling, every bottle was approached to the Bunsen burner to sterilize it before capping the bottle with a stopper.

Different aliquots of sample, covering all the bottling process (1, 2, 9, 10, 27, 28, 35, 36), were separated to the rest. These aliquots were used to test the homogeneity of the sample.

Differently from samples 1 and 2, urine samples 3 and 4 were only filtered before being spiked due to dead volume would have been bigger than the volume needed of the samples. These samples were pipette without sterile conditions. 2 mL of the sample were pipette in topaz bottles (see **Figure 3a**). A total of 36 bottles were prepared of every sample.

Different aliquots were stored at different conditions to be analyzed at 0, 3, 6, 12, 24 and 36 months to a future study of stability.

5.4.1.4. Lyophilisation process

The lyophilisation process of the samples was carried out at Mat Control Laboratory of Analytical Chemistry Department (UB).

The conditions of lyophilisation used were the following. First of all, the aliquots were frozen at -20 ºC at least overnight. Then, the samples were introduced into the apparatus. First, the temperature of the freeze-dryer reached -31 ºC and then the vacuum pump was turned on and the pressure of the freeze-dryer was reduced until 0,07 mBa. This succeeds while the temperature of the apparatus was achieving -41 ºC. The final conditions of lyophilisation were a temperature of -46 ºC or less, a pressure of 0,07 mBa and the process succeed during at least 24 hours (see **Figure 6**).

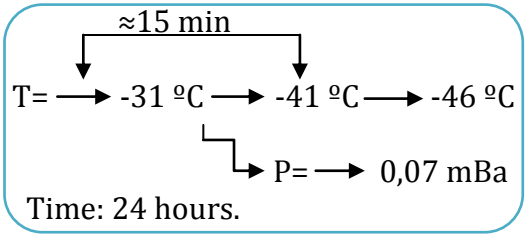


Figure 6. Lyophilisation process

5.4.1.5. Storage conditions

Lyophilized aliquots and fresh aliquots were stored at 4 ºC until analysis. Reference aliquots were stored at -20 ºC until analysis.

5.4.2. Quantification of THC-COOH

Fresh, lyophilized and reference samples were quantified in two different batches with two different groups of calibration tubes.

Lyophilized aliquots were first reconstituted with 15 mL of Milli Q water. Then, aliquots were mixed slowly after adding water, 5 minutes later samples were diluted mixing by inversion until complete dissolution.

In each batch of analysis, blank urines, samples under study and control urine samples were analyzed. Sample volumes analyzed were 3 mL in all cases. Samples under study were diluted by half (volume) with blank urine due to its target concentration wasn't into the concentration range of the calibration tubes.

For calibration, the suitable volume of standard was place in the calibration tubes to cover a concentration range of 50, 100, 150, 200 and 300 ng/mL.

50 µL of the standard solution of THC-COOH-d3 1 µg/mL were added to all tubes as internal standard.

Hydrolysis was performed by adding 300 µL of KOH 10M in all the tubes, mixing with a vortex and introducing all the tubes in a dry-bath 15 minutes at 61 ºC.

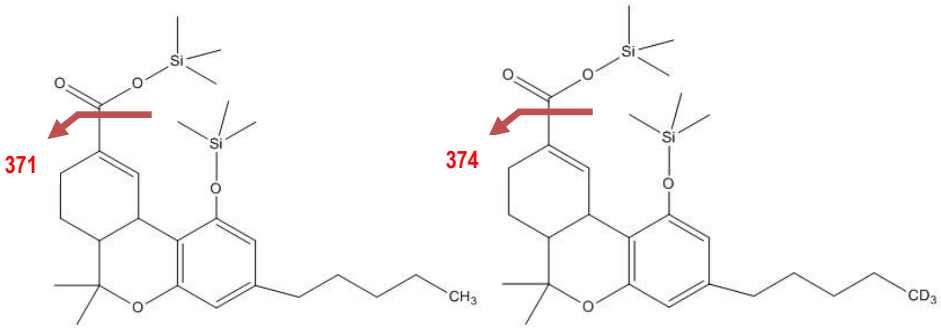
400 µL of glacial acetic acid and 3 mL of phosphoric acid 50mM were added in the tubes to adjust the pH to 4-5. Then, tubes were centrifuged at least 5 minutes at 2500 rpm.

Bond-Elut Certify columns were conditioned by washing with 2 mL of methanol and 2 mL of phosphoric acid 50 mM. The columns were prevented from drying. After adding the urine samples, columns were washed with 9 mL of phosphoric acid 50 mM and 3 mL of phosphoric acid 50 mM: methanol (80:20). The columns were dried at least 10 minutes. Then, 1 mL of hexane was added. Analytes were eluted with 2 mL of hexane: ethyl acetate (80:20).

Eluates were evaporated to dryness under a nitrogen stream in a water bath at 48 ºC and immediately, kept it in a vacuum oven with diphosphorus pentoxide (T = 40 ± 5 ºC) for at least 1 hour. Then, the derivatization was performed adding 50 µL of N- methyl- N-(trimethylsilyl)trifluoroacetamide (MSTFA):NH_4I:2-Mercaptoethanol. The solution was mixed at least 30 seconds and kept it in a dry bath at least 30 minutes at 61 ºC.

Finally, the solutions were transferred into the vials and injected into the chromatographic system.

The extracts were analyzed by gas chromatography coupled with mass spectrometry (GC/MS). Separation was performed using a methylsilicone capillary column (17 m x 0,2 mm id, 0,11 µm film thickness, HP-ULTRA1). Helium was used as a carrier gas at a flow rate of 0,9-1,1 mL/min. The oven was maintained at 200 ºC for 1 minute and the following rate was programmed: the temperature increased from 200 ºC to 280 ºC at 25 ºC/min, staying 2,8 minutes at 280 ºC with a total run time of 7 minutes. Samples were injected in split mode. The volume injection was 1 µL .The injector and the interface temperature were set at 280 ºC. The MS was operated in electron impact ionization (70 eV) and in selected ion monitoring acquisition mode (SIM). Three ions were monitored for each substance and used as qualifying ions for their identification. The ions used for quantification were *m/z* 371 for THC-COOH-bis-TMS derivative and *m/z* 374 for THC-COOH-d_3-bis-TMS derivative (see **Figure 7** and **Table 6**).



THC-COOH-bis-TMS THC-COOH-d3-bis-TMS

Figure 7. Product ions used for quantification of THC-COOH-bis-TMS and THC-COOH-d3-bis-TMS.

Analyte	**RT** [min]	***m/z***
THC-COOH-bis-TMS	4,3 ± 0,3	473, **371**, 488
THC-COOH-d3-bis-TMS	4,3 ± 0,3	476, **374**, 491

Table 6. Ions used for quantification of THC-COOH-bis-TMS and THC-COOH-d3-bis-TMS.

5.4.3. Quantification of Benzoylecgonine

Fresh, lyophilized and reference samples were quantified in two different batches with two different groups of calibration tubes.

Lyophilized aliquots were first reconstituted with 15 mL of Milli Q water. Then, aliquots were mixed slowly after adding water, 5 minutes later samples were diluted mixing by inversion until complete dissolution.

In each batch of analysis, blank urines, samples under study and control urine samples were analyzed. Sample volumes analyzed were 2 mL in all cases.

For calibration, the suitable volume of standard was place in the calibration tubes to cover a concentration range of 100, 480, 1000, 1400 and 2000 ng/mL.

50 µL of standard solution of benzoylecgonine-d3 10 µg/mL were added to all tubes as internal standard.

1 mL of phosphate buffer 0,1 M pH7 was added in the tubes to adjust the pH to 7, and the result was mixed. Then, it was centrifuged at 2500 rpm. at least 5 minutes.

Bond-Elut Certify columns were conditioned by washing with 2 mL of methanol and 2 mL of phosphate buffer 0,1 M pH7. The columns were prevented from drying. After adding the urine samples, columns were washed with 3 mL H_2O Milli Q, 3 ml of HCl 0,1 M and 9 mL of methanol. The columns were dried at least 2 minutes. Then, analytes were eluted with 2 mL of $CHCl_3$:iPrOH 80:20, NH_3 2 %.

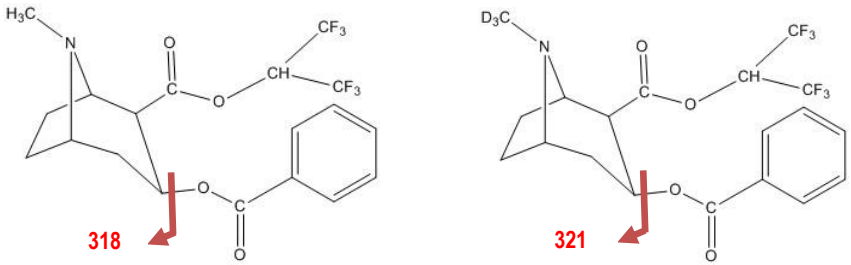
Eluates were evaporated to dryness under a nitrogen stream in a water bath at 38 ºC and immediately, kept it in a vacuum oven with diphosphorus pentoxide (T = 40 ± 5 ºC) for at least 1 hour. Then, the derivatization was performed adding 70 µL of Pentafluoropropionic anhydride (PFPA) and 30 µL of Hexafluoroisopropanol (HFIP). The solution was mixed and kept it in a dry-bath at least 10 minutes at 71 ºC.

The extracts were evaporated to dryness under a nitrogen stream in a water bath at 38 ºC. Then, dried extracts were reconstituted in 50 µL of ethyl acetate.

Finally, the solutions were transferred into the vials and injected into the chromatographic system.

The extracts were analyzed by gas chromatography coupled with mass spectrometry (GC/MS). Separation was performed using a methylsilicone capillary column (16,5 m x 0,2 mm

id, 0,11 µm film thickness; HP-ULTRA1). Helium was used as a carrier gas at a flow rate of 0,9-1,1 mL/min. Then the following rate was programmed: the temperature increased from 100 ºC to 280 ºC at 20 ºC/min, staying 4 minutes at 280 ºC with a total run time of 13 minutes. Samples were injected in split mode. The volume injection was 2 µL. The injector and the interface temperature were set at 280 ºC. The MS was operated in electron impact ionization (70 eV) and in selected ion monitoring acquisition mode (SIM). Three ions were monitored for each substance and used as qualifying ions for their identification. The ions used for quantification were *m/z* 318 for Benzoylecgonine-HFIP and *m/z* 321 for Benzoylecgonine-d_3-HFIP (see **Figure 8** and **Table 7**).



Benzoylecgonine-HFIP Benzoylecgonine-d_3-HFIP

Figure 8. Product ions used for quantification of Benzoylecgonine-HFIP and Benzoylecgonine-d3-HFIP.

Analyte	**RT** [min]	***m/z***
Benzoylecgonine-HFIP	6,15 ± 0,3	439, **318**, 82
Benzoylecgonine-d_3-HFIP	6,17 ± 0,3	442, **321**, 85

Table 7. Ions used for quantification of Benzoylecgonine-HFIP and Benzoylecgonine-d3-HFIP.

5.4.4. Quantification of Ethanol

Quantification of ethanol was performed by Instituto Nacional de Toxicología y Ciencias Forenses Departamento de Barcelona.

Fresh, lyophilized and reference samples were quantified in one batch.

Lyophilized aliquots were first reconstituted with 2 mL of Milli Q water. Then, aliquots were mixed slowly after adding water, 5 minutes later samples were diluted mixing by inversion until complete dissolution.

Briefly, to do the quantification of ethanol calibration samples and control samples were prepared.

To prepare the samples, 100 µL of internal patron as for example n-propanol or tert-butileter were added in a vial, then 100 µL of the sample were added. A piece of paper was introduced into the vial and the vial was sealed. Then, the vial was introduced in an oven during a determinate time.

Once the sample phase was introduced into the vial and the vial was sealed, volatile components diffused into the gas phase until the headspace had reached a state of equilibrium. The sample was then taken from the headspace.

Then, a headspace GC analysis was performed.

5.4.5. Quantification of Ethyl glucuronide and Ethyl sulphate

Lyophilized aliquots were first reconstituted with 2 mL of Milli Q water. Then aliquots were mixed slowly after adding water, 5 minutes later samples were diluted mixing by inversion until complete dissolution.

Fresh, lyophilized and reference samples were quantified in one batch.

In each series of analysis, blank urines, samples under study and quality control urine samples were analyzed. Sample volumes analyzed were 50 µL in all cases.

For calibration, the suitable volume of standard was place in the calibration vials to cover a concentration range of 5, 20, 50 and 100 µg/mL.

50 µL of the mixed standard solution of Ethyl glucuronide-d5 10 µg/mL and ethyl sulphate-d5 10 µg/mL were added to all vials.

Finally, the solutions were mixed at least 10 seconds, and 900 µL of H_2O with 0,1 % formic acid were added in all the vials.

The extracts were analyzed by liquid chromatography coupled with tandem mass spectrometry (LC/MS-MS).

Chromatographic separations were carried out using an Acquity UPLC BEH C18 column (2,1 mm x 100 mm i.d., 1,7 µm particle size). The column temperature was set to 55 ºC. The mobile phase was a mixture of methanol and water with gradient elution. The initial methanol content (1,0 %) was maintained for 0,5 minutes and then increase to 90,0 % in 4,5 minutes, maintained there for 0,5 minutes, decreased to 1,0 % in 0,5 minutes and stabilized for 1,5 minutes before the next injection. The flow rate of the mobile phase was maintained at 0,3 mL/min. A total run time of 7,5 minutes. The volume injection was 10 µL.

Drying gas as well as nebulising gas was nitrogen. The desolvation gas flow was set to 1200 L/h, and the cone gas flow was 50 L/h. The nitrogen desolvation temperature was 450 ºC, and the source temperature was 120 ºC.

The mass spectrometric conditions of the selected reaction monitoring (SRM) method are described in **Table 8.** Product ions used to quantify samples are in bold in **Table 8**.

Compound	Mode	RT [min]	Precursor Ion (*m/z*)	Product Ion (*m/z*)	CV [V]	CE [eV]
EtS	NEG	1,19	125 $[M-H]^-$	**97**, 80	20	10
EtS-d5	NEG	1,17	130 $[M-H]^-$	**98**	20	10
EtG	POS	1,44	240 $[M+ NH_4^+]^+$	141, **159**, 205	10	10
EtG-d5	POS	1,47	245 $[M+NH_4^+]^+$	141, **159**, 210	10	10

Table 8. Mass spectrometric conditions of the SRM method.

6. DISCUSSIONS AND RESULTS

6.1. PRELIMINARY TESTS FOR ESTABLISHING LYOPHILISING CONDITIONS

Different tests were carried out to choose the most suitable conditions and the best container and volume to lyophilize the samples without losses.

The first test demonstrated that the apparatus worked properly with urine samples. The time chosen to lyophilize the samples was not enough with 16 hours (**Figure 9**), therefore it was decided to lyophilize samples during at least 24 hours in the next tests to assure a completely lyophilisation.

Figure 9. Results obtained for Test 1.

Trying to correct the loss of samples (**Figure 9**) during the process, different combinations of volumes and bottles were tested to check what volume was the maximum volume advisable to fill the bottles and to assure no losses of sample during the process.

Observing that the matrix of the sample could be a problem for its density and viscosity, different tests were carried out with different dilutions of the blank urine to check if the dilution factor could have an effect on the lyophilisation process. It was decided that finally transparent bottles (with a capacity of 50 mL) can be filled with 15 mL of blank urine diluted 12,5 % with water to assure no losses of sample during the process. Topaz bottles (with a capacity of 10

mL) can be filled with 2 mL of blank urine diluted 12,5 % with water also. Finally, topaz bottles with a capacity of 30 mL were rejected because the geometry of this container away from the normally used by IMIM to prepare reference materials used in interlaboratory comparison.

All results obtained in tests are summarising in **Table 8** situated in appendix 1.

6.2. QUANTIFICATION STEP AND QUALITY CONTROL SAMPLES

Results obtained for calibration graphs for all samples are shown in **Table 9.** All coefficients of correlation obtained for each analyte are 0,999 or higher, therefore all calibration curves are suitable to calculate the concentrations of urine samples.

To assure that concentrations obtained were correct, results of quality control samples must have been into the acceptance range stipulated by IMIM. In general, the acceptance criterion applied is ±20 %, except for THC-COOH where ± 10 % is applied. These quality samples are prepared spiking blank urine with an adequate volume of the respective standard solutions, then the sample is divided in aliquots and it is introduced in the freezer. The obtained value after quantifying 6 aliquots is the nominal value where the acceptance criterion is applied in every following analysis until finishing the stored aliquots.

As it is shown in **Table 9,** all results obtained for quality control samples were into their respective acceptance range. Therefore, quantitative results were accepted.

Analyte	Calibration range	Coefficient of correlation	Results for quality samples	Acceptance range of quality samples
THC-COOH	50-300 ng/mL	0,999	208 ng/mL	192-234 ng/mL
BE	100-2000 ng/mL	0,999	484 ng/mL	354-532 ng/mL
EtG	5-100 µg/mL	1,000	92 µg/mL	68-102 µg/mL
EtS	5-100 µg/mL	1,000	75 µg/mL	60-90 µg/mL

Table 9. Calibration rage for every analyte, coefficient of correlation obtained, results for quality samples obtained and acceptance range of quality samples.

6.3. HOMOGENEITY STUDIES

To assure that samples could be used as reference material, samples must comply with an essential property, which is homogeneity.

To test the homogeneity of the fresh urine sample, 8 aliquots stored at 4 ºC were analyzed by duplicate (samples of THC-COOH and BE). Also 8 lyophilized aliquots stored at 4 ºC were analyzed by duplicate to test the homogeneity of every lyophilized urine sample.

Homogeneity was evaluated by applying the two criteria used in IMIM assuring homogeneity: a one-way ANOVA test (α = 5 %), and a precision Model of Horwitz.

According to the protocol established by IMIM if at least one criterion is accepted, the sample is regarded as homogeneous.

The concentrations obtained for every samples of THC-COOH, BE, EtG and EtS are shown in **Table 10 to Table 13** with their respective units.

Conc.	**Fresh aliquots of THC-COOH**							
ng/mL	**1**	**2**	**9**	**10**	**27**	**28**	**35**	**36**
Rep.1	133,4	120,6	130,7	132,6	134,7	131,0	124,7	125,4
Rep.2	136,6	114,4	131,7	129,1	132,7	129,2	122,5	125,3
Conc.	**Lyophilized aliquots of THC-COOH**							
ng/mL	**1**	**2**	**9**	**10**	**27**	**28**	**35**	**36**
Rep.1	137,2	133,3	132,8	141,8	131,2	131,8	134,5	136,0
Rep.2	139,8	135,1	135,8	138,5	131,9	134,1	130,7	134,3

Table 10. Concentrations [ng/mL] obtained for fresh and lyophilized aliquots of THC-COOH.

Conc.	**Fresh aliquots of BE**							
ng/mL	**1**	**2**	**9**	**10**	**27**	**28**	**35**	**36**
Rep.1	1341,2	1320,4	1304,7	1337,8	1330,6	1345,6	1348,5	1329,9
Rep.2	1345,7	1327,6	1321,1	1324,2	1333,3	1311,4	1365,7	1330,8

Table 11. Concentrations [ng/mL] obtained for fresh and lyophilized aliquots of BE.

Conc.	Lyophilized aliquots of BE							
ng/mL	1	2	9	10	27	28	35	36
Rep.1	1361,9	1344,7	1373,8	1326,6	1324,6	1249,5	1279,4	1329,5
Rep.2	1357,3	1352,6	1336,1	1304,6	1326,1	1270,2	1303,2	1321,4

Table 11 (cont.). Concentrations [ng/mL] obtained for fresh and lyophilized aliquots of BE.

Conc.	Lyophilized aliquots of EtG							
µg/mL	1	2	9	10	27	28	35	36
Rep.1	10,4	9,7	10,6	10,7	10,0	10,5	9,4	10,9
Rep.2	10,2	9,5	10,9	10,0	9,1	10,6	10,4	10,5

Table 12. Concentrations [µg/mL] obtained for lyophilized aliquots of EtG.

Conc.	Lyophilized aliquots of EtS							
µg/mL	1	2	9	10	27	28	35	36
Rep.1	8,6	8,6	8,2	9,5	7,2	7,9	6,8	9,2
Rep.2	8,4	7,0	8,1	7,7	7,4	9,3	8,0	7,5

Table 13. Concentrations [µg/mL] obtained for lyophilized aliquots of EtS.

Analyte		Fcal, Fcrit.	Test- F	$Ss/S_H<0,3$	Horwitz
THC-COOH	Fresh	15,04>3,50	NO OK	0,10<0,30	OK
	Lyophilized	5,27>3,50	NO OK	0,05<0,30	OK
BE	Fresh	2,80<3,50	OK	0,03<0,30	OK
	Lyophilized	12,07>3,50	NO OK	0,08<0,30	OK
EtG	Lyophilized	2,74<3,50	OK	0,16<0,30	OK
EtS	Lyophilized	1,38<3,50	OK	0,17<0,30	OK

Table 14. Results obtained for every sample applying criterion one (one-way ANOVA) and criterion two (Precision Model of Horwitz).

Results obtained for one-way ANOVA and for the precision Model of Horwitz for every sample aforementioned are shown in **Table 14**.

As it is shown in **Table 14** these samples were accepted at least by the second criterion. Therefore, it can be said that samples of THC-COOH and BE before being lyophilized are homogeneous and could be used as reference material without been lyophilized.

But one of the main points of this project is assure if prepared samples could be used as reference material after being lyophilized. As the results shown, all lyophilized samples listed in **Table 14** were accepted at least by the second criterion. Therefore, these samples could be used as reference material due to all samples are homogeneous even being lyophilized.

About ethanol, it was impossible to calculate the homogeneity because it was not detected in any of the aliquots (detection limit of 0,02 g/L). Ethanol was lost during the lyophilisation process.

6.4. BEHAVIOURS OF STUDIED ANALYTES DURING PROCESSING OF SAMPLES

It is important to know if during the processing samples a loss of the analyte was produced.

To study this fact, reference aliquots stored at -20 ºC, fresh aliquots stored at 4 ºC and lyophilized aliquots were quantified.

For THC-COOH and BE, 8 lyophilized aliquots were quantified in duplicate, 8 fresh aliquots were quantified in duplicate and finally 1 reference aliquot was quantified in triplicate. For the sample spiked with EtG and EtS, 8 lyophilized aliquots were quantified in duplicate, 1 fresh aliquot was quantified in triplicate and finally 1 reference aliquot was quantified in triplicate.

First of all, differences between fresh aliquots and reference aliquots were evaluated. The results are shown in **Table 15**. The results are presented with the mean and the standard deviation of every sample.

As it is shown in **Table 15**, lower differences between fresh and reference aliquots are observed. Therefore, it could be attributed to the variability of the method and not to a loss of the analyte.

	Reference aliquot	Fresh aliquots
	Mean ± sd	**Mean ± sd**
THC-COOH*	283 ± 3 ng/mL	257 ± 12 ng/mL
BE*	1344 ± 21 ng/mL	1332 ± 15 ng/mL
EtG	11,2 ± 0,3 µg/mL	10,8 ± 0,9 µg/mL
EtS	9,6 ± 0,5 µg/mL	8,8 ±0,7 µg/mL
EtOH	4,1 ± 0,1 g/L	4,0 ± 0,1 g/L

Table 15. Concentration of fresh aliquots (mean ± sd, n = 1 triplicate, *n =8 duplicate) and reference aliquots (mean ± sd, n = 1 triplicate).

To evaluate the possible loss of analyte during the process of lyophilisation, the percentage of change of lyophilized aliquots respect to reference aliquots was calculated (see **Table 16**).

	Lyophilized aliquots	
	Mean ± sd	**Change (%)**
THC-COOH	270 ± 6 ng/mL	-4,6
BE	1323 ± 34 ng/mL	-1,6
EtG	10,2 ± 0,5 µg/mL	-8,9
EtS	8,1 ± 0,8 µg/mL	-15,6
EtOH	-	-

Table 16. Concentration of lyophilized aliquots (mean ± sd, n = 8) and differences (percentage of change) respect reference aliquots.

Comparison of the concentration obtained for the lyophilized samples to that of the reference aliquots indicates the loss of some compounds during the process. A 5 % of variability of the methods was acceptance to evaluate the percentages of change obtained.

Minimal differences were detected for THC-COOH and BE, such differences were under the variability of the method aforementioned. Therefore, these differences could be attributed to the

variability of the method and not to a loss of the compound during the lyophilisation process. If these differences are maintained over time (3, 6, 12, 24 and 36 months), they can be attributed to the variability of the method. A future study of the stability of the samples would ensure this assumption.

However, higher differences were detected for EtG and EtS. In both cases, such differences were over the variability of the method. Due to the sample was homogeneous it couldn't be attributed to a physical loss of analyte during the process but it could be attributed to a chemical process. Therefore, part of the loss of these analytes seems to be related with the evaporation of them.

Percentages of change are represented in **Figure 10**.

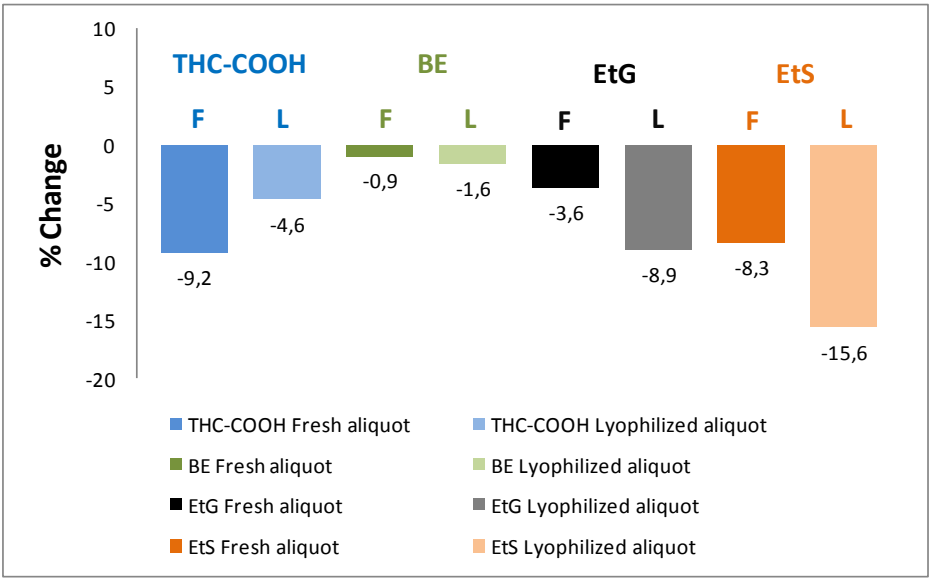


Figure 10. Representation of percentage of change for fresh and lyophilized aliquots.

About the sample of ethanol the only thing it could be said is that in the process of lyophilisation a higher loss of analyte was detected. The completely loss of ethanol is related to its high volatility.

To prepare reference materials of EtG and EtS, it would be necessary to fortify the sample taking into account the loss percentage observed.

7. CONCLUSIONS

- After carrying out different tests to choose the best container and volume to lyophilize the samples without losses, the transparent bottles filled with 15 mL of blank urine 12,5 % and topaz bottles (with a capacity of 10 mL) filled with 2 mL of blank urine 12,5 % were selected.
- The most suitable conditions to lyophilize these samples were a temperature of -46 ºC, a pressure of 0,070 mBa and a total time of at least 24 hours.
- Prepared sample of THC-COOH, BE, EtG and EtS were considered homogeneous after being lyophilized. These samples can be consider as reference materials.
- Differences detected in lyophilized samples of THC-COOH and BE were attributed to the variability of the method and not to a loss of the compound during the lyophilisation process.
- Differences detected in the lyophilized sample of EtG and EtS seem to be related with the evaporation of the analytes due to the homogeneity of it.
- Ethanol was completely lost during the lyophilisation process due to the high volatility of the analyte.
- Some bottles were prepared and stored to continue the long-term study of stability (0, 3, 6, 12, 24, 36 months) of the prepared samples.

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9. ACRONYMS

ABP Athlete Biological Passport

BE Benzoylecgonine

CE Collision Energy

CRM Certified Reference Material

CV Cone Voltage

ESI Electrospray ionization

EtG Ethyl glucuronide

EtOH Ethanol

EtS Ethyl sulphate

FAI The World Air Sports Federation

FIA *Federation Internationale de l'Automobile*

FIFA *Fédération Internationales de Football Association*

FIM International Motorcycling Federation

GC/MS Gas chromatography coupled with mass spectrometry

HFIP Hexafluoroisopropanol

IAAF International Association of Athletics Federations

IF International sport Federation

IMIM Institut Hospital del Mar d'Investigacions Mèdiques

IOC International Olympic Committee

ISO Intenational Organization for Standardization

LC/MS-MS Liquid chromatography coupled with tandem mass spectrometry

MSTFA N- methyl- N- (trimethylsilyl)trifluoroacetamide

PFPA Pentafluoropropionic anhydride

PTS Proficiency Testing Schemes

QCM Quality Control Material

RM Reference Material

RSD Relative standard deviation

RT Retention Time

SIM Selected ion monitoring

SRM Selected reaction monitoring

THC Δ^9- tetrahydrocannabinol

THC-COOH 11- nor- Δ^9- tetrahydrocannabinol- 9- carboxylic acid

TMS Trimethylsilyl

TQMS Triple quadrupole mass spectrometer

UB Universitat de Barcelona

UCI *Union Cycliste Internationale*

UIM *Union Internationale Motornautique*

UV Ultraviolet

WA World Archery Federation

WADA World Anti- Doping Agency

APPENDICES

APPENDIX 1: RESULTS FOR PRELIMINARY TESTS

	Topaz bottle (10 mL)			Topaz bottle (30 mL)				Transparent bottle (50 mL)					Tray
	5 mL	2 mL	1 mL	20 mL	15 mL	10 mL	5 mL	25 mL	20 mL	15 mL	10 mL	5 mL	80 mL
Milli Q water	OK	OK	-	OK	OK	-	-	-	OK	OK	-	-	-
Blank urine	NO OK	OK	-	NO OK	NO OK	NO OK	NO OK	-	NO OK	NO OK	NO OK	NO OK	OK
Untreated urine	NO OK	OK	-	-	-	NO OK	OK	-	-	-	-	-	-
Blank urine 50%	NO OK	NO OK	OK	-	-	NO OK	NO OK	-	-	-	NO OK	NO OK	-
Blank urine 25%	NO OK	NO OK	OK	-	-	NO OK	OK	NO OK	NO OK	NO OK	OK	OK	-
Blank urine 12,5%	NO OK	OK	OK	-	-	OK	OK	NO OK	OK	OK	OK	OK	-

Table 8. Results obtained for preliminary tests for establishing lyophilising conditions.