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NEW APPLICATION OF MASS SPECTROMETRY-BASED TECHNIQUES TO THE DETERMINATION OF DRUGS AND BIOMARKERS IN HAIR

S.S.D. MED/43

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ABBREVIATIONS

ACS	Acute coronary syndrome
CAD	Coronary artery disease
CAD*	Collision gas
CE	Collision energy
CI	Chemical ionization
CXP	Cell exit potential
CUR	Curtain gas
DAD	Diode array detection
DOA	Drugs of abuse
DP	Declustering potential
EI	Electron impact
EP	Entrance potential
ESI	Electrospray ionisation
EtG	Ethyl glucuronide
EtOH	Ethanol
EtS	Ethyl sulphate
EV	Elettronvolt
FDA	Food and drug administration
FRTIR	Fourier transform infrared
GC	Gas chromatography
GC-MS	Gas chromatography coupled with mass spectrometry
GC-MS/MS	Gas chromatography tandem mass spectrometry

GS	Ion source gas
HCl	Hydrochloric acid
hEtG	Hair ethyl glucuronide
HILIC	Hydrophilic interaction liquid chromatography
HMG-CoA	3-Hydroxy-3-methylglutaryl coenzyme A
HSS	High strength silica
IA	Immunoassay
IHD	Ischemic heart disease
IS	Internal standard
IS*	Ion spray voltage
LC	Liquid chromatography
LD	Limit of detection
LDL	Low-density lipoprotein
LLE	Liquid-liquid extraction
LLOQ	Lower limit of quantification
LC-MS	Liquid chromatography coupled with mass spectrometry
LC-MS/MS	Liquid chromatography tandem mass spectrometry
LOQ	limit of quantification
m/z	mass-to-charge ratio
ME	Matrix effect
MRM	Multiple reaction monitoring
MS	Mass spectrometry
NaOH	Sodium hydroxide
NCI	Negative chemical ionization mode

ON	Over night
PGC	Porous graphitic carbon
PREG	Polar retention effect on graphite
QC	Quality control
QTOF	Quadruple-TOF
QTRAP	Quadruple-ion trap
RE	Extraction recovery
RF	Radio frequency
RP	Reverse phase
RPM	Revolutions per minute
RSD	Relative standard deviation
S/N	Signal-to-noise
SD	Standard deviation
SIM	Selected ion monitoring
SoHT	Society of hair testing
SPE	Solid-phase extraction
TDM	Therapeutic drug monitoring
TEM	Temperature
THC	(−)-trans-Δ9-tetrahydrocannabinol
THC-COOH	11-nor-Δ9-tetrahydrocannabinol-9-carboxylic acid
TIC	Total ion chromatogram
TOF	Time of flight
tR	Retention time
TRAP	Ion trap

UHPLC	Ultra-high-performance liquid chromatography
UPLC	Ultra-performance liquid chromatography
UV	Ultra-violet
UV-VIS	Ultraviolet-visible absorption
V	Volts

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FRAMEWORK AND AIMS

Forensic toxicology is the discipline dealing with the analysis of biological samples for the presence of toxins and drugs. As a branch of medical sciences, it provides key information regarding the nature, effects, properties, and doses as well of toxic substances present in an individual, consistently with a therapeutic dosage or at harmful level. The use of forensic toxicology aids medical or legal investigation concerning a substance's potential effect on an individual's death, illness, mental or physical impairment, and drug abuse, making inferences combining knowledges of pharmacology, clinical, and analytical chemistry. The legal outcome of the toxicological investigation is not the primary concern, but rather the obtainment and the demonstration of the existence of a causal link between the diagnosis of poisoning due to the presence of a toxic agent, and the assessment of the state of intoxication, a lethal event, or a behavioural alteration. The interpretation of the analytical results and possibilities to properly obtain them are fundamental and linked to the type of biological sample to collect, the way in which those are acquired, stored, and handled, to the choice of the analytical procedures, and to the ability to assess the changes resulting from the state of degradation of the samples. The process of acquiring chemical-toxicological data can be done in a qualitative manner, when the nature of the exogenous toxicant is ascertained in a specific manner through a targeted investigation among substances belonging to the same group, or through generic investigation whether no clue on the nature of the substance is presented. Though, when it is necessary to assess whether the toxicant is present at therapeutic or toxic concentrations, a quantitative data is required and obtained by using suitable extraction and separation method, appropriate internal standard, and standardised detection methods with guaranteed analytical reliability and reproducibility. The assessment of the target compound in different biological fluids with optimum sensitivity, and specificity is of great importance. Among different techniques of interest in providing laboratory evidence, separation techniques coupled to mass spectrometry are predominantly utilized. Gas chromatography-mass spectrometry (GC-MS) is still considered as the “gold standard” for analytical forensic confirmatory analysis, as well as liquid

chromatography-mass spectrometry (LC-MS) represents a viable alternative. Typical run times by using currently available chromatographic and mass spectrometric systems have reached 10-15 minutes, rarely exceeding 20 minutes, which contributes to the slow turnaround times most forensic labs are facing. However, the major deficiency mostly related to separation techniques is the relatively low sample throughput. Recent efforts to improve and optimize these techniques have been done, from novel coupling strategies to integrating multiple degrees of separation and sample preparation.

This doctoral thesis includes two main parts. The former is dedicated to the description of the main characteristics of liquid chromatography coupled with mass spectrometry and to an overview of the general aspects of hair analysis, to provide a theoretical background for the second part, which is focused on two applications of hair analysis developed during the PhD course. In particular, the experimental part firstly concerned the optimization and validation of a LC-MS/MS method for the determination of ethyl glucuronide in hair (hEtG), as marker of chronic alcohol abuse. The second experimental application was the development and validation of a new LC-MS/MS method for the determination of statins in hair as a tool for the evaluation of adherence to therapy.

1. LIQUID CHROMATOGRAPHY APPLIED TO FORENSIC SCIENCES

1.1 LIQUID CHROMATOGRAPHY (LC)

Liquid chromatography (LC) is a widely used technique employed for the separation of compounds within a mixture according to the interactions and the affinity of the sample for a mobile, and a stationary phase. Basically, the separation occurs in the stationary phase, a fine adsorbent solid tightly packed to create a uniform distribution of material, as the mobile phase passes through making the components migrate into distinct bands and allowing them to be collected and easily analyzed separately. A uniform distribution of the packing minimizes the presence of air bubbles and dead volumes throughout the entire experiment when the solvents flow. The separation phenomenon is effective because different components are attracted to the adsorbent surface of the stationary phase with different degrees and depending on their unique structural features and interaction with the eluents. Moreover, the efficacy of the separation is dependent on the nature of the adsorbent solid used and the polarity of the mobile phase solvent. Compounds with a large distribution constant which are more attracted to the stationary phase will be more strongly retained in it and migrate at a slower rate than another one that has a higher affinity for the mobile phase. This time is also known as retention time (*t*R). Chromatographic separations in liquid chromatography generally take place in a column, commonly made of stainless steel with alumina or silica gel as a substrate. Several different solids and adsorbent materials may be employed as stationary phase, chosen based on their particle size and activity grade. Due to its high sample capacity, silica is one of the most popular adsorbent materials. Recently, the use of highly spherical, pure, and homogeneous silica particles has increased, improving the reproducibility, resolution, speed, and the efficiency of the analysis. The proper mobile phase is also chosen according to the features of the system, the sample, and the stationary phase. Mixture of aqueous and organic eluents such as methanol or acetonitrile, and additives as buffers are commonly used [1]. The solvents may also be changed during separation in order to change the polarity and elute the different components separately in a more timely manner. A liquid chromatograph is mainly

formed as following described [Fig.1]. An autosampler provides the sampling in temperature control way with high precision of variable injection volume, making also possible internal standard addition, or sample dilution in easily programed manners. The solvent delivery system consists of eluents reservoirs, a degasser and one or more pumps. The mixing of solvents in gradient separation may occur in a mixing chamber after the pumps, or prior to pumping. The vacuum degasser is usually incorporated into the pumps, which are currently made to provide variable flow rate. The sample flows then to the thermostatic compartment where the column is located, and the separation occurred. Depending on the diameter, analytical columns are classified into standard columns (typical internal diameter 4.6 or 4.0 mm), semi-micro columns (typical internal diameter 1–2 mm) and micro or capillary columns (internal diameter 0.1–1.0 mm) [2]. A guard or pre-column might also be used and placed before the analytical column to extend the life of the column. The inner composition should be the same as the column but with higher particle size to minimize pressure drop and to protect it from contaminants that could bind irreversibly to the stationary phase. Different column-packing materials with improved properties for separation of complex substance mixtures of polar and non-polar as well as hydrophilic and hydrophobic substances are nowadays available. According to the principle of separation, different strategies such as normal phase, reverse phase, ion exchange, size exclusion, and chirality modes are achievable [1]. The focus on following paragraph will be related mostly on reverse phase, and strategies that have been adopted for polar compounds separation. Furthermore, depending on the properties of the analyte and the required sensitivity, a great variety of detectors is nowadays available, such as ultraviolet-visible absorption (UV-Vis), diode array (DAD), electrochemical, fluorescence, Fourier transform infrared (FRTIR), and mass spectrometry (MS) [2][3].

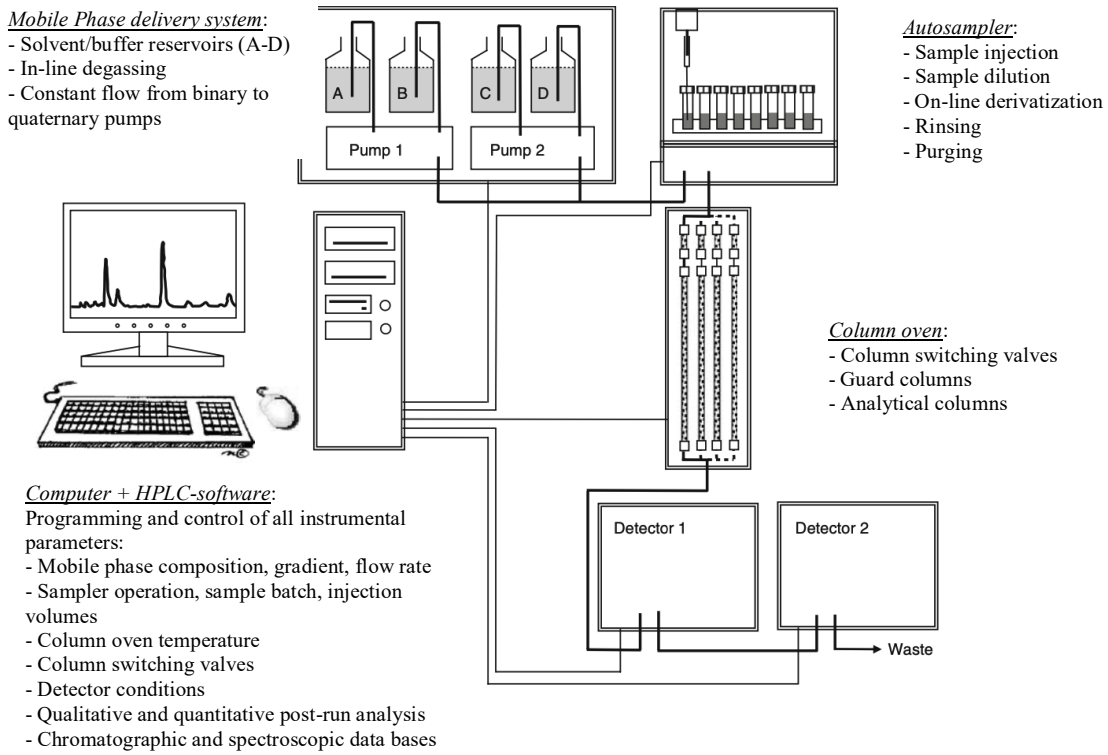


Fig. 1. A schematic of LC-system components [2].

1.1.1 REVERSED PHASE CHROMATOGRAPHY

Column-packing materials of the stainless-steel columns that are mainly used consist of silica gel particles modified by a lipophilic layer of alkyl groups bound to silanol (Si-OH) groups. In reversed phase (RP), material surfaces are covered by alkyl groups bound to silanols. Long alkyl chains (C18) are by far the most common to bind stronger low polarity organic molecules, letting pass through highly polar analytes. On the other hand, shorter alkyl groups from C1 to C8 have lower retention for low polarity analytes. Features and chemical composition of the ligands on the surface of the beads, the density, the capping chemistry, and the pore size of the beads must be considered. The particle size is highly related to the requirements of the separation. The larger the bead size is, the lower is the pressure affects the system. Preparative and analytical separations benefit with beads sizes in the 3–5 μm range, while smaller ranges are usually specified for MS detection.

Chromatographic performances may decrease due to non-uniform particle size or shape, residual non-bound Si-OH groups, and impurities which disturbed the separation by specific interaction with the analytes [2]. However, the use nowadays of highly pure and homogeneous silica particles has dramatically increased the speed and efficiency of separation, as well as the reproducibility of the analysis. Currently trends tend to the use of smaller particle diameters, especially in ultra-performance liquid chromatography (UPLC) systems.

1.1.2 POLAR COMPOUNDS SEPARATION

In the multi-component separation of analytes with different polarity, problems in the use of RP-C8 or -C18 strategies are related to the scarce retention and resolution of polar substances. To retain polar compounds on non-polar surfaces, trying to reduce the amount of organic in the mobile phase (i.e., making it weaker, e.g., 100% aqueous) may results in hydrophobic collapse of the particle surface. Polar imbedded phases are needed to enable a more uniform and increasing the retention of polar substances and avoiding excessive retention and wasted resolution of non-polar substances. Stationary silica phases, with SiOH groups which are functionalized by hydrophilic groups represent the base of the so-called hydrophilic interaction liquid chromatography (HILIC) [4]. Otherwise, straight chain of branched aliphatic, phenyl or fluorinated stationary phases represent valid alternative in terms of selectivity to traditional C8 and C18 [5][6].

1.2 LIQUID CHROMATOGRAPHY COUPLED WITH MASS SPECTROMETRY

The use of liquid chromatography coupled to mass spectrometry, as well as gas chromatography-mass spectrometry has increased considerably in forensic toxicology. Basically, by using LC-systems in combination with MS-detectors, thermolabile, polar and volatile compounds can be easily determined without time-consuming extraction and derivatization procedures. On the other hand, the separation efficiency is not as good as in GC, although this disadvantage is not critical because MS provides good specificity and selectivity. As already described, the LC-system consists of at least a binary pump, with one channel used for buffer

(i.e., volatile buffer, e.g., ammonium acetate) and one for organic solvent (e.g., acetonitrile, or methanol), a column oven and a vacuum degasser to keep the separation conditions stable. Separation is usually performed using a gradient run, adjusted to resolve most of the expected compounds in a sample from each other. Even when a mass spectrometer is used as a detector, several conditions must be considered to obtain reasonable total analysis time, retention and good a peak shape. The applications are several, both for screening procedures covering a wide range of different compounds, as well as quantitative methods for expected compounds. Moreover, liquid chromatography-tandem mass spectrometry (LC-MS/MS) techniques have provided the possibility of combining screening with simultaneous quantitation or confirmation tests, based also on spectrum library search. Commonly, three different approaches have been suggested: target screening, combined screening and confirmation by spectra, and screening based on accurate masses [7]. In target-screening methods, previously selected compounds are monitored using selected ion monitoring (SIM) or multiple reaction monitoring (MRM), while full scan detection mode of any compound at relevant intensity is applied in combined screening and confirmation methods, or in accurate mass-screening procedure. Many different strategies for qualitative and quantitative analysis of relevant drugs in forensic samples with LC-MS are possible, based on different mass analysers: single quadrupole, triple quadrupole, time-of-flight (TOF), ion trap (TRAP), or hybrid configurations (QTOF, QTRAP) especially for most recent screening methods in forensic toxicology. On the other hand, quadrupoles are known for showing high reliability in quantitative approaches. Single-quadrupole MS instruments are more affordable for routine laboratories. However, coeluting compounds and interfering spectra may occur by using these systems, making them unidentifiable from the libraries of pure compounds, and impossible to reproduce without extensive tuning of the instrument [8]. Triple quadrupole instruments [Fig.2] can be also used in SIM as a single quadrupole system, though is not very selective as all the compounds with the same parent ion (m/z value) can pass the survey scan, increasing the risk in getting false positives results. MRM mode in triple quadrupoles is more selective, due to the possibility to monitor multiple MRM transitions during the same run. Correct identifications are

commonly based on the identification of the right precursor ion, fragment ions (daughters) and retention time, also by splitting the chromatographic run into time windows to enhance the sensitivity [9]. The fewer transitions are monitored at the same time, the higher dwell times can be set to optimize the sensitivity and increase the number of compounds to be searched. With tandem-mass spectrometry, better specificity can be also achieved by optimising individually the collision energy for every ion originated only from the selected precursor.

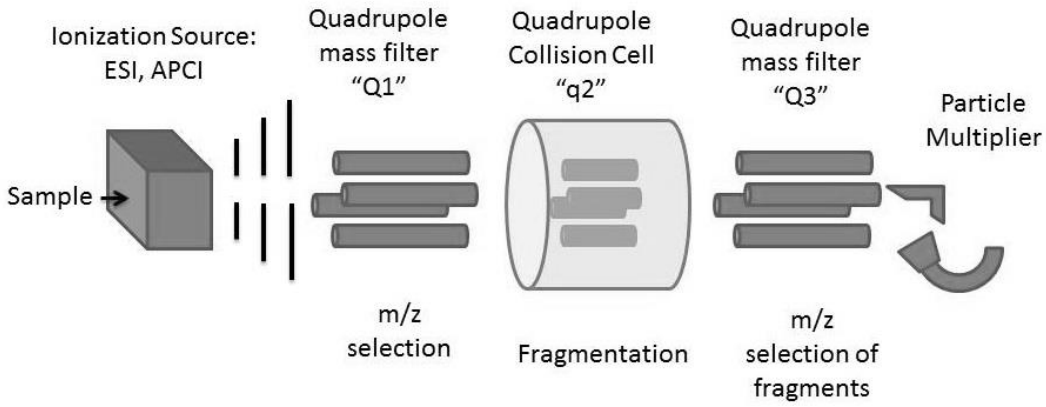


Fig. 2. A schematic of a triple quadruple MS-system.

1.2.1 ELECTROSPRAY IONISATION (ESI)

The electrospray ionisation (ESI) ion source consists of a capillary needle with an applied voltage, through which the sample solution from the LC-system [Fig.3]. The needle probe is surrounded by the nebulising gas (e.g., nitrogen), applied to promote droplet formation and evaporation, and splitting of the unnecessary eluent flow. In an ESI ion source, molecules are ionised in the liquid phase and evaporated to the gas phase under atmospheric pressure, then introduced into a mass spectrometer. ESI is considered a soft ionisation technique. So depending on the polarity of the electric field applied, substances are only slightly, or not at all, fragmented in protonated $[M+H]^+$ or deprotonated $[M–H]^-$ molecules. Within the

ion source, adduct ions might be formed due to the interactions of the compounds with other atoms or molecules. This phenomenon by the way, can be also occur in characteristic ways, and exploited for accurate identification. Typical adduct ions in the positive mode are ammonium $[M+NH4]^+$, sodium $[M+Na]^+$ and potassium $[M+K]^+$ adducts; acetate $[M+HCOO]^-$ and chlorine $[M+Cl]^-$ adducts are the most significant operating in negative mode. ESI source is suitable for a large range of molecules, from moderately non-polar to highly polar molecules, for high molecular weight compounds, or also for thermally labile substances.

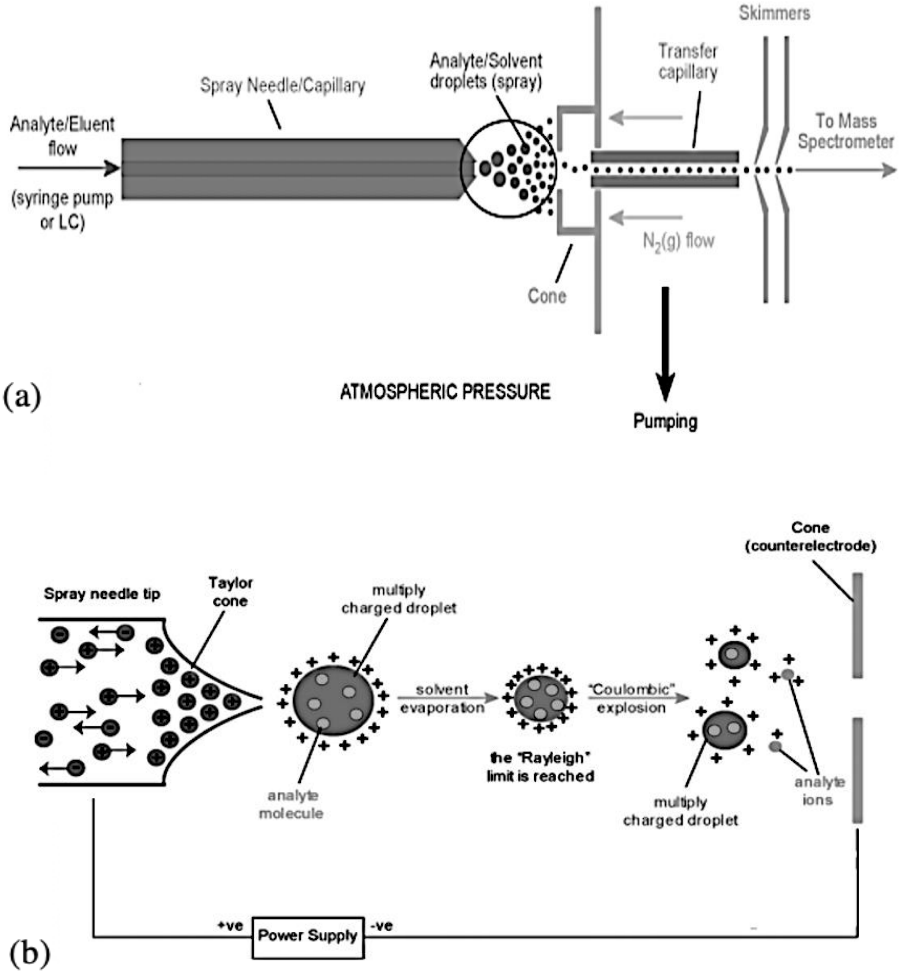


Fig. 3. A schematic of an ESI interface (**a**) and ion formation (**b**).

2. HAIR ANALYSIS FOR DRUGS AND MARKERS OF ABUSE

The use of hair in forensic sciences is important in both clinical and forensic toxicological situations [10]. Hair testing has gained increasing attention over the years especially for retrospective investigation of chronic drug abuse due to its unique ability to serve as a long-term storage site of foreign substances. A hair consists of keratinized cells glued by the cell membrane complex, forming three concentric structures: cuticle, cortex and medulla [11] [Fig.4]. It is already and clearly known indeed that hair differs from other biological matrices such as blood or urine for longer detection window, up to months and years. However, the precise mechanisms of drugs incorporation into hair remain difficult to explain. It is mostly assumed that chemicals enter the matrix by passive diffusion from blood capillaries into growing cells, though a variety of other possible mechanisms might be considered, such as incorporations from deep skin compartments, deposition by diffusion from sweat or sebum secretions, or from the external environment [Fig.5]. Improved chromatographic-mass spectrometric techniques with increased selectivity and sensitivity, and properly optimized methods of sample preparation have enhanced detection limits from the ng/mg range to below pg/mg. These technical advances resulted in several potential applications for many contexts of forensic interest, such as drug testing for case of addictions and criminal liability, driving ability examination, gestational drug exposure, diagnosis of chronic intoxication and in postmortem toxicology, therapy compliance control. Starting from this scenario, scientific research has led to the development of several strategies and standardized hair testing approaches, documented in consensus papers, written collection protocols, and official guidelines. Briefly, steps of hair analysis can be summarized as following described.

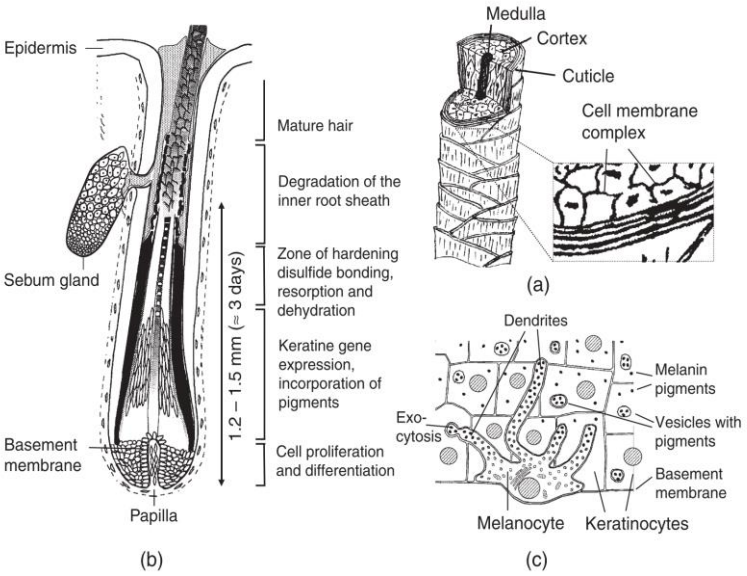


Fig. 4. Structure of the human hair shaft (**a**), and formation of hair in a follicle from matrix cells (**b**). Drug incorporation from blood should occur before completion of keratinisation (**c**) [11].

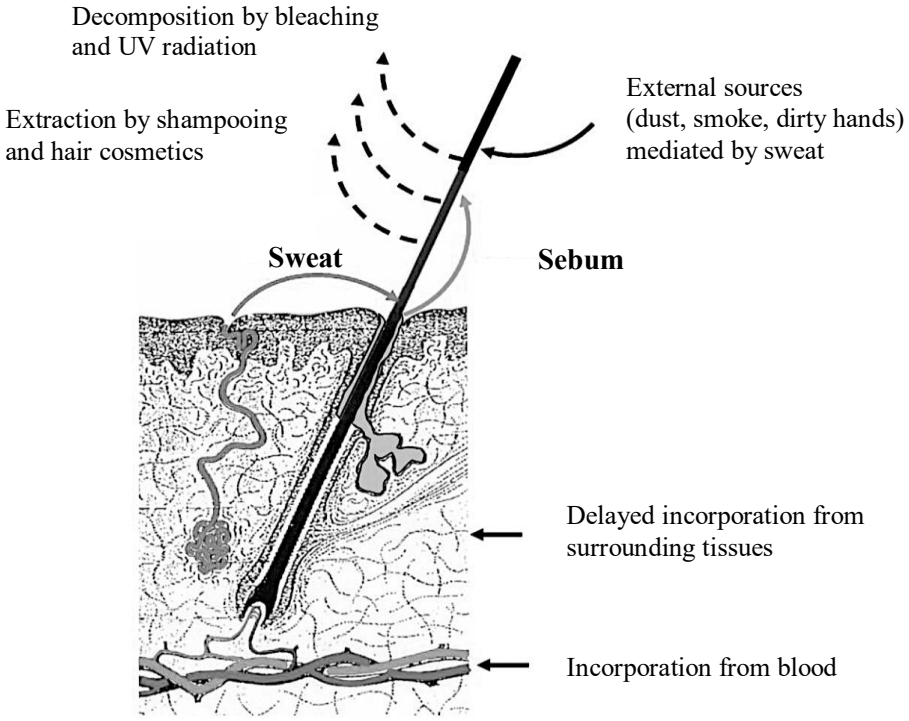


Fig. 5. Incorporation and elimination of drugs in hair [11].

2.1 SAMPLING AND SEGMENTATION

Hair segments to collect, the length and subsequent analytical methods depend on case history and hair sample characteristics. Further aspects that should be recorded include site of collection, colour, and history of cosmetic hair treatment. For forensic purposes, when it is possible at least two different hair samples with a length of 3 cm up to 6 cm should be collected, in order to save a second hair sample for possible future investigations. The preferred site on the scalp is the posterior vertex region of the head, as the region with the lowest portion of telogen hair, with a relatively uniform rate of growth. The appropriate segmental lengths and size varies considerably and reflect information according to time course of drug use. Each centimetre of a segment corresponds approximately to a month of growth. As reported by the Society of Hair Testing (SoHT), where head hair is not available, alternative sources including pubic, axillary and body hair can be collected, though the different physiology of non-head hair has to be considered during the interpretation [12].

2.2 DECONTAMINATION

The most crucial issue with hair analysis is facing possible false-positive results caused by passive exposure [13]. Increased analytical noise and background due to hair care products, sweat, sebum, and other residues present on hair lead potentially contribute to incorrect test results. Thus, decontamination and washing steps to remove external contamination without compromise levels of drugs from the hair matrix must be performed. However, there are no general guidelines on hair washing procedures. Solvents are commonly performed, included dichloromethane, methanol, water or sequential washing with different organic solvents [14], though it has been reported that the analytical outcome of hair analysis can be strongly affected by the wash procedure used [15].

2.3 CUTTING AND GRINDING

After decontamination, hair sample should be pulverized or cut into small pieces. Cutting the hair with scissors is a time-consuming process, especially when a huge number of routine samples should be analysed. To accelerate sample preparation, automated grinding homogenizer and process have been developed and optimized to obtain more homogenous matrices and better extraction yield. Moreover, it has also been demonstrated how detectable concentrations of a studied compound are significantly higher when analysing pulverized vs. cut hair samples, leading also to lower background noise [16]. Thus, when different methodologies are used, comparable results should be achieved and demonstrated through proficiency testing.

2.4 EXTRACTION

Drugs can be extracted from hair by solubilization or digestion of the matrix itself. A strategy that is compatible with almost all drug substances is methanol extraction. Methanolic incubation usually performed in an ultrasonic or thermostatic bath leading to swelling and drug liberation via diffusion, by dissolving neutral and lipophilic compounds. Especially for drugs at high concentrations, the methanol extract can be directly injected, though a disadvantage of this approach is the relatively high risk of impurity released by the matrix that can enter the system. Therefore, a secondary clean-up procedure, a liquid/liquid extraction (LLE), or solid-phase extraction (SPE) is generally recommended. Extraction by aqueous acids or buffer solutions may also be performed. Basic drugs such as cocaine and its metabolites, opiates, or amphetamines, are well extracted following HCl or phosphate buffer incubation [18][19]. On the other hand, for drugs that are stable under alkaline conditions such as (−)-trans-Δ9-tetrahydrocannabinol (THC) and 11-nor-Δ9-tetrahydrocannabinol-9-carboxylic acid (THC-COOH) [19], or antidepressants and neuroleptics, basic extraction in aqueous NaOH is convenient. Moreover, enzymatic digestion matrix can also be performed. Enzymes such as pronase and proteinase K, improved in the presence of dithiothreitol can be used to hydrolyse hair matrix proteins by reducing disulphide bonds.

2.5 DETECTION

Detection methods used in forensic toxicology for hair analysis must be suitable for unambiguous drug identification and quantitation. Issues in drug screening methods for hair are commonly related to low drug concentration and small sample size. Immunoassay (IA) kits might be affected by insufficient sensitivity and specificity, or available for a limited number of drugs or metabolites in hair. Sensitivity should be adequate avoid false negative, whereas positive results must be confirmed by a different analytical technique. The combination of liquid or gas chromatography coupled to mass spectrometry is useful increasingly used in hair analysis. The advantages of GC–MS include high resolution and specificity, with sufficient accuracy at very low concentration for volatile and stable at the high temperature substances. However, samples require derivatization steps prior to GC–MS analysis. Alternatively, LC-systems coupled with tandem-mass spectrometry can be used, also as valid alternative for screening methods.

2.6 PRACTICAL APPLICATIONS OF HAIR SAMPLE ANALYSIS

Benefits in the use of hair as a long-term index of drug use history have already been mentioned. Hair analysis provides a valid mechanism to monitor and control abuse of substances for many and several circumstances, such as workplace drug testing, driving ability examinations, diagnosis of drug abuse and chronic intoxication, gestational drug exposure, in postmortem toxicology, doping or therapy compliance control. In the present thesis, hair analysis was applied in two different applications. The first work concerns the detection of ethyl glucuronide (EtG) in hair, a metabolite and marker of chronic alcohol consumption. For the second application, the focus will be set particularly on the potential use of hair analysis in therapeutic drug control for individual adjustment of drug dosing and therapeutic drug monitoring (TDM). This part is about the development and validation of an analytical method for the determination of statins in hair, as a tool to monitor adherence to therapy to these medicinal drugs. Methods' applications will be also presented.

3. EXPERIMENTAL PART

3.1 INTRODUCTION AND AIMS

For many years hair analysis was successfully applied to the determination of drugs of abuse in different forensic contexts, such as driving licence re-granting, child custody and detoxification programs. However, more recently the spectrum of the applications of hair analysis has become larger, including the determination of therapeutical drugs and biomarkers. The aim of the research project carried out during the PhD course was to implement new analytical methods for the determination in hair of ethyl glucuronide (EtG), in the frame of the diagnosis of chronic alcohol abuse, and of cholesterol-lowering medications (statins), in the context of adherence to therapy monitoring.

3.2 ETHYL GLUCURONIDE IN HAIR (HETG)

Ethyl glucuronide (ethyl β-d-6-glucuronide, EtG) is a polar, slightly acidic and relatively stable minor non-oxidative ethanol metabolite resulting from enzymatic ethanol (EtOH) glucuronidation in the liver [20] [Fig.6]. Most of the ingested alcohol is metabolized in the liver in a two-stage enzymatically catalysed oxidation process [21]. Firstly, it is converted to acetaldehyde by alcohol dehydrogenase; then, it is furtherly metabolized to acetate by aldehyde dehydrogenase. A small amount is excreted unchanged in urine, sweat and expired air. It can be detected in several biological fluids and tissues such as blood and urine, commonly used to assess short-term consumption, or keratinized matrices, to monitor abstinence and chronic abuse over longer intervals of time [22]. The average time difference in reaching maximum blood ethanol concentrations and maximum plasma metabolite concentrations after the ingestion is approximately 2–3 h for EtG, detected in the urine for between 72 and 90 h, and 1–2 h for the ethyl sulphate (EtS) [23][24]. As an effective alcohol consumption marker, EtG is considered as the most reliable indicator of long-term drinking pattern in both clinical and forensic settings [25]–[27], and its relevance in the monitoring of heavy chronic drinking [28], traffic safety [29], workplace surveillance [30] has increased over the past years [31].

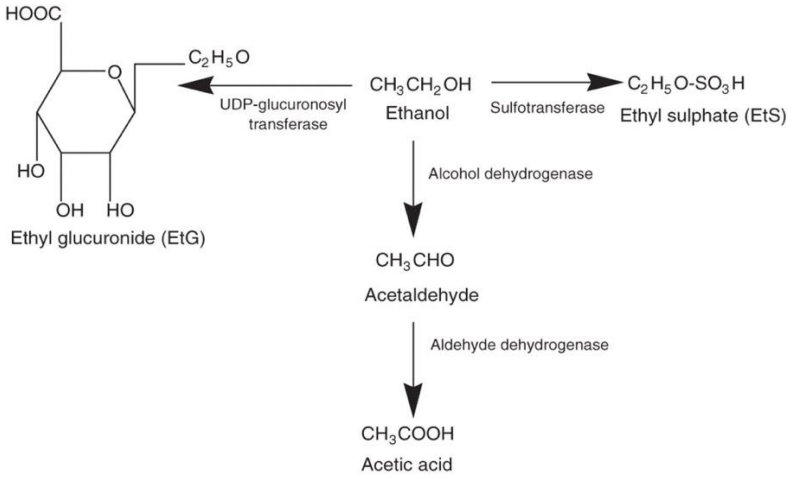


Fig. 6. Alcohol metabolism and formation of ethyl glucuronide [21].

The assessment of chronic alcohol use requires diverse and integrated clinical and analytical methodologies and standardized approaches. Several methods for the determination of EtG in hair (hEtG) are reported in the literature. It was firstly systematically measure in this matrix by Skopp et. al (2000) using gas chromatography coupled to mass spectrometry, and obtaining a LOD of 2200 pg/mg and a LLOQ of 5000 pg/mg [32]. Although GC-based analysis may guarantee very high analytical sensitivity [33][34], due to the time-consuming derivatization step required prior to GC analysis they are currently less used in routine hEtG analysis. Janda *et al*. (2002) developed the first LC-MS/MS method using isotopically labeled (deuterated) internal standard (EtG-D_5) and obtaining LOD = 51 pg/ mg and LLOQ = 102 pg/mg [35]. Moreover, in this work some important strategies are reported to improve sensitivity in the detection of EtG. Indeed, a post-column infusion of acetonitrile was adopted to improve the ionization process of this highly polar molecule, without influencing the chromatographic separation. A first highly sensitive LC-MS/MS method was developed by Morini *et al*. (2006) [28]. Using 100 mg of hair, LOD of 2 pg/mg and LLOQ of 3 pg/mg were achieved, providing the opportunity to investigate also

basal levels of hEtG in abstinent populations. The use of hEtG is generally grounded on interpretative concentration thresholds (cut-offs) established in the forensic toxicology community with the indications of the Society of Hair Testing (SoHT) regarding the correct classification of the severity of long-term alcohol intake. Already in 2009, a consensus on the cut-off concentrations of hEtG was proposed. Based on the World Health Organization guidelines which define "harmful" an alcohol consumption of 60 g of pure ethanol per day over several months, according to the Consensus a hEtG concentration above 30 pg/mg in the proximal 0-3 cm proximal segment of the hair strongly suggests chronic excessive alcohol consumption [36]. However, over the past few years this interpretative limit has been lively debated and different opinions on the possibility to re-establish cut-offs in this field have emerged. Some authors pointed out that the diagnostic specificity for hEtG is theoretically very high, since its formation was necessarily associated with ethanol, but a hEtG concentration below 30 pg/mg did not exclude alcohol abuse [37][38]. Since 2009, the SoHT Consensus has been updated about every two years (2011, 2012, 2014, 2016 and 2019). In the 2012 Consensus, it is reported that a concentration of EtG $\geq$ 7 pg/mg in the 0-3 up to 0-6 cm proximal scalp hair segment strongly suggests repeated alcohol consumption, either gas or liquid chromatography coupled with tandem-mass spectrometry with deuterated EtG as internal standard should be used to test for EtG in hair, and validated methods used for this purpose should have a LOQ $\leq$ 3 pg/mg [39]. In 2014, it was added a wording on concentrations < 7 pg/mg, which do not contradict the self-reported abstinence of a person during the corresponding time period before sampling [40]. Finally, in 2019 the cut-off for abstinence has been lowered to 5 pg/mg based on the fact that hEtG concentration lower than or equal to 5 pg/mg does not contradict self-reported abstinence. On the other hand, concentrations greater than 5 pg/mg strongly suggest repeated alcohol consumption [12]. According to the re-evaluation of the hEtG cut-offs over the years, another aspect must be mentioned. Currently, the analytical protocol for the determination of hEtG is far from being harmonized, and consequently the laboratories use different procedures that may yield different recoveries from real hair samples. It is also well known that different sample pre-treatments provide substantially different recoveries, leading to an inherent

analytical bias in result reporting. This variability in pre-analytical and analytical protocols is still strongly debated [41], as it limits the possibility of using fixed cut-off values, particularly when employed in highly contentious fields such as forensic and clinical chemistry. Variability on EtG concentrations in keratin matrix, particularly in hair samples, depending on sample pretreatment have been noticed and reported by several authors. Moreover, in the last revised SoHT Consensus on drugs of abuse (DOA) testing in hair of 2021, precise indications are reported on sample preparation and pre-analytical steps [42]. For instance, the preparation of hair samples should involve steps including washing, decontamination, and segmentation if required, aiming to obtain a representative sample from the available material. Moreover, before the extraction, hair samples must be homogenized by pulverization or digestion, or cut in small pieces, though for drug or metabolites that are present at low levels, pulverization is recommended. It has already been observed that the particle size of hair samples, as other factors such as, homogenization and incubation temperature and time, extraction solvents and ultrasonication steps, can influence the EtG concentrations and the extraction efficiency, which is despite all strongly increased in milled samples compared to cut hair [34][41][43][44]. Vignali *et al.* (2018), observed a mean increase of 20% in the concentration of EtG in pulverized hair, respect to the simple cutting procedure. The SoHT has already added to the consensus that pulverization should be chosen as the preferred pretreatment. Moreover, if non-pulverized samples or different methodologies are used for extraction, laboratories must demonstrate that comparable results are achieved through proficiency testing. At the Unit of Forensic Medicine of the University of Verona, EtG determination in hair was originally performed by using gas chromatography coupled with tandem-mass spectrometry (GC-MS/MS). In this PhD project, a specific study was planned and dedicated to the optimization of a LC-MS/MS method for the determination of EtG in hair, in order to reduce the analysis time and to improve the overall performance of the analysis. Part of this study was conducted at the Institute of Forensic Medicine of the Kantonsspital St.Gallen in St. Gallen, Switzerland.

3.3 OPTIMIZATION AND VALIDATION OF A LC-MS/MS METHOD FOR HETG

The measurement of EtG concentrations in hair is challenging due to the low concentrations recommended to discriminate [45]. GC–MS/MS methods generally show very high selectivity, sensitivity, and specificity. However, the most significant difference between GC-based and LC-based methods is related to the sample preparation prior the injections, which is rather laborious and time-consuming with GC–MS/MS. In the protocol previously reported, a derivatization step subsequently the SPE extraction of EtG from aqueous phase need to be carried out. This step also requires a further evaporation step of the derivatization reagent before the injection, taking of course quite a long time. From the literature, the LC–MS/MS strategy appears to be the first-choice method for hEtG quantitative analysis. The prevalence of this assay is attributed to the fact that no derivatisation steps are required. Currently in our laboratory, a LC-based assay is undergoing optimization and validation by using the UHPLC-MS/MS system composed by the ACQUITY UPLC™ system (Waters Corporation, Milford, USA) coupled to the 6500 QTrap mass spectrometer (Sciex, Warrington, UK). The chromatographic separation is achieved by injecting 5 µL of the samples into a 100 × 2.1 mm Acquity 1.8 µm HSS-T3 column (Waters Corporation, Milford, USA) held at 25°C. Gradient elution is set at a flow rate of 0.5 mL/min; solvent A consists of 5 mM ammonium formate containing 0.1% *v*/*v* formic acid, while solvent B is acetonitrile. The gradient profile is as follows: 0-2.50 min 0% B; 2.50-2.60 min linear gradient to 80% B, then held till 4.50 min; 4.50-4.60 min linear gradient from 80 to 0% B, then held till the end of the run for the re-equilibration prior the following injection. The total run time is 10 minutes. The MS-source operates in negative ESI ionization mode at a temperature (TEM) of 600°C (curtain gas, CUR: 30 L/hour; ion spray voltage, IS*: -4500 V; ion source gas GS1: 65 L/hour; GS2: 70 L/hour). Acquisition is performed in the multiple reaction monitoring (MRM) mode, by using optimized transition and mass parameters [Table 1].

Analyte	Precursor Ion (Da)	Daughter Ion (Da)	DP (V)	EP (V)	CE (V)	CXP (V)	Retention Time (min)
EtG	221.0	**75.0**	-42	-5	-19	-10	2.33
		85.0	-59	-5	-19	-10	
EtG-D_5	226.0	**75.0**	-23	-3	-21	-10	2.25
		85.0	-23	-3	-21	-10	

Table 1. Ionization mode of MRMs transitions, optimized mass parameters (declustering potential, DP; entrance potential, EP; collision cell exit potential, CXP; collision energy, CE) and retention times of the studied compound and its deuterated internal standard (IS) using the ACQUITY UPLC™ coupled to the 6500 QTrap mass spectrometer (quantifier transitions are in bold).

An important aspect which increased the attention on LC-MS/MS technique for hEtG is that liquid chromatography is more prone to the analysis of highly polar compounds in aqueous suspensions. Many protocols exploit columns based on RP-chemistry, requiring however high amounts of aqueous mobile phase to retain polar compounds, and strongly negatively affecting the ionization at the MS source level [46]. To overcome such limitation, post-column addition of solvents such as acetonitrile, or isopropanol is often used. Furthermore, more hydrophilic stationary phases for hEtG analysis have revealed successful [47]. Polar or ionized solutes can be separated on a polar stationary phase with polar solvents containing water as a minor constituent of the mobile phase. In this frame, with the aim to improve the separation in our LC-based protocol, in the context of the PhD Course an external working experience at the Institute of Forensic Medicine in St. Gallen, Switzerland has done. In the Laboratory of Forensic Toxicology of the Kantonsspital St.Gallen, the assay for hEtG currently applied is based on a HPLC-MS/MS system composed of an Agilent-1200 HPLC (Agilent Technologies, Santa Clara, USA) coupled to a 4000 QTrap mass spectrometer (Sciex, Warrington, UK). The chromatographic separation is achieved by injecting 20 µL of the samples into an Agilent 100 × 4.6

mm Zorbax Eclipse 1.8 μm XDB-C8 column provided with a Zorbax Fusion RP 4 x 2 mm precolumn, at 25 °C. Gradient elution is set at a flow rate of 0.75 mL/min; solvent A consists of 0.1% *v*/*v* formic acid in H_2O, while solvent B is 0.1% *v*/*v* formic acid in H_20/ACN (1:9). A post-column spray of isopropanol flowing at 0.1 mL/min is added. The gradient profile is as follows: 0-4 min 0% B; 4-5.50 min linear gradient to 20% B; 5.50-5.52 min linear gradient to 100% B, then held till 12.50 min; 12.50-12.60 min linear gradient from 100 to 0% B, then held till the end of the run for the re-equilibration prior the following injection. The total run time is 20 minutes. The MS-source operates in negative ESI ionization mode at 700°C (CUR: 40 L/hour; IS*: -4500 V; GS1: 85 L/hour; GS2: 85 L/hour). Acquisition is performed in the MRM-mode, by using optimized transition and mass parameters [Table 2]. A typical chromatogram obtained by injecting a standard mixture of the studied compound is reported in Figure 7.

Analyte	Precursor Ion (Da)	Daughter Ion (Da)	DP (V)	EP (V)	CE (V)	CXP (V)	Retention Time (min)
EtG	220.9	**84.9**	-24	-4.6	-45	-10	4.41
		74.9	-23	-4.1	-45	-10	
		56.9	-32	-4.1	-45	-10	
		54.9	-44	-4.1	-45	-10	
EtG-D_5	225.9	**84.9**	-24	-4.6	-45	-10	4.26

Table 2. MRMs transitions, optimized mass parameters and retention times of the studied compound and its deuterated internal standard (IS) using the Agilent-1200 HPLC coupled to the 4000 QTrap mass spectrometer (quantifier transitions are in bold).

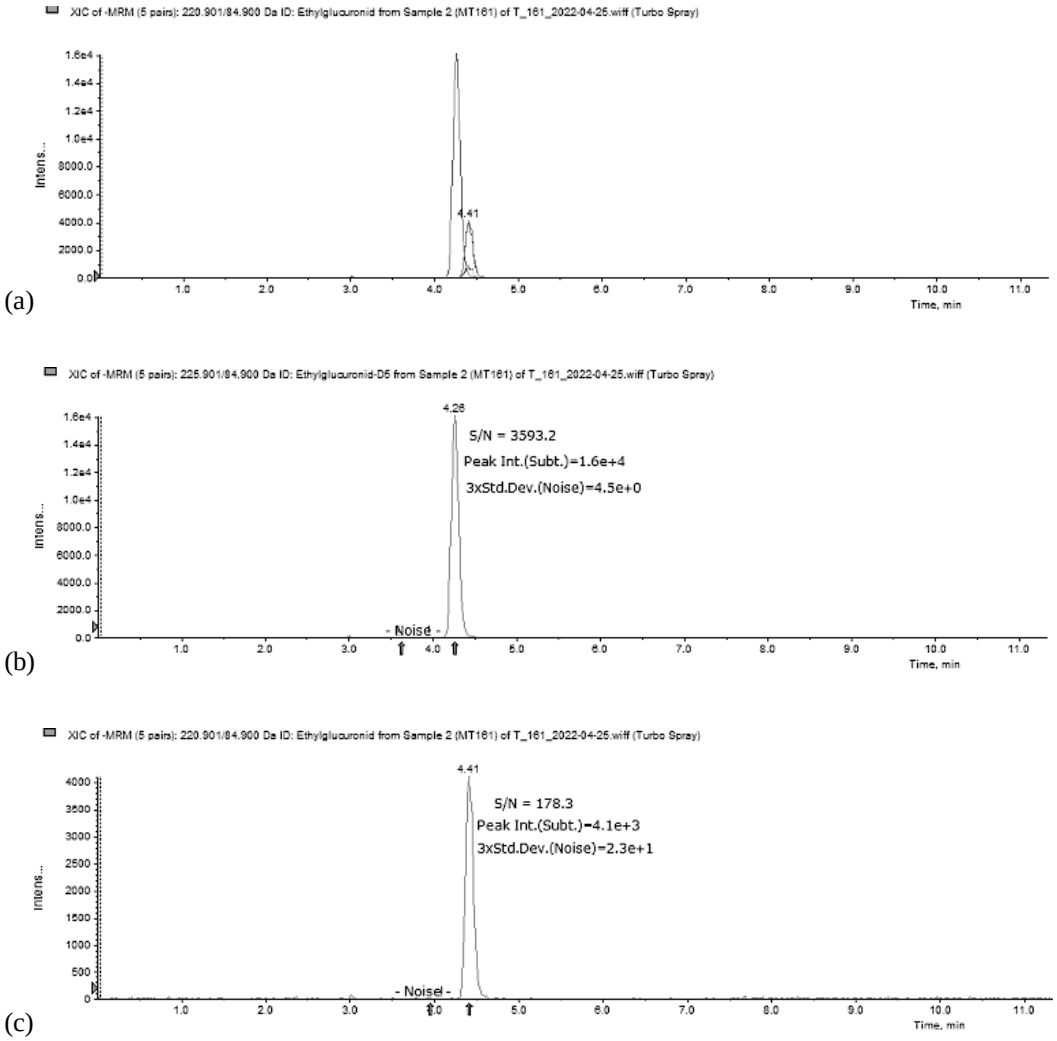


Fig. 7. Total ion chromatogram (**a**) of a standard mixture containing EtG at a concentration of 5 ng/mL (**c, quantifier ion**: m/z 220.9→84.9, tR: 4.41 min) and the IS EtG-D_5 at a concentration of 20 ng/mL (**b**: m/z 225.9→84.9, tR: 4.26 min) obtained using the Agilent-1200 HPLC coupled to the 4000 QTrap provided with the Agilent 100 × 4.6 mm Zorbax Eclipse 1.8 µm XDB-C8 column.

3.3.1 COMPARISON OF HPLC AND UHPLC FEATURES

High-performance chromatography allows to separate the maximum number of peaks in the shortest time frame. To do that, the choice of LC column becomes crucial, and the system hardware must be optimized to allow the column to deliver the required performances. HPLC is a robust, rugged methodology, able to remain unaffected by small, but deliberate variations in method parameters. On the other hand, UHPLC allows to separate sample compounds in a shorter time frame, owing to the use of smaller particle columns. In general, chromatographic peaks in UHPLC are narrower and sample throughput is higher. However, UHPLC demands highest quality solvents to be used, and properly extracted and filtered of particulates samples to be injected. Beside robustness, capital cost and operating cost for HPLC are lower. Another aspect to consider which affects both the system is related to the pressures occurred. HPLC fittings and connectors have been engineered to withstand higher pressures, but with the popularity of smaller HPLC column particle sizes due to the decrease in analysis time, HPLC system pressures have increased significantly. These smaller particles result in ads back pressure to the system and led to the development of LC-systems that can perform at higher pressures. UHPLC can withstand pressures up to 20,000 psi or 1,300 bar, while HPLC pressures are typically limited to 6,000 psi or 400 bar. During the experience conducted at the Kantonsspital St.Gallen, the necessity in the re-validation of the hEtG method on different systems raised basically on issues regarding short half-life of the column due to high back pressure (higher than 400 bar), long run time analysis, high volumes to inject and difficulties to find spare parts for a system starting to get old. As a result, two main backup systems have been optimized for further routine investigations, testing different chromatographic columns aiming for faster separation and less extract volume to use.

3.3.2 SAMPLE PREPARATION

Hair samples have been collected from subjects undergoing probation and DUI offenders, monitoring and zero tolerance alcohol rehabilitation programs, return-to-work programs, taken from the posterior vertex region of the head with the proximal hair segments of no more than 6 cm in length (3-5 cm long segments are

recommended). Samples have been washed and decontaminated with 10 mL H_20 milliQ, 10 mL of acetone (2 min, each step), and finally with 5 mL of petroleum ether for 4 min. Once completely dry, according to the sample features, pulverization has been obtained by using the Omni Bead Ruptor working at < 10°C with dry-ice/Isopropanol for 7 mL tubes containing 40 magnetic, or ceramic beads (3 mm∅; Speed: 5.65 m/s; Time: 30 sec; Cycles: 8; Pause: 1 min), or by using the Retsch MM400 for 2 mL eppendorf containing 2 magnetic beads of 7 mm∅ (Freq: 30 Hz; Time: 5 min; Cycles: 1). About 20 mg have been transferred into 2 mL eppendorf, spiked into 1.5 mL of deionized water, and let incubate for 1.5 h at room temperature on a shaker. After a centrifuge step at 13000 g for 5 minutes, the followed SPE procedure involves the following steps: Oasis-MAX cartridges (Waters Corporation, Milford, USA) are conditioned with 2 mL methanol and 2 mL water. After loading samples, cartridges are rinsed with 1 mL 1% ammonia in aqueous solution and 2mL methanol. Elution is finally performed using 1.5 mL of 2% formic acid in methanol directly into 2 mL V-Vials without the insert, making then the extracts evaporate and concentrate using the LabConco Centrivap vacuum concentrator system at 50°C for 75 min. Finally, 50 uL of eluent A (0.1% formic acid/H_20) are added prior the injection into the LC-MS/MS.

3.3.3 OPTIMIZATION OF THE CHROMATOGRAPHIC SEPARATION

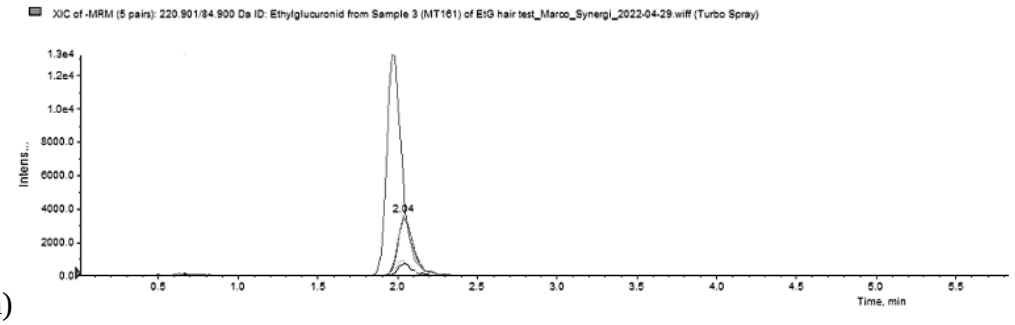
Method optimization experiments have been performed on two HPLC-MS/MS chosen as backup systems for the re-validation of the hEtG protocol; a Shimadzu Nexera LC-20ADxr HPLC (Shimadzu Corporation, Kyoto, JPN) coupled to a 5500 QTrap mass spectrometer (Sciex, Warrington, UK), and a Sciex ExionLC-20 AC HPLC coupled to a 6500+ QTrap mass spectrometer (Sciex, Warrington, UK). MS-working parameters are reported in Table 3. Same precursor and daughter ions reported in the Table 2 have been checked. However, the m/z 74.9 has been selected as quantifier as the ion which provided the highest signal with both the systems.

	Nexera LC-20ADxr HPLC - 5500 QTrap	ExionLC-20 AC HPLC - 6500+ QTrap
Curtain gas (CUR)	40 L/hour	35 L/hour
Collision gas (CAD*)	Medium	Medium
Ion spray Voltage (IS*)	-4500 V	-4500 V
Temperature (TEM)	700°C	700°C
Ion source gas 1 (GS1)	85 L/hour	70 L/hour
Ion source gas 2 (GS2)	85 L/hour	85 L/hour
Ion source polarity	Negative	Negative
MS-mode	MRM	MRM

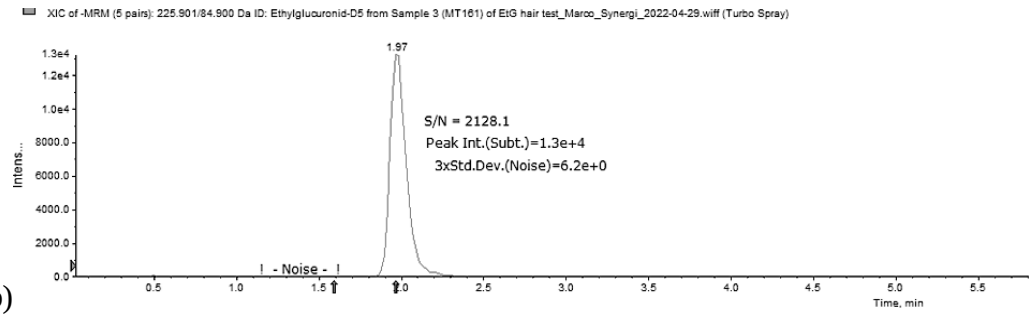
Table 3. Optimized mass parameters for the MRM-mode analysis using the chosen HPLC-MS/MS systems.

Chromatographic separation optimization has been planned by performing injections of pure standard solutions of EtG and EtG-D_5 on all the systems, testing also different columns suitable for improved separation of polar compounds such as EtG, according to the manufactures. Indeed, extremely polar analytes are not always retained and often do not separate well on conventional C18 columns. Firstly, a Synergi Hydro-RP 3.0 x 50 mm, 2.5 ųm provided with a AQ C18 4 x 2 mm precolumn (Phenomenex, Torrance, USA) has been tested. An example of a chromatogram obtained by injecting the same standard mixture shown in Figure 8 in the routine assay is reported as a comparison in Figure 9. As reported in the data sheet, this column is a polar endcapped C18 column that provides very high hydrophobic interactions and hydrogen donating capabilities, stable in 100% aqueous conditions limits until a maximum backpressure of 5,000 psi (345 bar),

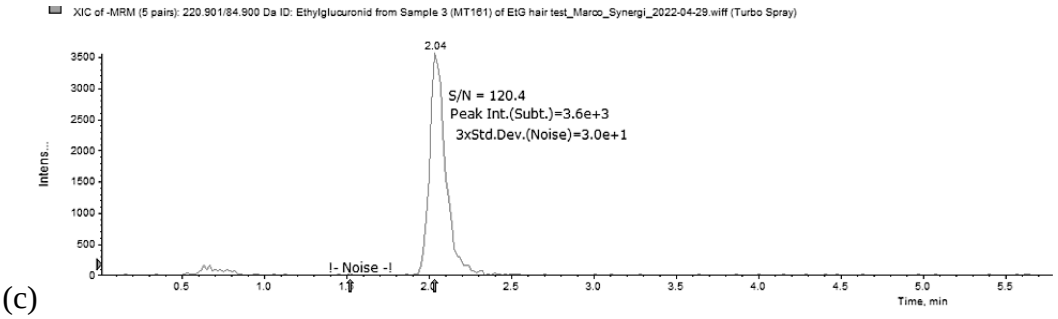
below a maximum temperature of 60 °C. Conventional C18 phases are poorly wetted by highly aqueous mobile phases causing the C18 ligands to mat down on the surface of the silica and, over time, retention is completely lost. The Synergi C18 bonded phase endcapped with a unique proprietary polar group to provide extreme retention of both hydrophobic as well as polar compounds. The net results in a very retentive C18 phase well suited to separating extremely polar analytes. Greater hydrophobicity is useful for many applications because higher percentage organic mobile phase can be used resulting in shorter run and re-equilibration times. This enhanced hydrophobicity results in analytes eluting at higher percentage organic mobile phase and improved sensitivity. Once adjusted the elution gradient, a faster elution, good peak shape and S/N ratio has been obtained by using this column, optimizing the flow and the working temperature respectively at 0.5 mL/min and 25°C. However, once tested for real extracted samples, bad background noise emerged especially at lower EtG concentrations [Fig.9]. As a result, compared to the routinely applied system no significant improvements in the chromatographic separations have been obtained by using this column.



(a)

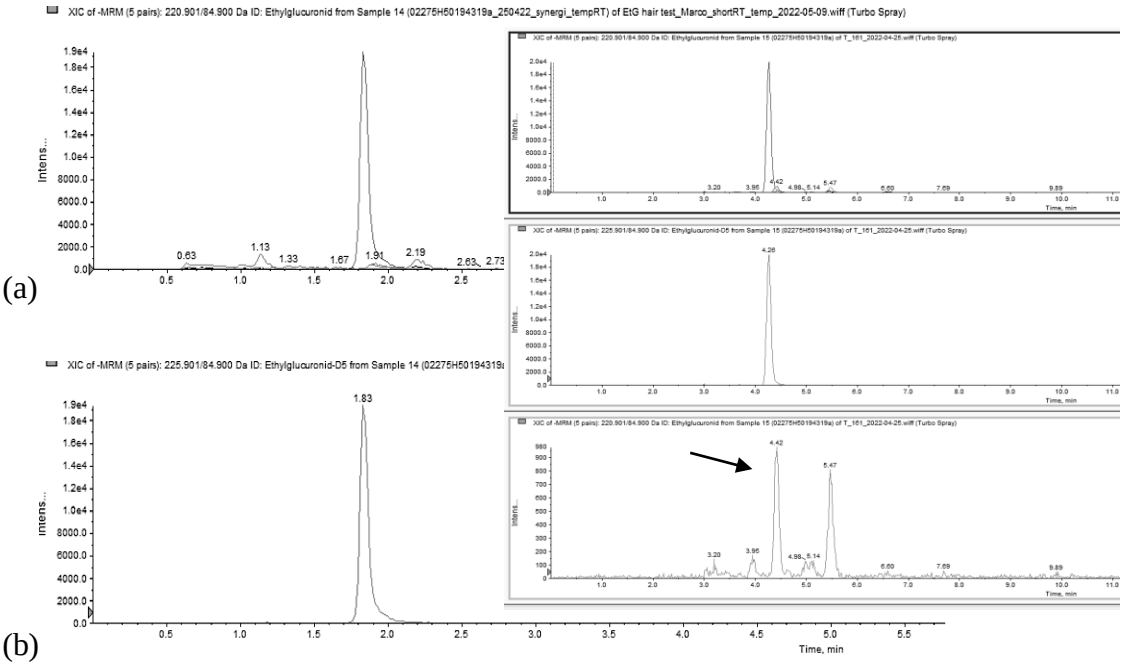


(b)



(c)

Fig. 8. Total ion chromatogram (**a**) of a standard mixture containing EtG at a concentration of 5 ng/mL (**c, quantifier ion**: m/z 220.9→84.9, tR: 2.04 min) and the IS EtG-D_5 at a concentration of 20 ng/mL (**b**: m/z 225.9→84.9, tR: 1.97 min) obtained using the Agilent-1200 HPLC coupled to the 4000 QTrap provided with the Phenomenex Synergi Hydro-RP 3.0 x 50 mm, 2.5 ųm at 25°C (Eluent A: 0.1% Form.Ac in H_20; Eluent B: 0.1% Form.Ac in H_20/ACN 1:9; Flow: 0.5 mL/min; post-column spray: 0.1 mL/min Isopropanol; Inj.volume: 20 ųL; Elution gradient: 0-2.50 min 0% B; 2.50-2.60 min linear gradient to 100% B, then held till 5.50 min; 5.50-5.60 min linear gradient from 100 to 0% B, then held till the end of the run for the re-equilibration prior the following injection; Total run time: 10 minutes).



(a)

(b)

(c)

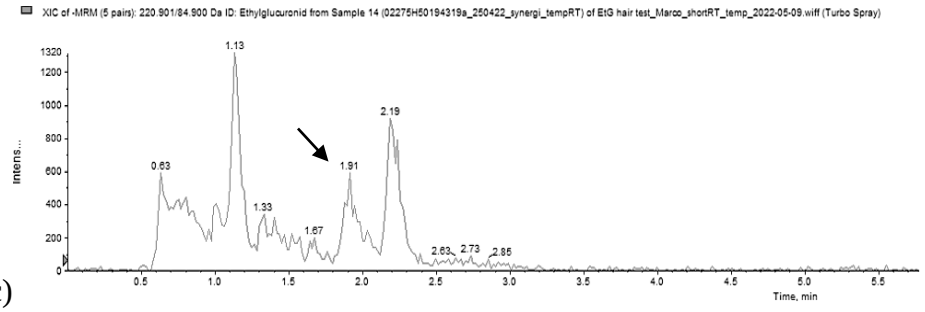


Fig. 9. Total ion chromatogram (**a**) of a real extracted sample containing EtG at a concentration of 4.75 pg/mg (**c, EtG**: m/z 220.9→84.9; **b, EtG-D$_5$**: m/z 225.9→84.9) obtained using the Agilent-1200 HPLC coupled to the 4000 QTrap provided with the Synergi Hydro-RP 3.0 x 50 mm, 2.5 µm at 25°C (Eluent A: 0.1% Form.Ac in H_20; Eluent B: 0.1% Form.Ac in H_20/ACN 1:9; Flow: 0.5 mL/min; post-column spray: 0.1 mL/min Isopropanol; Inj.volume: 20 µL; Elution gradient: 0-2.50 min 0% B; 2.50-2.60 min linear gradient to 100% B, then held till 5.50 min; 5.50-5.60 min linear gradient from 100 to 0% B, then held till the end of the run for the re-equilibration prior the following injection; Total run time: 10 minutes). On the top-right corner, it is reported what has been obtained by testing the same sample on the Agilent-1200 HPLC coupled to the 4000 QTrap and provided with the Agilent 100 × 4.6 mm Zorbax Eclipse 1.8 µm XDB-C8 during the routine analysis.

For these reasons, similarly an Acquity UPLC-HSS T3 2.1 x 100 mm, 1.8 µm (Waters Corporation, Milford, USA) has been tested. The HSS (High Strength Silica) is designed as a 100% silica-based bonded C18 phase, intended for use in applications up to 18000 psi (1240 bar). It is compatible with 100% aqueous mobile phase, suitable for the separation of both polar and non-polar compounds at recommended temperature of 20-45 °C. Also with the HSS, some unexpected results have been obtained, especially in terms of peak shape. As HPLC, the pressure that overalls the systems, set at a maximum of 400 bar, forced the use of this column close to the highest supported temperature, with a flow of 0.25 mL/min. At these conditions, not sufficiently good peak shapes have been obtained, as they resulted to be too much broad, and tailed compared to those obtained with the commonly used column [Fig.10].

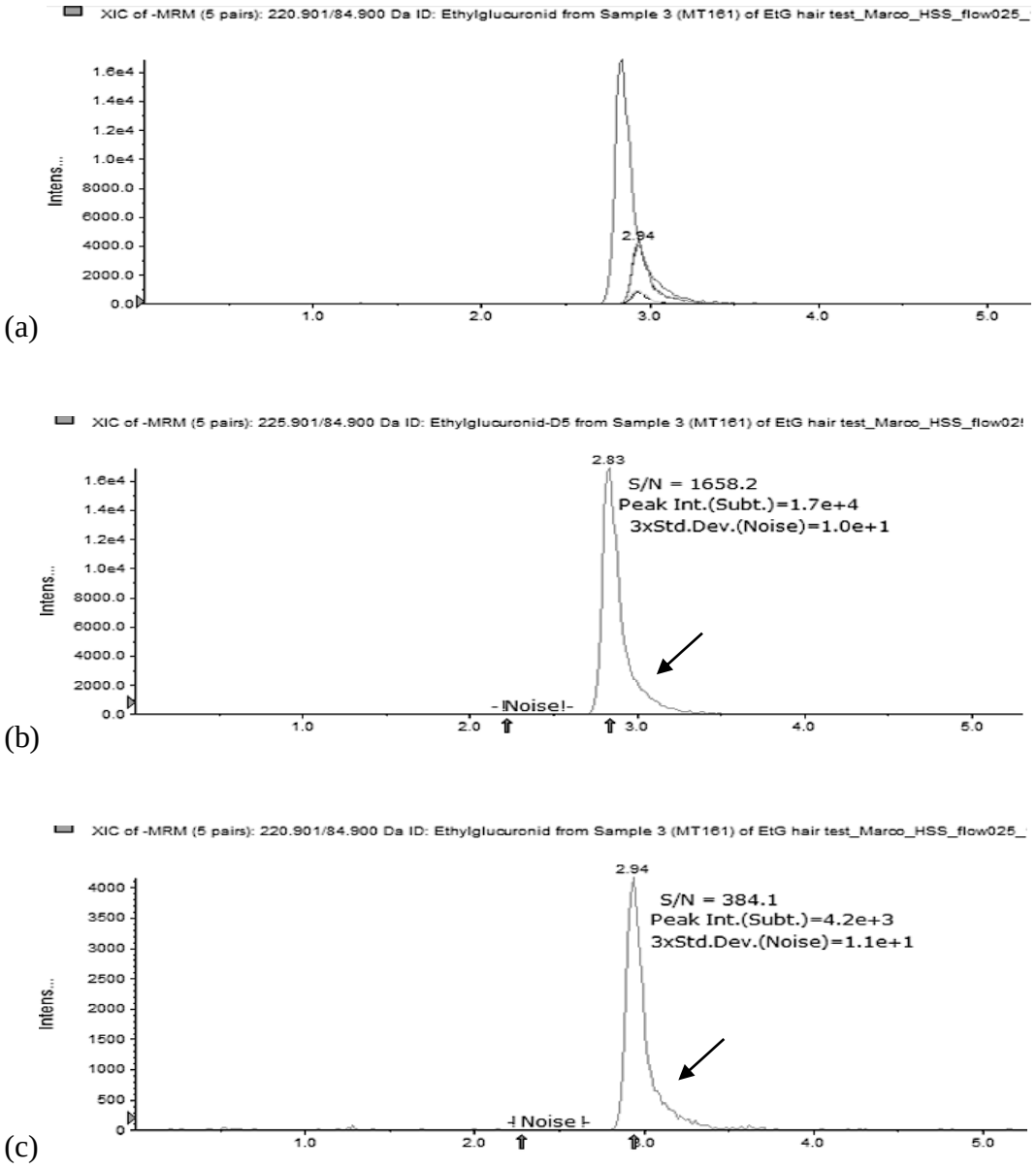


Fig. 10. Total ion chromatogram (**a**) of a standard mixture containing EtG at a concentration of 5 ng/mL (**c, quantifier ion**: m/z 220.9→84.9, tR: 2.94 min) and the IS EtG-D_5 at a concentration of 20 ng/mL (**b**: m/z 225.9→84.9, tR: 2.83 min) obtained using the Agilent-1200 HPLC coupled to the 4000 QTrap provided with the Acquity UPLC-HSS T3 2.1 x 100 mm, 1.8 µm at 40°C (Eluent A: 0.1% Form.Ac in H_20; Eluent B: 0.1% Form.Ac in H_20/ACN 1:9; Flow: 0.25 mL/min; post-column spray: 0.1 mL/min Isopropanol; Inj.volume: 15 µL; Elution gradient: 0-0.50 min 0% B; 0.50-2.00 min linear gradient to 20% B; 2.00-2.10 min linear gradient to 100% B, then held till 7.90 min; 7.90-8.00 min linear gradient from 100 to 0% B, then held till the end of the run for the re-equilibration prior the following injection; Total run time: 15 minutes). Tailed peaks shape and broadening effects are indicated.

The third and last column tested was the Hypercarb-PGC 3.0 x 100 mm, 3.0 µm provided with a Hypercarb 3 µm precolumn with 10 x 2.1 m cartridges (Thermo Fisher, Waltham, USA). The Hypercarb is designed as a 100% porous graphitic carbon (PGC) suitable for extended separation capabilities, high retention of very polar analytes, stable at wide pH range from 0 to 14 and ideal for high temperature applications. The PGC is a unique stationary phase composed of flat sheets of hexagonally arranged carbon atoms. It is unlike traditional silica bonded phases in both its structure and retentive properties, allowing for total stability and the retention highly polar species both reversed phase and normal phase applications. Hypercarb's mechanism of interaction is very dependent upon the polarity of the solute. These specific interaction mechanisms allow the successful retention and resolution of analytes that cannot be separated by typical reversed phase HPLC. In typical reversed phase chromatography, the retention of an analyte is directly related to its hydrophobicity: the more hydrophobic the analyte, the longer its retention. Conversely, as the polarity of the analyte increases, analyte-solvent interactions begin to dominate, and retention is reduced. As an exception to this rule, by using this column the retention may in some cases increase as the polarity of the analyte increases. This phenomenon is referred to as the "polar retention effect on graphite" (PREG), making the Hypercarb particularly useful for the separation of compounds that are normally difficult to retain and resolve on silica-based alkyl chain phases. As other useful features, the column can be used at very high temperatures (above 60°C to 80 °C). This may lead to several advantages, such as generally reduced mobile phase viscosity, and higher flow rates that can be used for fast separations, without compromising efficiency or exceeding the pressure limits of the HPLC system. The reduction of mobile phase viscosity at higher temperatures enhances the mass transfer of the solute between mobile and stationary phase, resulting in more efficient and sharper peaks and increased peak capacity. Sharper chromatographic peaks improve signal-to-noise ratios and sensitivity. According to the aims in choosing a different chromatographic column, the Hypercarb-PGC revealed to be the ideal to use for the further method re-validation. A chromatogram obtained by injecting the standard mixture in the routine assay is reported in Figures 11.

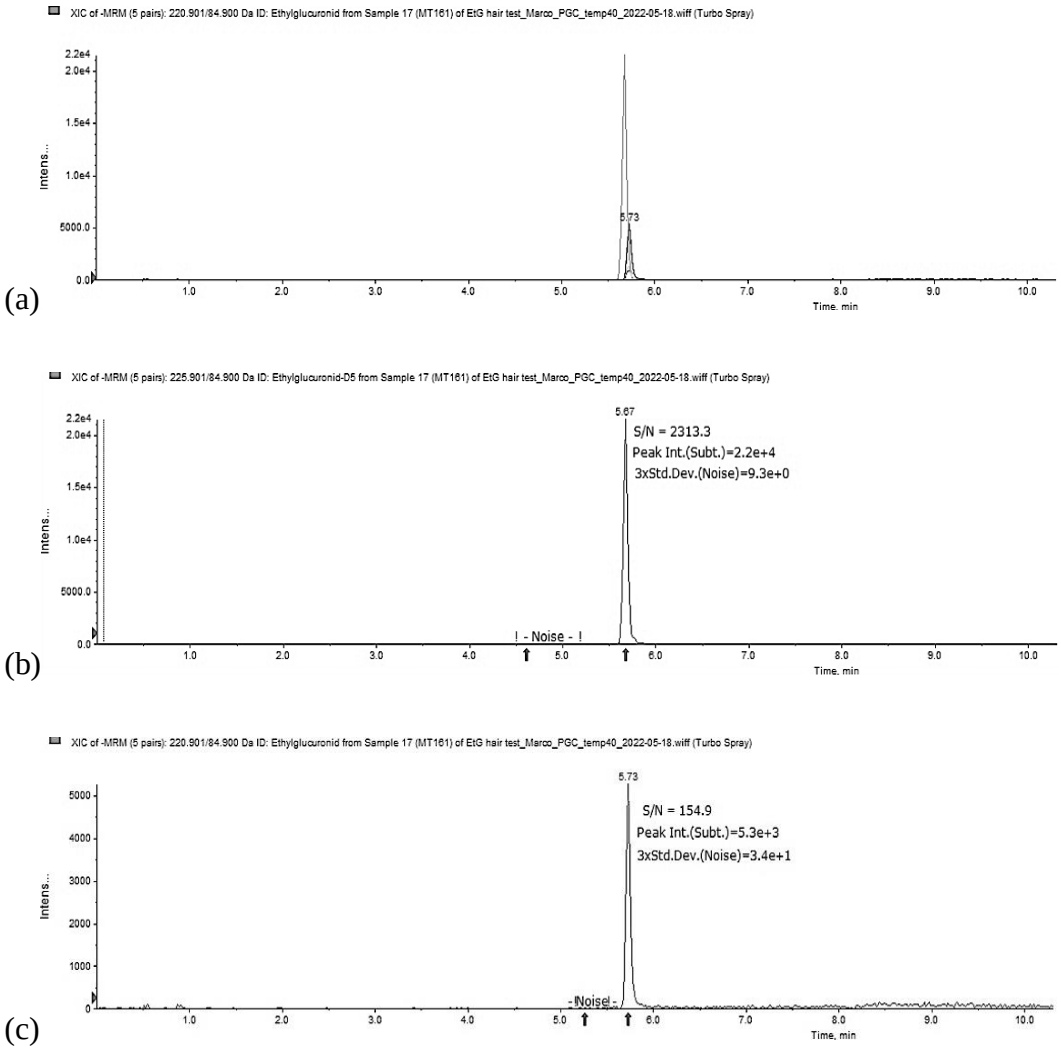


Fig. 11. Total ion chromatogram (**a**) of a standard mixture containing EtG at a concentration of 5 ng/mL (**c, quantifier ion**: m/z 220.9→84.9, tR: 5.73 min) and the IS EtG-D_5 at a concentration of 20 ng/mL (**b**: m/z 225.9→84.9, tR: 5.67 min) obtained using the Agilent-1200 HPLC coupled to the 4000 QTrap provided with the Hypercarb-PGC 3.0 x 100 mm, 3.0 ųm at 50°C (Eluent A: 0.1% Form.Ac in H_20; Eluent B: 0.1% Form.Ac in H_20/ACN 1:9; Flow: 0.75 mL/min; post-column spray: 0.1 mL/min Isopropanol; Inj.volume: 20 ųL; Elution gradient: 0-1.00 min 5% B; 1.00-6.00 min linear gradient to 40% B; 6.00-6.20 min linear gradient to 100% B, then held till 9.40 min; 9.940-9.50 min linear gradient from 100 to 5% B, then held till the end of the run for the re-equilibration prior the following injection; Total run time: 15 minutes).

3.3.4 METHOD VALIDATION

A methods shall be validated when it is necessary to verify a method's performance parameters are fit for use for a particular analysis [48]. Usually, a validation is planned for new analytical methods, for a protocol which undergoes modifications to improve performances or extend its use beyond that for which it was originally validated, or to demonstrate equivalence between an established system and a different one. The parameters to be evaluated for validation of methods depend on the circumstances in which the method is to be applied for. A validation plan should be placed prior to starting the validation, to provide directions for the experiments that will be performed and acceptance criteria for each parameter. These experiments are generally conducted using fortified samples of the matrix for which the method is intended or prepared from reference materials from different sources used to prepare calibration samples. Validation studies on different days must be planned, ensuring that instruments meet the same daily performance requirements as for casework. The method for the hEtG has been re-validated by using the *Valistat 2.0* validation software, according to the plan proposed by the GTFCh guideline on quality assurance of toxicological studies [49] [Fig.12]. The m/z 74.9 has been selected as quantifier as the ion which provided the highest signal with both the systems, once adjust the elution gradient for better retention [Fig.13-14]. The same experimental design has been followed for both the HPLC-MS/MS.

Run	Selectivity	Matrix effect/ Extraction efficiency/ Process efficiency	Processed sample stability	Linearity
0	10 sources of blank matrix 2 zero samples X spiked samples	30 samples (5 neat standards 5 spiked blank extracts 5 extracts. of spiked blanks, 2 conc. each)	16 injections of extracts (certain time intervals, 8 at each of 2 conc.)	36 calibration samples (6 conc. levels 6 replicates, each)
Total	94 (+ x)			

Run	Calibration samples, 6 levels	Validation samples								Total
		Low		Medium	High		optional	LLOQ	Dil.	
		P & A	F/T	P & A	P & A	F/T		P & A	P & A	
1	(6)	2	6	2	2	6		2	2	24 (+4)
2	(6)	2	-	2	2	-		2	2	12 (+4)
3	(6)	2	-	2	2	-		2	2	12 (+4)
4	(6)	2	6	2	2	6		2	2	24 (+4)
5	(6)	2	-	2	2	-		2	2	12 (+4)
6	(6)	2	-	2	2	-		2	2	12 (+4)
7	(6)	2	-	2	2	-		2	2	12 (+4)
8	(6)	2	-	2	2	-		2	2	12 (+4)
Total	120 (+32)									

Fig. 12. Experimental design for LC-MS method validation according to the GTFCh guideline on quality assurance of toxicological studies.

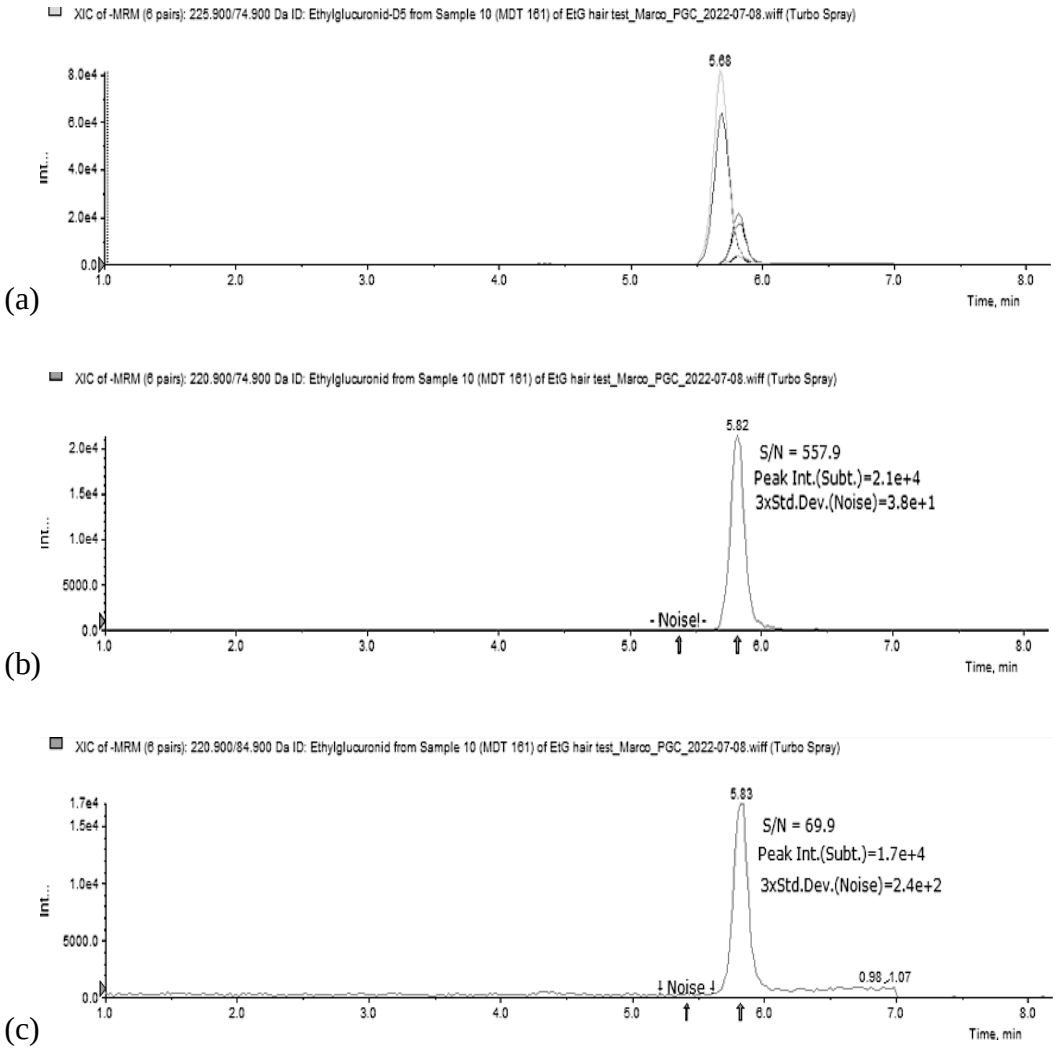


Fig. 13. Total ion chromatogram (**a**) of a standard mixture containing EtG at a concentration of 5 ng/mL (**b, quantifier ion**: m/z 220.9→74.9; **c, qualifier ion**: m/z 220.9→84.9) and the IS EtG-D_5 at a concentration of 20 ng/mL obtained using the Nexera LC-20ADxr HPLC coupled to the 5500 QTrap provided with the Hypercarb-PGC 3.0 x 100 mm, 3.0 ųm at 50°C (Eluent A: 0.1% Form.Ac in H_20; Eluent B: 0.1% Form.Ac in H_20/ACN 1:9; Flow: 0.75 mL/min; Inj.volume: 20 ųL; Elution gradient: 0-4.00 min 5% B; 4.00-8.00 min linear gradient to 40% B; 8.00-8.20 min linear gradient to 100% B, then held till 11.00 min; 11.00-11.20 min linear gradient from 100 to 5% B, then held till the end of the run for the re-equilibration prior the following injection; Total run time: 15 minutes).

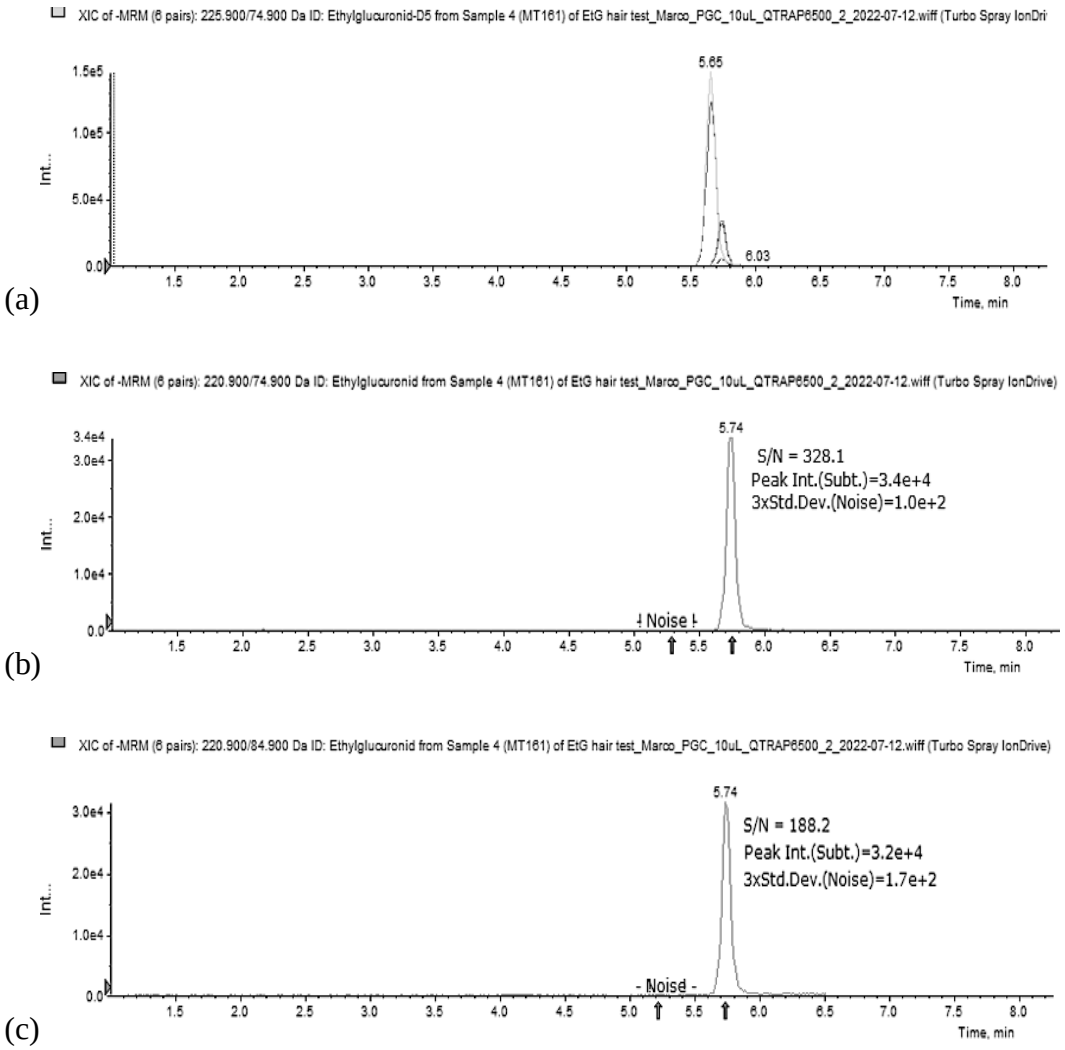


Fig. 14. Total ion chromatogram (**a**) of a standard mixture containing EtG at a concentration of 5 ng/mL (**b, quantifier ion**: m/z 220.9→74.9; **c, qualifier ion**: m/z 220.9→84.9) and the IS EtG-D_5 at a concentration of 20 ng/mL obtained using the ExionLC-20 AC HPLC coupled to the 6500+ QTrap provided with the Hypercarb-PGC 3.0 x 100 mm, 3.0 μm at 50°C (Eluent A: 0.1% Form.Ac in H_20; Eluent B: 0.1% Form.Ac in H_20/ACN 1:9; Flow: 0.75 mL/min; Inj.volume: 5 μL; Elution gradient: 0-4.00 min 5% B; 4.00-8.00 min linear gradient to 40% B; 8.00-8.20 min linear gradient to 100% B, then held till 11.00 min; 11.00-11.20 min linear gradient from 100 to 5% B, then held till the end of the run for the re-equilibration prior the following injection; Total run time: 15 minutes).

For the selectivity, or the extent to which other substances interfere with the determination of the studied compound according to the procedure, blank matrix samples of 10 different sources without the addition of the internal standard used in the method have been checked, in order to demonstrate the absence of possible common interferences from the matrix. For the evaluation of interferences from the stable-isotope internal standards, 2 different sources of blank matrix fortified with the IS have been tested [Fig.15-16]. This was planned as isotopically labelled compounds may contain non-labelled compound as an impurity. Additionally, the mass spectra of the labelled analogues may contain fragment ions with the same mass-to-charge ratios as the significant ions of the target analyte. In both instances, analyte identification and quantitation could be impacted. Interferences below the LOD have been considered insignificant. Likewise, 4 blank matrix samples fortified with the analyte at relatively high concentration, and the IS have been analysed to evaluate whether relevant amounts of the analyte impact the quantitation.

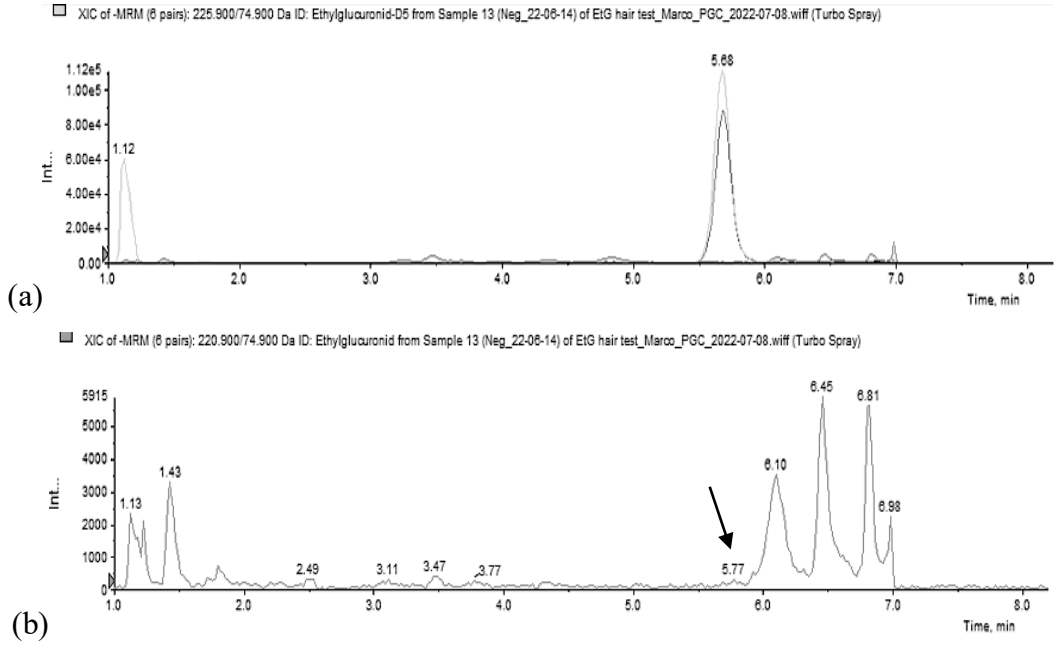


Fig. 15. Total ion chromatogram (**a**) of a blank hair sample fortified with the IS EtG-D_5 (**b, quantifier ion**: m/z 220.9→74.9) using the Nexera LC-20ADxr HPLC coupled to the 5500 QTrap provided with the Hypercarb-PGC 3.0 x 100 mm, 3.0 ųm.

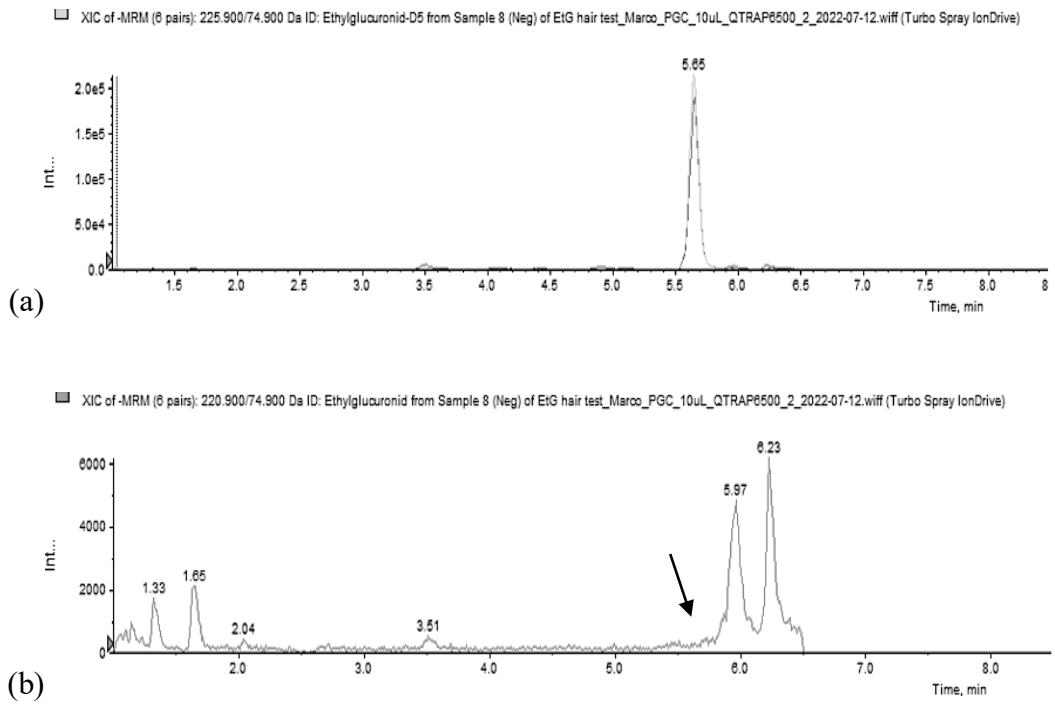


Fig. 16. Total ion chromatogram (**a**) of a blank hair sample fortified with the IS EtG-D_5 (**b, quantifier ion**: m/z 220.9→74.9) using the ExionLC-20 AC HPLC coupled to the 6500+ QTrap provided with the Hypercarb-PGC 3.0 x 100 mm, 3.0 ųm.

The linear relationship, or linearity has been evaluated across the range of the analytical procedure. The specified range is normally derived from linearity studies and depends on the intended application of the procedure. As reported by guidelines, it is established by confirming that the analytical procedure provides an acceptable degree of linearity, accuracy and precision when applied to samples containing amounts of analyte within or at the extremes of the specified range of the analytical procedure. For the establishment of linearity, testing a minimum of 6 concentrations is recommended. It may be demonstrated directly on the drug substance (by dilution of a standard stock solution), or separate weightings of synthetic mixtures of the drug product components. Linearity is usually evaluated by visual inspection of a plot of signals as a function of analyte concentration or content. Data from the regression line itself may be helpful to provide mathematical

estimates of the degree of linearity. Some analytical procedures, such as immunoassays, do not demonstrate linearity after any transformation. As a result, the analytical response should be described by an appropriate function of the concentration (amount) of an analyte in a sample. In this case, the linearity of the method has been assessed among 8 test days in the range of 2 to 100 pg/mg by injecting 6 replicates of each calibration point, for a total of 36 injections per day. The method resulted to be linear in the studied range, and the correlation coefficients were higher than 0.998. The evaluation of the detection limit is generally based on signal-to-noise (S/N) ratio by establishing the minimum concentration at which the analyte can be reliably detected, comparing the measured signals from samples with known low concentrations of analyte with those of blank samples, A ratio between 3 is generally considered acceptable for estimating the detection limit. With a similar approach, the quantification limit can be established as the minimum concentration at which the analyte can be reliably quantified, with a typical signal-to-noise ratio of 10:1. The limit of detection (LOD), or the lowest concentration level providing a S/N $\geq$ 3, and the lower limit of quantification (LLOQ) have been set respectively at 0.6 pg/mg and 2 pg/mg. Intra-day precision (repeatability) and day-to-day precision (reproducibility) have been also evaluated. Precision is commonly defined as the measure of the closeness of agreement between a series of measurements obtained from multiple samplings of the same homogenous sample and expressed as imprecision. The mean and standard deviation (SD) of the response is calculated for each studied concentration. The accuracy, expressed as *bias* is the closeness of agreement reported as a percent difference between the mean of the results of measurements of a measurand and the true (or accepted true) value of a measurand. Bias studies must be carried out for all quantitative methods and conducted concurrently with precision studies. In this study, intra-day precision and day-to-day precision have been calculated considering the peak area ratios of 4 chosen levels (LLOQ, low, medium, high) to the relative deuterated standard and running 2 replicates per days over 8 different days and expressed in terms of relative standard deviation per cent (RSD% $_{\text{intra-day}}$; RSD% $_{\text{inter-day}}$). The acceptance criteria were 20% RSD at the LLOQ, and 15% for the remaining concentrations. Accuracy (bias) has been calculated in terms

of percentage deviation from the real concentration of the same 4 levels in both intra- and inter-day tests. The acceptance criteria for the bias of the average results measured were set at ± 20% of the expected concentrations [Table 4]. In LC-MS applications, the enhancement or suppression of the analyte ionization resulting from the presence of co-eluting compounds is a commonly encountered phenomenon. Enhancement or suppression of ionization is most likely to impact the limit of detection, the limit of quantitation, and bias of a qualitative method. When the average suppression or enhancement exceeds +25%, no impact on other critical validation parameters should be demonstrated. In this study, the evaluation of matrix effect have been performed as proposed by Matuszewski et al. (2003) [50]. A first set of three standard lines (set A) has been prepared to evaluate the response for neat standards of the analytes injected in the mobile phase. The second set (set B, or *spiked-after*) has been prepared in plasma extracts and spiked after extraction, while the third set has been prepared in plasma from the same sources as in set B, but spiking the samples before extraction (set C, or *spiked-before*). In this manner, the results obtained allow determination of the matrix effect (ME), and the extraction recovery (RE) of the procedure, by comparing the absolute peak areas as follows: ME (%) = B/A × 100; RE (%) = C/B × 100. In our case, 6 neat STDs in water (set A), 6 spiked blank extracts (set B), and 6 extracts of spiked blanks (set C), have been tested for two different QCs among the calibration range [Table 5].

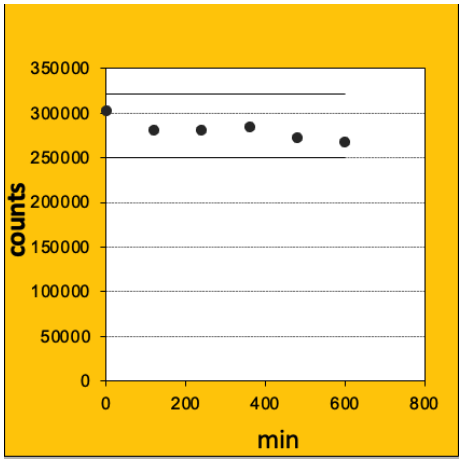
Analytical system	Levels (pg/mg)		Intraday-1 (n=2)		Intraday-2 (n=2)		Intraday-3 (n=2)		Intraday-4 (n=2)		Intraday-5 (n=2)		Intraday-6 (n=2)		Intraday-7 (n=2)		Intraday-8 (n=2)		Day-to-day	
			SD	BIAS %	SD	BIAS %	SD	BIAS %	SD	BIAS %	SD	BIAS %	SD	BIAS %	SD	BIAS %	SD	BIAS %	SD	BIAS %
HPLC -	low	25	1.72	1.2	0.81	0.5	0.55	0.2	2.89	0.7	1.07	1.0	0.66	0.1	1.46	0.2	2.12	1.8	3.13	0.62
5500 QTrap	middle	50	0.37	1.1	0.16	0.6	0.56	0.5	1.62	0.6	0.69	0.4	6.32	2.4	15.44	-0.7	5.06	1.1	9.53	0.94
	high	90	4.91	2.2	0.83	2.3	4.05	4.0	3.21	2.8	4.33	2.5	3.42	2.6	11.51	0.2	2.76	2.4	18.59	1.01
HPLC -	low	25	2.33	1.0	2.48	1.2	1.27	0.3	7.53	1.7	0.46	1.0	0.47	0.2	0.26	0.1	6.29	1.7	4.15	0.82
6500+ QTrap	middle	50	3.13	1.4	0.10	0.2	2.05	0.3	2.03	0.1	19.49	1.1	11.44	2.4	9.64	1.4	3.06	-0.1	10.21	1.01
	high	90	2.76	2.0	1.04	2.3	2.40	2.0	3.00	1.5	0.53	2.2	12.20	1.2	4.82	1.2	3.75	1.2	9.23	0.50

Table 4. Intra-day precision and accuracy expressed as relative standard deviation and bias (%) for the chosen levels using both the HPLC-MS/MS systems.

Analytical system	Levels (pg/mg)		Matrix Effect (ME)		Extraction recovery (RE)	
			ME %	RSD %	RE %	RSD %
HPLC - 5500 QTrap	QC low	50	105	2.71	80	2.02
	QC high	90	110	1.78	76	1.86
HPLC - 6500+ QTrap	QC low	50	113	2.16	81	2.65
	QC high	90	99	1.76	99	2.12

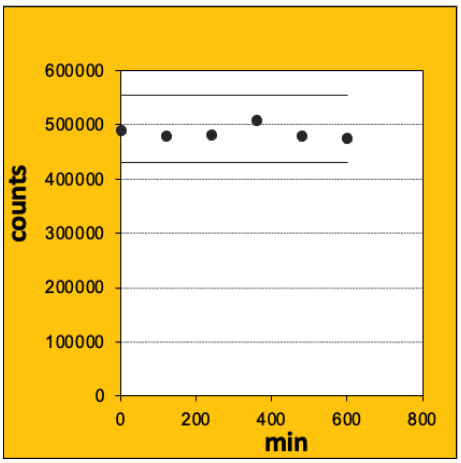
Table 5. Matrix effect (ME), extraction recovery (RE) and relative standard deviation (RSD%) experiments evaluated at the selected concentration levels.

Finally, processed sample stability has been studied to evaluate the length of time a processed sample can be maintained before it undergoes unacceptable changes in terms of reliable analyte detection, identification, or quantitation. For these reasons, 6 repeated injections for each of the two fortified chosen levels within the calibration range have been performed every 2 hours, for a total of 12 injections in an up to 24 hours experiment. The average responses at each time interval have been compared to the time zero responses, considering the analyte stable until the average signal remains among of acceptable bias of 25%. Figure 17 shows the stability obtained by using the ExionLC-20 AC HPLC coupled to the 6500+ QTrap, which was the system finally established as the main backup for the reasons explained in the following paragraph.



		QC-Pool
	min	counts
1	0,0	303200
2	120,0	281600
3	240,0	281000
4	360,0	284600
5	480,0	273100
6	600,0	267700

(a)



		QC-Pool
	min	counts
1	0,0	490200
2	120,0	479600
3	240,0	480900
4	360,0	509000
5	480,0	479100
6	600,0	476200

(b)

Fig. 17. Processed sample stability studied using the ExionLC-20 AC HPLC coupled to the 6500+ QTrap within the calibration range and among of acceptable bias of 25%. Two QCs (50 pg/mg, **a**; 90 pg/mg, **b**) have been injected every 2 hours, for a total of 6 injections each in an up to 24 hours experiment.

3.3.5 UNCERTAINTY OF MEASUREMENT

The uncertainty of measurement is closely linked to the measurement results and describes the range, and dispersions of the values that could reasonably be attributed to the measurand [51]. It is itself an estimation to assess the reliability and the confidence, in order to compare measurement results. Many components may be evaluated from the statistical distribution of the results of series of measurements and can be characterized by standard deviations (Type A estimations), or from assumed probability distributions based on experience or other information (Type B estimations). The most common uncertainty sources of a measurement process

are basically related to the sampling, matrix effects and interferences, environmental conditions, uncertainties of weights and volumetric equipment, uncertainties of reference values, approximations and assumptions in the measurement method and procedure, random variation. Estimating uncertainty using validation data can be considered as an alternative approach and takes into account long-term variation of results within one lab, including sample preparation (reproducibility, or $u_{(rep)}$), recovery ($u_{(rec)}$), and combined additional sources to expand the total uncertainty. Accordingly, the combined uncertainty is calculated as follows:

$$u_c = \sqrt{u_A^2 + u_B^2} = \sqrt{\sum_i u_X^2}$$

It follows that the expanded uncertainty can be expressed as $U = k \cdot u_c$, where the choice of the factor k is based on the level of confidence desired. For instance, the factor is normally set at 2 for an approximate level of confidence of 95%. The measure uncertainty of the method for hETG determination has to be considered when giving the quantitative results in the context of the assessment of problematic alcohol use. However, the uncertainty is expected to be different at different EtG concentrations. Thus, in order to obtain an acceptable profile, the expanded uncertainty U was estimated at 25, 50, and 90 pg/mg. The standard uncertainty of measurement was defined as: $u_{(rep)} = \frac{s_d}{\sqrt{n}}$, where S_d is the standard deviation and n is the number of measurements.

The expanded uncertainty U was obtained by multiplying $u_{(rep)}$ with the coverage factor k = 2, corresponding to 95% confidence intervals. The uncertainty of measurement for the whole range of EtG was always lower than 5%.

3.3.6 APPLICATION TO REAL CASES

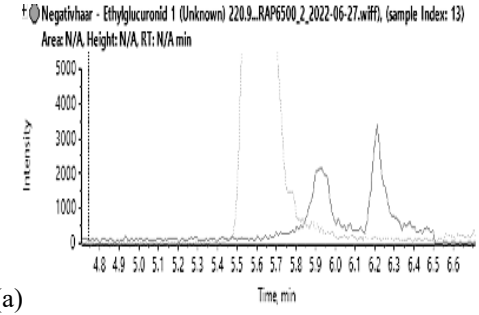
According to the aims already reported, the ExionLC-20 AC HPLC coupled to the 6500+ QTrap, provided with the Hypercarb-PGC column has been chosen as the main backup as able to face in a better way the back pressure of the system over the runs, allowing to inject less extract volume (5 uL) compared to the Nexera LC-20ADxr HPLC-5500 QTrap (20 uL). As a result, an entire batch of real samples from routine cases has been finally tested to check the accordance of the obtained quantifications with those provided by the routinely used instrumentation [Table 6]. Some processed chromatograms are reported [Fig.18].

Case **[20 mg]**	**Agilent-1200 HPLC - 4000 QTrap** [hEtG, pg/mg]	**ExionLC-20 AC HPLC - 6500+ QTrap** [hEtG, pg/mg]
Cal. 1 (2 pg/mg)	2.10	2.05
Cal. 2 (4 pg/mg)	3.74	4.00
Cal. 3 (25 pg/mg)	25.2	25.3
Cal. 4 (50 pg/mg)	51.6	50.7
Cal. 5 (75 pg/mg)	71.7	73.1
Cal. 6 (100 pg/mg)	101	102
Blk	N/A	N/A
Sample 1	4.83	4.49
Sample 2	N/A	N/A
Sample 3	N/A	N/A
Sample 4	N/A	N/A
Sample 5	10.5	10.6
Sample 6	39.7	38.1
Sample 7	30.9	33.3
Sample 8	N/A	N/A
Sample 9	N/A	N/A
Sample 10	N/A	N/A

Sample 11	N/A	N/A
Sample 12	N/A	N/A
Sample 13	5.40	5.44
Sample 14	N/A	N/A
Sample 15	N/A	N/A
QC_L (50 pg/mg)	52.5	52.1
Sample 16	N/A	N/A
Sample 17	N/A	N/A
Sample 18	N/A	N/A
Sample 19	N/A	N/A
Sample 20	2.83	3.19
Sample 21	25.3	25.9
Sample 22	34.9	32.7
Sample 23	3.26	3.49
Sample 24	18.1	17.9
Sample 25	N/A	N/A
QC_pos_1 (19.5 pg/mg)	18.9	19.6
Sample 26	5.39	5.70
Sample 27	N/A	N/A
Sample 28	N/A	N/A
Sample 29	N/A	N/A
Sample 30	2.97	3.16
Sample 31	N/A	N/A
Sample 32	N/A	N/A
Sample 33	27.1	40.5
Sample 34	N/A	N/A
Sample 35	4.51	4.90
QC_pos_2 (6.8 pg/mg)	7.03	7.57
Sample 36	N/A	N/A

Sample 37	2.68	2.81
Sample 38	N/A	N/A
Sample 39	N/A	N/A
Sample 40	N/A	N/A
Sample 41	94.7	89.1
Sample 42	N/A	N/A
Sample 43	5.33	5.59
Sample 44	N/A	N/A
Sample 45	2.15	2.03
QC_L (50 pg/mg)	52.3	51.4
Sample 46	52.1	43.2
Sample 47	2.63	2.85
Sample 48	N/A	N/A
Blk	N/A	N/A

Table 6. Comparison of real samples hEtG concentrations obtained by the routine batch using the routinely applied Agilent-1200 HPLC coupled to the 4000 QTrap, and the chosen backup system ExionLC-20 AC HPLC coupled to the 6500+ QTrap provided with the Hypercarb-PGC 3.0 x 100 mm, 3.0 µm.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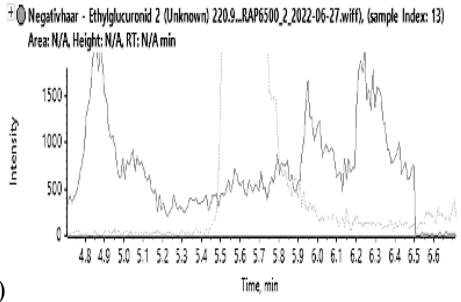
Area: N/A, Height: N/A, RT: N/A min
Intensity
Time, min

(a)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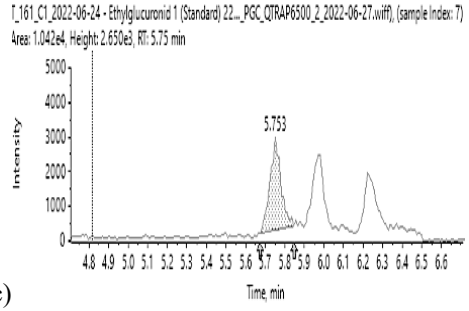
Area: N/A, Height: N/A, RT: N/A min
Intensity
Time, min

(b)


T_161_C1_2022-06-24 - Ethylglucuronid 1 (Standard) 22..._PGC_QTRAP6500_2_2022-06-27.wiff), (sample Index: 7)
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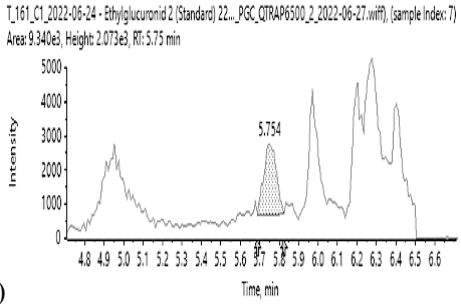
5.753
Intensity
Time, min

(c)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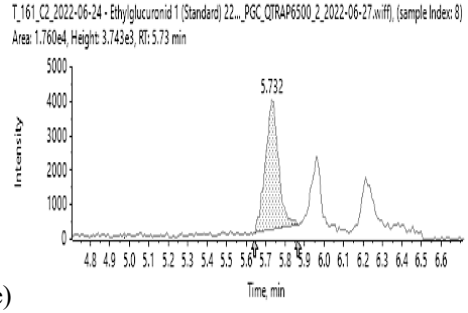
5.754
Intensity
Time, min

(d)


T_161_C2_2022-06-24 - Ethylglucuronid 1 (Standard) 22..._PGC_QTRAP6500_2_2022-06-27.wiff), (sample Index: 8)
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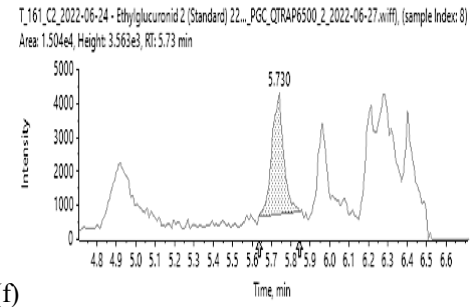
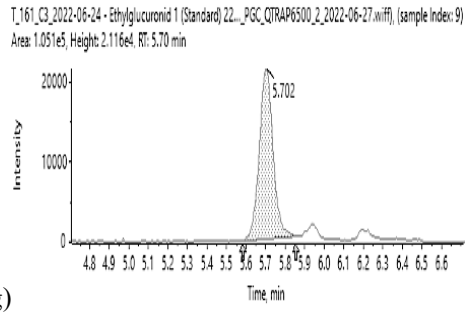
5.732
Intensity
Time, min

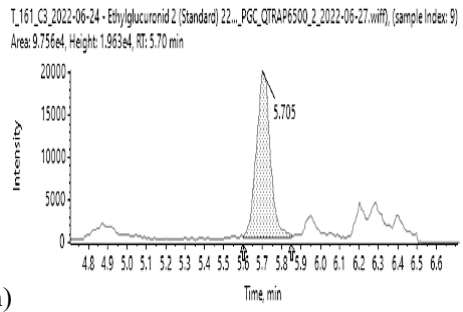
(e)


T_161_C2_2022-06-24 - Ethylglucuronid 2 (Standard) 22..._PGC_QTRAP6500_2_2022-06-27.wiff), (sample Index: 8)
Area: 1.504e4, Height: 3.563e3, RT: 5.73 min
5.730
Intensity
Time, min

(f)


T_161_C3_2022-06-24 - Ethylglucuronid 1 (Standard) 22..._PGC_QTRAP6500_2_2022-06-27.wiff), (sample Index: 9)
Area: 1.051e5, Height: 2.116e4, RT: 5.70 min
5.702
Intensity
Time, min

(g)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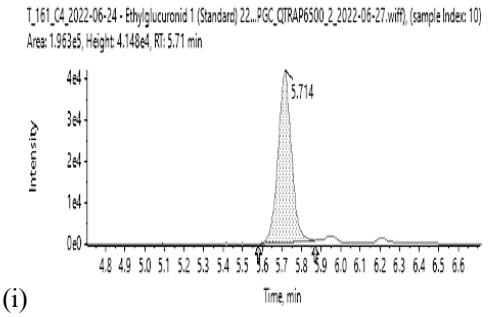
5.705
Intensity
Time, min

(h)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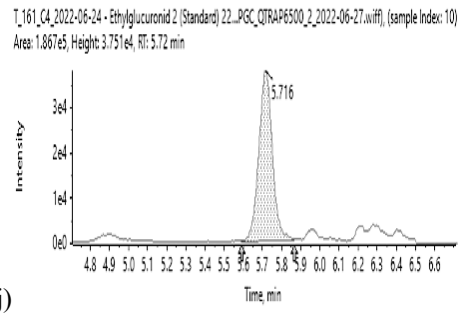
Area: 1.963e5, Height: 4.148e4, RT: 5.71 min
5.714
Intensity
Time, min

(i)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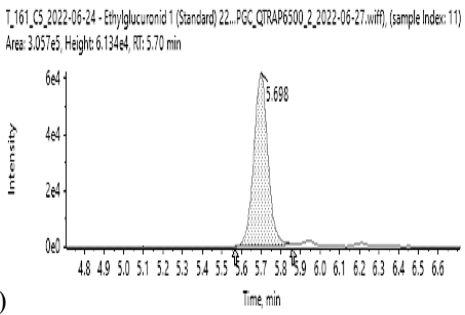
5.716
Intensity
Time, min

(j)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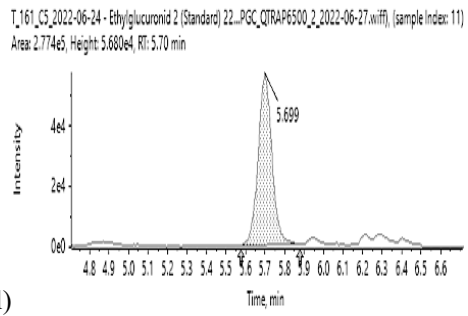
5.698
Intensity
Time, min

(k)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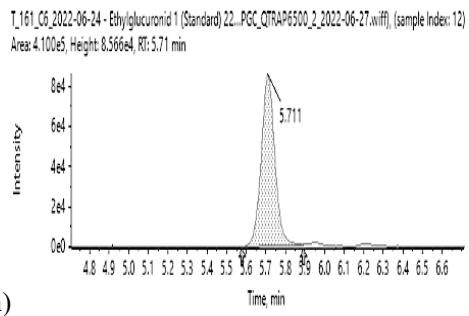
5.699
Intensity
Time, min

(l)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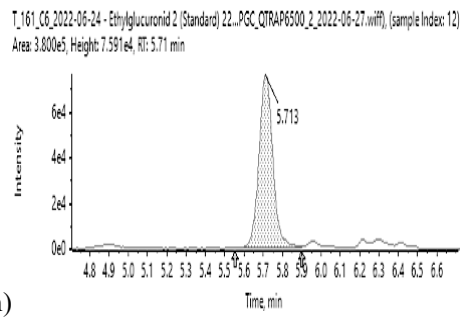
5.711
Intensity
Time, min

(m)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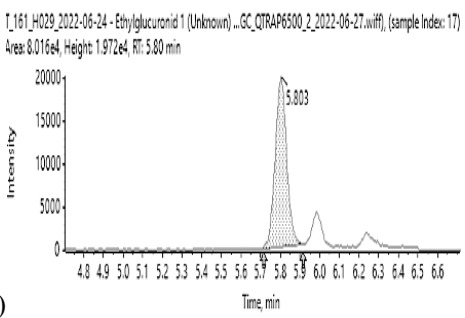
5.713
Intensity
Time, min

(n)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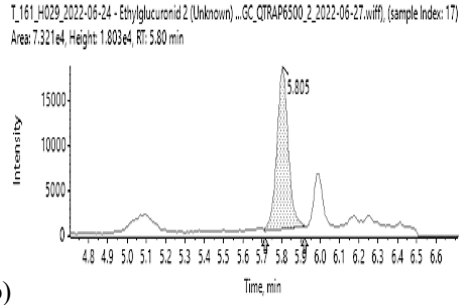
5.803
Intensity
Time, min

(o)


T_161_H029_2022-06-24 - Ethylglucuronid 2 (Unknown) ...GC_QTRAP6500_2_2022-06-27.wiff), (sample Index: 17)
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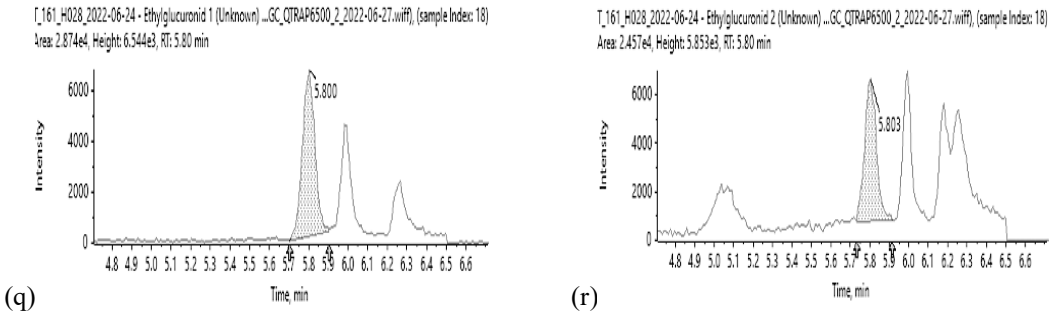
5.805
Intensity
Time, min

(p)



(q) (r)

Fig. 18. Chromatograms of real extracted samples tested using the ExionLC-20 AC HPLC coupled to the 6500+ QTrap provided with the Hypercarb-PGC 3.0 x 100 mm, 3.0 µm. **Blank** (**a**, quantifier ion: m/z 220.9→74.9; **b**, qualifier ion: m/z 220.9→84.9); **1st calibration point** (2 pg/mg; **c**, quantifier ion: m/z 220.9→74.9; **d**, qualifier ion: m/z 220.9→84.9); **2nd calibration point** (4 pg/mg; **e**, quantifier ion: m/z 220.9→74.9; **f**, qualifier ion: m/z 220.9→84.9); **3rd calibration point** (25 pg/mg; **g**, quantifier ion: m/z 220.9→74.9; **h**, qualifier ion: m/z 220.9→84.9); **4th calibration point** (50 pg/mg; **i**, quantifier ion: m/z 220.9→74.9; **j**, qualifier ion: m/z 220.9→84.9); **5th calibration point** (75 pg/mg; **k**, quantifier ion: m/z 220.9→74.9; **l**, qualifier ion: m/z 220.9→84.9); **6th calibration point** (100 pg/mg; **m**, quantifier ion: m/z 220.9→74.9; **n**, qualifier ion: m/z 220.9→84.9); **QC_positive_1** (19.5 pg/mg; **o**, quantifier ion: m/z 220.9→74.9; **p**, qualifier ion: m/z 220.9→84.9); **QC_positive_2** (6.8 pg/mg; **q**, quantifier ion: m/z 220.9→74.9; **r**, qualifier ion: m/z 220.9→84.9).

3.3.7 CONCLUDING REMARKS

The method for the quantification of EtG in human hair samples has been fully re-validated by using a properly established backup system for further routine analysis, allowing to inject less extract volumes. Moreover, the different analytical column tested allows for a rapid chromatographic separation and sensitive detection, showing the possibility in using it as a valid alternative for the separation of this highly polar compound. A complete and effective standardization of the analytical procedure for hEtG measurement and currently adopted cut-offs have been also discussed. Either gas or liquid chromatography coupled to mass spectrometry with deuterated internal standard can be used, though properly optimized and validated methods used for this purpose should have a LOQ ≤ 3 pg/mg. Inter-individual

differences in the incorporation rate of hEtG into hair and wash-out effects must be considered, as frequently produce “negative” results. Currently, analytical protocol for the determination of hEtG is far from being harmonized, and consequently the laboratories use different procedures that may yield different recoveries from real hair samples. Different sample pre-treatments (e.g., hair cutting in small pieces vs. pulverization) provide substantially different recoveries of the analyte from the hair matrix, leading to an inherent analytical bias in result reporting. Indeed, a significant increase in hEtG has been observed in pulverized samples vs. hair finely cut with scissors (without changing the extraction conditions). Moreover, the variability of this analysis is also quite high and strongly dependent on the preparation procedure and technology. As a result, the assessment of chronic alcohol abuse requires not only sensible analytical systems and properly optimized and validated methods, but also clinical and analytical standardized approaches and methodologies, which unfortunately to date are lacking. To face this problem, the use of real hair containing certified amounts of naturally embedded EtG from human donors should help in the standardization of the analytical procedures. Thus, only after a complete and effective standardization of the analytical procedure for hEtG measurement it will become possible for forensic and clinical laboratories to adopt fixed cut-offs with clinical and forensic defensibility, even in court cases.

3.4 HAIR ANALYSIS AS A NEW TOOL TO MONITOR ADHERENCE TO LONG-TERM THERAPY TO CHOLESTEROL-LOWERING MEDICATIONS (STATINS)

Adherence to long-term therapies has always been considered a worldwide spread problem with an impact that grows as the burden of chronic disease grows. Poor adherence to long term treatments has been reported as a well-known phenomenon for several pathological conditions, leading to critical consequences in the effectiveness of the treatment, population health perspective and life quality, adverse side effects and increasing health care costs [52]. It cannot be ruled out also how for many patients, particularly those requiring complex multi-therapies for which the care needed is based on patient self-management, the ability in following chronic treatment recommendations in an optimal manner is usually compromised by more than an aspect. Among these several factors, social and economic factors can also be included. So, the problem of compliance, as it is known, which has always been commonly associated to patients' bad behaviours in following strictly the therapy, should be extended in better ways also to the dynamic and complex changes many people need to maintain optimal health over long periods, even when affected by chronic diseases. Thus, the development of approaches aiming to monitor whether patients continue therapy for chronic conditions for long periods of time is nowadays of great interest not only for clinicians. However, there is not a single strategy that can be considered effective across all patients. Consequently, interventions that target adherence must be tailored to the illness-related demands experienced by the patient by accurately also assessing those factors that influence it. 3-Hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins) are cholesterol-lowering medications which are widely prescribed and used as first line for the treatment of hypercholesterolemia and hyperlipidaemia, aimed at reducing the risk of atherosclerotic diseases [52][53]. HMG-CoA reductase is the rate-limiting enzyme in cholesterol biosynthetic pathway which promote the conversion of HMG-CoA into a cholesterol precursor (mevalonate). By binding the active site of the enzyme, these compounds prevent the interaction of the reductase with its natural substrate, blocking the endogenous biosynthesis of cholesterol [Fig.19].

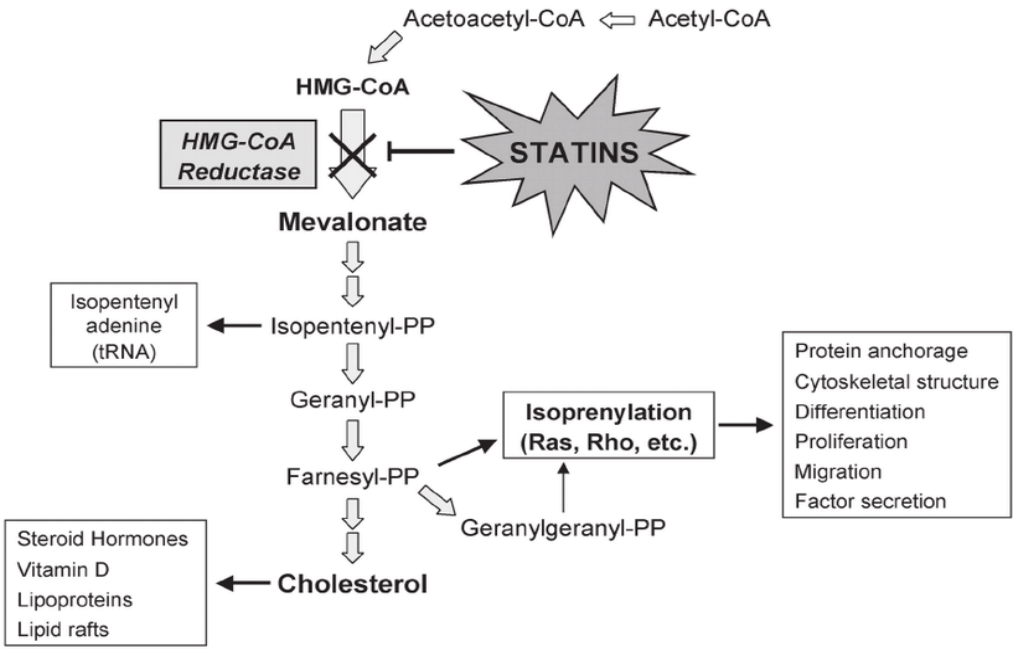


Fig. 19. The endogenous mevalonate pathway leading to cholesterol biosynthesis. Statins block the conversion of HMG-CoA to mevalonate by inhibiting the enzyme HMG-CoA reductase. This inhibition leads to decreased production of cholesterol and isoprenoid intermediates, such as farnesyl-PP and geranylgeranyl-PP. The inhibition of endogenous cholesterol synthesis by statins leads to lowered production of sterol products and impaired formation or disruption of lipid rafts [55].

Over the past years, atherosclerosis has been considered a cholesterol storage disease. Nowadays, the pathogenesis of atherosclerosis is well known to be such a complex phenomenon, not only due to a passive lipid accumulation in the vessels. It has been also demonstrated that immune cells and inflammation, especially in association with hyperlipidaemia and increased Low-Density Lipoprotein (LDL) [56], play a crucial role in mediating the stages of the disease, from the initiation through progression and also in the thrombotic complication of this condition [57]. Ischemic heart disease (IHD), also known as coronary artery disease (CAD), is one of the main clinical manifestations of atherosclerosis and the leading cause of death worldwide. CAD is characterized by a slow and progressive growth of

atherosclerotic plaque within the coronary arteries, with consequent reduced supply of oxygen to the heart which can lead to circulatory insufficiency (stenosis) and ischemic events at myocardial level [58]. The fibrous plaque rupture may lead to acute manifestations, resulting in thrombus formation that leads to acute coronary syndrome (ACS) [59]. Multifactorial conditions, both genetic and acquired risk factors are involved in the development of these conditions, such as age, sex, smoke, alcohol consumption obesity, familiarity for hypertension, dyslipidaemia, and inherited disturbances in lipid metabolism. Strong preventive measures to stop the onset of the disease and to decrease the risk of complications have been set over the years, as significant progresses made in the diagnosis and treatment of the pathology. The undoubted efficacy of statins in preventing cardiovascular events has already been shown and widely reported in literature, though the needed long-term treatment with these drugs is characterized by a high percentage of drop-out. Since first natural active metabolites were isolated, such as ML-236B (compactin, or mevastatin), many other extremely potent inhibitors of HMG-CoA showed significant effects in reducing total and LDL cholesterol concentration in plasma of patients with familial hypercholesterolemia [Fig.20] [60]. From these efforts, lovastatin, pravastatin, semi-synthetic simvastatin, and four synthetic statins (fluvastatin, atorvastatin, rosuvastatin, and pitavastatin) have been introduced into the market and commercialized over the years.

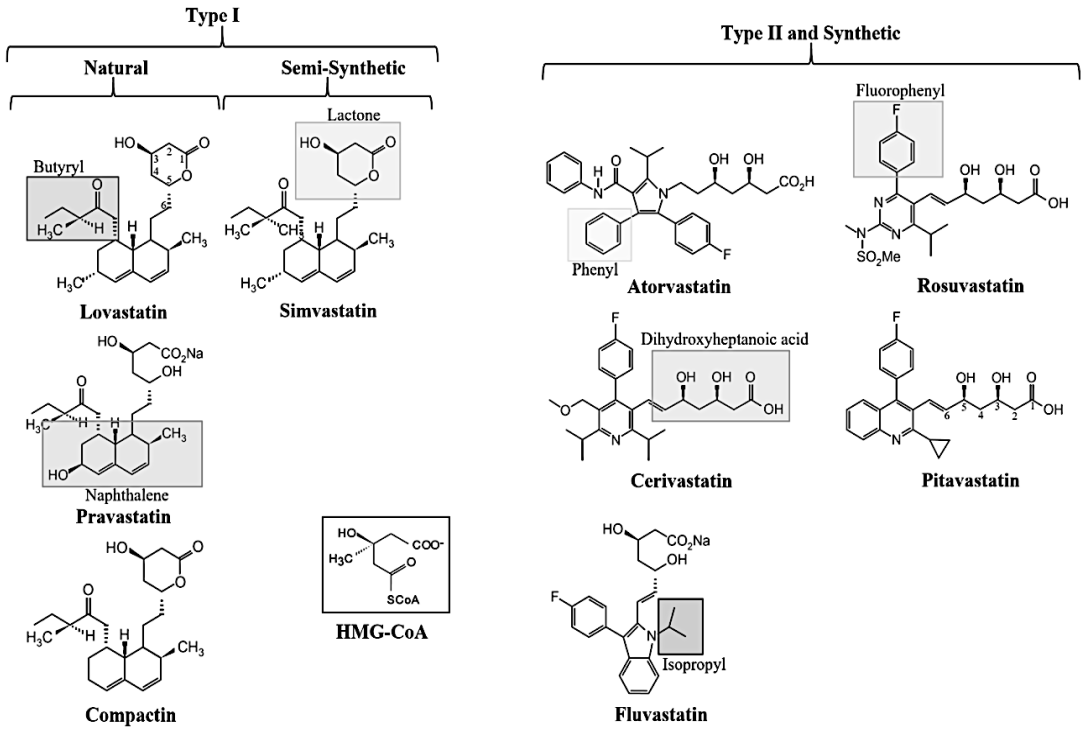


Fig. 20. Chemical structures of major HMG-CoA reductase inhibitors (statins). Among statins, pravastatin (c) is a derivative of fungal products, given in the active open acid form. Lovastatin and simvastatin are closed-ring lactone pro-drugs. Other newly developed statins atorvastatin, rosuvastatin, cerivastatin, pitavastatin and fluvastatin are completely chemically synthetized. The difference in structure accounts for their different solubility in water.

As described by Shitara *et al.* (2006), statins have different pharmacokinetic profiles that are associated with their physicochemical properties. The statin absorption rate of an ingested dose can vary from 40-75%, except for Fluvastatin which is almost completely absorbed, and characterized by a high first-pass extraction by the liver. Atorvastatin is a hydrophilic molecule actively taken up into the liver prior to the metabolism [60][61]. Rosuvastatin and pravastatin are not metabolized by P450, mainly taken up by the liver and eliminated from the body by a transporter-mediated excretion mechanism. The extraction occurs mostly in the bile, and for about 5-20% into urine. The range of concentrations that can be detected in the real samples for this specific class of compounds is wide, as they are

known to decay rapidly in serum/plasma media after the start of its prescription. Plasma half-life of these drugs ranges from 1-3 hours, except for atorvastatin and rosuvastatin (about 14h, and 19h respectively). Since cholesterol synthesis occurs predominately at night, statins are generally taken as a single daily dose in the evening, though different administration might be planned as for Atorvastatin. Doses vary according to the different prescribed compound. Therapy with statins is generally regarded as a chronic, safe, and well-tolerated pharmacological treatment. Their side effects are generally mild, being mainly represented by muscular symptoms (i.e., cramps and weakness sensation). However, severe outcomes may emerge in rare cases including myotoxic side effects, myopathy or rhabdomyolysis [63], considered also as the major cause of statin intolerance and therapy discontinuation. Consequently, an effective tool to verify the patients' compliance to statin therapy is required. In this context, the analysis for drugs and drug metabolites in the hair may represent an ideal tool to reflect the chronic intake of drugs and pharmaceuticals. During this PhD experience, a novel, specific and sensitive UHPLC-MS/MS method has been developed to determine six of the most prescribed statins and their metabolites in human hair. The results of the study show the possibility of using hair analysis as a new, and potentially almost ideal tool, to monitor patients' adherence to statin therapy.

3.4.1 STANDARD AND REAGENTS

Atorvastatin, rosuvastatin and pravastatin have been purchased from Sigma-Aldrich (St. Louis, Missouri, USA); (p)α-OH-atorvastatin-lactone, (o)α-OH-atorvastatin-lactone, N-desmethyl-rosuvastatin and hydroxy-simvastatin-D_6 from Alsachim (Illkirch-Graffenstaden, France), while atorvastatin-D_5 (calcium salt) from Cayman Chemical Company (Ellsworth Ann Arbor, Michigan, USA). Methanol and acetonitrile have been purchased from Merck KGaA (Darmstadt, Germany). Ultrapure water has obtained by a water purification system model PureLab Chorus-1 Complete (Elga Veolia, High Wycombe, UK). Separate stock solutions of all the statins have been prepared in methanol at a concentration of 1 mg/mL and used to prepare a working mixture containing all the compounds of

interest. Also, individual stock solutions of the two deuterated internal standards have been prepared, and kept refrigerated at -20 °C.

3.4.2 HAIR SAMPLE COLLECTION AND PREPARATION

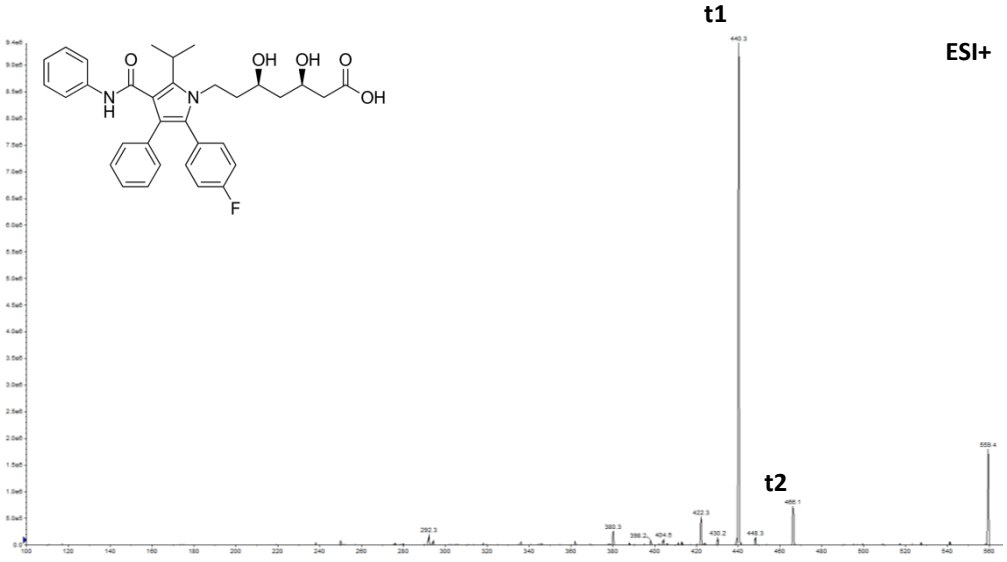
Blank hair for the method validation have been obtained from well-known subjects who declared no drugs taken in the previous 6 months. Those used for verifying the practical applicability of the method, and adherence to therapy have been collected from subjects under long-term therapy with statins. Samples have been collected by cutting as close as possible to the scalp a hair lock of about 4-5 cm, assuming a hair growth rate of 1 cm/month. After complete removal of possible external contaminants by washing twice with dichloromethane the solvent, the samples have been dried at room temperature and then manually cut in small segments with scissors. About 50 mg have been fortified with a mixture of the internal standards to a final concentration of 10 pg/mg, added with 1 mL of methanol and incubated overnight at room temperature. A six-point calibration curve has been prepared in this way by spiking drug free hair samples to mimic drug concentrations ranging from 0.75 to 20 pg/mg. The following day, a first ultrasonication step for 2 h, and a centrifugation at 13,000 rpm for 10 min prior the supernatant transfer into glass tubes have been performed, before a second ultrasonication step after the addition of one millilitre of methanol to the remaining hair. Once ultrasonicated again for 2 h and, finally the solvent has been pooled with the previous fraction and dried under gentle nitrogen stream. The residue has been reconstituted with 50 µL of mobile phase A/B (80:20) before the injection into the UHPLC-MS/MS system.

3.4.3 INSTRUMENTATION AND ANALYTICAL CONDITIONS

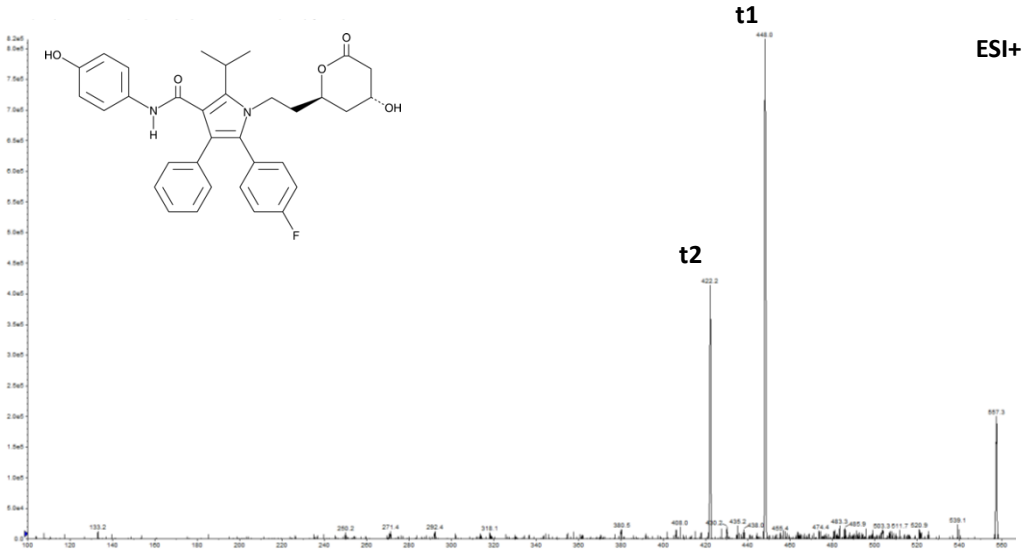
The analyses have been performed on the ACQUITY UPLC™ system (Waters Corporation, Milford, USA) coupled to the 6500 QTrap mass spectrometer (Sciex, Warrington, UK), achieving chromatographic separation by injecting 2 µL of the samples into a 50 × 2.1 mm Acquity 1.7 µm BEH Phenyl column (Waters Corporation, Milford, USA) held at 50°C. Gradient elution has been obtained at a flow rate of 0.5 mL/min. Solvent A consisted of 5 mM ammonium formate containing 0.01% *v*/*v* formic acid and solvent B of 0.1% *v*/*v* formic acid in

acetonitrile. The gradient profile was as follows: 0-1 min 20% B, 1-6 min linear gradient from 20 to 60% B, 6-8 min linear gradient from 60 to 90% B, then held for 1 minutes at 90% B, before re-equilibration at 20% B until the end of the run. The total run time was 10 minutes. The MS-source has been configured in the positive ESI ionization mode for atorvastatin, (p)α-OH-atorvastatin-lactone, (o)α-OH-atorvastatin-lactone, rosuvastatin, and N-desmethyl-rosuvastatin at 600°C (CUR:30 L/hour; IS*: 3000 V; GS1 60 L/hour; GS2 70 L/hour), and in the negative mode for pravastatin still at 600°C (CUR:30 L/hour; IS*: -4500 V; GS1 60 L/hour; GS2 70 L/hour) [Figure 21]. Acquisitions have been performed in the MRM-mode using optimized transition and mass parameters [Figure 22] [Table 7].

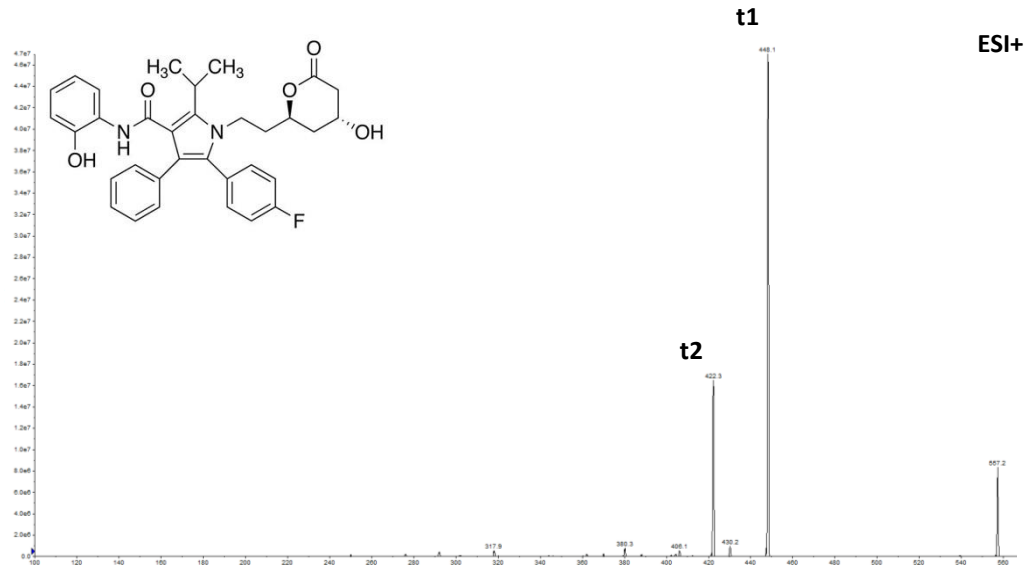
(a)



(b)

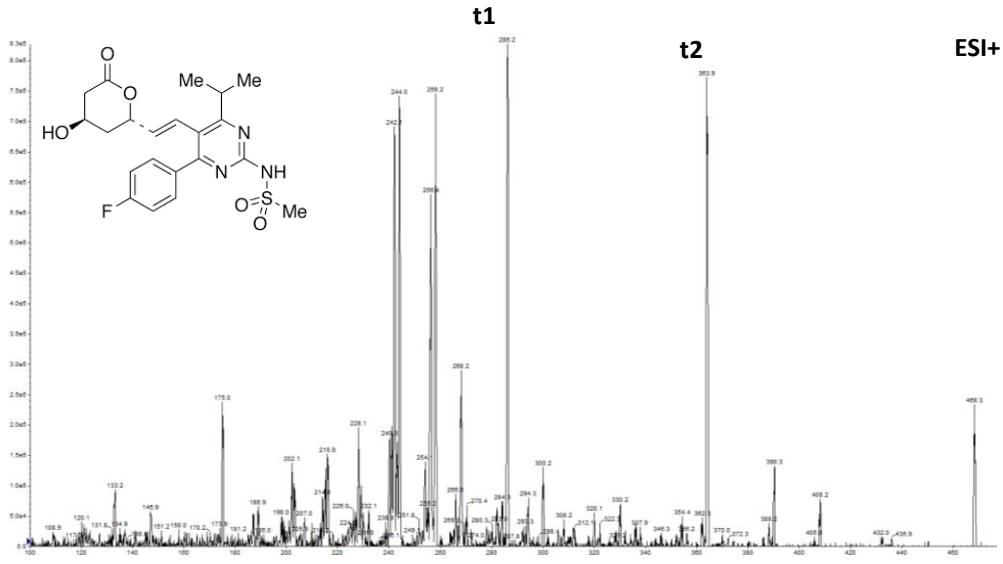


(c)



(d)

(e)



ESI-

(f)

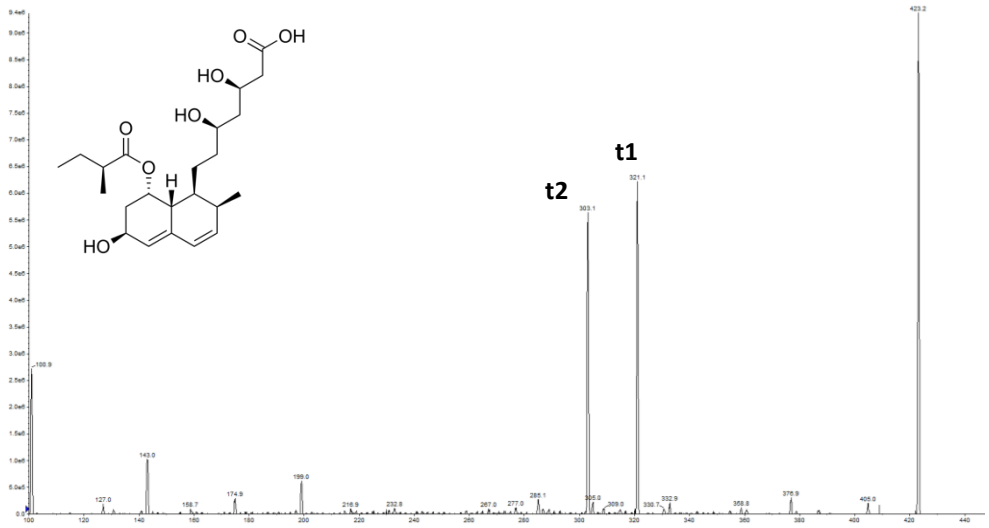


Fig. 21. Mass spectra of atorvastatin (**a,** m/z 559.2, **t1:** m/z 440.3, **t2:** m/z 466.3), (p)α-OH-atorvastatin-lactone (**b,** m/z 557.2, **t1:** m/z 448.2, **t2:** m/z 379.9), (o)α-OH-atorvastatin-lactone (**c,** m/z 557.2, **t1:** m/z 448.2, **t2:** m/z 422.1), rosuvastatin (**d,** m/z 482.2, **t1:** m/z 258.1, **t2:** m/z 300.3), N-desmethyl-rosuvastatin (**e,** m/z 468.2, **t1:** m/z 258.3, **t2:** m/z 384.1), and pravastatin (**f,** m/z 423.2, **t1:** m/z 320.9, **t2:** m/z 303.0).

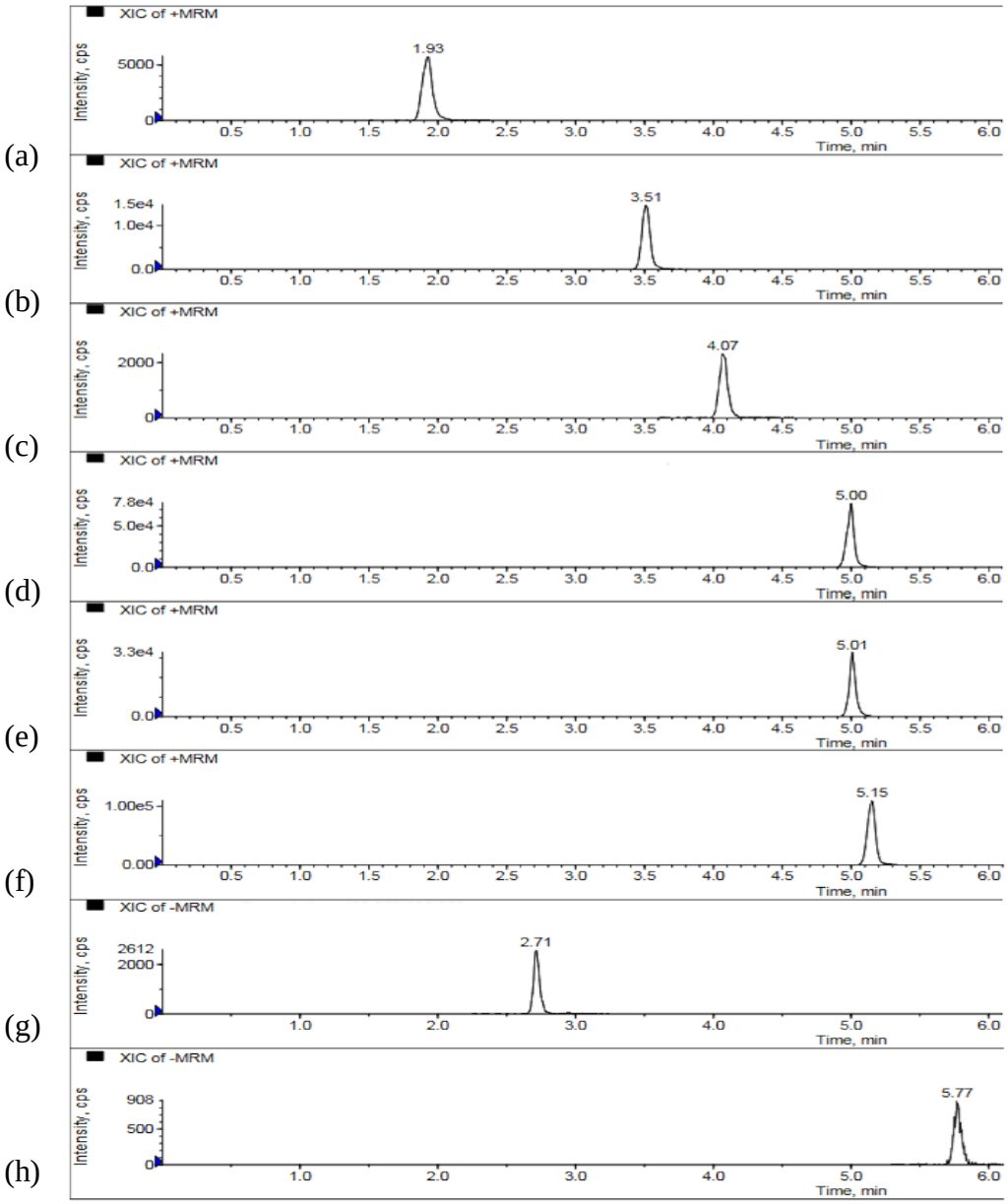


Fig. 22. Extract ion chromatograms of a mixture of the studied analytes each at the individual concentration of 20 pg/mg (**a.** N-desmethyl-rosuvastatin, m/z 468.2/258.3; **b.** rosuvastatin, m/z 482.2/258.1; **c.** (p)α-OH-atorvastatin lactone, m/z 557.2/448.2; **d.** atorvastatin-D_5, m/z 564.2/445.3; **e.** atorvastatin, m/z 559.2/440.3; **f.** (o)α-OH-atorvastatin lactone, m/z 557.2/448.2; **g.** pravastatin, m/z 423.2/320.9; **h.** $^2H_6$-OH-simvastatin, m/z 441.3/319.1).

Analyte	Precursor ion (Da)	Daughter ion (Da)	DP (V)	EP (V)	CE (V)	CXP (V)	Retention time (min)
Atorvastatin	559.2	**440.3**	115	7	32	13	5.01
		466.3	107	7	25	7	
(p)α-OH-atorvastatin lactone	557.2	**448.2**	88	10	26	30	4.07
		379.9	110	10	42	24	
(o)α-OH-atorvastatin lactone	557.2	**448.2**	98	9	26	13	5.15
		422.1	83	9	33	12	
Rosuvastatin	482.2	**258.1**	112	10	47	16	3.51
		300.3	112	10	47	16	
N-desmethyl-rosuvastatin	468.2	**258.3**	110	4	50	17	1.93
		384.1	98	4	46	10	
Pravastatin	423.2	**320.9**	-120	-4	-21	-11	2.71
		303.0	-120	-4	-25	-19	
Atorvastatin-D_5 (IS)	564.2	**445.3**	112	10	32	9	5.00
		471.3	118	9	24	17	
$^2H_6$-OH-simvastatin (IS)	441.3	**319.1**	-77	-5	-24	-17	5.77
		121.0	-77	-5	-31	-19	

Table 7. MRMs transitions, optimized mass parameters and retention times of the studied compounds and their internal standards using the UHPLC-MS/MS system (quantifier transitions are in bold).

3.4.4 METHOD VALIDATION

The method has been validated according to the guidelines of the U.S. Food and Drug Administration (FDA), in terms of selectivity, linearity, sensitivity, intra- and inter-day precision, accuracy (bias), matrix effect (ME), long-term storage and autosampler stability [64]. Selectivity has been assessed by testing 20 different hair samples collected from volunteers not taking statins during the period covered by study. No significant interferences for any of the elected transitions emerged from the analysis of the compounds of interest. Linearity has been determined in the range of 0.75 to 20 pg/mg for all the compounds, showing the best fit and a correlation coefficient higher than 0.996 for each compound. The LODs, that provide a S/N $\geq$ 3 were 0.23 pg/mg for all the statins, while the LLOQs were 0.75 pg/mg again for all the compounds. Intra-day and inter-day precision have been determined considering the peak area ratios of replicated levels to the relative deuterated standard and running 5 replicates per days over 4 different days and expressed in terms of relative standard deviation per cent (RSD% $_{\text{intra-day}}$; RSD% $_{\text{inter-day}}$). The acceptance criteria were 20% RSD at the LLOQs, and 15% for the remaining concentrations. Accuracy (bias) has been considered in terms of percentage deviation from the real concentration of the chosen levels (LLOQ, low, medium, high) in both intra- and inter-day tests, with the acceptance criteria for the average results measured set at ± 20% of the expected concentrations. All the tested analytes met the requested accuracy [Table 8]. Autosampler stability, or processed sample stability has been studied to evaluate the length of time a processed sample can be maintained before it undergoes unacceptable changes in terms of reliable analyte detection, identification, or quantitation. Indeed, circumstances may arise in which samples that have undergone routine preparation for instrumental analysis cannot be immediately analysed. For these reasons, repeated injections for each of the two fortified chosen levels within the calibration range have been performed every 2 hours in the same day, in an up to 24 hours experiment. The average responses at each time interval have been compared to the time zero responses, considering the analyte stable until the average signal remains among of acceptable bias of 25%. Finally, the matrix effect has been evaluated by comparing the slopes

of calibration curves obtained from samples consisting of standards spiked in hair matrix versus standards dissolved in methanol.

Analyte	Levels (pg/mg)	Intraday-1 (n=6)		Intraday-2 (n=6)		Intraday-3 (n=6)		Intraday-4 (n=6)		Day-to-day	
		RSD %	BIAS %	RSD %	BIAS %	RSD %	BIAS %	RSD %	BIAS %	RSD %	BIAS %
Atorvastatin	LLOQ 0.75	2.26	12.43	3.81	-7.56	14.45	-16.70	16.78	15.49	15.39	0.86
	low 1.25	1.44	7.41	1.74	-9.91	7.99	-11.23	10.07	-2.45	8.92	-4.05
	middle 5	2.99	0.09	1.52	2.44	1.88	0.25	3.21	-0.89	1.40	0.47
	high 10	4.77	1.52	2.74	1.38	7.79	-4.99	3.42	3.84	3.77	0.44
(p)α-OH - atorva.lactone	LLOQ 0.75	0.98	8.20	19.56	-17.02	11.16	12.76	10.12	-13.61	15.45	-2.42
	low 1.25	2.11	-13.91	12.16	-11.44	7.08	3.35	13.84	0.81	9.12	-5.30
	middle 5	11.04	-8.05	5.92	-5.09	11.29	3.20	7.43	0.49	5.25	-2.36
	high 10	7.35	-10.22	4.08	-9.44	2.95	8.76	5.83	-7.44	9.41	-4.58
(o)α-OH - atorva.lactone	LLOQ 0.75	3.05	4.80	1.44	10.17	6.42	-16.59	11.71	12.79	15.08	5.29
	low 1.25	6.90	0.97	4.66	-0.07	5.24	-14.34	5.50	9.78	10.07	-0.91
	middle 5	2.75	-5.14	2.14	2.37	9.82	8.69	3.27	-5.21	6.68	0.18
	high 10	6.21	-1.64	11.93	-12.42	7.71	-6.44	2.53	4.76	7.58	-3.94
Rosuvastatin	LLOQ 0.75	6.63	2.21	2.65	17.90	3.84	-18.80	15.90	-5.63	15.51	-1.08
	low 1.25	4.39	8.75	3.53	5.44	1.12	15.25	3.50	2.95	4.93	8.10
	middle 5	4.04	11.53	3.66	-0.82	4.00	-9.84	5.74	-1.36	8.81	-0.12
	high 10	8.32	8.83	8.63	-13.80	8.58	-11.03	1.56	-3.50	10.64	-4.87
N-desmethyl - rosuvastatin	LLOQ 0.75	7.32	-17.74	1.12	3.81	1.81	-16.24	11.33	-11.66	11.01	-10.46
	low 1.25	5.33	-4.14	3.62	7.56	7.33	12.81	3.94	2.49	6.91	4.68
	middle 5	13.48	0.26	2.49	-2.82	10.27	-6.36	4.75	-1.32	2.90	.2.56
	high 10	3.32	4.64	9.49	-9.35	9.94	-12.73	7.08	-6.70	8.02	-6.03
Pravastatin	LLOQ 0.75	10.40	-14.53	19.20	-14.20	8.90	3.88	6.91	-16.72	10.69	-10.39
	low 1.25	13.04	0.35	4.04	-7.87	10.45	1.87	14.93	-1.45	4.36	-1.78
	middle 5	10.23	11.82	5.58	10.17	10.69	-4.31	10.79	5.63	6.85	5.83
	high 10	7.56	15.45	8.68	8.19	8.94	-14.73	6.19	7.77	12.56	4.17

Table 8. Intra-day, day-to-day precision (RSD%) and accuracy (bias) of the studied analytes spiked at the chosen levels.

3.4.5 APPLICATION TO REAL CASES

The developed method has been preliminary tested for authentic hair samples collected from subjects under therapy [Figure 23] [Table 9]. Reliable information on the adherence to therapy of subjects were scarce, or not available. The range of concentrations found in the real samples is wide, but in most cases statins (and their metabolites) were present and detectable.

Case	Prescription	Atorvastatin (pg/mg)	(p)α-OH- (pg/mg)	(o)α-OH- (pg/mg)	Rosuvastatin (pg/mg)	N-des. (pg/mg)
a.	atorvastatin	2.34	2.23	0.26	/	/
b.	rosuvastatin	/	/	/	1.78	/
c.	atorvastatin	1.27	/	/	/	/
d.	atorvastatin	1.04	/	/	/	/
e.	rosuvastatin	/	/	/	/	/
f.	atorvastatin	10.7	18.1	1.90	/	/
g.	atorvastatin	9.20	1.92	0.27	/	/
h.	atorvastatin	6.09	3.31	0.49	/	/
i.	atorvastatin	0.25	/	/	/	/
l.	atorvastatin	12.0	1.40	0.98		

Table 9. Concentrations of detected compounds in real hair samples tested and collected from patients undergoing long term therapy with statins.

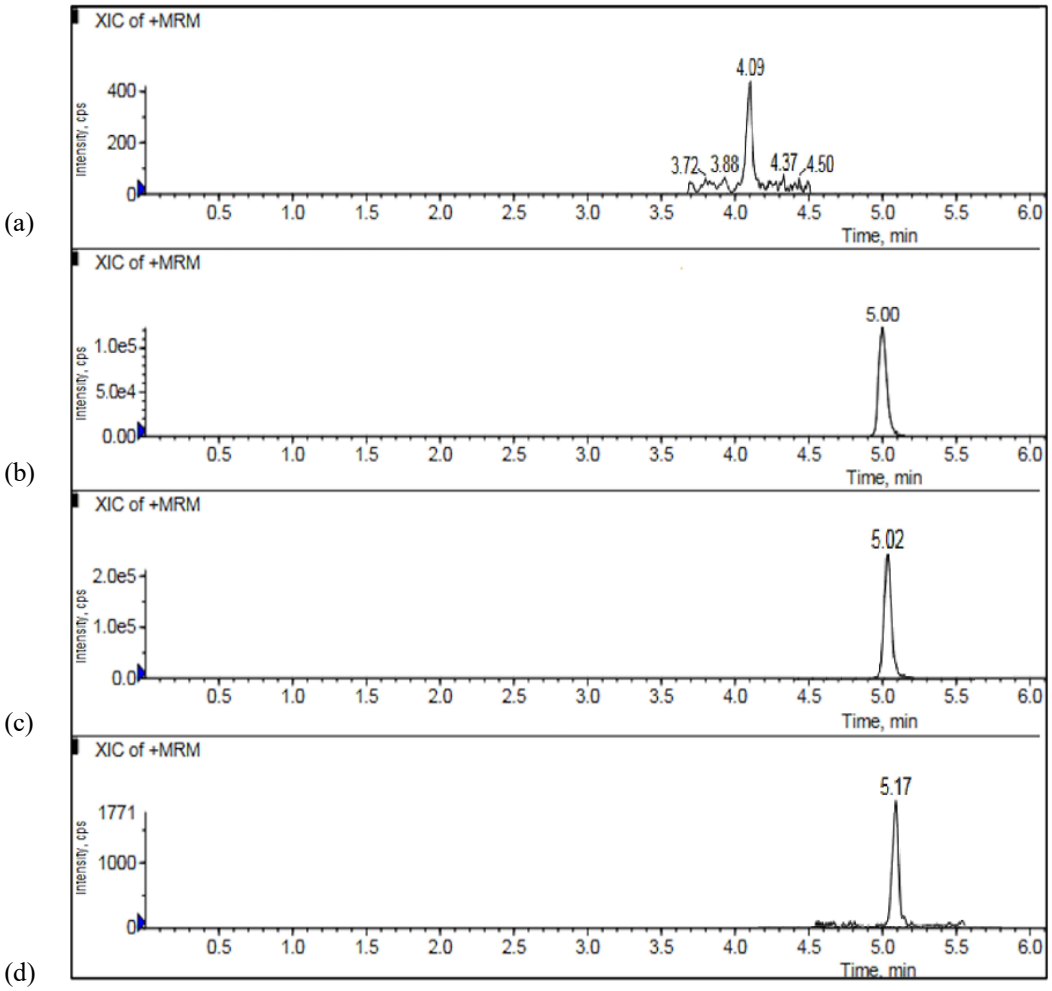


Fig. 23. Extract ion chromatograms of a real hair sample (case **g.**, Table 6) containing atorvastatin (**c**), (p)α-OH-atorvastatin lactone (**a**) and (o)α-OH-atorvastatin lactone (**d**) at the concentration of 9.20, 1.92 and 0.27 pg/mg, respectively (**b**: atorvastatin-D_5, IS).

3.4.6 CONCLUDING REMARKS

The fully validated UHPLC-MS/MS method developed for the quantification of statins in human hair samples allows for a rapid chromatographic separation and sensitive detection, showing the possibility of using hair analysis as a tool, to monitor patients' adherence to therapy for this specific class of compounds. However, as already reported, reliable information on the adherence to therapy for the tested cases were scarce, or not available. For this reason, this work should be considered a test of feasibility and not properly a form of clinical validation of this new tool of investigation. In conclusion, the results of the present study show for the first time the possibility of using hair analysis as a new, and potentially almost ideal tool, to monitor patients' adherence to therapy which, for this specific class of compounds, is known to decay rapidly after the start of its prescription. This clearly compromises the potential efficacy of statins in the prevention against atherosclerosis associated ischemic events, with consequent increases of deaths, disabilities, and costs for the health systems. Future perspectives are certainly to further investigate better extraction procedures to improve the signal detection of targets, excluding as well as possible matrix interferences. Furthermore, to validate the potential of mass spectrometry and setting up the hair analysis as an objective method to monitor adherence to therapy in chronic pharmacological treatments, not only for lipid-lowering medications such as statin therapy, but also in a context of a wider study with several class of drugs, such as beta-blockers and calcium-channel blockers (anti-hypertensive drugs).

4. CONCLUSIONS

The interpretation of the analytical results is of utmost importance in forensic toxicology depending also to the type of analysed biological sample, the way in which those are acquired, stored, and handled. Suitable extraction procedures, appropriate internal standard, and standardised detection methods which can provide reproducibility, adequate sensitivity and specificity are of great importance. Moreover, the choice of the proper analytical conditions appears to be also crucial. In the present PhD thesis, the analytical optimization of a method for the quantification of EtG in human hair has been presented, as a topic currently of great interest in the forensic field. The second part of this thesis faced the problem of poor adherence to long-term therapies proposing a novel method for the quantification of statins in human hair samples. The method has been fully validated showing for the first time the possibility of using hair analysis as a new, and potentially almost ideal tool, to monitor patients' adherence to therapy for this specific class of compounds.

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ORAL AND POSTER COMMUNICATIONS RELATED AND UNRELATED TO THE DOCTORAL THESIS

- Congress participation with poster presentation at *IATDMCT 2021 – 20^{h} International congress of therapeutic drug monitoring & clinical toxicology*. Prague, Czech Republic 18-21/09/2022.
  (M Ballotari, F Taus, G Tolle, E Danese, RM Dorizzi, F Tagliaro, R Gottardo. *Development of a new ultra-high-performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) method for the determination of digoxin and digitoxin in plasma: comparison with a clinical immunoassay).*

- Congress participation with oral presentation at *IATDMCT 2021 – 20^{h} International congress of therapeutic drug monitoring & clinical toxicology*. Rome, Italy 19-22/09/2021.
  (F Tagliaro, N Martinelli, F Taus, M Ballotari, R Gottardo. *Mass spectrometry applied to the monitoring of adherence to therapy in chronic pharmacological treatments).*

- Congress participation with oral presentation at *26th Society of Hair Testing (SoHT) and Italian Group of Forensic Toxicologists (GTFI) Meeting.* Verona, 8-10/06/2022.
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