Faculteit Farmaceutische, Biomedische en Diergeneeskundige Wetenschappen

Departement Farmaceutische Wetenschappen

Hair and nail analysis for the long-term monitoring of alcohol and drug consumption: focus on bioanalytical and correlation aspects

Haar- en nagelanalyse voor de lange termijnsopvolging van alcohol- en druggebruik: focus op bio-analytische en correlatie aspecten

Proefschrift voorgelegd tot het behalen van de graad van

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“Try not to become a man of success. Rather become a man of value.”

- Albert Einstein -

LIST OF ABBREVIATIONS

6-MAM	6-Monoacetylmorphine
Ac	Acetone
ACN	Acetonitrile
ADH	Alcohol dehydrogenase
ALAT	Alanine amino transferase
ALDH	Aldehyde dehydrogenase
AMP	Amphetamine
ASAT	Aspartate amino transferase
AUDIT	Alcohol Use Disorder Identification Test
BAC	Blood alcohol concentration
BE	Benzoylecgonine
BMI	Body mass index
CDT	Carbohydrate-deficient transferrin
COC	Cocaine
COD	Codeine
CV	Coefficient of variation
CYP2E1	Cytochrome P 450 2E1
DALYs	Disability-adjusted life years
DAST	Drug and Alcohol Screening Test
DCM	Dichloromethane
DHI	5,6-dihydroxyindole
DHICA	5,6-dihydroxyindole-2-carboxylic acid
DOPA	Dihydroxyphenylalanine
DSM	Diagnostic and Statistical Manual of Mental Disorders

EDDP	2-Ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine
EI	Electron impact
EMA	European Medicines Agency
EME	Ecgonine methyl ester
ESI	Electrospray ionization
EtG	Ethyl glucuronide
EtS	Ethyl sulphate
FA	Fatty acids
FAc	Formic acid
FAEE	Fatty acid ethyl esters
FDA	Food and Drug Administration
FN	Fingernails
GC	Gas chromatography
GGT	Gamma-glutamyl transferase
GHB	Gamma-hydroxybutyric acid
GTFCh	German Society of Toxicological and Forensic Chemistry
H	Hair
H_2O	Water
Hex	Hexane
HFBA	Heptafluorobutyric anhydride
HILIC	Hydrophilic interaction liquid chromatography
IQR	Interquartile range
LC	Liquid chromatography
LLE	Liquid-liquid extraction
LOD	Limit of detection
LP	Lipoproteins

LLOQ	Lower limit of quantification
mAMP	Methamphetamine
MCV	Mean corpuscular volume
MDEA	3,4-Methylenedioxy-ethylamphetamine
MDMA	3,4-Methylenedioxy-methamphetamine
MeOH	Methanol
MOR	Morphine
MRM	Multiple reaction monitoring
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MTD	Methadone
m/z	Mass-to-charge ratio
N_2	Nitrogen gas
NH_3	Ammonia
NH_4OH	Ammonium hydroxide
NICI	Negative ion chemical ionization
NPS	New psychoactive substances
PC	Phosphatidylcholine
PEth	Phosphatidylethanol
PL	Phospholipids
PLD	Phospholipase D
PFPA	Pentafluoropropionic anhydride
QC	Quality control
RSD	Relative standard deviation
SAMHSA	Substance Abuse and Mental Health Services Administration

SD	Standard deviation
SE	Standard error
SPE	Solid-phase extraction
SoHT	Society of Hair Testing
TG	Triglycerides
TN	Toenails
TLFB	TimeLine Follow-Back
UDP-glucuronosyltransferase	Uridine 5'-diphospho–glucuronosyltransferase
WHO	World Health Organization

TABLE OF CONTENTS

CHAPTER 1:

GENERAL INTRODUCTION

1.1. Hair

1.1.1. Introduction

The history of xenobiotic analysis in human hair traces back to 1858 when the first case of determination of a toxic metal (i.e. arsenic) in human hair was reported (Casper 1858). The researchers hypothesized that hair was a preferable matrix over blood and urine to document exposure to hazards as hair could store substances for an extended period of time. Heavy metals such as titanium, arsenic, lead and mercury were among the first substances to be detected in hair (Sachs 1997). Gradually, with the development of more sensitive bioanalytical technologies, the detection of organic substances, especially pharmaceuticals and drugs of abuse, in hair became possible (Pragst et al. 2006). In 1979, Baumgartner et al. were able to determine opiate content in hair of heroin users by radioimmunoassay (Baumgartner et al. 1979). In 1980, a chromatographic method was used for the first time to confirm radioimmunological results (Klug 1980). After matrix digestion with sodium hydroxide, Klug et al. were able to quantify opiates in hair using thin-layer chromatography with fluorescence detection (Klug 1980). Both studies found that opiate concentrations differed along the hair shaft and corresponded to the time of drug intake. These initial reports indicated the potential advantages of hair analysis and were followed by three decades of scientific research, which have led to the development and implementation of hair testing approaches. The first international meeting dealing with the subject of hair analysis was organized in 1992 in Genoa (Sachs 1997). In 1995, the Society of hair Testing (SoHT) was founded, which aims to define, update, and harmonize the requisites and procedures related to hair analysis.

1.1.2. Anatomy and physiology of hair

1.1.2.1. Structure of human hair

Hair is mainly composed of proteins (65-95%), primarily keratin, a protein containing large amounts of sulphur. Other constituents are water (15-35%), lipids (1-9%) and minerals (up to 1%) (Harkey 1993, Barbosa et al. 2013). A hair is not a homogenous fibre, but consists of keratinized cells that form three concentric structures: an outer cuticle which surrounds a central cortex which, in turn, may contain a central medulla (Figure 1.1a) (Harkey 1993, Pragst et al. 2006).

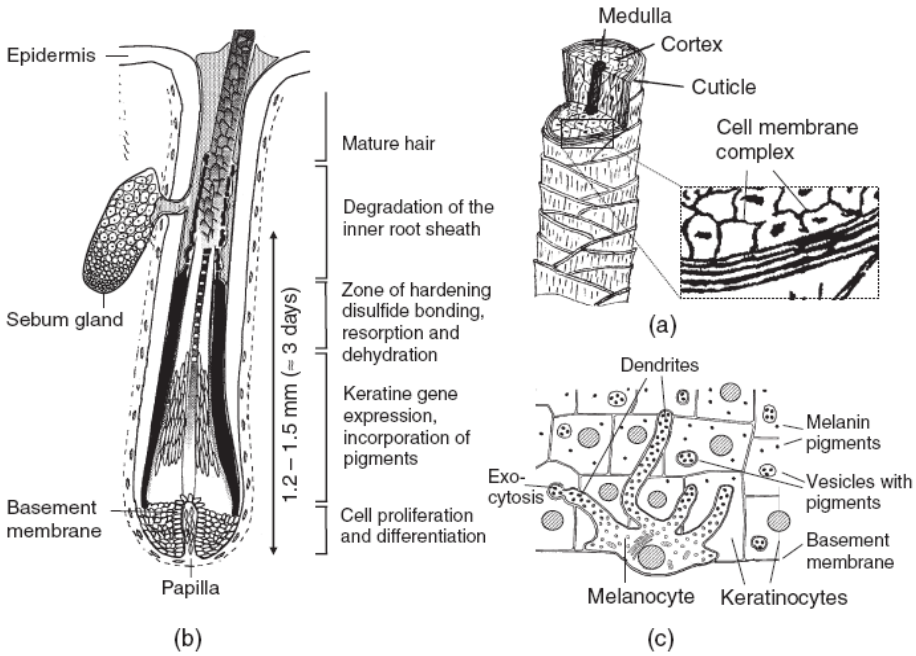

Figure 1.1: a) Structure of the human hair shaft; b) Formation of hair in a follicle from matrix cells located on the basement membrane; c) Melanocytes synthetize melanin in melanosomes that are discharged in vesicles into the keratinocytes (Pragst et al. 2006).

The cuticle, composed of overlapping elongated cells, is responsible for chemical and mechanical resistance, and prevents dehydration (Barbosa et al. 2013). The cortex forms the bulk of the hair shaft and consists of spindle-shaped keratinized cells which are formed into long fibres (Harkey 1993, Kintz 2007). It is responsible for stretching stability and colour composition through the presence of pigment granules in cortical cells. Melanin, the principle pigment of hair, is synthesized in specialized cells called melanocytes located within the hair bulb (Harkey 1993). Melanogenesis starts with hydroxylation and oxidation of L-tyrosine to dopaquinone through the action of the enzyme tyrosinase (Figure 1.2) (Harkey 1993, Slominski et al. 2005). Once dopaquinone is formed, melanogenesis proceeds further through oxido-reduction reactions, intramolecular transformations, and polymerisations to form two types of pigments: eumelanins and pheomelanins (Figure 1.2) (Slominski et al. 2005, Solano 2014). Structurally, eumelanins are heterogeneous polymers consisting of 5,6-dihydroxyindole (DHI) and 5,6-dihydroxyindole-2-carboxylic acid (DHICA) units, while pheomelanins are composed of benzothiazine,

benzothiazole, and isoquinoline units (Figure 1.2) (Kintz 2007). After pigment formation, the melanocytes transfer melanin to the migrating cortical cells from the hair follicle bulb (Figure 1.1c) (Kintz 2007). The amount, density and type of melanin in melanocytes determines the exact colour of hair. Eumelanins are dark brown/black pigments, while pheomelanins are reddish/yellow pigments (containing sulphur) (Slominski et al. 2005, Kintz 2007). The medulla forms the inner layer of the hair, but comprises only a small percentage of the hair mass and may even be absent (Harkey 1993).

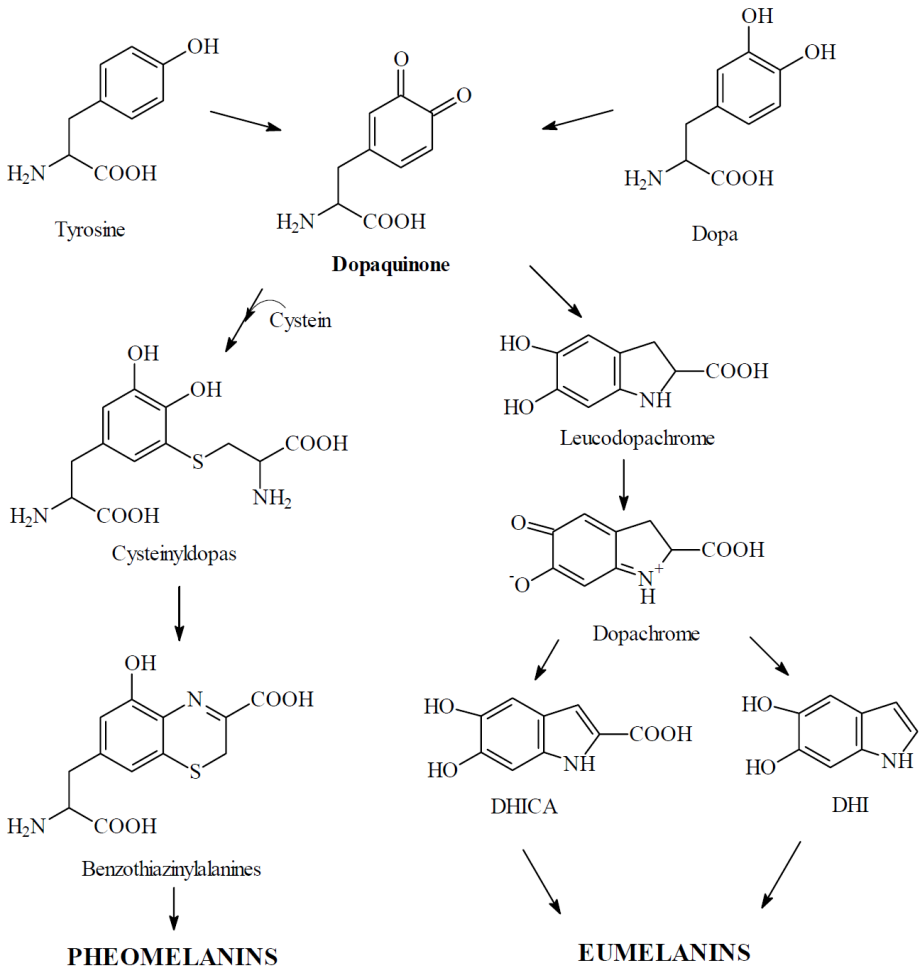

Figure 1.2: Simplified scheme illustrating in vivo biosynthesis of pheomelanins and eumelanins (Kintz 2007).

Abbreviations: DHI, 5,6-dihydroxyindole; DHICA, 5,6-dihydroxyindole-2-carboxylic acid; dopa, dihydroxyphenylalanine

1.1.2.2. Hair formation

Hair originates from the hair follicle located 3-5 mm below the skin surface (Figure 1.1b) (Harkey 1993, Pragst et al. 2006, Kintz 2007, Barbosa et al. 2013, Kintz et al. 2015). The innermost zone of the follicle is called the hair bulb with at its base the dermal papilla which contains the blood supply that provides the growing hair with the necessary metabolic material (Boumba et al. 2006). The cells located within the lower region of the bulb, the matrix cells (keratinocytes and melanocytes), are mitotically active and give rise to a stream of cells moving upward into the direction of the hair root mouth (Pragst et al. 2006, Kintz 2007, Kintz et al. 2015). The upper region of the bulb contains the keratinogenous zone where the hair undergoes hardening and solidification (Figure 1.1b) (Kintz et al. 2015). The final zone is the permanent hair shaft.

Hair follicles are closely associated with sebaceous glands and in some regions, like the axillary and pubic areas, with apocrine sweat glands. Eccrine sweat glands are located near the follicles but are not associated with them. The eccrine sweat glands secrete near the hair follicle exit, while the sebaceous and apocrine glands secrete directly into the hair follicle (Harkey 1993, Barbosa et al. 2013).

Hair grows in a cycle composed of three stages: the anagen, the catagen and the telogen stage (Figure 1.3) (Pragst et al. 2006). The anagen stage is the period of active growth during which the follicle develops and the hair is produced through rapid mitosis and hair growth (Kintz 2007). For scalp hair the duration is usually 4 to 8 years (Pragst et al. 2006). At any time, on average 85% of the approximately 1 million follicles of the adult scalp are in this growing stage (Pragst et al. 2006, Barbosa et al. 2013). The anagen stage is followed by the catagen or regression stage. During this short transition period, which lasts 2 to 3 weeks, the activity of the follicle bulb ceases, the hair shaft becomes fully keratinized and the blood supply ends (Boumba et al. 2006, Kintz 2007, Barbosa et al. 2013). During the telogen or resting stage the hair growth has completely stopped; the follicle is short and can be easily removed by pulling or by a new follicle arising in the same place (Barbosa et al. 2013). This period takes about 10 weeks for scalp hair but can take up to 2 to 6 years for body hair. After this rest phase the follicle will start a new cycle and produce a new hair. In humans, each hair follicle has his own cycle independently of its neighbours (Kintz 2007). The growth rate of hair and the proportion of anagen/telogen hair vary according to the anatomic region, race, gender and age (Pragst et al. 2006). For scalp hair, an average growth of 1 cm per

month is generally accepted (Cooper et al. 2012). However, variations in growth rate have been reported ranging from 0.6 to 3.36 cm per month depending on the region of the scalp (Table 1.1) (Harkey 1993, Barbosa et al. 2013). It is considered that hair from the vertex posterior region of the head (back of the head) provides the lowest variability in growth rate. In general, body hair grows slower and has a lower percentage of follicles in the anagen stage than scalp hair (Table 1.1) (Barbosa et al. 2013, Kintz et al. 2015).

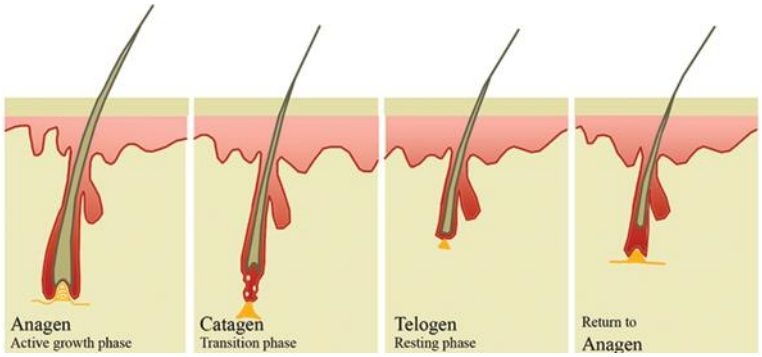

Figure 1.3: Overview of the hair growth cycle composed of the anagen, catagen and telogen stages.

Table 1.1: Reported growth rates for different types of hair (Kintz et al. 2015).

Hair type	Growth rate ranges (cm/month)
Head	0.60 – 3.36
Pubic	0.60 – 0.90
Axillary	0.87 – 1.00
Beard	0.75 – 1.20
Chest	0.66 – 0.96
Arm hair	1.05
Leg hair	0.39 – 1.05

1.1.3. Compound incorporation into hair

The exact mechanisms involved in the incorporation of compounds into hair are not yet fully understood and require further investigation. The most realistic and generally accepted model is the multi-compartment model which hypothesizes that incorporation of compounds into hair occurs through three main routes: (I) diffusion from blood; (II) diffusion from sweat or sebum; and (III) external contamination (Figure 1.4) (Henderson et al. 1996).

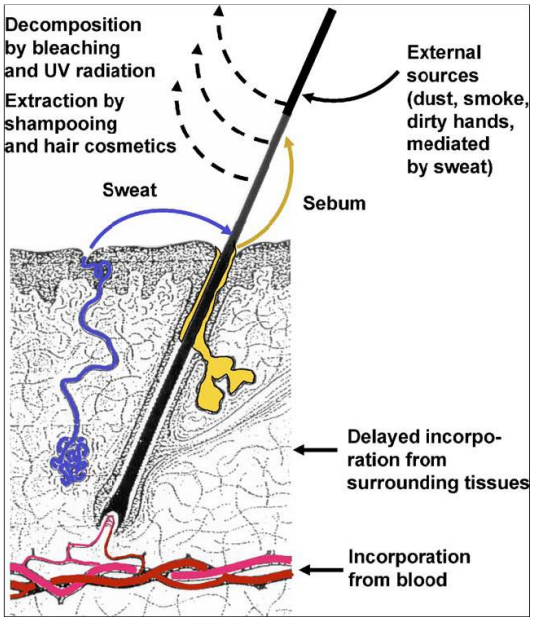

Figure 1.4: Drug incorporation routes into hair (Pragst et al. 2006).

First, compounds can incorporate into the growing hair through diffusion from the blood flow of dense capillary network feeding the dermal papilla of the hair follicle (Kintz 2007, Barbosa et al. 2013). In order to enter the matrix cells of the growing hair, compounds have to diffuse across the cell membrane. The rate of diffusion is related to the lipid solubility of the compound, and the concentration- and pH-gradient between the plasma and the cells (Kintz 2007). Neutral and lipophilic molecules are more likely to diffuse across the membrane. As human Phase I and Phase II metabolites are generally more polar than their parent compound, they are less prone to passive diffusion and lower concentrations are expected to accumulate in hair compared to

parent compounds (Barbosa et al. 2013). The pH gradient from plasma (pH = 7.3) to more acidic conditions within the melanocytes and keratinocytes (pH = 3-6) favours the diffusion and accumulation of basic compounds in hair (Barbosa et al. 2013, Kintz et al. 2015). Once in the matrix cells, the binding of compounds to the cell proteins may enhance this effect, as the compound concentration in the cytosol decreases when the compounds are associating with structures within the cell (Kintz 2007).

Second, compounds can incorporate in hair through their secretion into sweat or sebum followed by diffusion into the growing or mature hair. As sweat is more acidic (pH $\cong$ 6.3) than blood, alkaline compounds accumulate in sweat, and are more concentrated in sweat than in blood (Henderson et al. 1996, Barbosa et al. 2013).

Third, incorporation of compounds in hair can occur through external contamination of the mature hair fibre (e.g. vapours, powders or liquids) (Kintz 2007). This is particularly relevant when smoked or inhaled drugs, such as cannabis, cocaine, nicotine and heroin, are considered.

The relative importance of each route depends on the physicochemical properties of the incorporated compound as well as on the physiological characteristics of the individual (Barbosa et al. 2013). For the latter two incorporation routes, the porosity of the hair is of importance. The porosity of the hair is mainly influenced by cosmetic treatments that are performed on the hair (Pragst et al. 2006). Cosmetic treatments, such as colouring, bleaching with hydrogen peroxide or perming, can influence the incorporation of compounds in two ways. First, the reactive molecules used in those treatments can react with the compounds present in the hair, which results in biotransformation and degradation. Second, the cuticle gets damaged and the porosity increases facilitating incorporation of compounds into hair (Kintz 2007, Barbosa et al. 2013).

1.1.4. Applications of hair analysis

Several classes of compounds have been detected in hair and the applications of hair analysis are widespread, Table 1.2 presents a non-limitative overview of the applications of hair analysis.

Table 1.2: Non-limitative overview of the compounds detected in hair and their applications.

Class of compounds	Examples of compounds	Applications	References
Metals	Arsenic, mercury, cadmium, lead, copper, zinc	Biomonitoring of human exposure	(Wilhelm et al. 1991, Liu et al. 2001, Strumylaite et al. 2004, Berglund et al. 2005)
Pollutants	Polychlorinated biphenyls, polycyclic aromatic hydrocarbons, polybrominated diphenyl ethers, organophosphate esters, flame retardants, perfluoroalkyl substances, phthalate esters	Biomonitoring of human exposure	(Ohgami et al. 1989, Toriba et al. 2003, Zhao et al. 2008, Schummer et al. 2009, Alves et al. 2014, Zheng et al. 2014, Kucharska et al. 2015)
Alcohol biomarkers	EtG, FAEE	Gestational drug exposure, workplace drug testing, driving ability examination, liver and kidney transplantations	(Pragst et al. 2010, Andresen-Streichert et al. 2015, Cappelle et al. 2015, Oppolzer et al. 2017)
Illicit drugs	Cocaine, opioids, cannabinoids, amphetamines, GHB	Gestational drug exposure, workplace drug testing, driving ability examination, chronic intoxication, drug abuse history and withdrawal control, criminal liability, drug facilitated crimes, post-mortem toxicology	(Dufaux et al. 2012, Concheiro et al. 2013, Imbert et al. 2014, Joya et al. 2015, Busardo 2016, Cappelle et al. 2017, Taylor et al. 2017, Wang et al. 2018)
NPS	Phenethylamines, ketamine, synthetic cathinones, synthetic cannabinoids		(Strano-Rossi et al. 2014, Salomone et al. 2016, Alvarez et al. 2017, Kyriakou et al. 2017, Salomone et al. 2017)

Abbreviations: EtG, ethyl glucuronide; FAEE, fatty acids ethyl esters; GHB, gamma-hydroxybutyric acid; NPS, new psychoactive substances

Table 1.2: Continued.

Class of compounds	Examples of compounds	Applications	References
Pharmaceuticals	Benzodiazepines, z-drugs, antidepressants, antipsychotics, methadone	Workplace drug testing, therapy compliance control, criminal assaults, drug-facilitated crimes, post-mortem toxicology	(Dufaux et al. 2012, Concheiro et al. 2013, Binz et al. 2014, Ramirez Fernandez et al. 2015, Ramirez Fernandez et al. 2016, Sim et al. 2017, Hoiseth et al. 2018, Wang et al. 2018)
Hormones	Cortisol, cortisone	Measuring chronic stress, Cushing's disease	(Manenschijn et al. 2011, Wells et al. 2014, Mwanza et al. 2016)
Doping agents	Anabolic androgenic steroids, clenbuterol, beta-2 agonists, ephedrine	Doping control	(Kintz et al. 2000, Thieme et al. 2000, Dumestre-Toulet et al. 2002, Strano-Rossi et al. 2013)
Other compounds	Nicotine, cotinine, caffeine, paraxanthine	Smoking behaviour, cytochrome P450 1A2 phenotyping, consumption monitoring	(Antunes et al. 2015, De Kesel et al. 2015, Bertol et al. 2017)

Abbreviations: EtG, ethyl glucuronide; FAEE, fatty acids ethyl esters; GHB, gamma-hydroxybutyric acid; NPS, new psychoactive substances

1.2. Nails

1.2.1. Introduction

Nails are keratinized matrices capable of storing substances for long periods of time (Palmeri et al. 2000, Daniel et al. 2004). Since 1965, nails have been used to detect arsenic intoxication and exposure to metals, such as cadmium, copper, lead, zinc, iron and magnesium (Lander et al. 1965, Pounds et al. 1979, Alexiou et al. 1980, Wilhelm et al. 1991, Gerhardsson et al. 1995). Over the years, nail analysis has expanded towards the detection of drugs of abuse, pharmaceuticals and their metabolites. For example, nails have been employed for the therapeutic drug monitoring of antimycotics and for the detection of amphetamine-like substances, cocaine and opiates (Suzuki et al. 1984, Palmeri et al. 2000).

1.2.2. Anatomy and physiology of the nail

1.2.2.1. Structure of the nail

The human nail acts as a protective covering to the delicate tip of fingers and toes, and facilitates grasping and tactile sensitivity (Gupchup et al. 1999). Similarly to hair, the main chemical constituent of the human nail is keratin. Other constituents of nails are water (18%), lipids (less than 5%) and minerals (Verma et al. 2013). Three major nail components can be distinguished, starting from the outer structure, they are the nail plate, the nail bed, and the germinal matrix (Figure 1.5) (Gupchup et al. 1999, Jenkins 2007).

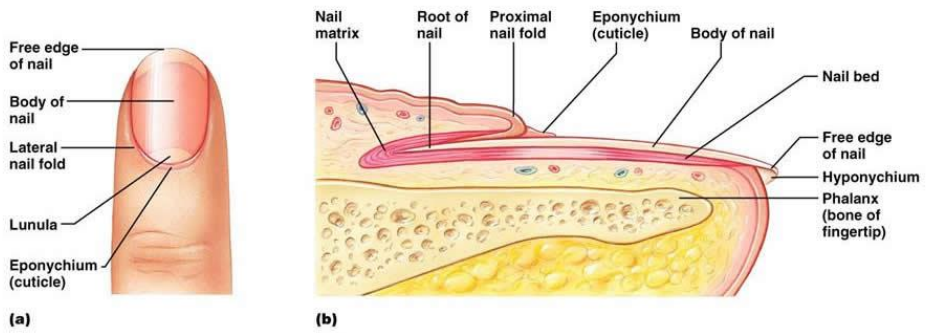

Figure 1.5: a) Dorsal view of the nail; b) Sagittal section of the nail showing the structural components of the nail (Marieb 2012).

1.2.2.1.1. The germinal matrix

The germinal matrix, or the nail matrix, is a highly proliferative epidermal tissue and is mainly responsible for the formation of the nail plate or the body of nail (Gupchup et al. 1999, Palmeri et al. 2000). It lies beneath the skin, forms the floor of the proximal nail groove, and is protected by the proximal nail fold (Figure 1.5). As the germinal matrix produces new keratin cells, they push the older ones out through the cuticle where they flatten, undergo cell nuclear fragmentation and cytoplasmic condensation, and form the translucent nail plate. The germinal matrix contains few melanocytes, cells responsible for producing melanin, and hence nails lack pigmentation. The size and shape of the germinal matrix determine the shape and thickness of the nail plate and may vary between individuals. The edge of the germinal matrix is seen as a white crescent-shaped structure called the lunula (Figure 1.5a). It corresponds to the area where the matrix extends beyond the proximal nail fold (Gupchup et al. 1999, Jenkins 2007).

1.2.2.1.2. The nail bed

The nail bed extends from the lunula to the hyponychium (Figure 1.5) (Gupchup et al. 1999). The hyponychium refers to the area under the nail plate, where the nail bed ends and the normal epidermis begins. The nail bed has an extensive vascular network and consists of longitudinal epidermal ridges and dermal papillae. It acts mainly as a holder and slide for the nail plate (Jenkins 2007). Although it does not contribute much to the formation of the nail plate, keratin production in the nail bed occurs synchronously with extension of the nail plate (Gupchup et al. 1999). The nail bed is thus responsible for the increasing thickness of nail by formation of ventral layers during growth from the lunula to the free edge (Palmeri et al. 2000, Jenkins 2007).

1.2.2.1.3. The nail plate

The nail plate is the hard, flat, translucent material known more simply as the nail (Figure 1.5). It is approximately 0.5 mm thick and composed of dead, adherent, keratinous cells without nuclei or organelles, but with prominent cell borders (Johnson et al. 1993, Gupchup et al. 1999). The nail plate has a three layer structure: the dorsal and intermediate nail, formed from the matrix; and the ventral nail, formed from the nail bed (Figure 1.6). The dorsal nail is a few cell layers thick

and contains hard keratin, while the intermediate nail contains softer keratin and accounts for 75% of the whole nail thickness. The ventral nail is one to two cell layers thick and is comprised of soft, hyponychial keratin (Gupchup et al. 1999). The nail plate sits on the nail bed in three grooves made by three folds of skin: the proximal nail fold (or nail wall) and two lateral nail folds (Figure 1.5). The nail folds act as protective barriers against bacteria for the matrix and the nail bed. The nail plate grows from under the proximal nail fold, which is an extension of the epidermis and dermis and contains sweat glands. The epithelium of the proximal nail fold, called the eponychium, attaches the nail plate and moves with it as it grows (Figure 1.5) (Jenkins 2007).

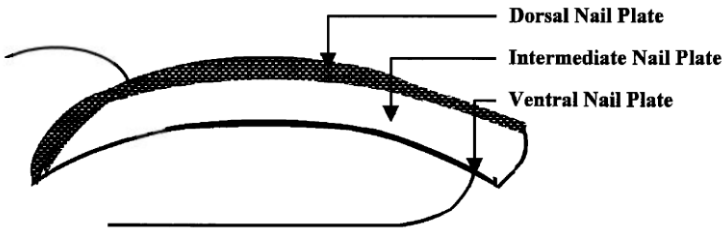

Figure 1.6: Longitudinal section of the nail plate showing the three layers: the dorsal nail, the intermediate nail, and the ventral nail (Gupchup et al. 1999).

1.2.2.2. Blood supply to the nail

The nail matrix and nail bed have a rich blood supply that originates from two digital arteries. In addition, there is an extensive capillary blood supply to the tissues around the nail; in particular, a capillary loop system supplies blood to the whole nail fold (Gupchup et al. 1999).

1.2.2.3. Nail formation

Nails grow at a continuous rate without resting stages in contrast to hair. The average growth rate of fingernails is 3.0 mm per month, while toenails grow at an average rate of 1.1 mm per month (Fleckman 1985, Gupchup et al. 1999). The regeneration time (i.e., the time to grow from the germinal matrix to the nail's free edge) is 3 to 5 months and 8 to 16 months for finger- and toenails, respectively (Fleckman 1985, Palmeri et al. 2000, Lin et al. 2004). As previously

mentioned, nails grow according to two different directions, length and thickness (Johnson et al. 1991). The germinal matrix is responsible for the distal growth of the nail and accounts for about 80% of the total nail mass. Through the activity of the nail bed the nail plate increases in thickness from the proximal to the distal end (Palmeri et al. 2000). The thickening rate is constant and slow, with a mean value of 0.02 mm/mm (0.22% per each 1% increment in length) (Johnson et al. 1993).

1.2.3. Compound incorporation into nails

Incorporation of substances into nails mainly occurs through diffusion of compounds from the rich blood supply, which deposits substances to both the germinal matrix and the nail bed on the underside of the nail plate, thus allowing incorporation in both a vertical and horizontal way during nail formation (Gupchup et al. 1999) (Figure 1.7). Other mechanisms of incorporation that have been proposed are: incorporation through diffusion from biological fluids such as sweat, sebum and saliva (Ropero-Miller et al. 2000, Hang et al. 2013, Madry et al. 2014), and incorporation through diffusion from the external environment (Engelhart et al. 2002). This latter pathway of incorporation was deemed to be minimal (Engelhart et al. 2002). However, mechanisms for substance incorporation into nails have only been scarcely investigated, and the relative importance of the different mechanisms as well as the quantitative differences with incorporation into hair are not clear yet.

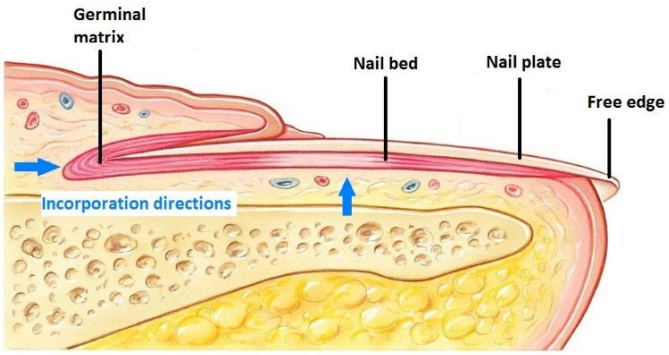

Figure 1.7: Drug incorporation in nails (adapted from (Marieb 2012)).

1.2.4. Applications of nail analysis

Table 1.3 presents an overview of the compounds that have been detected in nails together with their applications. Compared to hair, less compounds and less applications have been investigated in nails. Moreover, the number of articles reporting on a given compound is smaller for nails than for hair.

Table 1.3: Non-limitative overview of the compounds detected in nails and their applications.

Class of compounds	Examples of compounds	Applications	References
Metals	Arsenic, mercury, cadmium, lead, copper, zinc	Biomonitoring of human exposure	(Pounds et al. 1979, Alexiou et al. 1980, Wilhelm et al. 1991, Morton et al. 2004, Alves et al. 2014)
Pollutants	Polybrominated diphenyl ethers, organophosphate esters, flame retardants, perfluoroalkyl substances	Biomonitoring of human exposure	(Alves et al. 2014, Liu et al. 2015, Alves et al. 2017)
Alcohol biomarkers	EtG	Gestational drug exposure	(Jones 2012, Morini et al. 2012, Morini et al. 2013, Cappelle et al. 2016)
Illicit drugs	Cocaine, opioids, cannabinoids, amphetamines	Gestational drug exposure, chronic intoxication, drug abuse history and withdrawal control, criminal liability, drug-facilitated crimes, post-mortem toxicology	(Suzuki et al. 1984, Lemos et al. 1999, Ropero-Miller et al. 2000, Shen et al. 2014)
NPS	Ketamine		(Shu et al. 2015)
Pharmaceuticals	Benzodiazepines, zolpidem, antidepressants, antipsychotics, methadone, antimycotics	Therapy compliance control, criminal assaults, drug facilitated crimes, post-mortem toxicology	(Lemos et al. 2000, Palmeri et al. 2000, Irving et al. 2007, Chen et al. 2012, Hang et al. 2013, Krumbiegel et al. 2014, Madry et al. 2014, Moretti et al. 2018)
Hormones	Cortisol, cortisone	Measuring chronic stress	(Ben Khelil et al. 2011)
Doping agents	Anabolic androgenic steroids	Doping control	(Choi et al. 2001, Brown et al. 2011)
Other compounds	Nicotine, cotinine, caffeine	Smoking behaviour, gestational exposure	(Al-Delaimy et al. 2002, Al-Delaimy et al. 2008, Mari et al. 2008)

Abbreviations: EtG, ethyl glucuronide; NPS, new psychoactive substances

1.3. Analysis of keratinized matrices

Due to the complexity of the keratinized matrix and the low concentrations of incorporated compounds that are often observed (pg/mg to ng/mg range), an extensive sample preparation strategy as well as a specific and sensitive bioanalytical technique are required (Figure 1.8). A typical sample preparation procedure of keratinized matrices comprises the following steps: decontamination, homogenization, extraction and clean-up. Decontamination prior to analysis has two main purposes. First, to remove residues of daily care products, sweat, sebum or surface material that may interfere with the analysis or that may reduce extraction recovery. Second, to remove potential external contaminants from the environment (Kintz 2007, Cooper et al. 2012). Decontamination involves the use of washing solvents (e.g., water, acetone and methanol) for a few minutes at room temperature. Next, samples should be cut into smaller pieces or pulverized to powder to be sure a representative and homogenous sample aliquot is obtained for analysis. Pulverisation allows the achievement of small particle sizes and a higher surface area. Samples are then accurately weighed. Substances trapped into the matrix need to be extracted from the matrix by either direct extraction/solubilisation or digestion. Further clean-up, usually through liquid-liquid extraction (LLE) or solid-phase extraction (SPE), is necessary to eliminate interferences and to concentrate the compounds of interest. Most of the analytical methods for hair and nail analysis are based on chromatographic separation (i.e. gas chromatography (GC) or liquid chromatography (LC)) coupled to mass spectrometric (MS) detection to achieve sufficient specificity and sensitivity.

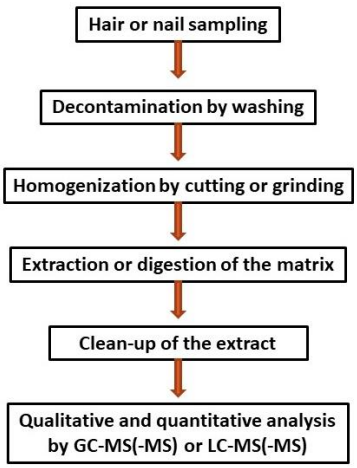

Figure 1.8: Pre-analytical an analytical steps in hair and nail analysis.

1.4. Advantages of keratinized matrices

Keratinized matrices, such as hair and nails, have important advantages compared to the standard matrices blood and urine (Table 1.4). Since compounds stably accumulate in hair and nails, these matrices have a longer window of detection (months to years, Figure 1.9) from which information on xenobiotic exposure or ingestion can be retrieved (Palmeri et al. 2000, Daniel et al. 2004, de la Torre et al. 2004). Sample collection is non-invasive, easy to perform, does not require medically trained personnel, and can be achieved under close supervision to prevent adulteration. Additionally, hair and nails can be transported and stored at room temperature. Furthermore, the detection profile in keratinized matrices is similar to blood, which means that a relatively high concentration of parent compounds is present compared to the concentration of metabolites, while urine has an opposite detection profile. The predominant presence of parent compounds facilitates the interpretation of the results. The presence of metabolites and the ratio between parent compound and metabolites are important to discriminate between actual consumption and external contamination. Finally, segmental analysis of hair can provide temporal information about the exposure to or consumption of xenobiotics. A commonly used segment length is the 3 cm proximal portion from the vertex posterior representing hair grown during the last 3 months (Cooper et al. 2012). However, depending on the situation, different strategies of segmentation may apply. Several short segments can provide a more detailed historical profile of an individual's xenobiotic exposure than a single segment. This can be relevant in cases of drug-facilitated sexual assault where a single administration of a central nervous system depressant drug (e.g. GHB) at the time of the assault is suspected (Pragst et al. 2006). The accuracy of segmental analysis depends on both the sampling and the segmentation procedure. Poor sampling procedures with shifted hair strands may severely lower the segmental resolution (Cooper et al. 2012, Kintz et al. 2015). Sampling of nails can be performed in two ways: vertical segmentation achieved by nail clipping, or horizontal segmentation by nail scraping. Scraping off the upper nail layer can be interesting to reduce external contamination, and thus increase the reliability of the obtained results.

Table 1.4: Comparison of the advantages and disadvantages between blood, urine, hair and nails (Kintz 2007).

Parameter	Blood	Urine	Hair and nails
Sampling	Invasive	Non-invasive	Non-invasive
Analysis of	Parent compounds + metabolites	(Parent compounds +) metabolites	Parent compounds + metabolites
Analytical techniques	Immunoassays, GC-(MS/)MS, LC-(MS/)MS		GC-(MS/)MS, LC-(MS/)MS
Detection window	Hours - days		Months – years (depending on the length)
Falsification	Difficult	Possible	Difficult
Type of measurement	Incremental		Cumulative

Abbreviations: GC-(MS/)MS, gas chromatography (tandem) mass spectrometry; LC-(MS/)MS, liquid chromatography (tandem) mass spectrometry

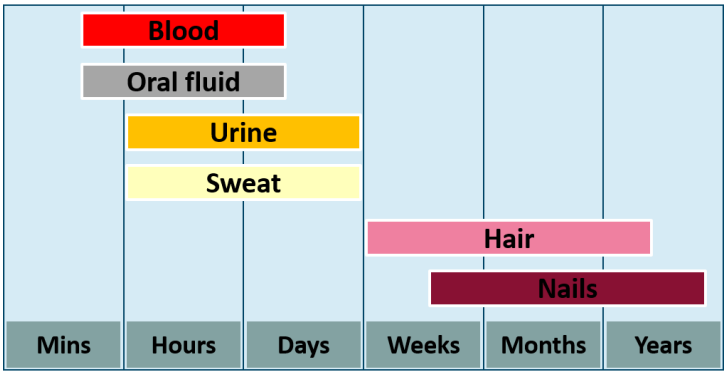

Figure 1.9: Comparison of the windows of detection in blood, oral fluid, urine, sweat, hair and nails.

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CHAPTER 2:

OBJECTIVES AND OUTLINE

CHAPTER 2

Forensic toxicology is used to aid medical and legal investigation of drug use, poisoning and death. Different types of matrices can be analysed to identify and quantify the substances present in a person under investigation. At the moment, blood and urine are the preferred matrices for analysis thanks to the widely established and validated analytical procedures and the available expertise in interpreting the measured concentrations. However, the use of blood and urine in forensic toxicology has also some disadvantages: the collection of blood is invasive and requires medically trained personnel, urine samples can be adulterated, and the presence of substances of interest (alcohol, drugs of abuse, pharmaceuticals, poisons, …) can only be detected in blood and urine in the subsequent hours to days after intake. This small window of detection is a major drawback when information regarding long-term use (i.e., weeks to months) is required, or when the substances of interest are only detectable for a very short period of time in blood and urine (e.g., alcohol, gamma-hydroxybutyric acid (GHB)). In several circumstances, such as drug-facilitated crime, post-mortem toxicology, driver's license regranting procedures, liver and kidney transplantation processes, and *in utero* exposure, detection of alcohol, drugs and pharmaceuticals over longer time periods is crucial.

To obtain conclusive information in these situations, it is imperative to explore alternative matrices that have a longer detection window (weeks to months). As such, two matrices of interest deserve further investigation: hair and nails. These keratinized matrices stably accumulate ingested substances and their metabolites over longer periods of time and can thus expand the detection window to months or even years. As a result, the analysis of hair and nails allows for a retrospective investigation of previous drug consumption after their elimination from the body. Combined analysis of classical and keratinized matrices gives complementary information on substance use over an extended timeframe. Nevertheless, keratinized matrices can also be used in the standalone analysis, when collection relates to an event that occurred several weeks or months earlier, or when retrospective information over a large period of time is required. Other advantages include the easy, non-invasive collection of samples which does not require qualified medical staff and can be performed under close supervision to prevent adulteration, as well as the transport and storage at room temperature.

The main objective of this PhD is to investigate the use of hair and nails as matrices for the long-term follow-up of alcohol and drug consumption, thereby expanding the detection window compared to the traditional matrices blood and urine. This main objective is subdivided into two aims. The first aim is to develop and to validate bioanalytical assays to measure biomarkers of alcohol and drugs of abuse consumption in hair and nails (**Part I**), while the second aim is to investigate the correlation between reported consumption of these substances and the presence of their biomarkers in hair and nails, on the one hand, and to evaluate the correlation between concentrations measured in both matrices, on the other hand (**Part II**).

Due to the complexity of keratinized matrices and the low concentrations of compounds present, an optimized sample preparation (i.e., decontamination, pulverisation, extraction and clean-up), and a sensitive and specific detection method are necessary (**Part I**). **Chapter 3** focuses on the development and validation of bioanalytical methods to measure alcohol biomarkers in hair and nails. Two direct alcohol biomarkers are investigated: ethyl glucuronide (EtG) and ethyl sulphate (EtS). In contrast to indirect biomarkers, EtG and EtS are exclusively formed upon the consumption of alcohol, and are therefore specific for alcohol consumption. EtG is known to accumulate in keratinized matrices, and is currently used as long-term marker for the detection of alcohol consumption. For EtG the objectives were to develop more sensitive and specific methods for its quantification in hair and nails, and to apply these methods to hair and nail samples from different types of alcohol consumers. Additionally, in nails, the influence of several sample preparation steps (including decontamination and pulverisation) on EtG concentrations is evaluated. EtS has been analysed in blood and urine but has never been reported in hair so far. Thus, Chapter 3 investigates whether EtS can be detected in hair and presents the development and validation of a method for the quantification of EtS in hair. The purpose of **Chapter 4** is to develop and validate a method for the simultaneous detection of nine drugs of abuse and their metabolites in hair and nails. The focus lies on a simple and straightforward sample preparation to reduce costs, and to allow application in routine laboratory practice.

In **Part II**, the developed methods are used to acquire more knowledge on different types of relationships. Correlations between drinking behaviour and EtG concentrations in hair have been reported but a large variability exists, and few information is available on the correlation

between low/moderate alcohol consumption and EtG concentrations in hair. The aim of **Chapter 5** is to gain more knowledge on the correlation between drinking behaviour and EtG concentrations in hair. First, a prospective controlled alcohol-dosing study in healthy volunteers consuming either no alcohol, either low/moderate amounts of alcohol is performed. Next, retrospective studies in non-excessive alcohol consumers and alcohol-dependent patients are conducted. In addition, the influence of gender on the correlation between alcohol consumption and EtG concentrations measured in hair is evaluated in both populations. The main purpose of **Chapter 6** is to assess the differences between hair and nails for the accumulation of compounds, and to evaluate the possibilities of nails as an alternative to hair for the long-term monitoring of alcohol and drug consumption. To this end, this chapter focuses on the differences and correlations between hair and nails (finger- and toenails). First, the correlation between hair and nails for EtG as a marker for alcohol use is investigated. Next, the correlation between drugs of abuse concentrations in hair and nails is evaluated.

The PhD thesis concludes with a critical discussion of the obtained results and recommendations for further research (**Chapter 7**).

PART I:

METHOD DEVELOPMENT AND VALIDATION FOR BIOMARKERS OF ALCOHOL AND DRUGS IN HAIR AND NAILS

CHAPTER 3:

BIOMARKERS OF ALCOHOL IN HAIR AND NAILS

Based on the following papers:

Cappelle D, Neels H, Yegles M, Paulus J, van Nuijs ALN, Covaci A and Crunelle CL. Gas chromatographic determination of ethyl glucuronide in hair: Comparison between tandem mass spectrometry and single quadrupole mass spectrometry. *Forensic Science International* 249 (2015) 20-24.

Cappelle D, Neels H, Yegles M, Fransen E, Dueffels K, Bremenfeld S, Maudens KE, van Nuijs ALN, Covaci A and Crunelle CL. Ethyl glucuronide in nails: method validation, influence of decontamination and pulverization, and particle size evaluation. *Forensic Toxicology* 34(1) (2016) 158-165.

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3.1. Ethyl glucuronide in hair and nails

3.1.1. Introduction

The harmful use of alcohol is a worldwide problem and causes a large disease, social and economic burden in societies. Alcohol abuse is directly and indirectly responsible for more than 40 diseases most notably alcohol use disorders, liver cirrhosis, cancers and injuries. Overall, 5.1% of global mortality and 4.1% of disability-adjusted life years (DALYs) lost are directly attributable to alcohol (World Health Organization 2011, Rehm et al. 2017). Moreover, both heavy drinking and alcohol use disorders are associated with a range of negative socio-economic effects, e.g. diminished work productivity, traffic accidents, and criminality (World Health Organization 2014).

There is a growing need for objective, specific and sensitive long-term alcohol markers for the detection and monitoring of harmful and/or chronic alcohol consumption. Clinical and forensic applications for these alcohol markers are widespread: from the assessment of excessive alcohol use (e.g. in treatment settings and in forensic cases) to the monitoring of alcohol abstinence, (e.g. during pregnancy, in child custody cases and in liver transplant procedures due to alcoholic liver failure).

Screening for alcohol abuse through questionnaires can be problematic due to participant's denial or recall bias. Indirect alcohol biomarkers, such as aspartate amino transferase (ASAT), alanine amino transferase (ALAT), gamma-glutamyl transferase (GGT) and mean corpuscular volume (MCV) are mainly correlated to liver damage, and are influenced by age, gender and somatic pathologies (Conigrave et al. 2003, Hock et al. 2005). As carbohydrate-deficient transferrine (CDT) has a short half-life (8-12 days), its use to detect chronic alcohol consumption is limited. Direct alcohol markers, which are formed only after alcohol consumption, have the advantage of reflecting direct alcohol presence without the biases that influence indirect measures (Crunelle et al. 2014b).

After alcohol consumption, ethanol is readily absorbed from the stomach and the small intestine into the blood stream. The blood alcohol concentration (BAC) depends on the amount and type (beer, wine, spirit) of alcohol consumed but is also influenced by age, body weight and gender (Zakhari 2006, Cederbaum 2012). Only a small percentage (2 - 5%) of the

ingested ethanol is excreted unchanged in the urine, breath and sweat (Figure 3.1). About 90 - 98% of the consumed ethanol undergoes biotransformation via oxidative metabolism (Phase I) in the liver where ethanol is transformed into acetic acid via acetaldehyde. The enzymes involved are alcohol dehydrogenase (ADH), aldehyde dehydrogenase (ALDH), cytochrome P450 2E1 (CYP2E1) and catalase (Figure 3.1) (Zakhari 2006, Jones 2010). A limited proportion (about 1%) of the ingested ethanol is eliminated by non-oxidative metabolism (Phase II) and results in the formation of ethyl glucuronide (EtG), ethyl sulphate (EtS), phosphatidylethanol (PEth) and fatty acid ethyl esters (FAEE) (Figure 3.1) (Jones 2010, Maenhout et al. 2013).

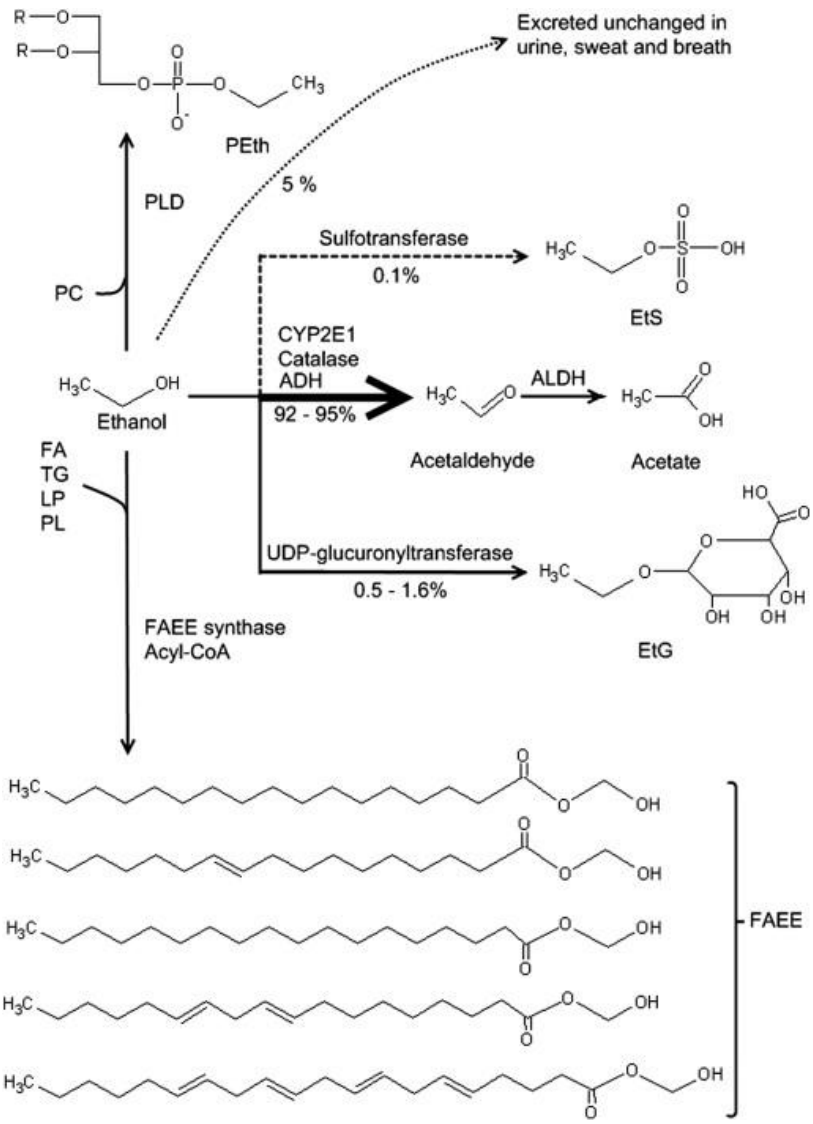

Figure 3.1: Alcohol metabolism and formation of non-oxidative ethanol metabolites (Maenhout et al. 2013).

Abbreviations: ADH, alcohol dehydrogenase; ALDH, acetaldehyde dehydrogenase; CYP2E1, cytochrome P 450 2E1; FA, fatty acids; LP, lipoproteins; PC, phosphatidylcholine; PL, phospholipids; PLD, phospholipase D; TG, triglycerides; UDP-glucuronosyltransferase, uridine 5'-diphospho–glucuronosyltransferase

EtG is formed through a conjugation reaction catalysed by uridine 5'-diphospho–glucuronosyltransferase (UDP-glucuronosyltransferase) (Figure 3.1) (Schmitt et al. 1995, Seidl et al. 2001, Maenhout et al. 2013). It accumulates in keratinous matrices, where it remains

detectable for several months (Crunelle et al. 2014b, Cappelle et al. 2015b). EtG has proved to be a specific and sensitive marker for alcohol consumption (Wurst et al. 2003).

The aim of the first part of this section was to develop a sensitive method for EtG in hair using gas chromatography coupled to tandem mass spectrometry (GC-MS/MS) operated in the negative ion chemical ionization (NICI) mode. Sensitive methods are required to differentiate teetotallers from moderate drinkers according to the established cut-off (i.e., 7 pg/mg hair). Moreover, the validated method was applied to hair samples from teetotallers, moderate and excessive alcohol consumers, and results were compared to a previously validated GC-NICI-MS method.

In the second part, an analytical method for the determination of EtG in nails using GC-NICI-MS/MS is developed and validated. Furthermore, the influence of sample preparation on EtG concentrations is evaluated.

3.1.2. Ethyl glucuronide in hair: method validation, and comparison between single and triple quadrupole mass spectrometry

3.1.2.1. Preface and aims

The biotransformation of ethanol to EtG represents approximately 1 % of the total alcohol elimination (Maenhout et al. 2013). Consequently, only small amounts of EtG accumulate in hair, providing EtG concentrations in the lower pg/mg range: > 30 pg/mg hair in alcohol-dependent individuals (Crunelle et al. 2014a, Crunelle et al. 2014b), between 7 and 30 pg/mg hair for moderate alcohol consumers, and < 7 pg/mg hair for teetotallers (Society of Hair Testing 2016). Sensitive analytical methods are thus required for the reliable determination of such low EtG concentrations. The current methods offer lower limits of quantification (LLOQs) varying between 2 and 5000 pg/mg, depending on the method applied, with LLOQs generally higher (> 10 pg/mg hair) for liquid chromatography (LC) methods compared to gas chromatography (GC) methods (< 10 pg/mg hair; for a review see Crunelle et al., 2014). The use of negative ion chemical ionization (NICI) instead of electron impact (EI) improves the sensitivity of GC-MS methods, with LLOQs of 5000 (Skopp et al. 2000) and 300 (Álvarez et al. 2009) pg/mg hair for EI compared to LLOQs of 6 (Yegles et al. 2004) and 2.3 (Kerekes et al. 2009) pg/mg hair for NICI. Tandem mass spectrometry (MS/MS) minimizes background interferences and allows multiple reaction-monitoring (MRM) and should thus result in higher sensitivity and selectivity. Indeed, GC-MS/MS methods operated in EI mode showed an improved sensitivity (Shi et al. 2010, Paul et al. 2011), but this increase could not be observed between GC-NICI-MS and GC-NICI-MS/MS methods (Kharbouche et al. 2009, Agius et al. 2010).

This part describes the validation of a GC-NICI-MS/MS method for the determination of EtG in hair after heptafluorobutyric anhydride (HFBA) derivatisation. By analysing hair samples from teetotallers, moderate, and excessive alcohol consumers, the applicability of the method should be demonstrated. In addition, the GC-NICI-MS/MS method was compared to a GC-NICI-MS method, in order to assess the possible advantages of MS/MS and MRM in the determination of EtG in hair from teetotallers, moderate and excessive alcohol consumers.

3.1.2.2. Materials and methods

3.1.2.2.1. Reagents and materials

EtG and the internal standard ethyl glucuronide-D_5 (EtG-D_5) were purchased from Medichem (Stuttgart, Germany). HFBA was obtained from Sigma Aldrich (Bornem, Belgium). Methanol, ammonium hydroxide solution (25%), ethyl acetate, formic acid (98% - 100%), and acetone were supplied by Biosolve (Valkenswaard, the Netherlands). All chemical and reagents were of analytical purity grade.

Oasis® MAX (60 mg, 3 mL) extraction cartridges were acquired from Waters (New Bedford, MA, USA). A ball mill of type MM2 (Retsch, Haan, Germany) was used for the pulverisation of the hair samples. Extraction was performed with an ELMA TI-H-15 ultrasonication bath (Elma Hans Schmidbauer GmbH & Co. KG, Singen, Germany) and centrifugation with a Sigma centrifuge (Osterode am Harz, Germany). A Supelco Visiprep™ SPE Vacuum Manifold (Bellefonte, CA, USA) with 24 ports was employed to load the hair samples and to dry the cartridges. Solvent evaporation was achieved with a Pierce Reacti-Therm III Heating Module (Rockford, IL, USA).

3.1.2.2.2. Standard solutions

Stock solutions of EtG (1 mg/mL) and EtG-D_5 (0.2 mg/mL) were prepared in methanol. The working solutions were prepared in methanol by further dilution of the stock solutions. All solutions were stored at -20 °C.

3.1.2.2.3. Samples

Hair samples together with self-reported data about alcohol consumption were obtained from 58 volunteers: teetotallers (no alcohol consumption; n = 2), moderate alcohol consumers (< 60 g alcohol/day; n = 20), and excessive alcohol consumers (> 60 g alcohol/day; n = 36). Twenty-six volunteers were female and 32 volunteers were male, aged 21 to 58 with a mean age of 42 ± 9.7 years. The study was approved by the Ethical Committee of the University Hospital of Antwerp (Belgian registration number B300201215554–12/43/333).

Samples were collected from the vertex posterior of the head, and the first 3 cm segment from the proximal end was used for further analysis.

3.1.2.2.4. Sample preparation

Hair samples were processed according to a previously described method (Kerekes et al. 2013). Briefly, the samples were washed with water and acetone, dried, cut into 1 - 2 mm pieces, and pulverized in a ball mill. Then, 2 mL of water and 2 ng of EtG-D_5 as internal standard were added to an accurately weighed portion of approximately 30 mg of the pulverized samples. Next, the samples were ultrasonicated for 1.5 h, vortexed, and centrifuged at 5000 rpm for 10 min. Solid-phase extraction was then performed by transferring the supernatant to the cartridges, previously conditioned with 2 mL of methanol and 2 mL of water. The columns were washed with 1 mL of water/ammonia (5%) solution and 2 mL of methanol, and subsequently dried for 5 min. Elution was performed using 2 mL of 2% formic acid in methanol and the eluate was evaporated to dryness under a nitrogen gas stream at 37 °C. The residues were derivatised with HFBA (30 min, 60 °C) (Figure 3.2), dried under a nitrogen gas stream, and then reconstituted in 50 µL of ethyl acetate. Finally, the extracts were transferred to vials and 1 µl was injected twice into the GC-MS/MS system (for GC-NICI-MS analysis and for GC-NICI-MS/MS analysis).

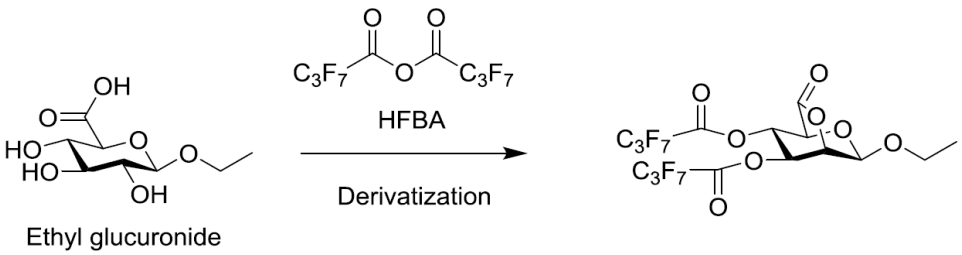

Figure 3.2: Derivatisation of ethyl glucuronide with heptafluorobutyric anhydride (HFBA) (Lin et al. 2016).

3.1.2.2.5. GC-MS/MS instrumentation and conditions

Both GC-NICI-MS and GC-NICI-MS/MS analyses were carried out on a GC-MS/MS system consisting of a 7890A gas chromatograph, equipped with an automatic injector AS 7693 and coupled to a 7000C triple quadrupole mass spectrometer (Agilent Technologies, Waldbronn, Germany). Chromatographic separation was achieved on a HP-5 MS (5% phenyl methyl siloxane) column (length 30 m × internal diameter 0.25 mm × film thickness 0.25 µm).

The injector temperature was set at 250 °C. The carrier gas was helium with a flow rate of 1 mL/min. The oven was initially held at 100 °C for 2 min, heated to 170 °C at a rate of 10 °C/min, and then programmed at a final temperature of 300 °C at 40 °C/min. The detector was operated in the negative ion chemical ionization (NICI) mode and the detector temperature was 280 °C. The GC-NICI-MS analyses were done as published elsewhere (Kerekes et al. 2013). For the GC-NICI-MS/MS analyses data acquisition was performed in the multiple reaction-monitoring (MRM) mode. The monitored ion transitions were *m/z* 596 → 213 (quantifier) and 397 → 213 (qualifier) for EtG, and *m/z* 601 → 213 for EtG-D_5.

3.1.2.2.6. Validation

Validation of the analytical method was based on the international guidelines of the European Medicines Agency (EMA) and the Food and Drug Administration (FDA) (Food and Drug Administration 2001, European Medicines Agency 2011). The following quality criteria were taken into account: recovery, linearity, accuracy, precision and sensitivity limits (LLOQ, limit of detection (LOD)).

The extraction recovery was calculated as the percentage response in extracted samples relative to samples where the standard was added after extraction. A calibration curve, consisting of blank hair spiked at 7 different concentrations (2, 4, 10, 50, 100, 200, 400 pg/mg), was constructed to evaluate linearity. Accuracy and precision were assessed for replicated quality control (QC) samples at a concentration of 25 pg/mg. Intra-day precision and accuracy were assessed by analysing 10 QC samples in the same analytical run. Inter-day precision and accuracy were calculated on QC samples analysed in 6 different runs, on separate days. Acceptance criteria were 1) a bias less than 15% for accuracy, and 2) a relative

standard deviation (RSD) lower than 15% for precision (Food and Drug Administration 2001, European Medicines Agency 2011).

The LLOQ was defined as the lowest concentration producing peaks with a signal to noise ratio of at least 10, and with an accuracy and precision within ± 20% of the reference concentration. The LOD was calculated as the lowest concentration producing peaks with a signal to noise ratio of at least 3 (Food and Drug Administration 2001, European Medicines Agency 2011).

3.1.2.2.7. Data analysis

Statistical analysis was performed using IBM® SPSS® Statistics version 22. The normality of the data was evaluated, followed by a parametric paired-sample t-test on the normally distributed data to assess the differences in concentrations obtained with GC-NICI-MS and GC-NICI-MS/MS. Regression analysis was performed to evaluate the correlation between both methods. A p-value < 0.05 was considered as statistically significant.

3.1.2.3. Results and discussion

3.1.2.3.1. Method validation

Mean overall extraction recovery was 98.8% (n = 3). The calibration curve (7-point model) was linear in the range from 2 to 400 pg/mg hair. As presented in Table 3.1, intra- and inter-day accuracy and precision for replicated QC samples at 25 pg/mg were within the acceptance criteria of 15%.

Table 3.1: Accuracy and precision measured at a concentration of 25 pg/mg.

	Accuracy (% bias)	Precision (% RSD)
Intra-day (n=10)	1.7	3.3
Inter-day (n=6)	3.6	5.5

The LOD and LLOQ obtained for the GC-NICI-MS/MS method were 0.05 pg/mg and 0.2 pg/mg hair, respectively. Thus, when compared to GC-NICI-MS (LOD = 0.5 pg/mg; LLOQ = 1.5 pg/mg),

the use of GC-NICI-MS/MS enables the achievement of a lower LOD and LLOQ (Kerekes et al. 2013). Furthermore, the LOD and LLOQ obtained with the presented GC-NICI-MS/MS method are lower than those reported by previously published GC-NICI-MS/MS methods with LLOQs of 8.4 pg/mg (Kharbouche et al. 2009) and 2.8 pg/mg (Agius et al. 2010). As a consequence, and in contrast to previous publications (Kharbouche et al. 2009, Agius et al. 2010), the current method demonstrates the higher sensitivity of MS/MS.

3.1.2.3.2. Application to real hair samples

Hair samples, collected from teetotallers (n = 2), moderate (n = 20) and excessive (n = 36) alcohol consumers, were analysed with the developed GC-NICI-MS/MS method and the results were compared with those obtained using the GC-NICI-MS method published by Kerekes et al. (Kerekes et al. 2013) (Table 3.2).

Table 3.2: Hair ethyl glucuronide concentrations for different drinking behaviours obtained with GC-NICI-MS and GC-NICI-MS/MS.

Drinking behaviour	**Concentration obtained by GC-NICI-MS (pg/mg hair)**		**Concentration obtained by GC-NICI-MS/MS (pg/mg hair)**	
	Mean ± SD	Range	Mean ± SD	Range
Teetotallers (n=2)	0.9 ± 0.4	0.6 - 1.1	0.8 ± 0.2	0.7 - 1.0
Moderate drinkers (n=20)	5.4 ± 4.4	1.2 - 19.6	5.4 ± 4.4	1.0 - 19.4
Excessive drinkers (n=36)	193.0 ± 159.0	31.2 - 667.0	192.0 ± 160.0	30.7 - 674.0

Hair EtG concentrations obtained by GC-NICI-MS and GC-NICI-MS/MS did not differ significantly from each other (paired-sample t-test, $p > 0.05$, see Table 3.3). Calculation of the coefficient of variation (CV) of the obtained EtG concentrations for each sample showed that the results were similar for both methods (mean CV = 1.0%). The concentrations of EtG obtained by GC-NICI-MS/MS were highly correlated to those obtained by GC-NICI-MS ($R^2 = 0.999$, $p < 0.01$, see Figure 3.3). Thus, using one method instead of the other would not change the interpretation of the EtG concentrations regarding the differentiation between teetotallers, moderate and excessive alcohol consumers.

Table 3.3: Overview of the ethyl glucuronide (EtG) concentrations in hair for the different drinking behaviours obtained with GC-NICI-MS and GC-NICI-MS/MS.

Nr.	Drinking behaviour	EtG concentration (pg/mg hair)	
		GC-NICI-MS	GC-NICI-MS/MS
1	Teetotaller	1.11	0.96
2		0.61	0.68
3	Moderate drinker	3.99	3.64
4		1.59	1.43
5		4.97	4.98
6		2.90	3.10
7		2.88	2.35
8		3.03	2.96
9		2.20	2.21
10		4.32	4.37
11		1.23	1.03
12		9.14	9.55
13		1.50	1.64
14		8.71	8.60
15		4.44	4.32
16		12.6	12.6
17		2.82	2.97
18		5.74	5.54
19		6.57	6.12
20		3.49	3.59
21		7.03	7.27
22		19.6	19.4
23	Excessive drinker	115	118
24		31.2	32.3
25		34.7	34.8
26		63.7	62.8
27		99.2	99.8
28		77.6	79.5
29		368	368
30		202	207
31		155	162

Table 3.3: Continued.

Nr.	Drinking behaviour	EtG concentration (pg/mg hair)	
		GC-NICI-MS	GC-NICI-MS/MS
32	Excessive drinker	549	552
33		72.1	74.4
34		208	207
35		70.5	70.6
36		176	174
37		110	106
38		87.9	87.0
39		662	674
40		63.3	61.8
41		182	184
42		321	320
43		76.7	78.6
44		462	468
45		180	175
46		39.4	36.1
47		306	303
48		31.7	30.7
49		157	150
50		497	481
51		233	219
52		95.0	91.2
53		289	296
54		94.2	91.5
55		175	168
56		92.8	91.8
57		404	402
58		161	159

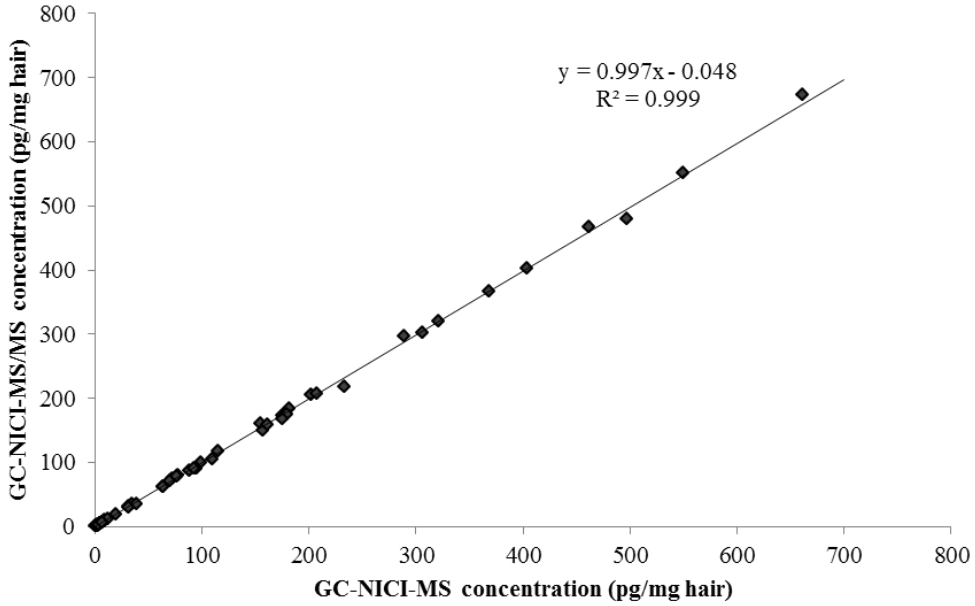

Figure 3.3: Comparison of hair ethyl glucuronide concentrations of teetotallers, moderate and excessive alcohol consumers obtained by GC-NICI-MS and GC-NICI-MS/MS.

The differences between both methods were larger for EtG concentrations < 30 pg/mg hair (mean CV = 1.7%; see Figure 3.4a) than for EtG concentrations > 30 pg/mg hair (mean CV = 0.7%; see Figure 3.4b). In contrast to GC-NICI-MS, using GC-NICI-MS/MS no decrease of sensitivity was observed at lower concentrations. These findings suggest a better selectivity of GC-NICI-MS/MS at lower concentrations. However, the results should be interpreted with caution as these CVs may fall within the analytical uncertainty of the method.

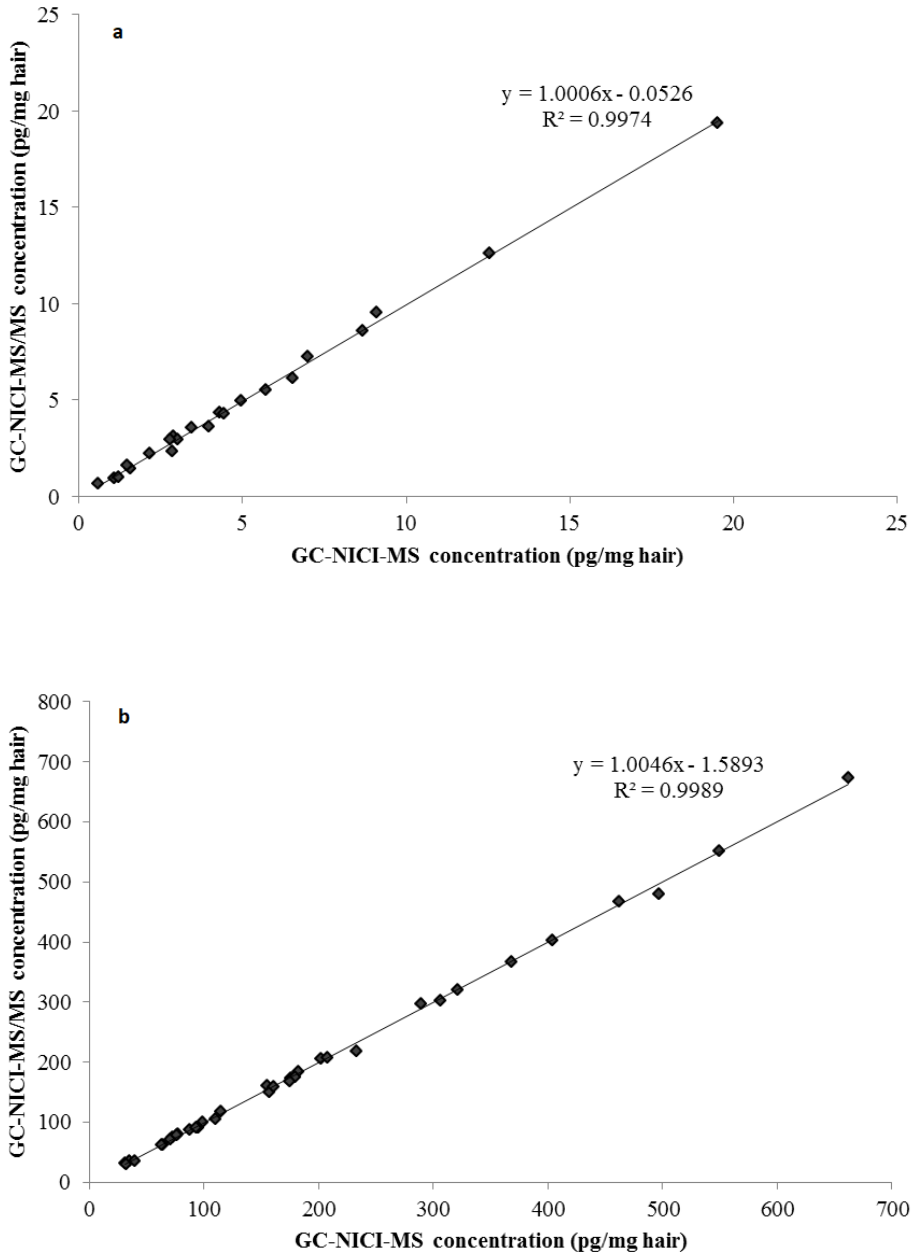

Figure 3.4: Comparison of hair ethyl glucuronide (EtG) concentrations obtained by GC-NICI-MS and GC-NICI-MS/MS. (a) Hair EtG concentrations of teetotallers and moderate alcohol consumers (concentrations < 30 pg/mg hair). (b) Hair EtG concentrations of excessive alcohol consumers (concentrations > 30 pg/mg hair).

Furthermore, a low background noise was observed using GC-NICI-MS/MS through the use of specific MRM transitions (see Figure 3.5).

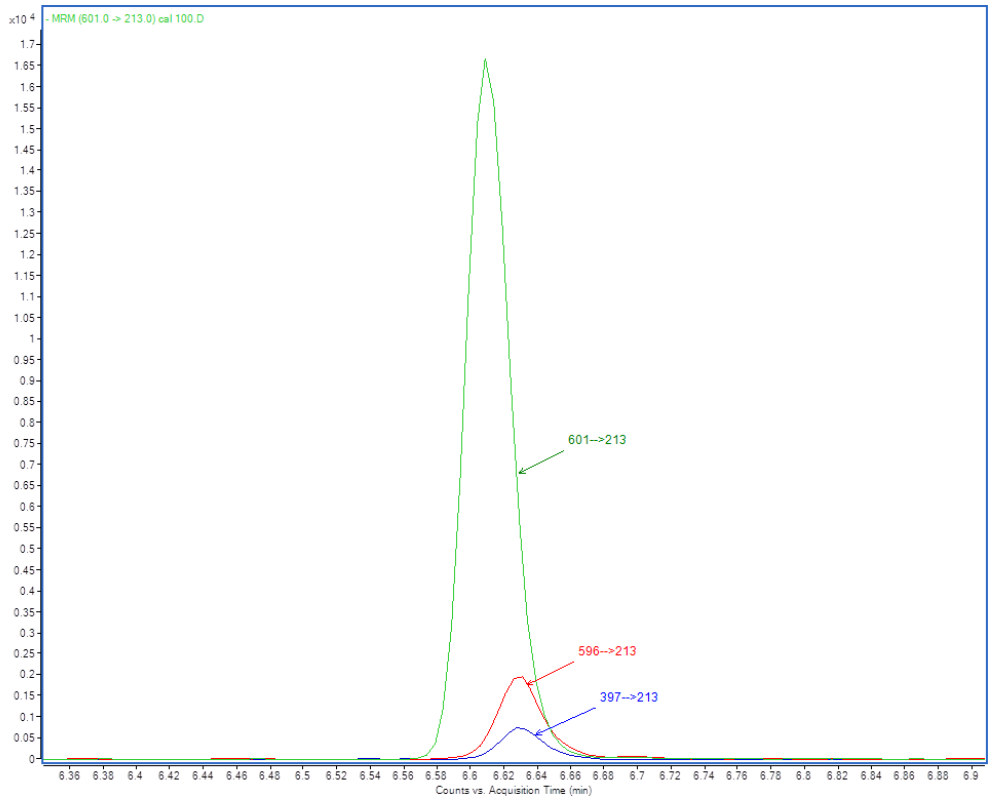

Figure 3.5: Chromatogram obtained by GC-NICI-MS/MS at an ethyl glucuronide (EtG) concentration of 4 pg/mg hair (*m/z* 596 → 213 (quantifier); 397 → 213 (qualifier)) and an EtG-D_5 concentration of 20 pg/mg hair (*m/z* 601 → 213).

This higher selectivity of GC-NICI-MS/MS at lower concentrations in combination with its high sensitivity, underlines the usefulness of GC-NICI-MS/MS instead of GC-NICI-MS for the differentiation between teetotallers and moderate alcohol consumers.

3.1.2.4. Conclusions

A GC-NICI-MS/MS method for the determination of EtG in hair was validated, applied to hair samples, and compared to a previously published GC-NICI-MS method. Using the presented GC-NICI-MS/MS method, a better analytical selectivity and an improved signal to noise ratio were achieved, compared to earlier published methods. Therefore, this GC-NICI-MS/MS method provides sensitive and reliable results, and should preferentially be used for the determination of EtG in hair, especially when differentiating between teetotallers and moderate alcohol consumers according to the current cut-off (i.e., 7 pg/mg hair).

3.1.3. Ethyl glucuronide in nails: method validation, influence of decontamination and pulverisation, and particle size evaluation

3.1.3.1. Preface and aims

So far, research has primarily focused on the detection of EtG in hair (Crunelle et al. 2014b), whereas studies on its measurement in nails are sparse (Jones 2012, Morini et al. 2012, Keten et al. 2013, Morini et al. 2013, Berger et al. 2014). However, nail analysis offers advantages compared to hair analysis. First, nails grow slower than hair (3 mm/month for fingernails and 1 mm/month for toenails compared to 1 cm/month for head hair (Barbosa et al. 2013)), and would thus allow a larger accumulation of EtG. This can be relevant in situations where low EtG concentrations need to be determined, e.g., for the differentiation between teetotallers and moderate alcohol consumers. Second, nails provide an alternative in cases where hair is not (sufficiently) available (e.g., alopecia, newborns). Third, cosmetic hair treatments have been proven to reduce EtG content in hair (Morini et al. 2010), which would be avoided through nail analysis.

Currently available methods for the detection of EtG in nails include liquid chromatography (LC) coupled to tandem mass spectrometry (MS/MS) operated in electrospray ionization (ESI) mode (Jones 2012, Morini et al. 2012, Keten et al. 2013, Morini et al. 2013, Berger et al. 2014). While no gas chromatography coupled to mass spectrometry (GC-MS) methods are available for EtG in nails, derivatisation followed by a GC-MS determination may improve the analytical sensitivity as has been demonstrated for hair (Crunelle et al. 2014b).

In addition to the analytical method, the sample preparation of complex keratinous matrices is crucial. It includes decontamination, homogenization, extraction, and clean-up steps. In a study on cocaine and opioid determination, over 98% of external contamination could be removed with two methanol washes (Engelhart et al. 1998). Lemos and colleagues developed a valuable decontamination procedure consisting of subsequent washes with 0.1% sodium dodecyl sulphate, water, and methanol. The efficiency of this procedure was confirmed by negative final methanol washes and it was applied for the detection of cocaine, opioids, and cannabinoids (Lemos et al. 1999, Lemos et al. 2000a, Lemos et al. 2000b). Decontamination by washing with water and methanol was proved sufficient to remove external contamination prior to amphetamines and cannabinoids analysis (Kim et al. 2008). With

regard to the analysis of EtG in nails, two different procedures for decontamination have been described including, dichloromethane and methanol (Morini et al. 2012, Morini et al. 2013) or dichloromethane, methanol and hexane (Jones 2012, Berger et al. 2014) as solvents. In neither EtG methods, the efficiency of the decontamination was evaluated. Homogenization of the sample allows optimal extraction of the compound of interest from the matrix. In hair, mechanical pulverisation increased the extraction efficiency compared to scissor cut samples (Albermann et al. 2012, Mönch et al. 2013). Studies in nails have shown that cryogenic pulverisation leads to a higher surface area and smaller particles compared to cut samples (Takaichi 2004, Shen et al. 2014). For homogenization prior to EtG analysis in nails, studies employ either no homogenization procedure (Morini et al. 2012, Morini et al. 2013), or a mechanical pulverisation of the nails (Jones 2012, Keten et al. 2013, Berger et al. 2014), but without any comparison.

This part presents the development and validation of a specific and sensitive GC-MS/MS method for the determination of EtG in nails. Several decontamination procedures have been also evaluated, next to the influence of mechanical pulverisation on EtG extraction efficiency.

3.1.3.2. Materials and methods

3.1.3.2.1. Reagents and materials

Reagents and materials were the same as described in section 3.1.2.2.1. Additionally, particle size measurements were performed using a Camsizer XT (Retsch Technology GmbH, Haan, Germany) equipped with Camsizer XT software 6.5.1.

3.1.3.2.2. Standard solutions

Stock solutions of EtG (10 ng/µL) and EtG-D_5 (10 ng/µL) were prepared in methanol. The working solutions were prepared in methanol by further dilution of the stock solutions. All solutions were stored at -20 °C.

3.1.3.2.3. Samples

Nail samples were obtained by clipping of the distal edge of finger- and toenails. For method validation, blank nail samples were collected from teetotallers. For decontamination and homogenization experiments, nail clippings were obtained from five volunteers who reported regular alcohol consumption (at least once a week) over the past year without binge drinking events. Nails were collected every 2 weeks over several months, in order to obtain a large quantity of nails per subject (300 – 700 mg). Per subject, finger- and toenails were collected and stored separately, resulting in 5 sets of fingernails and 5 of toenails.

3.1.3.2.4. Sample preparation

The sample preparation steps prior to injection on the GC-MS/MS system are summarized in Table 3.4.

Table 3.4: Workflow of nail analysis. Enumeration of the different steps included in the sample preparation of nail analysis.

Sample preparation step
– H_2O wash for 1 min
– Acetone wash for 1 min
– Drying of the nails
– Pulverisation for 6 min at 30 Hz
– 30 mg of powdered nails + 2 mL of H_2O
– Spiking with 500 pg of EtG-D_5
– Ultrasonication for 2 h at room temperature
– Centrifugation at 5000 rpm for 10 min
– Conditioning of Oasis® MAX extraction cartridge with 2 mL of MeOH and 2 mL of H_2O
– Transfer of supernatant to Oasis® MAX extraction cartridge
– Washing with 1 mL of 5% NH_3 in H_2O and 2 mL of MeOH
– Vacuum drying for 5 min
– Elution with 2 mL of 2% FAc in MeOH
– Evaporation of the eluent to dryness with N_2 at 37 °C
– Derivatisation with 100 µL of HFBA at 60 °C for 30 min
– Evaporation to dryness with N_2 at 37 °C
– Reconstitution in 50 µL of ethyl acetate
– 2 µL injection in GC-MS/MS system

Abbreviations: EtG, ethyl glucuronide; FAc, formic acid; GC-MS/MS, gas chromatography tandem mass spectrometry; H_2O, water; HFBA, heptafluorobutyric anhydride; MeOH, methanol; N_2, nitrogen gas; NH_3, ammonia

3.1.3.2.5. GC-MS/MS instrumentation and conditions

The GC-MS/MS instrumentation and conditions were as described in section 3.1.2.2.5.

3.1.3.2.6. GC-MS/MS validation

Validation of the analytical method was performed based on the 'Guidelines on bioanalytical method validation' provided by the EMA with modifications (European Medicines Agency 2011). The following performance criteria were evaluated: linearity, calibration range, selectivity, accuracy, precision, LLOQ, and recovery.

Calibration curves were prepared by spiking blank matrix samples with EtG-D_5 and five different levels of EtG ranging from 2 to 100 pg/mg (2, 8, 20, 40, 100 pg/mg). In addition, zero blanks (processed matrix sample without EtG and without EtG-D_5), procedural blanks (processed matrix with EtG-D_5) and QC samples were included. Calibration curves were constructed by plotting the ratio between the peak area of EtG and EtG-D_5 against the spiked concentration, and were 1/x weighted. Selectivity was assessed through evaluation of blank samples for interfering components.

Accuracy and precision were evaluated by analysing replicated blank matrix samples spiked at concentration levels of 2 pg/mg (LLOQ samples) and 40 pg/mg (QC samples). Within-run accuracy and precision were determined by analysing four samples in a single analytical run. Between-run accuracy and precision were calculated from samples analysed in different runs on three separate days. Acceptance criteria were: 1) accuracy (bias) within 15% of the nominal value for QC samples and within 20% for LLOQ samples, and 2) precision (RSD) lower than 15% for QC samples and lower than 20% for LLOQ samples. The extraction recovery was calculated by comparison of responses from standards spiked before extraction to standards spiked after extraction.

3.1.3.2.7. Decontamination experiment

Three different procedures were tested on five sets of nails, either finger- or toenails, each in triplicate on 30 mg of sample. The first two procedures were retrieved from earlier

publications, and consisted of washing with dichloromethane and methanol (DCM-MeOH) (Morini et al. 2012, Morini et al. 2013) or washing with hexane, dichloromethane and methanol (Hex-DCM-MeOH) (Jones 2012, Berger et al. 2014). The third procedure was based on a method for EtG analysis in hair (Cappelle et al. 2015a) and included washing with water and acetone (H_2O-Ac). The decontamination protocol was similar between procedures and consisted of subsequent addition of 1 mL of washing solvent, vortex mixing for 30 s and solvent removal. For each procedure, the washing solvents were collected, combined, evaporated to dryness, derivatised with HFBA, and injected into the GC-MS/MS system.

3.1.3.2.8. Homogenization experiment

The homogenization experiment was performed on another 30 mg of nail samples (five sets of fingernails and five sets of toenails) previously washed with water and acetone. Per set of nails, five procedures were compared: cut nails without further pulverisation (T_p = 0 min), 3 min of pulverisation (T_p = 3 min), 6 min of pulverisation (T_p = 6 min), 9 min of pulverisation (T_p = 9 min), and 12 min of pulverisation (T_p = 12 min). Each procedure was performed in duplicate or triplicate, depending on the amount of sample remaining. After homogenization, samples were further analysed for EtG concentrations as presented in Table 3.4.

3.1.3.2.9. Particle size measurements

From the samples pulverized for 3 min, 6 min, 9 min, and 12 min, approximately 5 mg was collected for particle size measurements. As described in the standard ISO 13322-2 (The International Organization for Standardization 2006), particle size was assessed by dynamic digital image analysis and using wet dispersion. Briefly, a pulverized sample was added to the dispersion area with approximately 300 mL isopropanol as dispersion medium. The dispersion medium was degassed to remove possible air bubbles, and agglomerates were broken down by ultrasound. A centrifugal pump was used to keep the particles in suspension and prevent sedimentation during analysis. A gap width of 4 mm was set as spacing through which the flow was optically assessed and analysed, and a total number of 20000 image frames per sample were recorded. The areal density was set between 0.4 and 0.6%. Three

measurements were performed for each sample in order to assess repeatability. After each sample, the liquid was drained, and the area was rinsed three times and was subsequently refilled with medium for the next sample. From the obtained measurements, Camsizer XT software was used to assess the shape, calculate the mean and median diameter, and the range of the diameter of particles within each sample.

3.1.3.2.10. Data analysis

Statistical analysis was performed using R (version 3.1.2., The R Foundation for Statistical Computing). Normality was tested on raw data and log-transformed data. For all statistical tests, a p-value < 0.05 was considered statistically significant. Results are presented as mean ± standard error (SE). For the decontamination experiment, the EtG concentrations in the washing solutions were analysed using logistic regression, with the outcome variable being the presence (yes/no) of detectable EtG (i.e., > LLOQ). All other outcome variables (EtG concentrations in the nail samples, influence of pulverisation and origin of the nails (finger or toe) on EtG concentrations, and effect of pulverisation time on particle sizes) were analysed using linear mixed models, accounting for the dependence between observations within the same individual. Significance of the fixed effects was tested using an F-test with Kenward-Roger correction for multiple testing. A log-transformation was carried out if the outcome variable was not normally distributed. Post-hoc analysis was performed using a Tukey test for multiple testing.

3.1.3.3. Results

3.1.3.3.1. Method validation

As the response in blank samples was less than 20% of the LLOQ (2 pg/mg) for EtG and less than 5% for EtG-D_5, the absence of interfering substances was accepted. The calibration curve was linear (R^2 > 0.99) in the range from 2 to 100 pg/mg. The back-calculated concentrations of all the calibrators were within ± 15% of the nominal value.

The within- and between-run accuracy and precision results are presented in Table 3.5 and are within the acceptance criteria of 15% for the QC samples and 20% for the LLOQ samples. For QC samples (n = 12) within- and between-run accuracy (% bias) ranged between 0.8 and 1.4, and within- and between-run precision (% RSD) ranged between 3.1 and 6.7. For LLOQ samples (n = 12) within- and between-run accuracy (% bias) ranged between 6.5 and 10.5, and within- and between-run precision (% RSD) ranged between 3.3 and 8.2. Mean overall SPE recovery was 88.5 ± 1.8%.

Table 3.5: Results for accuracy and precision.

Ethyl glucuronide concentration	**Accuracy (% bias)**		**Precision (% RSD)**	
	Within-day	Between-day	Within-day	Between-day
2 pg/mg (n = 12)	10.5	6.5	3.3	8.2
40 pg/mg (n = 12)	0.8	1.4	3.1	6.7

3.1.3.3.2. Decontamination experiment

EtG concentrations in the washing solutions and the respective nail samples are presented in Table 3.6 as mean of the experiments in triplicate. The raw (untransformed) data showed a non-normal distribution. The data on EtG concentrations in the nails were normalized by a logarithmic transformation. However, the data on EtG concentrations in the washing solutions remained non-normal due to the presence of multiple samples < LLOQ. The outcome variable was therefore recoded into a binary outcome variable indicating whether or not the EtG concentrations reached the LLOQ, and analysed using logistic regression. The effect of decontamination procedure on the outcome was not significant ($p = 0.13$), which means that the probability to reach the LLOQ did not differ significantly between the three procedures. The data on EtG concentrations in the nails were analysed using a linear mixed model with the log-transformed EtG concentrations as outcome variable, and the decontamination procedure as fixed effect. The effect of the procedure on the EtG concentrations in nails was significant ($p = 0.01$). Post-hoc analysis showed that the Hex-DCM-MeOH procedure led to significantly higher EtG concentrations compared to the DCM-MeOH procedure ($p = 0.007$). Ratios between EtG concentrations in the nails compared to EtG

concentrations in the washing solutions were stable and ranged between 0.13 and 0.21 (mean 0.17 ± 0.03), with one exception for set 4 which had a higher nail/washing EtG ratio of 0.63.

Table 3.6: EtG concentrations in the washing solutions ("washes", in pg/mL) and in the corresponding nails ("nails", in pg/mg) from 3 different decontamination procedures. All data are presented as mean from the experiments performed in triplicate.

Decontamination		DCM-MeOH	Hex-DCM-MeOH	H_2O-Ac
#1	Washes	< LLOQ	< LLOQ	< LLOQ
	Nails	9	11	12
#2	Washes	< LLOQ	< LLOQ	52
	Nails	< LLOQ	12	6
#3	Washes	< LLOQ	< LLOQ	< LLOQ
	Nails	5	6	6
#4	Washes	28	< LLOQ	60
	Nails	15	13	11
#5	Washes	163	264	189
	Nails	26	39	21

Abbreviations: Ac, acetone; DCM, dichloromethane; EtG, ethyl glucuronide; H_2O, water; Hex, hexane; LLOQ, lower limit of quantification; MeOH, methanol

3.1.3.3.3. Homogenization experiments

The results of the homogenization experiment are presented in Table 3.7 as the mean of the experiments performed in duplicate or triplicate. The influence of pulverisation and origin of the nails (finger or toe) on EtG concentrations, was modelled using linear mixed models. Fixed effects included origin, pulverisation time and their interaction. The interaction between the origin of the nails and pulverisation time was not significant ($p = 0.14$). This means that the EtG concentrations between the five homogenization procedures are not significantly different for finger- and toenails. This non-significance is not surprising, due to the large variances between the individuals. Although one model could theoretically fit for both finger- and toenails, the effect of pulverisation time was studied separately. For toenails, the effect

of pulverisation time on EtG concentration was not significant (p = 0.29). In fingernails, the effect of pulverisation time on EtG concentration was significant (p = 0.045). Post-hoc testing with Tukey correction showed that the no pulverisation led to a lower EtG concentration compared to the four other times (i.e., 3 min, 6 min, 9 min and 12 min). The difference in EtG concentration of no pulverisation with 3 and 6 min was marginally significant (p = 0.07 and p = 0.06, respectively), and the difference with 9 and 12 min was significant (p = 0.03 and p = 0.02, respectively). No significant differences were observed between the other pulverisation times. EtG concentration versus the time of pulverisation is visualized in Figure 3.6. Compared to no pulverisation, EtG concentration increased with pulverisation. Although there is a lot of variance between subjects, in general pulverisation for longer than 3 min did not led to strong increases in EtG concentrations.

Table 3.7: Ethyl glucuronide concentrations (in pg/mg) in nail samples for 5 different homogenization procedures: no pulverisation (T_p = 0 min), 3 min (T_p = 3 min), 6 min (T_p = 6 min), 9 min (T_p = 9 min), and 12 min (T_p = 12 min) of pulverisation. All data are presented as means of the experiments performed in duplicate or triplicate.

Homogenization		**T_p = 0 min**	**T_p = 3 min**	**T_p = 6 min**	**T_p = 9 min**	**T_p = 12 min**
Fingernails	#1	13	19	24	25	22
	#2	21	102	106	114	123
	#3	2	23	14	15	17
	#4	< LLOQ	11	10	9	9
	#5	5	18	23	26	24
Toenails	#1	15	14	37	92	21
	#2	24	48	31	46	83
	#3	2	16	17	14	20
	#4	< LLOQ	12	6	7	11
	#5	6	14	14	13	4

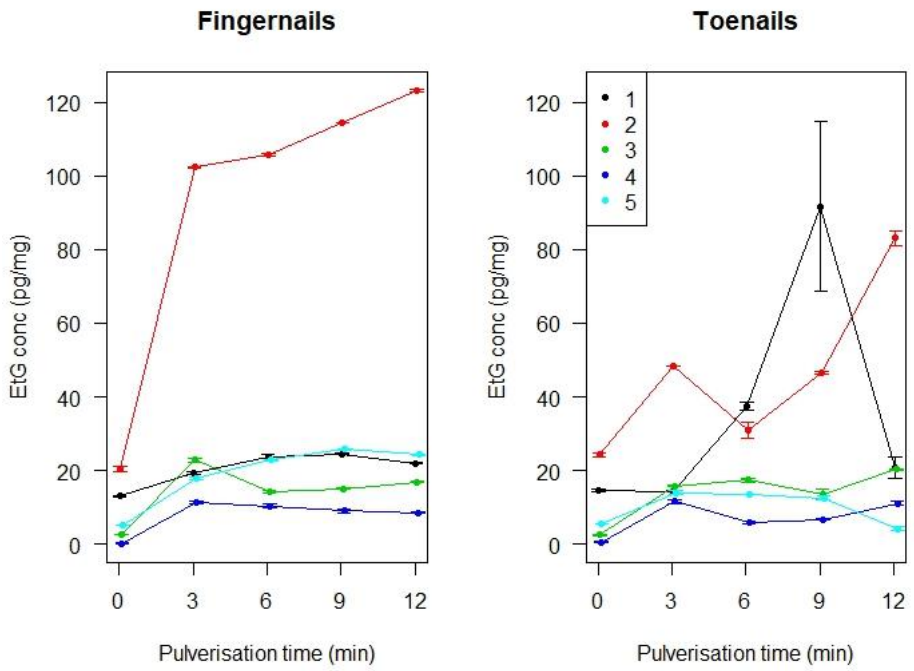

Figure 3.6: Scatterplot of the ethyl glucuronide (EtG) concentrations (pg/mg) in finger- and toenails as a function of pulverisation time (min). Each line represents data from one subject.

The results from particle size measurements after 3 min, 6 min, 9 min, and 12 min of pulverisation are presented in Table 3.8. The effect of pulverisation time on particle size was analysed using a linear mixed model with the fraction of particles < 600 µm as outcome variable. There was no significant interaction between the origin of the nails (finger or toe) and the pulverisation time ($p = 0.43$). The particle sizes did not differ significantly between the pulverisation times, but this non-significance is probably due to the low number of observations. Therefore, finger- and toenails were analysed in separate models. These models showed that neither in finger-, nor in toenails, there is a significant effect of pulverisation time on particle size (fingernails: $p = 0.11$, toenails: $p = 0.72$). Figure 3.7 shows the fraction of particles < 600 µm as a function of pulverisation time. For most subjects, the highest fraction of particles < 600 µm (smallest particle sizes) were reached after 9 min of pulverisation. Additional pulverisation to 12 min, however, led to decrease in fraction of particles < 600 µm in a more or less pronounced way, depending on the subject.

Table 3.8: Particles sizes obtained after 3 min (T_p = 3 min), 6 min (T_p = 6 min), 9 min (T_p = 9 min), and 12 min (T_p = 12 min) pulverisation of finger- and toenails from five subjects. Particle sizes are presented as % of particles < 600 µm and as mean particle size ± standard deviation (SD).

Pulverisation time		**Tp = 3 min**		**Tp = 6 min**		**Tp = 9 min**		**Tp = 12 min**	
		Particles < 600 µm (%)	Mean ± SD particle size (µm)	Particles < 600 µm (%)	Mean ± SD particle size (µm)	Particles < 600 µm (%)	Mean ± SD particle size (µm)	Particles < 600 µm (%)	Mean ± SD particle size (µm)
Fingernails	#1	89	259 ± 251	96	208 ± 178	100	74 ± 109	96	111 ± 186
	#2	99	92 ± 122	100	79 ± 105	100	89 ± 122	99	140 ± 150
	#3	95	141 ± 179	100	57 ± 63	100	40 ± 51	98	93 ± 150
	#4	98	100 ± 135	100	85 ± 117	100	78 ± 110	100	115 ± 132
	#5	100	79 ± 84	100	72 ± 87	98	101 ± 152	97	112 ± 168
Toenails	#1	98	127 ± 154	94	252 ± 206	99	98 ± 130	100	143 ± 138
	#2	96	99 ± 170	97	62 ± 140	100	41 ± 60	100	36 ± 48
	#3	100	38 ± 41	100	33 ± 51	100	77 ± 96	99	92 ± 124
	#4	99	67 ± 105	98	76 ± 144	99	70 ± 116	100	65 ± 96
	#5	100	57 ± 60	100	64 ± 80	100	75 ± 93	90	257 ± 234

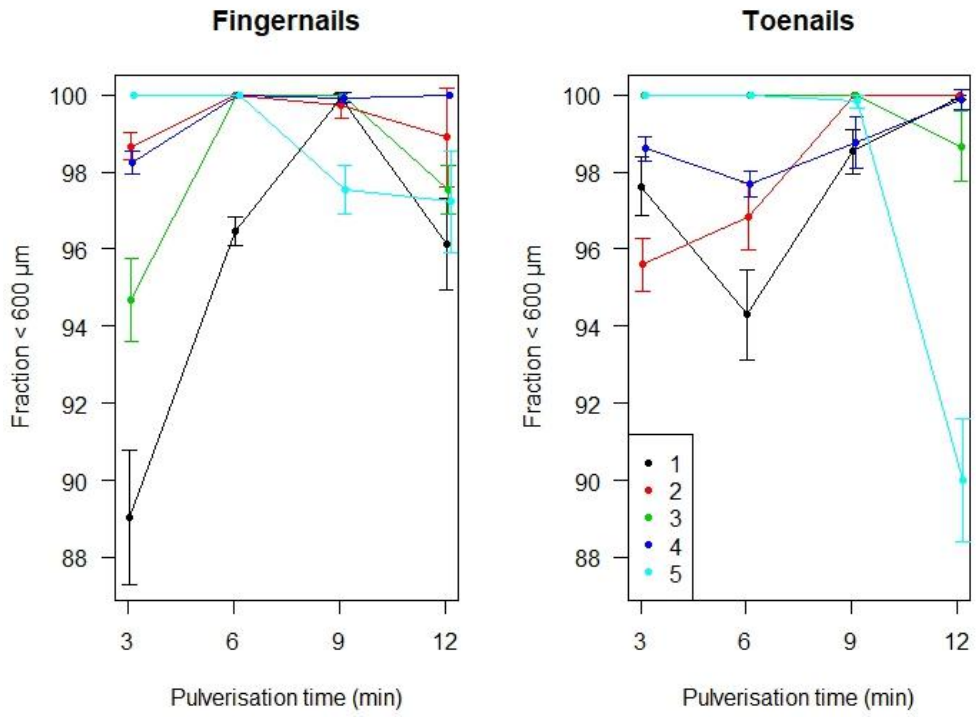

Figure 3.7: Scatterplot of the fraction of particles < 600 µm in finger- and toenails as a function of pulverisation time (min). Each line represents data from one subject.

3.1.3.4. Discussion

Only three studies have investigated the detection of EtG in nails as a marker for monitoring alcohol consumption over extended periods of time (Jones 2012, Morini et al. 2012, Keten et al. 2013, Morini et al. 2013, Berger et al. 2014). These studies provided the first evidence that EtG could be detected in fingernails using LC-MS/MS. Moreover, EtG concentrations in fingernails were found to be correlated with those in hair, and suggested a higher sensitivity of fingernails compared to hair (Morini et al. 2012). To the best of our knowledge, the current study is the first to report on the detection of EtG in toenails. Although differences between finger- and toenails were not significant, this may be due to a low number of samples. More research is required to investigate whether finger- and toenails are substitutable. Furthermore, comparison of concentrations in finger- and toenails could be interesting in the evaluation of the extent of incorporation through sweat and external contamination, and

deserves further investigation. In contrast to the published procedures, the current study comprises the development and validation of a GC-MS/MS based method. Advantages of GC-MS/MS compared to LC-MS/MS are a less expensive apparatus, less pronounced matrix effects and enhanced sensitivity through derivatisation. Indeed, the method presented here with HFBA derivatisation has a lower LLOQ compared to the other studies on EtG in nails that encompassed a full validation using similar criteria (2 pg/mg compared to 10 and 8 pg/mg). As in hair, the cut-off for discriminating between teetotallers and moderate drinkers has been established at 7 pg/mg (Kintz 2015), this drop in LLOQ below 7 pg/mg may be of significant importance for discrimination between the two categories.

Procedures for decontamination and homogenization of nails were evaluated. The choice of decontamination solvents was based on previous publications of EtG detection in nails (Jones 2012, Morini et al. 2012, Morini et al. 2013, Berger et al. 2014) and on a recently published method for EtG detection in hair (Cappelle et al. 2015a). There was no significant difference in EtG concentrations in the washing solutions among the three procedures, indicating that none of the procedures removed significantly more EtG from the nails. The effect of the decontamination procedure on EtG concentrations in nails was significant: decontamination using the Hex-DCM-MeOH procedure resulted in significantly higher EtG concentrations compared to the DCM-MeOH procedure. The ratios between EtG concentrations in the nails compared to EtG concentrations in the washing solutions were stable among the decontamination procedures, which suggests there is no overall difference between procedures. These experiments underscore the need for an adequate decontamination procedure. Decontamination should remove only external contamination, and not incorporated EtG, thus higher EtG concentrations in washing solutions are not always desired. As a result, the choice of an optimal decontamination procedure is not straightforward, and as the current study does not provide enough data to draw definitive conclusions, further research is required. To this end, concentrations in nails should be compared to alcohol consumption and reference material for nails should be developed.

Pulverisation (in comparison to no pulverisation) allows the achievement of small particle sizes and a homogeneous powder, thereby enhancing the extraction efficiency, reducing the extraction time from the matrix, and improving accuracy in the determination of EtG concentrations in nails. As such, cutting of nail samples without further pulverisation may

underestimate the actual EtG concentrations, and consequently, influence the interpretation of results. Due to the large variances between subjects, it is difficult to draw definitive conclusions on optimal pulverisation time. In theory, longer pulverisation times would result in smaller particle sizes and higher EtG concentrations. Experimental data, however, suggest that this theory is only true until a certain point after which longer pulverisation times results in bigger particles and stable or decreased EtG concentrations. Possible explanations are the formation of agglomerates and the degradation of EtG during extensive pulverisation.

To minimize the measuring variation, all experiments were performed in duplicate or triplicate. Limitations of the study include the small number of subjects. In addition, further research is needed to confirm and elaborate upon these findings, and to establish optimal decontamination and homogenization procedures.

3.1.3.5. Conclusions

This part presents the development and validation of a sensitive GC-MS/MS method for the determination of EtG concentration in both finger- and toenails. The results from decontamination and homogenization experiments underline the importance of washing and pulverisation steps for achieving accurate and reliable nail EtG results that can be used to evaluate alcohol consumption over extended periods of time.

3.2. Ethyl sulphate in hair

3.2.1. Preface and aims

As introduced previously, the abuse of alcohol is a worldwide problem associated with negative effects on health and society (World Health Organization 2011, Rehm et al. 2017). To detect and monitor harmful and/or chronic alcohol consumption, there is need for objective, specific and sensitive alcohol biomarkers.

EtG and EtS are two key non-oxidative metabolites of ethanol (see Figure 3.1). They are formed through Phase-II conjugation reactions catalysed by uridine 5'-diphospho–glucuronosyltransferase (UDP-glucuronosyltransferase) and sulfotransferase (Schmitt et al. 1995, Seidl et al. 2001, Helander et al. 2004, Helander et al. 2005). EtG was first detected in human urine in 1995 by Schmitt et al. (Schmitt et al. 1995), while the presence of EtS in human urine after ingestion of ethanol was not reported until 2004 by Helander and Beck (Helander et al. 2004).

Direct human biotransformation products of ethanol, such EtG, EtS and FAEE, are considered specific for alcohol consumption (Seidl et al. 2001, Wurst et al. 2006, Pragst et al. 2010). This is in contrast to the traditional alcohol markers, such as ASAT, ALAT, GGT and MCV which are considered as indirect biomarkers, and are influenced by age, gender and somatic pathologies (Conigrave et al. 2003, Hock et al. 2005). Another advantage is that EtG and EtS are detectable in blood and urine for a longer period of time than ethanol itself, implying a higher sensitivity for the detection of recent alcohol drinking (Helander et al. 2009, Hoiseth et al. 2009).

The window of detection of alcohol consumption can further be extended through the use of hair as a matrix. EtG is known to accumulate in hair where it remains detectable for several weeks to months depending on the length of the hair (Pragst et al. 2006). Other advantages of hair compared to the more traditional matrices (e.g. blood and urine) are the easy, non-invasive collection of samples as well as the transport and storage at room temperature. Moreover, hair collection does not require qualified medical staff and can be performed under close supervision to avoid adulteration. Over the past 20 years, monitoring alcohol consumption through the detection of EtG in hair has been widespread since EtG in hair has

proved to be a reliable, specific and sensitive long-term biomarker for the detection of chronic alcohol consumption (Boscolo-Berto et al. 2014, Crunelle et al. 2014b). The Society of Hair Testing (SoHT) has established guidelines for the determination of EtG in hair and defined cut-off values to discriminate between abstinence, social drinking, and excessive drinking (Society of Hair Testing 2016).

Compared to EtG, there is a significant research gap on measuring EtS in hair for the detection of alcohol use. So far, there are no analytical methods available for assessing this biomarker in hair, nor is there any information on the relationship between EtS and EtG in hair. Though, the concurrent determination of EtS and EtG in hair may improve the sensitivity of the detection, enhance the reliability of the results, and facilitate the data interpretation. To our best knowledge, the presence of EtS in hair has never been reported.

This section presents the first development and validation of an assay for quantifying EtS in hair using LC-MS/MS. After optimization of the sample preparation, chromatographic and mass spectrometric parameters, the method is fully validated according to the 'Guidelines on bioanalytical method validation' of the EMA (European Medicines Agency 2011). To evaluate the applicability of EtS as a biomarker of alcohol consumption, the method is applied to hair samples obtained from patients treated for alcohol use disorders. These results are compared to EtG concentrations in hair from the same patients (Cappelle et al. 2017).

3.2.2. Materials and methods

3.2.2.1. Reagents and materials

Reference standards (purity ≥ 95%) of EtS and the internal standard EtS-D_5 were purchased from Athena Enzyme Systems (Baltimore USA) as sodium salts. Ultrapure water was prepared using an Elga Purelab Flex water purification system (Veolia Water Technologies, Tienen, Belgium). Acetone (for gas chromatography ECD and FID SupraSolv®) was acquired from Merck KGaA (Darmstadt, Germany). Methanol (MeOH, LC-MS grade) was purchased from Fisher Scientific UK (Loughborough, United Kingdom). Ammonium hydroxide solution (NH_4OH, 25%) was bought from Sigma-Aldrich Chemie GmbH (Steinheim, Germany), formic acid (FAc, 98-100%) was supplied by Biosolve (Valkenswaard, the Netherlands), and acetic

acid was obtained from Merck KGaA (Darmstadt, Germany). Hair was pulverized using a ball mill Retsch MM 400 (Retsch Benelux, Aartselaar, Belgium). Extraction was performed in an ELMA TI-H-15 ultrasonication bath (Elma Hans Schmidbauer GmbH & Co. KG, Singen, Germany) and centrifugation with a Sigma centrifuge (Osterode am Harz, Germany). Oasis® solid-phase extraction (SPE) cartridges (60 mg, 3 mL), including WAX and MAX, were acquired from Waters (New Bedford, MA, USA). The SPE procedure was executed using a Supelco Visiprep™ SPE Vacuum Manifold (Bellefonte, CA, USA) with 24 ports and a self-cleaning vacuum system. Solvent evaporation was achieved with a Pierce Reacti-Therm III Heating Module (Rockford, IL, USA).

3.2.2.2. Standard solutions

Starting from the purchased dry powders, stock solutions of EtS (1 µg/mL) and EtS-D_5 (1 µg/mL) were prepared in MeOH. The working solutions were prepared in MeOH by further dilution of the stock solutions. Three standard working solutions were prepared in MeOH at concentrations of 100 pg/µL, 10 pg/µL and 1 pg/µL. The internal standard working solution was prepared in MeOH at a concentration 10 pg/µL and 150 µL of this solution was added to each sample. All solutions were stored in amber bottles at -20 °C.

3.2.2.3. Samples

For validation, blank hair samples were collected from strict teetotallers. Care was taken that cosmetic hair treatment and consumption of any alcohol were excluded. The validated method was applied to hair samples (n=40) from patients engaged in treatment for alcohol use disorders at the psychiatric centre of ZNA Stuivenberg together with an informed consent. The Ethical Committee of the University Hospital of Antwerp (UZA) and the local Ethical Committee of ZNA Stuivenberg approved the study (Belgian registration number B30020169233).

Hair samples were collected from the vertex posterior region of the head and cut as closely to the scalp as possible. The first 3 cm segment from the proximal end was used for further analysis. Samples were stored in aluminium foil at room temperature until analysis.

3.2.2.4. Sample preparation

To remove external contamination, collected hair samples were first washed; once in water and once in acetone, both for 1 min. Samples were air-dried, and subsequently pulverized for 5 min at 30 Hz using a ball mill. Then, an amount (20-35 mg) of powdered sample was weighted and transferred to a test tube. In this tube, 150 µL of EtS-D_5 as internal standard, and 2 mL of ultrapure water were added. Next, samples were ultrasonicated for 2 h, and centrifuged at 5000 rpm for 10 min. Solid-phase extraction was then performed by transferring the supernatant to Oasis® WAX cartridges, pre-conditioned with 2 mL of MeOH and 2 mL of ultrapure water. The cartridges were washed with 2 mL of MeOH, and subsequently dried for 5 min. Elution was performed using 2 mL of 5% NH_4OH/MeOH and the eluate was evaporated to dryness under a nitrogen gas stream at 37 °C. The residues were reconstituted in 50 µL of ultrapure water/MeOH (95/5, v/v). Finally, the extracts were transferred to vials and 8 µL was injected into the LC-MS/MS system.

3.2.2.5. LC-MS/MS instrumentation and conditions

The LC-system consisted of an Agilent Infinity 1200 (Agilent, Diegem, Belgium) equipped with a binary mixing pump, vacuum membrane degasser, thermostatic column compartment, and an autosampler. Chromatographic separation was achieved on a Waters Atlantis T3 column (150 mm x 2.1 mm x 3 µm, Waters, Zellik, Belgium) in gradient mode. The mobile phase was composed of (A) 0.1% acetic acid in ultrapure water, and (B) 0.1% acetic acid in MeOH. The optimized gradient was as follows: 0-0.50 min 5% B, 0.50-5.50 min increase to 20% B, 5.50-5.60 min increase to 98% B, 5.60-9 min 98% B, 9-9.10 min decrease to 5% B, and 10-14 min 5% B. The column temperature was set at 40°C. The flow rate was maintained at 0.3 mL/min. The injection volume was set at 8 µL. EtS had a retention time of 3.8 min, and the total run-time including column equilibration was 14 min. The MS-system consisted of an Agilent 6460 triple quadrupole mass spectrometer equipped with an electrospray interface operating in negative ionization mode. Nitrogen was used as nebulizing, sheath and collision gas. Source parameters, including ion spray voltage, temperature, nebulizing gas flow and pressure, were optimized. Quantitative analyses were performed in multiple reaction monitoring (MRM) mode. The MRM parameters, including fragmentor voltage, collision energy, and cell

accelerator voltage, were optimized for EtS and EtS-D_5. The two most abundant transitions (one quantifier and one qualifier) were recorded. Table 3.9 gives an overview of the MS parameters, and the recorded transitions for EtS and its internal standard.

Table 3.9: Liquid chromatography-tandem mass spectrometry parameters for ethyl sulphate (EtS) and its internal standard (EtS-D_5).

Analyte	Precursor (*m/z*)	Fragmentor voltage (V)	Cell accelerator voltage (V)	Quantifier		Qualifier	
				Product ion (*m/z*)	Collision energy (V)	Product ion (*m/z*)	Collision energy (V)
EtS	124.9	84	4	96.9	14	80.0	38
EtS-D_5	130.0	90	4	97.9	14	80.0	38

3.2.2.6. Quantification and method validation

The most abundant MRM transitions of EtS and EtS-D_5 were used for quantification (quantifier), while the other transition (qualifier) was used for identity confirmation. For confirmation of the detected compound, both retention times and quantifier/qualifier ratios were used. A tolerance of ± 5% deviation of compound retention time from the reference standard retention time was accepted. Variations of relative quantifier/qualifier intensities were considered acceptable within ± 20% with respect to the reference standard (Paul et al. 2009).

Selectivity, LLOQ, linearity, accuracy, precision, carry-over, matrix effects, recovery and stability were validated according to the 'Guidelines on bioanalytical method validation' of the EMA (European Medicines Agency 2011). On the first attempt, these parameters were examined using blank hair matrix (i.e. hair from teetotallers). However, due to the presence of EtS in the blank matrix samples, the linearity, accuracy and precision were eventually assessed using solvent, as was also applied in previous studies (Been et al. 2017, González-Mariño et al. 2017, Lai et al. 2017).

The LLOQ is defined as the lowest concentration of compound that can be determined with an acceptable accuracy and precision (i.e., within 20% bias and within 20% relative standard deviation (RSD)), and is included as the lowest calibration point of the calibration curves.

Based on the expected concentration range, a six-point calibration curve (5-500 pg/mg) was constructed at different concentrations to evaluate linearity. According to the EMA criteria, the back-calculated concentrations of the calibration standards should be within ± 15% of the nominal concentration, except at the LLOQ where it should be within ± 20%.

Accuracy and precision were assessed for replicated QC samples at three concentration levels (low QC: 15 pg/mg; mid QC: 75 pg/mg; and high QC: 350 pg/mg) covering the entire calibration range. Within-run precision and accuracy were assessed by analysing five samples per each QC concentration level prepared at the same day, and analysed in the same analytical run. Between-run precision and accuracy at each level were calculated on QC samples prepared and analysed in three different runs, on three separate days. Acceptance criteria were a bias less than 15% and a RSD lower than 15%.

Carry-over was assessed by injection of solvent samples after the highest calibration standard. The analyte response in the solvent sample should not be higher than 20% of the response of the LLOQ.

For the determination of matrix effects and recovery, three sets of samples (set 1, set, 2 and set 3) were prepared. In the first set (set 1), samples without matrix (neat mobile phase samples) were spiked with internal standard. The second set (set 2) included matrix samples from six different sources spiked *after* extraction with internal standard, while in the third set (set 3) matrix samples from six different sources were spiked with internal standard *before* extraction. Matrix effects could be evaluated through comparison of responses obtained in set 2 with those obtained in set 1 as the only difference between both is the presence or absence of matrix.

$$matrix\ effects\ (\%) = \frac{mean\ of\ responses\ set\ 2}{mean\ of\ responses\ set\ 1} \times 100 \qquad \textit{Equation 1}$$

The absolute recovery was calculated by comparison of responses obtained in set 3 with those obtained in set 2.

$$recovery\ (\%) = \frac{mean\ of\ responses\ set\ 3}{mean\ of\ responses\ set\ 2} \times 100 \qquad \textit{Equation 2}$$

The autosampler stability of processed samples was evaluated at the different QC levels (LLOQ, low QC, mid QC and high QC). These samples were analysed immediately and after remaining 24 h and 48 h on the autosampler at room temperature. The stability of EtS and EtS-D_5 in the working solutions was tested after 4 months at the QC levels (LLOQ, low QC, mid QC and high QC).

3.2.2.7. Data analysis

Statistical analysis was performed using R (version 3.3.1., The R Foundation for Statistical Computing). Normality was tested through histograms and QQ-plots of the raw data. Due to non-normality of the data, further analyses were performed with non-parametric statistics. The association between EtS and EtG in hair was expressed using a Spearman correlation. For all statistical tests, a p-value < 0.05 was considered statistically significant. Results are presented as mean ± standard deviation (SD) or as median [interquartile range (IQR)].

3.2.3. Results and discussion

3.2.3.1. Method development and optimization

In order to isolate EtS from the hair matrix, two different types of SPE sorbents were tested. Mixed-mode strong anion-exchange (Oasis® MAX) and mixed-mode weak anion exchange (Oasis® WAX) were evaluated. Both cartridges have a mixed-mode sorbent which includes reversed-phase and ion-exchange functionality, and are capable of retaining acidic analytes. Matrix effects and absolute recoveries were assessed to select the most suitable SPE cartridge. The matrix effect was assessed by comparing hair samples spiked with internal standard after SPE to neat standards spiked at the same concentration level (Equation 1). The recovery was calculated by comparing the analyte area between hair samples spiked with internal standard before and after SPE (Equation 2).

For the MAX cartridges, the sorbent was conditioned with 2 mL of MeOH followed by 2 mL of water. After sample loading, the sorbent was washed with 1 mL of 5% NH_4OH/water and 2 mL MeOH. The cartridges were then dried under vacuum and eluted with 2 mL 2% FAc/MeOH.

The WAX cartridges were conditioned similarly to the MAX cartridges. Following sample loading, the sorbent was washed with 2 mL of 2% FAc/MeOH, dried under vacuum and eluted with 2 mL of 5% NH_4OH/MeOH.

The MAX sorbent gave less than 5% recovery, and therefore was not considered anymore. Further optimization was performed with WAX cartridges. Table 3.10 outlines the three different procedures that were tested using the WAX cartridges. Procedure 1 had a recovery of less than 5% and therefore was discarded. Procedure 2 resulted in 51% recovery and -88% matrix effect (ion suppression), whilst procedure 3 showed 83% recovery and -16% matrix effect. Thus, procedure 3 was chosen as the final method since it provided a better recovery and less matrix suppression.

Table 3.10: Different solid-phase extraction procedures tested using WAX cartridges.

	Procedure 1	**Procedure 2**	***Procedure 3****
Condition	2 mL MeOH	2 mL MeOH	2 mL MeOH
	2 mL H_2O at pH 8.2[a]	2 mL H_2O	2 mL H_2O
Load sample			
Wash	3 mL H_2O at pH 8.2	2 mL 2% FAc in MeOH	2 mL MeOH
Vacuum drying	5 min	5 min	5 min
Elute	2 mL MeOH	2 mL 5% NH_4OH in	2 mL 5% NH_4OH in
	2 mL 5% NH_4OH in MeOH	MeOH	MeOH

[a] H_2O adjusted to pH 8.2 using NH_4OH; * ***Selected optimized method***

Abbreviations: FAc, formic acid; H_2O, water; MeOH, methanol; NH_4OH, ammonium hydroxide

3.2.3.2. EtS traces in blank matrix samples

At the first attempt to validate the method, the method selectivity was assessed by individually analysing six different sources of blank hair from teetotallers. According to the EMA guidelines, interfering components are considered absent when the response (i.e., peak area) is less than 20% of the LLOQ for the given compound. However, the response in the six different blank matrices was more than 20% of the mean response at the LLOQ calculated over the three validation days. This implied that basal levels of EtS were present in the hair of teetotallers, comparable to what was earlier reported for EtG (Pirro et al. 2013). This could be due to exposure to a range of products such as ethanol-containing food, prescriptions, cosmetics and body care products (Goodguide, Martins Ferreira et al. 2012, Sporkert et al. 2012). Another possible explanation is the *in vivo* formation of EtS for example by metabolic biotransformation of food or beverages (Musshoff et al. 2010, Agius et al. 2012).

3.2.3.3. Method validation

Figure 3.8 shows a chromatogram obtained at mid QC level (75 pg/mg), and Table 3.11 presents an overview of the performance of the developed method.

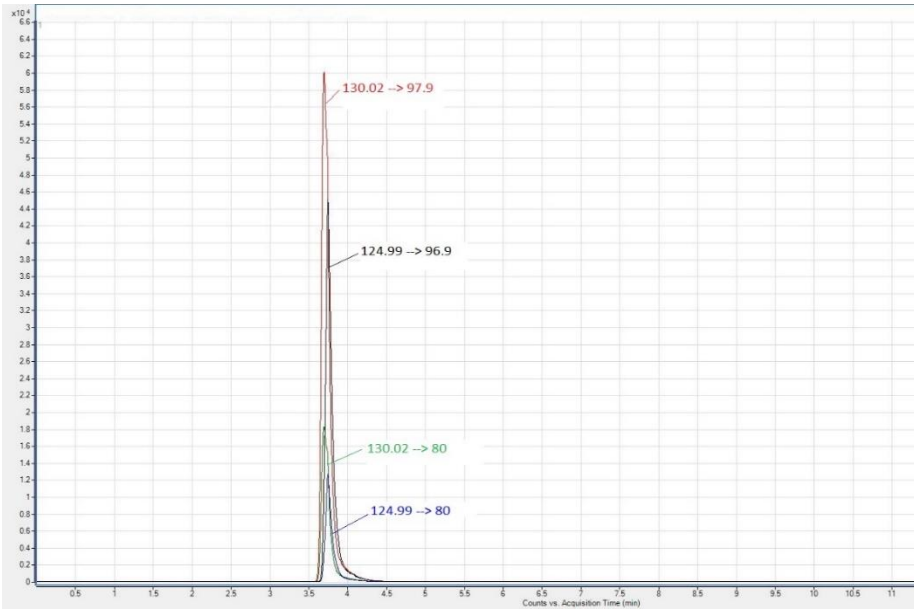

Figure 3.8: Chromatogram achieved for ethyl sulphate (EtS) at mid QC level (75 pg/mg). EtS peaks are shown in black (quantifier) and blue (qualifier), EtS-D_5 peaks are shown in red (quantifier) and green (qualifier).

The LLOQ in hair was estimated at 5 pg/mg based on low concentration hair samples showing a signal-to-noise ratio of 10. Without hair matrix, the LLOQ was determined at 5 pg/mg. With low matrix effect and high recovery (see results below), it is logical that both LLOQs are comparable. Within- and between-run accuracy, expressed as bias, was below 20%. For within- and between-run precision, the RSD was lower than 20% (see Table 3.11).

The six-point calibration curve was linear in the investigated range (5-500 pg/mg). When a least-squares linear regression is used to compute a calibration curve, data at the high end of the calibration curve tend to dominate the calculation of the linear regression as the absolute variation is larger for higher concentrations. Therefore, a weight factor of $1/x^2$, which weights the data inversely with the concentration to compensate for the excessive error at the lower concentration, was used (Gu et al. 2014). All calibrators had their back-calculated concentrations (mean over three days) of the calibration standards within ± 15% of the nominal concentration, or within ± 20% at the LLOQ.

For all concentration levels (low, mid and high QC), within- and between-run accuracy was within the limits of ± 15% bias. Within- and between-run precision was lower than 15% RSD for all concentration levels (Table 3.11).

Table 3.11: Accuracy and precision of the optimized analytical method.

EtS concentration	Accuracy (% bias)		Precision (% RSD)	
	Within-run (n = 5)	Between-run (n = 15)	Within-run (n = 5)	Between-run (n = 15)
LLOQ (5 pg/mg)	4.8	2.7	9.1	11.2
Low QC (15 pg/mg)	6.7	5.0	3.0	8.2
Mid QC (75 pg/mg)	4.2	2.5	4.5	5.4
High QC (350 pg/mg)	1.6	2.3	2.0	3.6

Abbreviations: EtS, ethyl sulphate; LLOQ, limit of quantification; RSD, relative standard deviation; QC, quality control

Carry-over was assessed by injection of blank samples after the highest calibration point. The area of EtS in the blank sample injected after the highest calibrator was not higher than 20% of the LLOQ.

Matrix effect and recovery were re-evaluated during method validation to confirm the results obtained during method development and optimization. The matrix effect was -20% (RSD = 6%), while the absolute recovery of the internal standard was 63% (RSD = 7%).

No significant instability of the processed samples in the autosampler was observed after 24 and 48 h. The working solution of EtS and EtS-D_5 remained stable after 4 months.

3.2.3.4. Method application

Authentic hair samples (n=40) were analysed with the described, validated method to demonstrate its applicability for EtS measurements. Data on EtG concentrations in hair from the same patients were retrieved from our previous study (Cappelle et al. 2017). Analyses of both EtG and EtS in the hair samples allow assessing the relationship between these two markers in hair.

EtS concentrations in hair ranged from 24 to 1776 pg/mg (mean = 212 ± 302 pg/mg, median = 101 pg/mg, IQR [62; 272]), while EtG concentrations in hair ranged from 1 to 1149 pg/mg (mean = 196 ± 223 pg/mg, median = 147 pg/mg, IQR [40; 274]).

There was a significant and positive correlation between EtS and EtG in hair (ρ = 0.66, p-value < 0.001, Figure 3.9). Due to the presence of basal EtS levels in hair, caution was taken for the interpretation of the observed correlation. To do this, samples classified in the same quartile for both EtG and EtS were selected to investigate if there was a correlation among these samples within each quartile. Our data suggest that the best agreement (i.e. the best correlation) was obtained for EtG concentrations between 74 and 1149 pg/mg and EtS concentrations between 82 and 1776 pg/mg (corresponding to the range from the second to fourth quartile for both compounds). This result also confirmed our observations from the selectivity experiments; samples with EtG concentrations corresponding to abstinence (< 7 pg/mg) had EtS concentrations from 25 to 84 pg/mg.

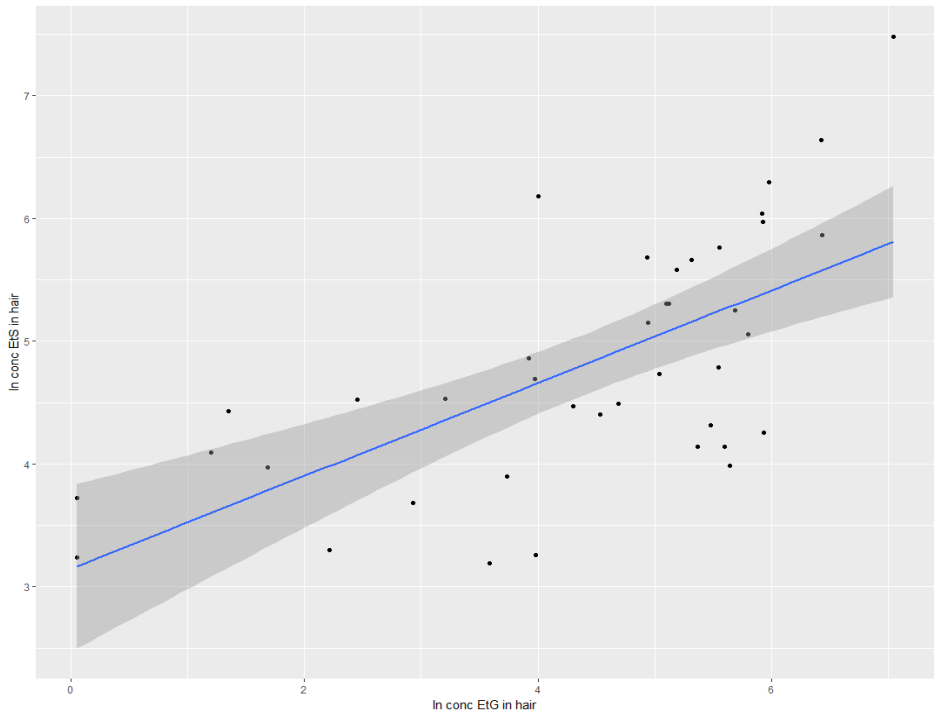

Figure 3.9: Correlation between ethyl sulphate (EtS) and ethyl glucuronide (EtG) concentrations in hair samples (ρ = 0.66, p-value < 0.001). The values for both variables are log-transformed.

Although more research is needed before establishing cut-off concentrations for EtS in hair, the results from this study indicate that the cut-off levels for EtS hair will be higher than those for EtG. This follows from the observation that the basal levels of EtS in hair are higher than the basal levels of EtG in hair. The basal concentrations may arise from the exposure to exogenous sources other than alcoholic beverages or from *in vivo* EtS formation from various food or beverages (Goodguide, Musshoff et al. 2010, Agius et al. 2012, Martins Ferreira et al. 2012, Sporkert et al. 2012, Pirro et al. 2013). Another possible hypothesis to explain the higher basal concentrations of EtS might be that the incorporation of EtS in hair is more efficient than the one of EtG, which could be explained by differences in physicochemical properties between both compounds. This deserves further investigation in future studies with higher numbers of samples.

For now, our results suggest that it is necessary to take into account the background level of EtS in hair when it is to be used to assess alcohol consumption or define cut-offs for alcohol consumption. Nevertheless, there could be some advantages to measure EtS in hair for the detection of alcohol use. Since EtS is present in hair in relatively higher concentrations than EtG, the related analytical challenges appear reduced in measuring EtS compared to EtG, e.g. one needs less sensitive assays to measure EtS in hair for a preliminary assessment of alcohol use. Methods capable of detecting both EtG and EtS should be developed. Using these methods, EtS results could complement EtG measurements to improve the sensitivity of detection, enhance the reliability of results, and facilitate the data interpretation. However, it may be challenging to develop a single sample preparation procedure (e.g. using the same SPE sorbents) for EtG and EtS.

3.2.4. Conclusions

The current article is the first to report on the presence of EtS in hair. For the measurement of EtS, a LC-MS/MS method was developed and validated according to the EMA guidelines. The method is robust and sensitive with a LLOQ of 5 pg/mg. It provides an additional option to prove alcohol consumption based on hair analysis. The developed method was successfully applied to quantify EtS in hair samples from patients treated for alcohol use disorders, and these EtS concentrations were compared to EtG concentrations in hair of the same patients.

EtS in hair was found to significantly correlate with EtG in hair, suggesting that EtS can be used as a biomarker for alcohol consumption. However, care must be taken regarding the relatively high basal levels of EtS present in alcohol-abstinent persons and this finding needs to be further investigated in the future.

CHAPTER 4:

BIOMARKERS OF DRUGS OF ABUSE IN HAIR AND NAILS

Based on the following paper:

Cappelle D, De Doncker M, Gys C, Krysiak K, De Keukeleire S, Maho W, Crunelle CL, Dom G, Covaci A, van Nuijs AL and Neels H. A straightforward, validated liquid chromatography coupled to tandem mass spectrometry method for the simultaneous detection of nine drugs of abuse and their metabolites in hair and nails. *Analytica Chimica Acta* 960 (2017) 101-109.

4.1. Straightforward determination of nine drugs of abuse and their metabolites in hair and nails

4.1.1. Preface and aims

Despite the emergence of new psychoactive substances, amphetamine-like stimulants, cocaine, and opioids continue to be in high demand and supply, with major impacts on public health (European Monitoring Centre for Drugs and Drug Addiction 2017). According to the European Drug Report 2016, cocaine remains the most commonly used illicit stimulant drug in Europe (European Monitoring Centre for Drugs and Drug Addiction 2017). The combined consumption of several drugs of abuse, i.e. polysubstance abuse, is a prevalent pattern of substance use and represents a significant health concern. The monitoring of these patients within a therapeutic or forensic/legal procedure, represents a particular challenge that requires multi-analyte methods capable of detecting several compounds in a single run using a straightforward and simple procedure.

The importance of *hair* as a matrix for illicit drug testing has substantially increased in recent years, e.g., in driver's license regranting procedures and in investigations related to causes of death (Gambelunghe et al. 2005, Kłys et al. 2007). In this context, several analytical methods for the measurement of drugs of abuse in hair have been described mainly using gas chromatography (GC) or liquid chromatography (LC) coupled to (tandem) mass spectroscopy ((MS/)MS). Most of these methods focus on the validation of a semi-quantitative screening method (Kronstrand et al. 2004, Hegstad et al. 2008, Broecker et al. 2012), and/or on a limited number (i.e. only basic substances (Miyaguchi et al. 2011)) of compounds (Quintela et al. 2010, Miguez-Framil et al. 2011, Alves et al. 2013), while quantitative multi-target methods are rather scarce (Aleksa et al. 2012, Zhu et al. 2012) and often require expensive instrumentation (Favretto et al. 2011, Miyaguchi et al. 2011). Recently, two articles have reported upon the validation of an LC-MS/MS method for the quantification of amphetamines, cocaine, opioids and metabolites in hair (Fernandez et al. 2014, Imbert et al. 2014). However, the disadvantage of both methods is that they involve a time consuming, expensive and laborious solid-phase extraction step as part of the sample preparation. A possible alternative has been proposed by Chang et al. (Chang et al. 2014) in their development of a method based on microwave assisted extraction for the detection of

amphetamines and opiates in hair. Together with the increased use of hair samples, scientific organizations, such as the Society of Hair Testing (SoHT) (Cooper et al. 2012) and Substance Abuse and Mental Health Services Administration (SAMHSA) (Department of Health and Human Services 2014), have published guidelines for hair analysis and recommendations for confirmatory cut-offs to distinguish positive from negative samples. On the other hand, studies on *nail* analysis for drugs of abuse are limited, and they only reported on a limited number of substances, e.g. amphetamine-like stimulants or cocaine (Valente-Campos et al. 2006, Kim et al. 2010). Neither guidelines nor cut-off values are available for nail analysis, and therefore interpretation of results is often complicated. Very few articles have developed methods for both *hair and nails*, and these methods are only applicable for one class of substances (Shen et al. 2014, Madry et al. 2016).

This chapter aims at the development of a straightforward analytical procedure for the simultaneous detection of relevant drugs of abuse in keratinized matrices. The investigated drugs of abuse and their metabolites are presented in Figure 4.1 and Table 4.1 together with the recommended cut-off concentrations in hair. The development of a single method for the simultaneous analysis of these compounds was challenging as the analytes under investigation belong to different classes (amphetamine-type stimulants, opioids, and cocaine) and have different physicochemical characteristics. The method includes a simple sample preparation step, thereby reducing costs and allowing application in routine laboratory practice. The method was validated for both hair and nails, which allows comparison between the two matrices, and enhances the confidence level of the results. Moreover, it extends the window of detection even more, as nails are growing slower compared to hair. In addition, as a result of their slower grow rate and increased drug accumulation, nails may enhance the method sensitivity. Finally, the presented method offers the possibility to analyse paired hair and nails samples, and to use nails as an alternative matrix whenever hair is not available (e.g., alopecia, newborn) or not reliable (e.g., bleached hair) and vice versa to analyse hair samples in cases of cosmetic nail treatment (e.g. nail polishing, nail coating).

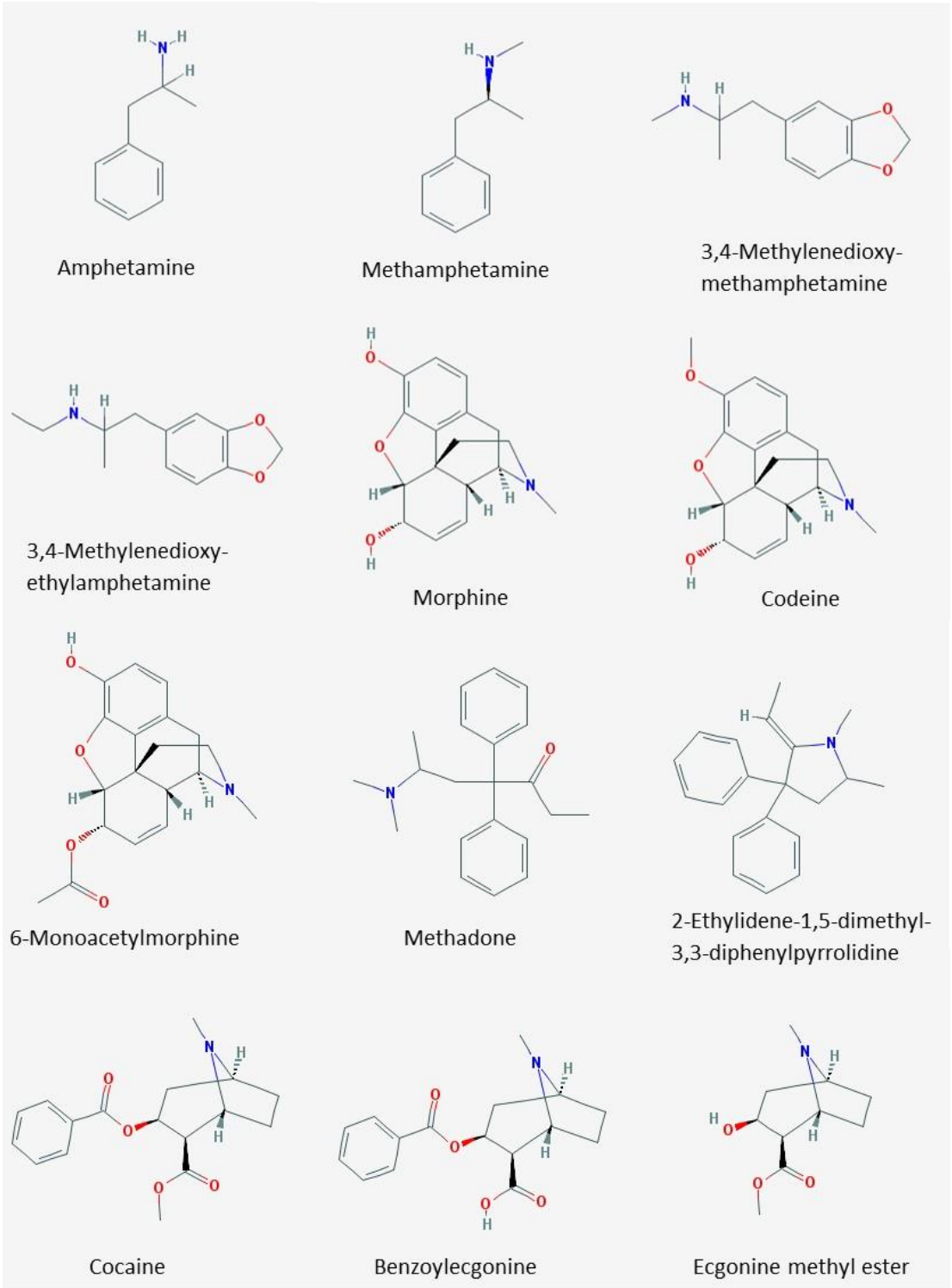

Figure 4.1: Structures of the investigated drugs of abuse and their metabolites.

Table 4.1: Overview of the nine drugs of abuse (indicated in bold), their metabolites, and the corresponding cut-off values.

Compound	Abbreviation	Recommended cut-off concentrations in hair (pg/mg)		Notes
		SoHT	SAMHSA	
Amphetamine-type stimulants				
Amphetamine	AMP	200	300	
Methamphetamine	mAMP	200	300	
3,4-Methylenedioxy-methamphetamine	MDMA	200	300	
3,4-Methylenedioxy-ethylamphetamine	MDEA	200	300	
Opioids				
Morphine	MOR	200	200	Parent compound, but also metabolite of **heroin** and codeine
Codeine	COD	200	200	
6-Monoacetylmorphine	6-MAM	200	200	Minor but exclusive metabolite of heroin
Methadone	MTD	200	/	
2-Ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine	EDDP	50	/	Metabolite of methadone
Cocaine				
Cocaine	COC	500	500	
Benzoylecgonine	BE	50	> 5% COC level	Metabolite of cocaine
Ecgonine methyl ester	EME	50	/	Metabolite of cocaine

Abbreviations: SAMHSA, Substance Abuse and Mental Health Administration; SoHT, Society of Hair Testing

4.1.2. Materials and methods

4.1.2.1. Reagents and materials

Ultrapure water was prepared using an Elga Purelab Flex water purification system (Veolia Water Technologies, Tienen, Belgium). Acetone (for gas chromatography ECD and FID SupraSolv®) was acquired from Merck KGaA (Darmstadt, Germany). Methanol (MeOH; LC-MS grade) and acetonitrile (ACN; HPLC grade) were purchased from Fisher Scientific UK (Loughborough, United Kingdom). Formic acid was obtained from Merck-Schuchardt OHG (Hohenbrunn, Germany). Ammonium formiate (97%) was bought from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). The following analytical standards were acquired from Cerilliant (Round Rock, TX, USA) as solutions at concentrations of 1.0 mg/mL in MeOH, unless otherwise stated: 6-monoacetylmorphine (6-MAM; in ACN), amphetamine (AMP), benzoylecgonine (BE), cocaine (COC; in ACN), codeine (COD; 100 µg/mL), 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP) perchlorate, ecgonine methyl ester (EME; in ACN), methamphetamine (mAMP), 3,4-methylenedioxyethylamphetamine (MDEA), 3,4-methylenedioxymethamphetamine (MDMA), and morphine (MOR). The methadone (MTD) standard was purchased as a solution of 1.0 mg/mL in MeOH from LGC GmbH (Luckenwalde, Germany). For each analyte, the corresponding deuterated analogue was included and used for quantification. The deuterated internal standards were obtained from Cerilliant as solutions in concentrations of 1.0 mg/mL in MeOH, unless otherwise stated: 6-MAM-D_3 (in ACN), AMP-D_8, BE-D_3, COC-D_3 (in ACN), COD-D_6 (100 µg/mL), EME-D_3 (100 µg/mL in ACN), MDEA-D_6 (100 µg/mL), MDMA-D_5, MTD-D_9, mAMP-D_{11} (100 µg/mL) and MOR-D_3. The deuterated internal standard EDDP-D_3 perchlorate was bought from LGC GmbH as a solution of 1.0 mg/mL. Through participation in a proficiency testing program organized and realized by Arvecon GmbH (Walldorf, Germany), the laboratory received authentic standard hair specimens for testing. For pulverisation of hair and nail samples, a ball mill Retsch MM 400 (Retsch Benelux, Aartselaar, Belgium) was employed. Incubation was performed using an Eppendorf Mixmate (Eppendorf Belgium, Rotselaar, Belgium), and centrifugation was done using a Beckman Coulter Microfuge 18 (Analis SA, Namen, Belgium). Mini-UniPrep vials for filtration of the supernatant after incubation and centrifugation were acquired from Agilent (Diegem, Belgium).

4.1.2.2. Standard solutions, calibrators and quality control samples

Starting from the purchased stock solutions, working solutions for both standards and internal standards were prepared in order to contain all analytes in the same concentrations. Three standard working solutions were prepared in MeOH at concentrations of 1 ng/µL, 100 pg/µL, and 10 pg/µL. Calibrators and quality controls (QCs) were prepared by spiking blank matrix samples with one of those solutions. The internal standard working solution was prepared in MeOH at a concentration 100 pg/µL and 100 µL of this solution was added to each sample. All solutions were stored at -20°C.

4.1.2.3. Samples

For validation, blank (i.e., drug-free) hair and nails samples were obtained from healthy volunteers together with self-reports. Care was taken that cosmetic hair treatment, nail polishing, and consumption of any drug of abuse were excluded. Hair samples were collected from the vertex posterior region of the head and cut as closely to the scalp as possible. Nail samples were obtained by clipping of the distal edge of finger- and toenails. Samples were stored in aluminium foil at room temperature until analysis.

In addition, hair samples from the proficiency tests DHF 3/15 and DHF 2/16 organized by Arvecon GmbH were analysed with the method to test its performance. The validated method was applied to hair and nail samples from different drug-dependent individuals. The hair samples were obtained from forensic and toxicological cases. The nail samples were collected from patients engaged in treatment for substance use disorders at the psychiatric Centre Multiversum together with a detailed anamnesis of past drug consumption. The Ethical Committee of the University Hospital of Antwerp (UZA) and the local Ethical Committees of Multiversum and ZNA Stuivenberg approved the study (Belgian registration number: B30020169233).

4.1.2.4. Sample preparation

To remove external contamination, collected hair and nail samples were first washed; once in water and once in acetone, both for 1 min. Samples were air dried, and subsequently pulverized for 5 min at 30 Hz using a ball mill. Approximately 20 mg of powdered sample was then accurately weighed, and transferred into a 2 mL Eppendorf tube. To this Eppendorf, 100 µL of internal standard solution and 500 µL of the extraction solution [MeOH: ACN: 2 mM ammonium formiate in ultrapure water (25:25:50, v/v), pH 5.3] were added, and the mixture was incubated for 18 h at 500 rpm in an Eppendorf Mixmate. After centrifugation for 10 min at 13500 rpm, the supernatant was filtrated using Agilent Mini-UnipPrep vials and the vials were placed on the LC-MS/MS instrument for injection.

4.1.2.5. LC-MS/MS instrumentation and conditions

The LC-system consisted of an Agilent 1200 series binary pump and autosampler (Agilent, Diegem, Belgium). Chromatographic separation of the drugs was achieved on an EZ:faast C18 (250 mm x 2.0 mm x 4 µm) column (Phenomenex, Utrecht, the Netherlands) in the gradient mode. The mobile phase was composed of (A) 5 mM ammonium formiate and 0.01 % formic acid in ultrapure water, and (B) 0.01% formic acid in ACN. The optimized gradient was as follows: 0-1 min 1% B, 1-5 min increase to 15% B, 5-9 min increase to 25% B, 9-10 min increase to 50% B, 10-10.5 min increase to 100% B, 10.5-14 100% B, 14-14.5 decrease to 1% B, and 14.5-21 min 1% B. The flow rate was 0.3 mL/min and the injection volume was set at 1 µL. All compounds were eluted between 2.5 and 14 min, and the total run-time including column equilibration was 21 min. The MS-system consisted of an Agilent 6410 triple quadrupole mass spectrometer equipped with an electrospray interface operating in positive ionization mode. Nitrogen was used as drying gas at a temperature of 350 °C and a flow of 10 mL/min. Quantitative analyses were performed in dynamic multiple reaction monitoring (MRM) mode and the three most abundant transitions (one quantifier and two qualifiers) were recorded for each compound. Table 4.2 gives an overview of the MS parameters, and the retention times for all compounds and internal standards.

Table 4.2: Overview of liquid chromatography-tandem mass spectrometry (LC-MS/MS) parameters for the selected drugs of abuse and their metabolites.

Compound	Internal Standard[a]	Retention time (min)	Precursor ion (*m/z*)	Fragmentor voltage (V)	Quantifier		Qualifiers	
					Product ion (*m/z*)	Collision energy (V)	Product ion (*m/z*)	Collision energy (V)
EME	*EME-D_3*	2.83	200.2	124	182.1	14	82.1	22
			203.2		*185.1*		65.1	46
MOR	*MOR-D_3*	8.35	286.1	160	165.0	45	157.0	41
			289.1		*165.0*		44.1	41
COD	*COD-D_6*	10.30	300.2	144	115.1	46	152.0	74
			306.2		*152.1*		165.0	86
AMP	*AMP-D_8*	10.75	136.1	64	91.1	5	119.1	13
			144.2		*97.1*		65.1	41
6-MAM	*6-MAM-D_3*	11.35	328.2	162	165.0	40	152.0	76
			331.1		*165.1*		43.1	72
mAMP	*mAMP-D_{11}*	11.50	150.2	92	91.1	9	119.1	13
			161.2		*97.1*		65.1	45
MDMA	*MDMA-D_5*	11.70	194.2	92	163.0	9	105.1	21
			199.1		*165.1*		77.1	45
BE	*BE-D_3*	12.30	290.1	124	168.1	14	105.1	30
			293.2		*171.1*		77.1	58
MDEA	*MDEA-D_6*	12.57	208.2	88	163.0	9	105.1	21
			214.1		*166.1*		77.1	49
COC	*COC-D_3*	13.05	304.2	124	182.1	14	82.1	66
			307.1		*185.1*		77.1	30
EDDP	*EDDP-D_3*	13.76	278.1	178	234.1	31	219.1	43
			281.3		*243.1*		115.1	79
MTD	*MTD-D_9*	13.86	310.4	112	105.1	61	77.1	25
			319.3		*105.1*		91.1	41

[a] Values for internal standards are shown in italic

4.1.2.6. Quantification and method validation

The stability of the compounds during the extraction was evaluated. Three Eppendorfs (in triplicate) were spiked at mid QC level and the extraction solution was added. From these, aliquots were taken at different time points during extraction: 0 h, 1 h, 4 h, 9 h and 18 h. Internal standard was added after aliquot withdrawal. The stability was evaluated by comparing the concentration at each time point compared to the initial concentration at time point 0.

For each compound, the most abundant MRM transition was used for quantification (quantifier), while two other transitions (qualifiers) were used for identity confirmation. For the deuterated internal standards, only one transition was used. For confirmation of the detected compounds retention times and quantifier/qualifier ratios were evaluated. A tolerance of ± 5% deviation of compound retention time from the reference standard retention time was accepted (GTFCh Scientific Committee Quality Control 2009). The ratio quantifier/qualifier in the processed samples should not be outside the range of ± 20% of the same ratio acquired from a reference standard (GTFCh Scientific Committee Quality Control 2009).

The analytical method was validated according to the 'Guidelines on bioanalytical method validation' of the European Medicines Agency (EMA). The following parameters were assessed to evaluate the performance of the method: selectivity, lower limit of quantification (LLOQ), linearity, accuracy, precision, carry-over, matrix effects, recovery, and process efficiency.

The method selectivity was assessed using six different sources of blank hair and nails, spiked with internal standards and analysed individually. The absence of interfering components is accepted where the response (i.e., peak area) is less than 20% of the LLOQ for a given compound.

The LLOQ is defined as the lowest concentration of compound that can be determined with an acceptable accuracy and precision (i.e., within 20% bias and within 20% relative standard deviation (RSD)), and is included as the lowest calibration point of the calibration curves.

Based on the expected concentration range, a seven-point calibration curve was constructed by spiking blank matrix samples at different concentrations to evaluate linearity. According to the EMA criteria the back-calculated concentrations of the calibration standards should be within ± 15% of the nominal concentration, except at the LLOQ where it should be within ± 20%.

Accuracy and precision were assessed for replicated QC samples at three concentration levels covering the entire calibration range (low QC, mid QC, and high QC). Within-run precision and accuracy were assessed by analysing five QC samples per concentration level prepared at the same day, and analysed in the same analytical run. Between-run precision and accuracy at each level were calculated on QC samples prepared and analysed in three different runs, on three separate days. Acceptance criteria were: 1) a bias less than 15%, and 2) a RSD lower than 15%.

Carry-over was assessed by injection of blank hair and nails after the highest calibration standard. The response of the analyte in the blank sample should not be higher than 20% of the response of the LLOQ.

As proposed by Matuszewski et al. (Matuszewski et al. 2003), three sets of samples (set 1, set, 2 and set 3) were prepared for the determination of matrix effects, recovery and process efficiency. In the first set (set 1), samples without matrix (neat mobile phase samples) were spiked at low and high QC concentration. The second set (set 2) included blank matrix samples from six different sources spiked *after* extraction at low and high QC concentration, while in the third set (set 3) blank matrix samples from six different sources were spiked at the same concentrations but *before* extraction. For each concentration level, two replicates were included and analyte responses were normalized with the responses of the internal standards. Matrix effects could be evaluated through comparison of normalized responses obtained in set 2 with those obtained in set 1 as the only difference between both is the presence or absence of matrix. A deviation of maximum ± 15% for the normalized matrix effect is tolerated.

$$matrix\ effects\ (\%) = \frac{mean\ of\ normalized\ responses\ set\ 2}{mean\ of\ normalized\ responses\ set\ 1} \times 100$$

The extraction recovery was calculated by comparison of normalized responses obtained in set 3 with those obtained in set 2 and the relative standard deviations were calculated.

$$recovery\ (\%) = \frac{mean\ of\ normalized\ responses\ set\ 3}{mean\ of\ normalized\ responses\ set\ 2} \times 100$$

Recovery and matrix effect were combined in order to calculate the overall process efficiency and the relative standard deviations were calculated.

$$process\ efficiency\ (\%) = \frac{mean\ of\ normalized\ responses\ set\ 3}{mean\ of\ normalized\ reponses\ set\ 1} \times 100$$

4.1.3. Results and discussion

4.1.3.1. Method development and optimization

As outlined in the introduction, several methods for keratinized matrices are available but often require (i) a complicated sample preparation procedure; (ii) only include a restricted amount of target analytes; (iii) are only applicable for screening purposes; or (iv) require expensive instrumentation (e.g. high resolution mass spectrometry). The novelty of the proposed methodology lies in the *combination* of: a simultaneous quantification of drugs of abuse from several classes, an easy (very few handlings) and straightforward sample preparation, quantification limits below the cut-offs of the SoHT and applicability to hair and nails samples, with a standard LC-MS/MS instrument in order to allow application in routine laboratories. To the best of our knowledge, there are no scientific papers that focused on the detection and quantification of a broad range of drugs of abuse and relevant metabolites in hair as well as in nails. In this study, attention was paid to the ease by which the method could be applied, the possibility to quantify several common drugs of abuse in one run, and the method sensitivity. The sample preparation is simple and straightforward, requires only minimal manual handling and the extraction can easily run overnight, which allows application in routine laboratory practice and reduces costs of consumables and personnel. Moreover, it enables the sensitive and specific quantification of nine drugs of abuse and their relevant metabolites in hair and nails.

Incubation time was based on a previous article by Nielsen et al. (Nielsen et al. 2010), in which the extraction yield was increased up to 100% when the time of extraction increased from 2 to 18 h. Moreover, Kronstrand et al. (Kronstrand et al. 2004) reported a good recovery and stability for a.o. MOR, COD, 6-MAM, AMP, mAMP, MDMA, BE, COC after 18 h of extraction. In addition, 18 h is a practical timescale in the laboratory since the extraction can be performed overnight.

Regarding the experiments on the stability of the compounds during extraction, no significant decrease in concentration was detected for any of the compounds under investigation.

Mini-UniPrep vials (Figure 4.2) were chosen for filtration of the supernatant, as they provide a quick, economical, and environmentally conservative way to remove particulate matter from samples. These preassembled filtration devices replace the combination of syringe filters, auto-sampler vials, septa, and caps with a single disposable unit. Moreover, as filtration is accomplished by simply pressing the plunger (upper part) through the liquid placed into the chamber (lower part), the vials do not require specifically trained lab personnel.

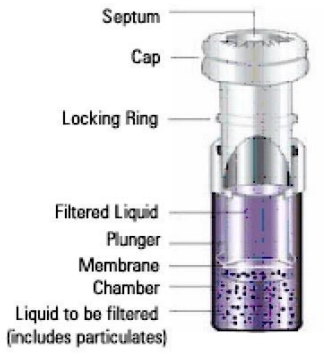

Figure 4.2: Schematic representation of a Mini-UniPrep vial. Each vial consists of two parts: a chamber and a plunger. The design incorporates a filtration membrane on the one end of the plunger and a pre-attached cap with septum on the other. By pressing the plunger through the liquid placed into the chamber, positive pressure forces the filtrate up into the reservoir of the plunger. Air escapes through the vent hole until the evaporation seal is engaged, providing an airtight seal. The Mini-UniPrep can then be placed into the autosampler for automated injection into the instrument.

Peak shapes and chromatographic separation of the target analytes were compared and evaluated on several types of columns with different dimensions: hydrophilic interaction liquid chromatography (HILIC), biphenyl, C8, and C18. The EZ:faast C18 (250 mm x 2.0 mm, 4 µm) column was selected for its sharp and narrow peaks, and sufficient chromatographic separation of the analytes (although complete separation is not necessary due to the use of specific MRM transitions for each compound). Special attention was paid to EME as this analyte shows only little retention on reversed phase columns. The gradient of the mobile phase and the flow rate were optimized for the best results in an acceptable time frame of 21 min (including column equilibration) and at a tolerable back-pressure (± 200 bar). After optimization, reproducible retention times, good peak shapes, and sufficient resolution and sensitivity were obtained.

Specific MS parameters such as fragmentor voltage, collision energy and ionization mode were optimized for each compound separately by injecting standard solutions without an LC-column. Dynamic MRM was chosen over normal MRM as it allowed enhancing the method sensitivity by a factor three. In dynamic MRM the mass spectrometer only monitors MRM transitions of the compounds that are eluting from the column at that particular time point. As a result, fewer transitions are recorded during each MS scan which permits the mass spectrometer to use a longer dwell time and more data points per peak. In comparison to MRM, dynamic MRM provides more reliable and better quality data, and eliminates background and interferences. Consequently dynamic MRM is suitable for monitoring of many compounds at low concentrations.

4.1.3.2. Method validation

Figure 4.3 shows a chromatogram achieved at mid QC level (750 pg/mg) in hair, and Tables 4.3, 4.4, 4.5 and 4.6 present an overview of the performance of the developed method.

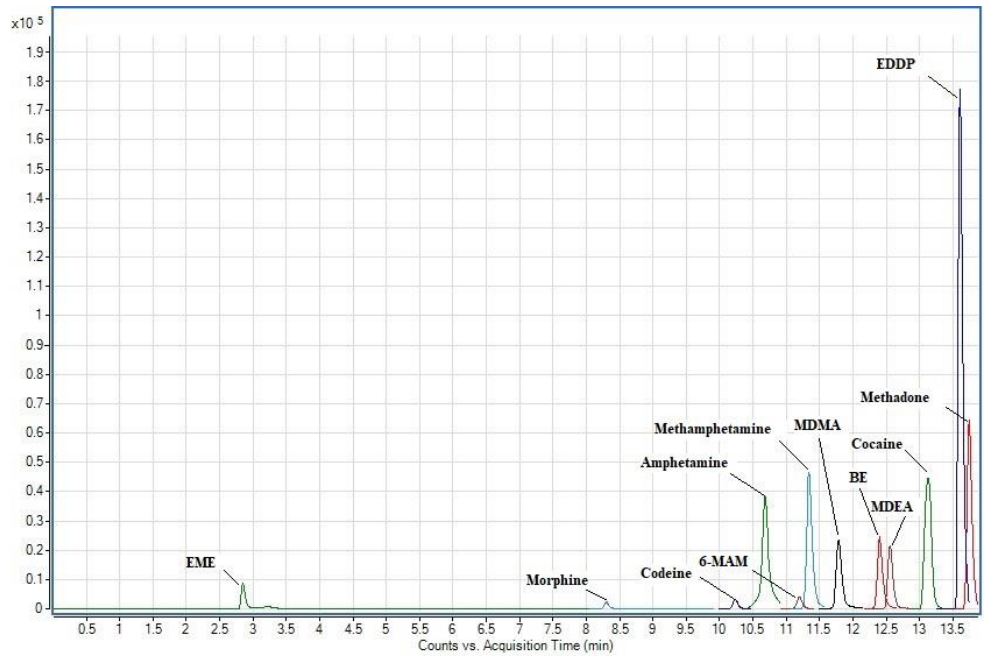

Figure 4.3: Chromatogram of a mid quality control sample in hair (750 pg/mg). The peaks of the quantifier transitions for all compounds are shown.

The response in six different blank matrices, spiked with internal standard, was less than 20% of the mean response at the LLOQ calculated over the three validation days for all analytes except for MDMA in hair (38%). To meet the selectivity criteria, the LLOQ for MDMA in hair could be set to 50 pg/mg instead of 25 pg/mg, which is still below the recommended cut-offs of SoHT and SAMHSA, 200 pg/mg and 300 pg/mg respectively.

The LLOQ in hair was 25 pg/mg (except for MDMA: 50 pg/mg) and 50 pg/mg in nails. Within- and between-run accuracy, expressed as bias, was below 20% for all compounds. For within- and between-run precision, the RSD was lower than 20% for all analytes. As presented in Table 4.1 and 4.3, the LLOQs of the developed method are below the cut-offs recommended by the SoHT and the SAMHSA. By establishing specific cut-offs for hair testing, these scientific instances provide guidance to laboratories interested in hair testing. The cut-off concentration levels are set to determine whether the sample is positive or negative for the presence of a drug.

For all compounds, the seven-point calibration curves were linear in the investigated ranges in both hair (25 – 20 000 pg/mg) and nails (50 – 20 000 pg/mg) (Table 4.3). When a least-squares linear regression is used to compute a calibration curve, data at the high end of the calibration curve tend to dominate the calculation of the linear regression as the absolute variation is larger for higher concentrations. Therefore, a weight factor of $1/x^2$, which weight the data inversely with the concentration to compensate for the excessive error at the lower concentration, was used (Gu et al. 2014). All calibrators had their back-calculated concentrations (mean over three days) of the calibration standards within ± 15% of the nominal concentration, or within ± 20% at the LLOQ.

For the analysis of BE in nails, the within- and between-run accuracy at low QC exceeded 15% bias. A LLOQ of 75 pg/mg instead of 50 pg/mg could therefore be proposed. For all other compounds and all other concentration levels, within- and between-run accuracy was within the limits of ± 15%. Within- and between-run precision was for all compounds in hair and nails lower than 15% RSD and this for all concentration levels (Table 4.4).

Table 4.3: Linearity, lower limit of quantification (LLOQ), accuracy and precision at the LLOQ for the developed method.

Matrix	Compound	Linearity (pg/mg)	LLOQ (pg/mg)	Accuracy (% bias)		Precision (% RSD)	
				Within-run (n = 5)	Between-run (n = 15)	Within-run (n = 5)	Between-run (n = 15)
Hair	EME	25 - 20 000	25	12.4	6.7	3.6	6.7
	MOR			15.5	7.0	12.8	14.2
	COD			1.2	4.4	9.7	9.8
	AMP			0.5	2.2	12.3	12.2
	6-MAM			7.7	5.3	3.1	9.6
	mAMP			10.9	7.4	5.0	6.3
	MDMA			1.5	2.7	13.8	10.5
	BE			13.2	8.6	2.3	6.9
	MDEA			5.7	7.3	6.2	5.6
	COC			5.8	9.3	4.3	3.9
	EDDP			7.9	5.6	1.8	2.9
	MTD			1.0	3.6	2.8	3.1
Nails	EME	50 - 20 000	50	2.6	3.8	8.7	9.3
	MOR			3.2	3.2	6.2	8.0
	COD			3.5	4.5	9.3	10.1
	AMP			3.2	2.9	5.4	4.7
	6-MAM			3.7	3.9	5.0	7.0
	mAMP			2.3	2.2	5.7	5.1
	MDMA			11.4	12.9	8.1	14.1
	BE			15.3	17.9	7.0	11.0
	MDEA			14.9	19.7	3.4	17.6
	COC			2.9	3.0	3.8	4.0
	EDDP			2.3	2.6	1.8	1.7
	MTD			1.1	1.4	3.5	4.1

Table 4.4: Accuracy and precision of the developed method.

Compound		Accuracy (% bias)						Precision (% RSD)					
		Within-run (n = 5)			Between-run (n = 15)			Within-run (n = 5)			Between-run (n = 15)		
		Low QC [a]	Mid QC [b]	High QC [c]	Low QC	Mid QC	High QC	Low QC	Mid QC	High QC	Low QC	Mid QC	High QC
EME	hair	1.8	5.5	2.3	3.4	5.1	2.9	5.8	3.6	1.5	5.4	3.0	3.1
	nails	11.3	10.3	10.6	5.6	10.4	11.2	7.3	2.9	3.1	6.9	3.7	4.4
MOR	hair	0.5	3.5	1.2	0.6	3.8	0.1	9.1	2.2	4.1	6.5	2.7	3.1
	nails	0.0	8.7	5.9	1.9	6.4	7.2	10.6	2.8	4.2	7.3	5.2	4.5
COD	hair	0.7	6.9	3.6	1.1	6.3	2.2	4.7	4.3	1.9	5.4	4.4	6.0
	nails	0.2	9.3	4.8	3.9	8.0	5.5	5.7	2.0	4.1	5.9	4.9	3.9
AMP	hair	7.4	1.8	0.7	5.6	2.0	0.8	5.0	7.2	2.0	9.5	5.0	3.2
	nails	6.8	4.8	7.9	5.8	3.8	9.9	2.3	3.6	2.7	3.2	2.7	2.9
6-MAM	hair	1.2	5.7	1.3	0.9	6.1	1.0	6.2	7.3	6.2	6.7	6.5	4.5
	nails	15.1	4.5	5.7	8.9	6.8	9.8	4.4	5.9	2.2	6.3	6.8	3.8
mAMP	hair	3.7	3.3	1.2	3.5	1.7	3.3	3.2	3.9	2.9	2.4	4.8	3.8
	nails	6.1	8.0	10.5	7.0	7.1	12.8	2.2	2.8	2.8	2.8	4.2	3.5
MDMA	hair	4.4	5.2	1.5	0.0	4.4	0.5	1.9	1.6	1.8	5.3	3.4	3.3
	nails	8.5	12.0	3.9	6.8	11.5	7.1	7.5	2.6	1.7	9.0	4.3	3.7
BE	hair	2.9	2.8	1.5	3.8	3.1	2.0	4.0	1.5	2.2	3.5	2.2	3.0
	nails	19.6	14.6	1.4	22.7	5.8	7.5	14.9	1.6	1.3	15.0	8.0	7.8
MDEA	hair	5.3	4.9	0.9	7.3	7.2	0.2	2.9	2.0	2.3	2.7	2.9	3.6
	nails	19.1	7.7	17.4	15.1	11.6	11.0	5.1	4.8	3.2	11.7	6.5	5.6
COC	hair	2.1	3.3	4.6	0.2	0.1	2.6	2.2	2.3	2.5	3.4	3.4	3.2
	nails	4.6	10.1	12.6	4.6	8.4	13.1	2.2	2.9	1.9	2.4	3.2	1.9
EDDP	hair	5.3	3.8	1.6	6.6	4.7	1.8	2.8	2.8	2.6	2.4	2.5	2.6
	nails	4.3	10.3	4.8	5.2	8.3	6.9	3.9	4.4	2.2	4.5	3.6	2.4
MTD	hair	4.2	2.6	1.4	5.5	3.3	0.3	3.1	2.8	1.9	3.5	2.2	2.2
	nails	6.5	11.4	5.6	5.9	9.1	8.4	1.8	2.1	2.3	4.6	4.2	4.2

[a] Low QC hair 75 pg/mg, low QC nails 100 pg/mg; [b] mid QC hair and nails 750 pg/mg; [c] high QC hair and nails 5000 pg/mg

Carry-over was assessed by injection of blank samples after the highest calibration point. The carry-over in the blank sample should not be greater than 20% of the LLOQ. In hair samples, carry-over limits were exceeded for 6-MAM, MDMA, MDEA and COC. In nails, MDMA, BE, COC had a carry-over above 20%. Supplementary experiments showed that by successive injection of two blank samples carry-over remained well below 20%. These results underline the importance of including blank samples after samples with a high concentration in a sequence.

The results obtained for matrix effects, recovery and process efficiency are outlined in Tables 4.5 and 4.6. In hair, absolute matrix effects ranged between +5% and +12% at high QC level and RSDs were lower than 9%. Matrix effects for nails ranged between -11% and +8% at low QC level (RSDs lower than 8%). At high QC level in nails, matrix effects were between -17% and +16% (RSDs lower than 8%), and thus slightly above the ±15% limit.

Depending on the compound, recovery in hair samples was between 92% and 99% at high QC (RSDs below 6%). In nails recovery at low QC was between 94% and 115% (RSDs below 10%), and between 90% and 109% at high QC (RSDs below 8%). Recovery at low and high QC concentration was calculated as the ratio of blank matrix samples spiked *before* and *after* extraction. The EMA guidelines do not include specific recommendations for recovery, however it is important for the recovery to be high (around 100%) and reproducible (RSD below 15%). This was true for both hair and nail samples along with RSD values below 10%.

Matrix effects and recovery can be combined to calculate process efficiency. Process efficiency at low QC level ranged between 91% and 105%, and between 93% and 110%, in nails and hair, respectively. At high QC level, process efficiency was between 88% and 120% in nails, and between 99% and 105% in hair. There are no specific EMA recommendations for process efficiency, but as the percentages are around 100% and the RSDs are low (below 16%), it can be concluded that the method has an excellent process efficiency.

Table 4.5: Overview of the results for matrix effect, recovery and process efficiency in hair.

Hair	Matrix effect		Recovery		Process efficiency			
	High level		High level		Low level		High level	
	Mean (%)	RSD (%)	Mean (%)	RSD (%)	Mean (%)	RSD (%)	Mean (%)	RSD (%)
EME	111.9	4.5	93.8	0.8	110.3	4.2	104.9	3.7
MOR	108.7	7.8	93.4	6.3	97.7	16.1	101.3	1.5
COD	108.2	3.3	94.2	5.3	100.7	4.2	101.9	2.0
AMP	108.1	5.4	93.3	3.7	93.0	0.5	100.8	1.7
6-MAM	105.8	2.8	98.7	2.0	98.0	8.2	104.5	4.8
mAMP	112.3	1.0	92.9	0.2	100.6	0.8	104.3	0.8
MDMA	105.2	3.9	96.0	2.0	100.3	3.5	100.9	1.9
BE	109.2	3.6	91.7	2.6	100.9	0.2	100.1	1.0
MDEA	106.1	9.2	93.8	4.7	100.6	0.0	99.3	4.4
COC	110.2	6.5	92.4	3.4	102.2	3.4	101.7	3.1
EDDP	108.5	1.8	91.8	1.4	101.6	1.1	99.6	0.3
MTD	106.1	3.2	93.7	2.4	97.9	4.3	99.4	0.8

Low level: 75 pg/mg; high level: 5000 pg/mg

Table 4.6: Overview of the results for matrix effect, recovery and process efficiency in nails.

Nails	Matrix effect				Recovery				Process efficiency	
	Low level		High level		Low level		High level		Low level	High level
	Mean (%)	RSD (%)	Mean (%)	RSD (%)	Mean (%)	RSD (%)	Mean (%)	RSD (%)	Value (%)	Value (%)
EME	95.4	8.2	97.2	6.9	97.5	1.8	106.4	4.4	97.4	104.0
MOR	103.0	4.9	83.9	3.6	103.1	2.2	89.8	7.6	101.1	87.5
COD	96.4	6.9	93.4	7.2	94.4	4.1	97.9	1.5	100.3	91.5
AMP	93.7	3.0	98.5	6.5	100.8	0.6	100.0	0.0	92.4	116.7
6-MAM	95.9	8.3	88.7	8.4	106.1	4.2	108.7	5.9	105.3	105.0
mAMP	101.6	3.1	116.3	5.2	98.7	0.9	94.5	4.0	103.0	120.1
MDMA	104.7	7.2	98.9	5.9	96.6	2.5	97.9	1.5	102.2	112.2
BE	104.5	7.1	101.9	4.3	98.6	1.0	98.9	0.8	119.0	109.8
MDEA	89.6	3.4	93.3	7.8	114.7	9.7	104.0	2.7	103.5	106.2
COC	107.6	6.1	100.2	4.5	95.8	3.0	95.9	3.0	105.1	106.4
EDDP	89.5	4.3	93.7	6.8	102.5	1.8	97.3	2.0	91.2	100.5
MTD	103.0	5.5	99.9	8.3	99.4	0.4	101.9	1.3	103.1	110.5

Low level: 100 pg/mg; high level: 5000 pg/mg

4.1.3.3. Method application

4.1.3.3.1. Participation to proficiency tests DHF 3/15 and DHF 2/16

By means of participating to proficiency tests, laboratories can assess and control the accuracy of their measurements and get an idea regarding their own analytical performance. Together with 77 other laboratories, our laboratory participated to the proficiency tests DHF 3/15 and DHF 2/16 in which each time two hair samples provided by Arvecon GmbH were analysed with the optimized and validated method. The z-scores were calculated by means of the standard deviation according to Horwitz, using the following formula.

$$z - score = \frac{individual\ result - target\ value}{standard\ deviation\ according\ to\ Horwitz}$$

Concentrations of all compounds were within the acceptance range for a successful participation (|z-score|≤ 2) except for EDDP, for which the Z-score was -3.00 (DHF 3/15) and -2.37 (DHF 2/16).

4.1.3.3.2. Application to real samples

After validation, authentic hair (n = 11) and nail (n = 5) samples were analysed with the described method to demonstrate its applicability. Table 4.7 presents the results obtained from the hair and nail samples, while Figure 4.4 shows a chromatogram from a subject. The concentrations were in accordance with previously reported concentrations in hair from regular drug users (Fernandez et al. 2014, Imbert et al. 2014). The results of the analysis of nail samples were in agreement with data available from a comprehensive self-report for each subject (Table 4.7).

Table 4.7: Reported consumption and concentrations (pg/mg) detected in hair samples from forensic and toxicological cases, and in nail samples from patients engaged in treatment for substance use disorders.

Matrix	Subject nr.	Compound concentration (pg/mg)												Reported consumption
		EME	MOR	COD	AMP	6-MAM	mAMP	MDMA	BE	MDEA	COC	EDDP	MTD	
Hair	1		45		151 000	140	360		183		1167			Heroin, cocaine, amphetamine
	2	58			339			501	839		8270			Cocaine, amphetamine, MDMA
	3	27							81		499			Cocaine
	4							224	70		282			Cocaine, MDMA
	5								122		789			Cocaine
	6				102			1150						Amphetamine, MDMA
	7										296			Cocaine
	8		1769	612		1880					90	254	4012	Heroin, cocaine, methadone
	9	230						125	1210		9590			Cocaine, MDMA
	10				2926				683		3910			Cocaine, amphetamine
	11	64			2140			86	772		3320			Cocaine, amphetamine, MDMA
Nails	12				74			326	2450		283			Cocaine, amphetamine, MDMA
	13				5695				473		829			Cocaine, amphetamine
	14				287				793		126			Cocaine, amphetamine
	15	311			10504				22421		11811			Cocaine amphetamine
	16		124					50	107		51	131	1951	Cocaine, methadone

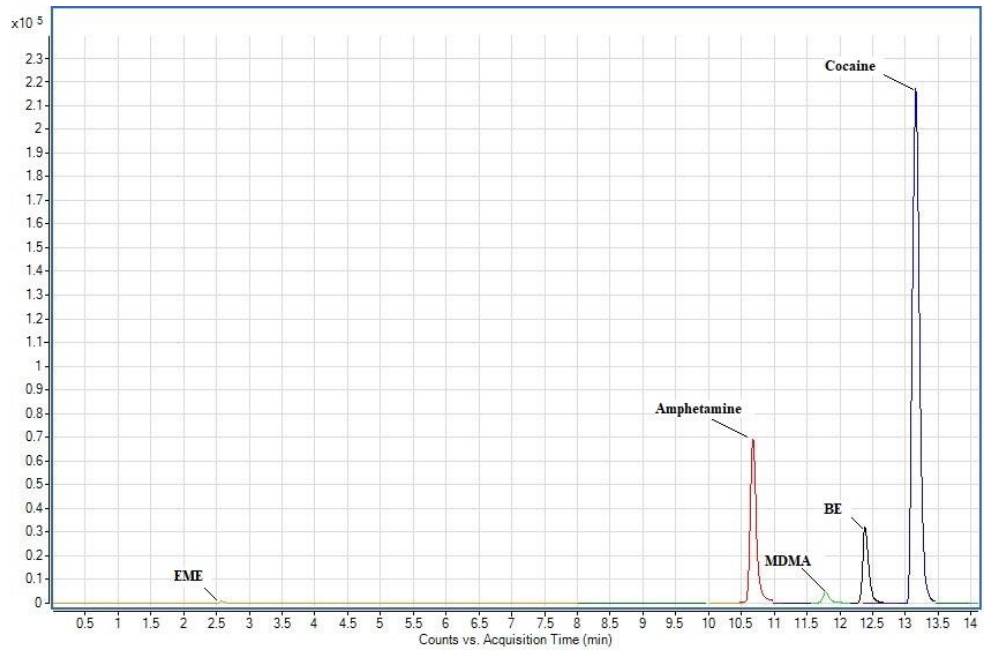

Figure 4.4: Chromatogram of the hair sample from subject 11. The peaks of the quantifier transitions of the detected compounds are shown.

4.1.4. Conclusions

A LC-MS/MS method for the simultaneous determination of nine widely used drugs of abuse and their relevant metabolites in hair and nails was developed and fully validated according to the EMA guidelines. Specificity, LLOQ, linearity, accuracy, precision, carry-over, matrix effects, recovery, and process efficiency were assessed. Important advantages of the developed method are the easy and straightforward sample preparation, the simultaneous detection of several drugs with different physicochemical properties, the LLOQs below the recommended SoHT cut-off concentrations, and the applicability to both hair and nails. Future work will apply this method in correlating paired hair and nail samples which can aid in defining cut-off levels for nail analysis.

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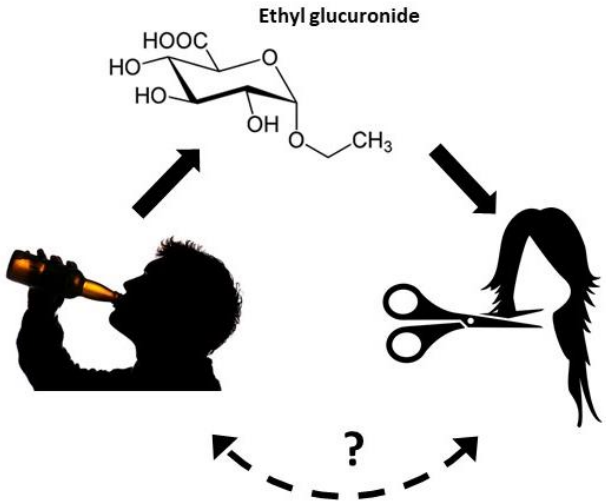
PART II:

CORRELATIONS FOR BIOMARKERS OF ALCOHOL AND DRUGS IN HAIR AND NAILS

CHAPTER 5:

CORRELATIONS BETWEEN ALCOHOL CONSUMPTION AND ETHYL GLUCURONIDE CONCENTRATIONS IN HAIR



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Crunelle CL, **Cappelle D***, Yegles M, De Doncker M, Michielsen P, Dom G, van Nuijs ALN, Maudens KE, Covaci A and Neels H. Ethyl glucuronide concentrations in hair: a controlled alcohol-dosing study in healthy volunteers. *Analytical and Bioanalytical Chemistry* 408(8) (2016) 2019-2025. ***Shared first co-author**

Crunelle CL, **Cappelle D**, Flamand E, Cox J, Covaci A, De Doncker M, van Nuijs ALN, Michielsen P, Yegles M and Neels H. Ethyl glucuronide in hair of non-excessive alcohol consumers: correlations and gender influence. *Forensic Toxicology* 34(1) (2016) 186-190.

Crunelle CL, **Cappelle D**, Covaci A, van Nuijs ALN, Maudens KE, Sabbe B, Dom G, Michielsen P, Yegles M and Neels H. Hair ethyl glucuronide as a biomarker of alcohol consumption in alcohol-dependent patients: Role of gender differences. *Drug and Alcohol Dependence* 141 (2014) 163-166.

5.1. Introduction

Ethyl glucuronide (EtG) is a minor phase II metabolite of alcohol that incorporates in hair after alcohol consumption. In contrast to traditional alcohol markers, such as carbohydrate-deficient transferrin (CDT), EtG is a direct marker of alcohol that is exclusively produced upon alcohol consumption (Hoiseth et al. 2009). Hair provides an easy and non-invasive matrix for stable accumulation of compounds over extended periods of time. Thus, EtG in hair is proposed as a reliable marker to quantify the retrospective consumption of alcohol over a relatively long period of several months (Palmer 2009). According to the Society of Hair Testing (SoHT) guidelines, an EtG concentration > 7 pg/mg hair strongly suggests repeated alcohol consumption, and a concentration > 30 pg/mg hair indicates excessive chronic alcohol consumption of > 60 g per day over a period of several months (Society of Hair Testing 2016).

Correlations between drinking behaviour and EtG concentrations in hair have been reported but large variability exists. In addition, few studies have reported on the correlation between EtG in hair and non-excessive alcohol consumption. This chapter addresses those issues. First, by performing a prospective controlled alcohol-dosing study in healthy volunteers. Secondly, by investigating the influence of gender on the correlation between alcohol consumption and EtG concentrations in hair of non-excessive alcohol consumers and alcohol-dependent patients.

5.2. Ethyl glucuronide concentrations in hair: a controlled alcohol-dosing study in healthy volunteers

5.2.1. Preface and aims

In humans, using self-report questionnaires and medical records, correlations between EtG concentrations in hair and alcohol consumption have been reported (Politi et al. 2006, Appenzeller et al. 2007a, Kerekes et al. 2009, Crunelle et al. 2014a). Though, in participants consuming either 16 g or 32 g daily alcohol, hair EtG concentrations were detected in only 24% of alcohol consumers (Kronstrand et al. 2012). These studies show important inter-individual variability in hair EtG concentrations even when using data normalized for body weight (Politi et al. 2006) and for participants with similar alcohol consumption reports (Crunelle et al. 2014a). This variability in hair EtG concentrations may be related to EtG incorporation or accumulation in hair (e.g., cosmetic hair treatments (Kerekes et al. 2013)), but could also be related to differences in alcohol consumption profiles or due to the inaccurate reporting of alcohol consumption prior to hair sample collection. Evidence for the presence of a linear correlation between hair EtG concentrations and ingested amounts of ethanol was also suggested from rat studies (Kharbouche et al. 2010, Malkowska et al. 2012), where the reporting of alcohol consumption cannot influence correlation measures. However, the extrapolation of animal results to humans should be done with care: differences in metabolism and hair structure between rat and humans may have implications on the adsorption and accumulation of chemicals. This section presents the data of EtG concentrations in hair following the controlled consumption of 0, 100 or 150 g of alcohol per week in a randomized 12-week controlled alcohol-dosing study in human volunteers matched on age and gender. The primary research goal was to assess whether amounts of alcohol consumed correlated with EtG concentrations in hair. Additionally, the use of doses of alcohol reflecting a mean daily consumption of 1.5 to 2 standard alcoholic drinks allows determining whether the current applied cut-off value of 7 pg/mg hair is adequate to assess the regular consumption of low-to-moderate amounts of alcohol.

5.2.2. Materials and methods

5.2.2.1. Participants

A total of 30 participants were recruited through leaflets distributed at the University of Antwerp (Belgium), and through mouth-to-ear advertisement. Participants were included when aged between 20 – 70 years old, when consuming alcohol on a regular basis (between 2 and 10 alcohol units per week over the past six months), and when being able to understand study procedures and sign informed consent. Participants were excluded when: (i) having (a history of) alcohol or other drug abuse or dependence according to DSM-IV criteria, (ii) using pharmacotherapy that could influence the effects of alcohol, alcohol metabolism or alcohol excretion in the past 30 days, (iii) having a severe illness (diseases of the gastro-intestinal system, and/or kidneys, cardiovascular disease) or (a history of) psychiatric treatment that could make participation in the study hazardous, or (iv) having any contra-indication for the use of alcohol (e.g., hypertension, pregnancy). The Alcohol Use Disorders Identification Test (AUDIT) was used to assess harmful and hazardous alcohol use. The short version of the Drug and Alcohol Screening Test (DAST) was used to additionally assess harmful alcohol or drug use. The study was performed according to the Helsinki Declaration on Scientific Research with Humans and approved by the Ethical Committee of the University Hospital of Antwerp (Belgian registration number B300201215554). Participants gave written informed consent.

5.2.2.2. Study design

The design was a 12-week controlled alcohol-dosing study with 30 age- and gender-matched healthy individuals assigned to one of three study conditions: consuming either no alcohol (N = 10), 100 g (N = 10) or 150 g (N = 10) of alcohol per week for 12 consecutive weeks. The first eight included participants were attributed to a study group based on their personal preference for one of the three study groups, after which other participants were matched for age and gender and attributed to one of the groups.

Participants consumed alcohol during five days per week with two alcohol abstinence days per week (NHMRC 2001), resulting in daily alcohol consumption of 20 and 30 g pure alcohol, respectively. The alcohol was provided to the participants as red wine from the same batch.

In three bottles, the alcohol concentration was measured with a validated analytical method based on head-space gas chromatography with flame ionization detection: 13.6 ± 0.3 (%, *v/v*). A glass with a measuring line was provided to the study participants to concur with the necessary daily volumes of wine (resp. 186 mL and 279 mL) that had to be consumed.

To control for study compliance, urine samples were collected every three days and EtG in urine was measured using a liquid chromatography tandem mass spectrometry method (LC-MS/MS) (adapted from (Beyer et al. 2011)). Briefly, 20 µL of EtG-D_5 20 µg/mL (internal standard) and 360 µL of mobile phase A were added to 20 µL of urine sample. After vortexing and centrifugation 100 µL of the upper layer was transferred to a LC vial. Ten microliters were then injected on an Agilent 6430 LC-MS/MS system equipped with an electrospray interface operating in negative mode. The column used was a Luna® Phenyl-Hexyl column (150 mm x 4.6 mm, 5 µm; Phenomenex) operated at a flow rate of 0.6 mL/min with a total run time of 8 min. A linear gradient consisting of mobile phase A (5 mM aqueous ammonium acetate adjusted to pH 5.6) and mobile phase B (methanol) was used. The lower limit of quantification (LLOQ) of the method was 0.1 µg/mL.

At study start, hair samples were collected by fixing approximately 100 mg of hair at the vertex posterior region. The samples were cut as close as possible to the scalp and the proximal 3 cm lengths were analysed to assess pre-study alcohol consumption. Following the 12-week alcohol-dosing regimen, the newly grown hairs at that location (a total length of about 3 cm) were cut and analysed for concentrations of EtG. This approach minimizes the influence of EtG content that might have been present pre-study in the 6 – 18% non-growing hairs (in catagen and telogen phase; (Pragst et al. 2006)) and reduces the error on the segmentation process when hair growth would not have been exactly 3 cm (i.e., 1 cm/month). Hair bleaching, permanent colouring, hair perming and hair straightening are proposed to affect hair EtG content and were therefore not allowed during the study period (Crunelle et al. 2015). Hair melanin content does not influence the incorporation of EtG in hair (Appenzeller et al. 2007b). Therefore, no inclusion criteria were set regarding hair colour. Participants were instructed not to go to the hairdresser during the study period.

5.2.2.3. Ethyl glucuronide in hair

Sample preparation was as described in section 3.1.2.2.4. Briefly, hair samples were decontaminated with water and acetone and pulverized using a ball mill (Type MM2, Retsch GmbH & Co. KG, Haan, Germany). About 30 mg of pulverized hair was carefully weighted and used for further analysis. Samples were extracted with 2 mL of ultrapure water during 1.5 h ultrasonication, followed by solid-phase extraction on Oasis® MAX cartridges, evaporation of the eluate and derivatisation with heptafluorobutyric anhydride (HFBA). Quantification was performed using gas chromatography-tandem mass spectrometry with negative ion chemical ionization (GC-NICI-MS/MS; Agilent 7000C). The method was linear in the range from 2 to 400 pg/mg hair, and had a limit of detection (LOD) of 0.05 pg/mg and a LLOQ of 0.2 pg/mg. For a full description and validation of the analytical method, see section 3.1.2 (Cappelle et al. 2015a). The EtG concentrations obtained from the hair samples collected at 12 weeks were the primary outcome measure.

5.2.2.4. Demographic variables and questionnaires

The variables assessed included age and gender (for matching purposes), body mass index (BMI), pre-study alcohol consumption, and hair treatment (hair colour, pre-study cosmetic treatments) using an in-house questionnaire. The Timeline Follow-Back method (TLFB; (Sobell et al. 1992)) was used to keep track of daily alcohol consumption during the 12-weeks trial, as a way of monitoring the obligatory alcohol-free days per week. Regular telephone calls and house visits were made to increase study compliance.

5.2.2.5. Data analysis

Data were assessed on normality using Shapiro-Wilk tests. Differences between the three groups on hair EtG concentrations, demographic variables, and questionnaires were analysed using repeated measures ANOVA tests or Kruskal-Wallis tests for nonparametric data in SPSS version 22 (Statistical Package for the Social Sciences). Statistically significant differences between two groups were addressed post-hoc (using t-tests for parametric and Mann-Whitney U tests for nonparametric data). A Bonferonni correction for multiple testing was

applied. According to the distribution of the data, correlations between EtG measures in hair and alcohol (volume) intake was assessed using nonparametric correlation analysis (Spearman's rho). Data are presented as mean ± standard error (SE) or as median ± interquartile range (IQR) where appropriate, with a p-value < 0.05 considered statistically significant.

5.2.3. Results

5.2.3.1. Participants

Participants' characteristics are presented in Table 5.1. Participants were matched on age and gender. BMI, alcohol consumption in the 3 months prior to study start, hair colour and the occurrence of cosmetic hair treatments prior to study start did not differ between groups (see Table 5.1). Self-reported alcohol consumption in the prior 3 months ranged between 0 and 3300 g alcohol/3 months (see Table 5.1). Hair EtG concentrations in the hairs cut at study start were 2.7 ± 7.7 pg/mg (range 0 – 83.0 pg/mg; see Table 5.1) and correlated with the self-reported alcohol consumption of the past 3 months (Spearman's rho $r = 0.742$; $p < 0.001$). The DAST was negative (score 0) for all participants, indicating no harmful drug use. Mean AUDIT scores were 3.7 ± 2.6 and did not differ between groups (ANOVA $p = 0.234$).

Table 5.1: Baseline participants' characteristics across the three study groups of 12-week non-alcohol consumers (N = 10), 100 g alcohol per week consumers (N = 10) and 150 g alcohol per week consumers (N = 10).

	No alcohol (N = 10)	**100 g/week (N = 10)**	**150 g/week (N = 10)**	**p-value**
Age (years) [a]	44 ± 5	49 ± 5	46 ± 5	NS [a]
Gender (males/females) [a]	2/8	3/7	3/7	NS [a]
BMI	26 ± 1	25 ± 2	27 ± 1	NS
Pre-study alcohol consumption (g/3 months)	75 ± 314 [b] (range 0-1020)	415 ± 978 [b] (range 0-1040)	460 ± 1075 [b] (range 0-3300)	NS
Hair colour				NS
Blond (N)	4	4	2	
Brown (N)	5	3	5	
Red (N)	0	2	2	
Grey/white (N)	1	1	1	
Pre-study hair colouring (N)	2	3	3	NS
Pre-study EtG concentration (pg/mg hair)	0.9 ± 1.1 [b] (range <LLOQ-14.0)	3.4 ± 5.8 [b] (range <LLOQ-11.9)	7.4 ± 22.3 [b] (range 1.3-83.0)	0.003

[a] Age and gender were matched across groups
[b] Data are presented as mean ± standard error, or as median ± interquartile range
Abbreviations: BMI, body mass index; EtG, ethyl glucuronide; NS, non-significant; LLOQ, lower limit of quantification: 0.2 pg/mg

5.2.3.2. Hair EtG following 12-week controlled alcohol-dosing

EtG concentrations following the 12-week controlled alcohol intake are presented in Table 5.2. In the non-drinking group, median EtG concentrations were 0.5 pg/mg (IQR 1.7 pg/ mg; range <LLOQ – 4.5 pg/mg). In the group of participants consuming 100 g and 150 g alcohol per week, median EtG concentrations were 5.6 pg/mg (IQR 4.7 pg/mg; range 2.0 – 9.8 pg/mg) and 11.3 pg/mg (IQR 5.0 pg/mg; range 7.7 – 38.9 pg/mg), respectively. Hair EtG concentrations were significantly different between the three study groups of non-drinkers, 100 g per week alcohol consumers and 150 g per week alcohol consumers (Kruskal-Wallis $p < 0.001$; post-hoc 0 g vs. 100 g: $p = 0.001$; 100 g vs. 150 g: $p < 0.001$; 0 g vs. 150 g: $p < 0.001$; see Figure 5.1). In the group of participants consuming 150 g alcohol per week, one participant had a high EtG concentration of 38.9 pg/mg (Figure 5.1).

Urine EtG analysis revealed negative EtG concentrations in the non-drinkers, and provided evidence for alcohol consumption (however with large variation) in the alcohol consumers (see Table 5.2).

Table 5.2: EtG concentrations in the 0-3 cm hair segments of participants following a 12-week controlled alcohol dosing regimen (4th and 5th column). Urine EtG concentrations, not corrected for creatinine, are also provided (last column), as well as pre-study alcohol consumption (mean weekly consumption for comparison purposes; 2th column), and pre-study hair EtG results (3th column).

Participant nr.	Pre-study alcohol consumption (g/week)	Pre-study hair EtG (pg/mg)	Controlled study alcohol consumption (g/week)	Post-study hair EtG (pg/mg)	Urine EtG during controlled alcohol intake (ranges) (µg/mL)
#1 (♀, 28)	10	2.2	0	0.9	< 0.1
#2 (♀, 32)	85	14.0	0	4.5	< 0.1
#3 (♂, 35)	<10	0.4	0	0.2	< 0.1
#4 (♂, 40)	0	< LLOQ	0	0.2	< 0.1
#5 (♀, 28)*	0	0.9	0	0.6	< 0.1
#6 (♀, 52)	<10	< LLOQ	0	0.2	< 0.1
#7 (♀, 41)	30	0.5	0	0.5	< 0.1
#8 (♀, 48)	<10	1.15	0	3.7	< 0.1
#9 (♀, 68)*	<10	0.8	0	< LLOQ	< 0.1
#10 (♀, 64)	30	1.1	0	1.4	< 0.1
#11 (♂, 52)	0	< LLOQ	100	9.4	< 0.1 – 5.1
#12 (♀, 35)*	40	6.3	100	5.5	< 0.1 – 3.8
#13 (♂, 52)	80	3.6	100	3.2	n.a.
#14 (♀, 68)*	85	4.2	100	2.8	< 0.1 – 7.3
#15 (♀, 58)	85	11.9	100	5.6	< 0.1 – 9.0
#16 (♀, 31)	n.a.	2.6	100	2.7	< 0.1 – 33.1
#17 (♀, 41)*	25	1.0	100	9.8	< 0.1 – 27.8
#18 (♀, 64)	15	11.1	100	2.0	< 0.1 – 16.3
#19 (♂, 62)	n.a.	3.2	100	6.9	n.a.

* Participants who coloured or cosmetically treated their hair pre-study

Abbreviations: EtG, ethyl glucuronide; LLOQ, lower limit of quantification: 0.2 pg/mg; n.a., not available

Table 5.2: Continued.

Participant nr.	Pre-study alcohol consumption (g/week)	Pre-study hair EtG (pg/mg)	Controlled study alcohol consumption (g/week)	Post-study hair EtG (pg/mg)	Urine EtG during controlled alcohol intake (ranges) (µg/mL)
#20 (♀, 30)	0	1.9	100	6.8	n.a.
#21 (♀, 59)*	20	1.8	150	10.5	< 0.1 – 59.5
#22 (♀, 66)*	75	5.5	150	7.7	< 0.1 – 60.5
#23 (♀, 29)	0	1.3	150	11.7	n.a.
#24 (♀, 45)	60	7.9	150	10.9	< 0.1 – 84.2
#25 (♀, 42)	275	83.0	150	38.9	< 0.1 – 61.9
#26 (♂, 49)	n.a.	30.3	150	10.1	< 0.1 – 33.5
#27 (♀, 31)	35	23.0	150	12.9	< 0.1 – 30.2
#28 (♀, 49)*	40	2.8	150	14.8	n.a.
#29 (♂, 23)	30	21.3	150	15.2	< 0.1 – 89.6
#30 (♂, 62)	150	6.9	150	9.3	< 0.1 – 95.0

* Participants who coloured or cosmetically treated their hair pre-study

Abbreviations: EtG, ethyl glucuronide; LLOQ, lower limit of quantification: 0.2 pg/mg; n.a., not available

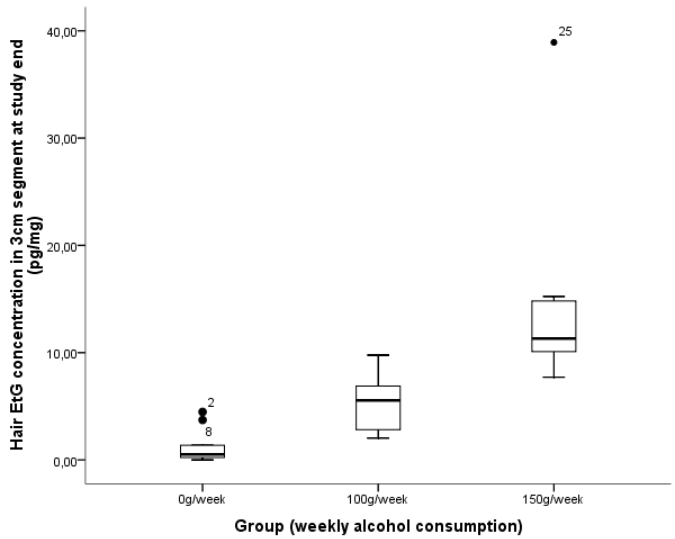


Figure 5.1: Hair ethyl glucuronide concentrations (pg/mg hair) of participants consuming no alcohol (N = 10), 100 g alcohol per week (N = 10) or 150 g alcohol per week (N = 10) for 12 consecutive weeks.

5.2.4. Discussion

This section presents findings of hair EtG concentrations following a controlled alcohol-dosing study in 30 healthy volunteers matched on age and gender, consuming either 0 , 100 or 150 g of pure ethanol per week. The latter two groups represent an average daily consumption of 1.5 and 2 alcoholic drinks over several months, being a good representation of daily low-to-moderate-alcohol consumption.

The results provide evidence that EtG concentrations in hair increase with increasing doses (amounts) of alcohol consumed, and that a distinction can be made in terms of amounts of alcohol consumed and abstinence, even when low-to-moderate doses of alcohol are consumed. However, still some variation was noted: in participants consuming no alcohol, all but one had very low, but measurable (above the LLOQ of 0.2 pg/mg), EtG concentrations while having consumed no alcohol in the prior months. As the method used in this paper (GC-MS/MS) is an outmost sensitive one, especially in the lower concentration ranges, these EtG concentrations might not have been detected using other methods for the detection of EtG in hair, such as GC-MS and LC-MS/MS with higher LLOQs. Methods with low LLOQs are published by us and other groups (Crunelle et al. 2014b, Cappelle et al. 2015b), and improve the interpretation of results closer to the low cut-off values. With increased sensitivity of the method comes a better assessment at lower concentrations, resulting in lower alcohol use that can be detected as shown in this section.

One controlled study in humans reported on 44 participants that consumed 16 g or 32 g red wine (calculated according to the concentration provided on the wine label) daily for three months, but EtG concentrations were detectable in only 5 of 21 alcohol consumers (Kronstrand et al. 2012). In that study, all females drank 16 g alcohol per day, while all males drank 32 g alcohol per day, providing a gender bias. Methodologically, low sample weights between 7 and 26 mg hair were used for analysis, and cut hair samples were used instead of pulverized hair, such that EtG from the hair matrix would not have been extracted optimally (Albermann et al. 2012), which may have contributed to the relatively high number of non-detects**.**

The presence of low amounts of EtG was however noted in abstinent participants. Because hair samples were cut prior to the study start, and the newly re-grown hairs were cut to be

analysed at study end, the impact of possibly alcohol-containing non-growing hairs present pre-study was minimised. However, below the scalp surface, still a minimal amount of hair remains, which might explain the presence of EtG concentrations in the group of participants consuming no alcohol during the study. We suggest this could be the case for the outlier #2 and #25, who both consumed relatively large amounts of alcohol pre-study (respectively 1020 g and 3300 g pure alcohol in the three months prior to study inclusion). Other possibilities would include non-reported (additional) consumption of alcohol during the study or intake through food. Study adherence is thereby an important factor to take into consideration in lengthy controlled alcohol-dosing human studies. An attempt was made to improve study compliance through the monitoring of urine EtG, although it would have been more interesting to control study compliance by measuring creatinine-corrected urine EtG concentrations. Also, a possible endogenous presence of EtG cannot be excluded and should be investigated further.

In all participants consuming no alcohol, all hair EtG values were below 4.5 pg/mg. In participants consuming 100 g pure alcohol per week for 3 months, EtG concentrations lower than 7 pg/mg were still observed in 8 out of 10 participants (between 2.7 and 6.9 pg/mg). This raises questions whether the 7 pg/mg hair cut-off value proposed by the SoHT to assess repeated alcohol consumption might not be too high. In light of the obtained results, we suggest the cut-off should be re-evaluated and perhaps be lowered. This deserves further research on a large series of teetotallers.

In support of earlier studies (Appenzeller et al. 2007a, Kerekes et al. 2009, Crunelle et al. 2014a, Crunelle et al. 2014b), the analyses of the hairs cut pre-study showed a strong positive correlation between hair EtG values in the proximal 0-3 cm hair segment and the amounts of alcohol consumed in the past three months before the study.

In this study, participants were well characterized on the volumes of alcohol consumed prior to study start using a validated questionnaire (TLFB) and were of similar BMI. Also hair colour and pre-study cosmetic treatments were mapped in detail and all were matched on gender and age, such that gender-bias was kept to a minimum. For reasons of feasibility and ethics, only a limited number of dosing groups were investigated.

5.2.5. Conclusions

Concentrations of EtG in hair correlate with the amounts of alcohol consumed, and can be used to differentiate between repeated (low-to-moderate) amounts of alcohol consumed over a long time period. For the assessment of repeated alcohol use, we propose that the current cut-off of 7 pg/mg should be re-evaluated.

5.3. Ethyl glucuronide in hair as a biomarker of alcohol consumption: correlations and gender differences

5.3.1.1. Preface and aims

Some studies showed a correlation between EtG concentrations in hair and amounts of alcohol consumed (Politi et al. 2006, Appenzeller et al. 2007a, Kerekes et al. 2009), while other studies found no or modest correlations (Yegles et al. 2004, Stewart et al. 2013). The lack of correlation between EtG concentrations in hair and amounts of alcohol consumed could be explained by several factors, such as hair treatments, pathophysiological conditions, or factors underlying alcohol metabolism such as age and gender. Of these, gender can influence EtG incorporation in hair, but, in contrast to other variables, the influence of gender on measured EtG concentrations in hair has not been investigated. Upon similar alcohol consumption, females have higher blood alcohol concentrations (BAC) than males (Sutker et al. 1983), and presumably eliminate alcohol faster than males (Jones 2010), resulting in shorter but higher BAC peaks. As EtG is incorporated into hair through the blood supply, the question arises whether, as a result of differences in BACs, males and females differ in their EtG incorporation in hair.

This section summarizes the results of two retrospective studies which evaluated the correlation between alcohol consumption and EtG concentrations in hair, and the influence of gender on this correlation. The first study was performed in non-excessive alcohol consumers (< 60 g/day), while the second was done in alcohol-dependent patients.

5.3.1.2. Methods

EtG was measured by gas chromatography coupled to tandem mass spectrometry in the hair (first 3 cm) of 130 non-excessive (< 60 g/day) alcohol consumers, and 36 alcohol-dependent patients (25 males/11 females) starting an alcohol detoxification program (Kerekes et al. 2009, Kerekes et al. 2013). Factors that could possibly influence EtG content in hair (except age and gender) were excluded. Daily alcohol consumption over a period of three months prior to hair collection was assessed using the Timeline Follow-Back interview (Sobell et al. 1992).

5.3.1.3. Results

Non-excessive alcohol consumers showed large variation in consumption, ranging from 10 g (~1 unit) to 3300 g (~330 units), during the prior three months. Eight individuals abstained from alcohol and served as a control group. EtG concentrations varied from < LLOQ to 29.8 pg/mg, and showed a linear correlation with the amount of alcohol consumed ($r = 0.81$; $p < 0.001$). Gender had no influence on this correlation ($p = 0.113$).

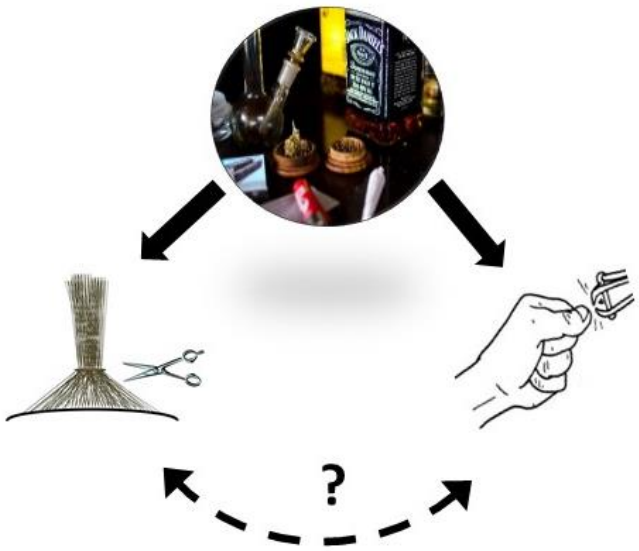
In alcohol-dependent patients, median total alcohol consumption over three months was 13,050 g pure alcohol (range 60–650 g/day). EtG concentrations in hair varied between 32 and 662 pg/mg. There was a statistically significant linear and positive correlation between EtG concentrations in hair and amounts of alcohol consumed (Pearson $r = 0.83$; $p < 0.001$), in both males (Pearson $r = 0.83$; $p < 0.001$) and females (Pearson $r = 0.76$; $p = 0.007$). There was no effect of gender on the correlation between EtG concentrations in hair and amounts of alcohol consumed ($p = 0.947$).

5.3.1.4. Conclusions

In both non-excessive alcohol consumers and alcohol-dependent patients, there is a positive linear correlation between the amount of alcohol consumed in the last three months and the concentrations of EtG in hair. Gender has no influence on EtG concentrations in hair, such that the interpretation of EtG results in hair can occur independently of gender, and using the same cut-offs for male and female subjects.

CHAPTER 6:

CORRELATIONS BETWEEN HAIR AND NAILS



Based on the following papers:

Cappelle D, Neels H, De Keukeleire S, Fransen E, Dom G, Vermassen A, Covaci A, Crunelle CL and van Nuijs ALN. Ethyl glucuronide in keratinous matrices as biomarker of alcohol use: a correlation study between hair and nails. *Forensic Science International* 279 (2017) 187-191.

Cappelle D, De Keukeleire S, Neels H, Been F, De Doncker M, Dom G, Crunelle CL, Covaci A and van Nuijs ALN. Keratinous matrices for the assessment of drugs of abuse consumption: a correlation study between hair and nails. *Drug Testing and Analysis* 10(7) (2018) 1110-1118.

6.1. Introduction

Hair and nails are capable of storing substances for extended periods of time, and can provide long-term information about exposure to and consumption of xenobiotics. Due to their keratinized nature, hair and nails have several characteristics in common but also differ in some aspects. Incorporation of compounds in hair and nails can occur via the bloodstream, the sebaceous and eccrine secretions, and external contamination. In nails, incorporation via blood occurs through a dual mechanism via the germinal matrix and the nail bed, while in hair it only takes place via the blood stream feeding the hair follicle. The growth rate of nails is not only slower than hair (3 mm/month for fingernails and 1 mm/month for toenails compared to 1 cm/month for head hair), but also occurs at a constant rate, while hair is characterized by a cyclic growth rate with different stages. In contrast to hair, nails lack melanin, which can influence the amount of incorporation of some compounds. As a result of these differences, a variation in the extent of incorporation between both matrices can be expected. Correlation studies comparing hair and nails are a good way to gain more information on the practical implications of these differences and are of great interest, but are very scarce.

This chapter investigates the correlations between hair, nails and the consumption of alcohol (6.2) and drugs of abuse (6.3). These correlation studies are particularly important to achieve more knowledge on the use of nails. In recent years, hair has gained importance as an alternative matrix, whilst nails have only been investigated scarcely. The Society of Hair Testing (SoHT) has established guidelines on how to analyse hair samples and interpret the obtained results. For several compounds in hair external quality control samples are available, and proficiency tests are being organized yearly. For nails, no guidelines, external quality controls or proficiency tests are available. Through the investigation of correlations between both matrices, differences and similarities between both matrices can be identified, and the possibilities of nails as an alternative to hair can be discovered.

6.2. Ethyl glucuronide in keratinous matrices as biomarker of alcohol use: a correlation study between hair and nails

6.2.1. Preface and aims

Ethyl glucuronide (EtG) accumulates in keratinized matrices, such as hair and nails, where it remains detectable for several weeks to months depending on the length of the hair or nail (Daniel Iii et al. 2004). Over the past years, the monitoring of alcohol consumption through the detection of EtG in hair has been widespread, since EtG in hair has proved to be a reliable specific and sensitive long-term biomarker for the detection of alcohol consumption (Morini et al. 2009, Crunelle et al. 2014b). The SoHT has stated its confidence in EtG as a marker for alcohol and has established guidelines and cut-off values for EtG concentrations in hair (Kintz 2012). An EtG concentration in hair of > 30 pg/mg suggests chronic excessive alcohol consumption, a concentration between 7 and 30 pg/mg corresponds to moderate alcohol consumption, and an EtG concentration of < 7 pg/mg does not contradict self-reported abstinence (Society of Hair Testing 2016).

Research has primarily focused on the detection of EtG in hair, whereas studies on its measurement in nails are sparse (Morini et al. 2012, Keten et al. 2013). However, nail analysis offers multiple advantages over hair analysis (Baumgartner 2014, Cappelle et al. 2015c). First, nails grow slower than hair (3 mm/month for fingernails and 1 mm/month for toenails compared to 1 cm/month for head hair), and would thus allow a more significant accumulation of EtG (Gupchup et al. 1999). This can be relevant in situations where low EtG concentrations need to be determined, e.g., for the differentiation between teetotallers and moderate alcohol consumers. Second, nails can provide an alternative option in cases where hair is not (sufficiently) available (e.g., alopecia, newborns). Third, cosmetic hair treatments have been proven to reduce EtG content in hair, which would be avoided through nail analysis (Palmeri et al. 2000, Cappelle et al. 2015c). Possible disadvantages of nails can be insufficient sample amount in cases of (finger)nail biting, presence of nail diseases (e.g. bacterial or fungal infections), and effects of nail polishing or cleaning.

To the best of our knowledge, only two studies compared the presence of EtG in hair and nails (Jones et al. 2012, Berger et al. 2014). The studies included hair and fingernails collected from a group of college-students with a limited alcohol consumption. Only Berger et al. 2014

considered cosmetic treatment of hair and nails. In this section, we assess EtG concentrations in paired hair and nail samples (both finger- and toenails), from alcohol-dependent patients, to investigate the differences and correlations between both matrices. In this way, we evaluate the applicability of nail samples as an alternative to hair for the monitoring of long-term alcohol consumption.

6.2.2. Materials and methods

6.2.2.1. Reagents, standard solutions and materials

Reagents and materials were as described under 3.1.2.2.1 and 3.1.2.2.2. Pentafluoropropionic anhydride (PFPA), obtained from Sigma Aldrich (Bornem, Belgium), was used as derivatisation agent instead of HFBA.

6.2.2.2. Sample collection

Hair and nail samples were collected from patients engaged in treatment for alcohol use disorders at the psychiatric centres of ZNA Stuivenberg and Multiversum together with an informed consent. The Ethical Committee of the University Hospital of Antwerp and the local Ethical Committees of ZNA Stuivenberg and Multiversum approved the study (Belgian registration number B30020169233). Hair samples were collected from the vertex posterior region of the head and cut as closely to the scalp as possible. The first 3 cm segment from the proximal end was used for further analysis. Nail samples were obtained by clipping of the distal edges of all ten finger- and toenails. Finger- and toenail samples were collected and analysed separately. Samples were stored in aluminium foil at room temperature until analysis.

6.2.2.3. Data on alcohol consumption

For all patients, the diagnosis of alcohol use disorder was made by the treating psychiatrist. Together with the hair and nail samples, a detailed anamnesis of past alcohol consumption was taken. Patients were asked about their alcohol consumption in the past 12 weeks using either the Timeline Follow-Back (TLFB) method or an in-house questionnaire. The total amount of alcohol (in grams) consumed in the past 12 weeks, and per week, was calculated for each subject.

6.2.2.4. Data on cosmetic treatment

Patients were asked whether or not they had bleached, dyed, permed or thermally straightened their hair in the past year, as cosmetic hair treatment may lead to a degradation or removal of EtG in hair (Morini et al. 2010, Society of Hair Testing 2016). Nail polishing, the use of acetone and other nail treatment were also recorded. Patients reporting any cosmetic treatment of the hair or nails were excluded from the study.

6.2.2.5. Sample preparation and instrumental analysis

The sample preparation and the gas chromatography coupled to tandem mass spectrometry (GC-MS/MS) analysis were performed according to previously described methods (see 3.1.2.2.4 and 3.1.2.2.5) (Cappelle et al. 2015b, Cappelle et al. 2016).

Briefly, samples were washed with water and acetone, dried, and pulverized in a ball mill. Then, an accurately weighed portion between 15 and 35 mg of each sample was spiked with EtG-D_5 as internal standard, and the samples were ultrasonicated for 2 h in water. Next, samples were centrifuged and solid-phase extraction was performed using Oasis® MAX (60 mg, 3 mL) cartridges. The eluate was evaporated to dryness under a nitrogen gas stream. The residues were derivatised with PFPA, dried under a nitrogen gas stream, and then reconstituted in ethyl acetate. Finally, 1 µL was injected into the GC-MS/MS system.

The gas chromatographic system consisted of a 7890A gas chromatograph, equipped with an automatic injector AS 7693 and coupled to a 7000C triple quadrupole mass spectrometer

(Agilent Technologies, Waldbronn, Germany). Chromatographic separation was achieved on a HP-5 MS (5% phenyl methyl siloxane) column (15 m × 0.25 mm, 0.25 µm). The injector temperature was set at 150 °C. The carrier gas was helium with a flow rate of 1.2 mL/min. The oven was initially held at 100 °C for 2.25 min, heated to 170 °C at a rate of 20 °C/min and then programmed at a final temperature of 310 °C at 50 °C/min. The ionization source was operated in negative ion chemical ionization (NICI) mode at 150 °C. Data acquisition was performed in multiple reaction-monitoring (MRM) mode. The monitored ion transitions were *m/z* 496 → 163 (quantifier) and 347 → 163 (qualifier) for EtG, and *m/z* 501 → 163 for EtG-D_5.

6.2.2.6. Data analysis

Statistical analysis was performed using R (version 3.3.1., The R Foundation for Statistical Computing). Normality was tested through histograms and QQ-plots of the raw data. Due to non-normality of the data, further analyses were performed with non-parametric statistics. Differences in median concentrations were assessed using Wilcoxon signed rank test. The associations between hair and nails (finger- and toenails) were expressed using Spearman correlation. For the correlation between hair and fingernails, an 80% prediction interval was calculated. In addition, using the regression coefficients β0 and β1, the existing cut-off concentrations for EtG in hair (Society of Hair Testing 2016) were translated to preliminary cut-off concentrations in fingernails. For all statistical tests, a p-value < 0.05 was considered statistically significant. Results are presented as mean ± standard deviation (SD) or as median [interquartile range (IQR)].

6.2.3. Results

A total number of 45 patients engaged in treatment for alcohol use disorders were included in the study. Eleven participants were female and 34 participants were male. Patients were between 18 and 77 years old with a mean age of 45.3 ± 13.1 years. Nine patients provided hair, finger- and toenail samples, 32 patients provided hair and fingernail samples, and 4 patients provided hair and toenail samples. To collect information on past alcohol

consumption the TLFB method was used in 26 patients, while the in-house questionnaire was used in 19 patients. Median alcohol consumption in the past 12 weeks was 11507 g (IQR [5475; 18927]). Median alcohol consumption per week was 959 g (IQR [456; 1577]).

6.2.3.1. Comparison of medians

Hair EtG concentrations ranged from < LLOQ to 1149 pg/mg (median = 164 pg/mg, IQR [42; 283]). Fingernail EtG concentrations ranged from < LLOQ to 4090 pg/mg (median = 250 pg/mg, IQR [74; 645]). Toenail EtG concentrations ranged from 127 to 3792 pg/mg (median = 687 pg/mg, IQR [379; 1370]). The differences in median EtG concentrations between paired hair and fingernail samples, and between paired hair and toenail samples were significant (p-values < 0.001).

6.2.3.2. Correlations

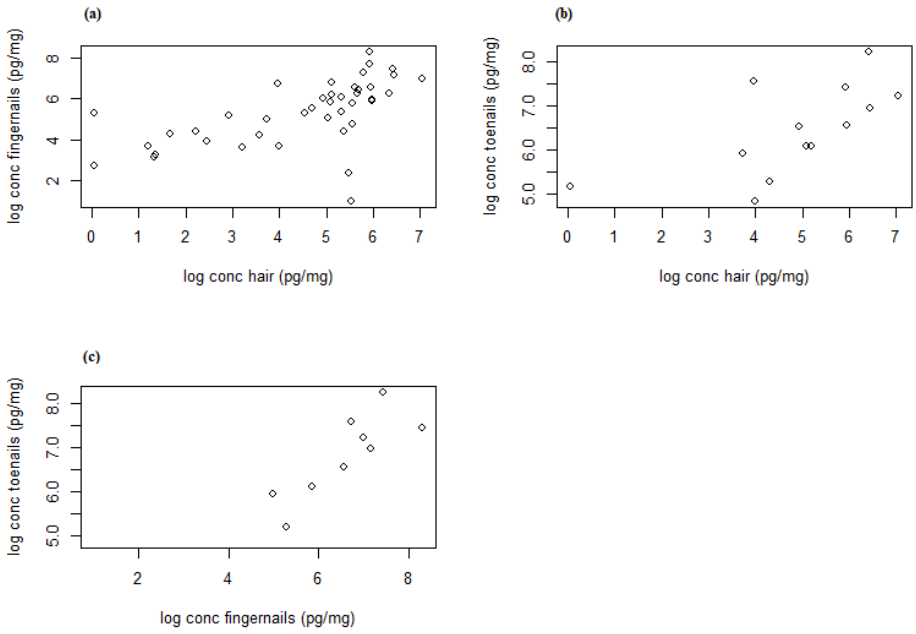


Figure 6.1: Matrix of scatterplots showing the different correlations in EtG concentrations investigated: (a) hair versus fingernails, (b) hair versus toenails, and (c) fingernails versus toenails. The values for the variables were log-transformed.

Figure 6.1 displays the scatterplots of the three correlations. Two of these were statistically investigated, and were found positive and significant. The Spearman-correlation coefficient between EtG concentrations in hair and in fingernails was 0.70 (p-value < 0.001, β0 = 3.11, β1 = 0.50, see Figure 6.2). For this correlation, the 80 % prediction interval was calculated (see Figure 6.3). This interval around the regression line contains 80% of the individuals' observations, which means that for a given EtG concentration in hair, the prediction interval delineates the range in which the corresponding EtG concentration in fingernails will be situated for 80% of the individuals. Thus, the prediction interval in combination with the regression coefficients β0 and β1 enables to make some preliminary predictions of cut-off concentrations for fingernail analysis based on those that exist for hair analysis (see Table 6.1). The correlation coefficient between EtG concentrations in hair and toenails was 0.62 (p-value = 0.02, β0 = 4.96, β1 = 0.36).

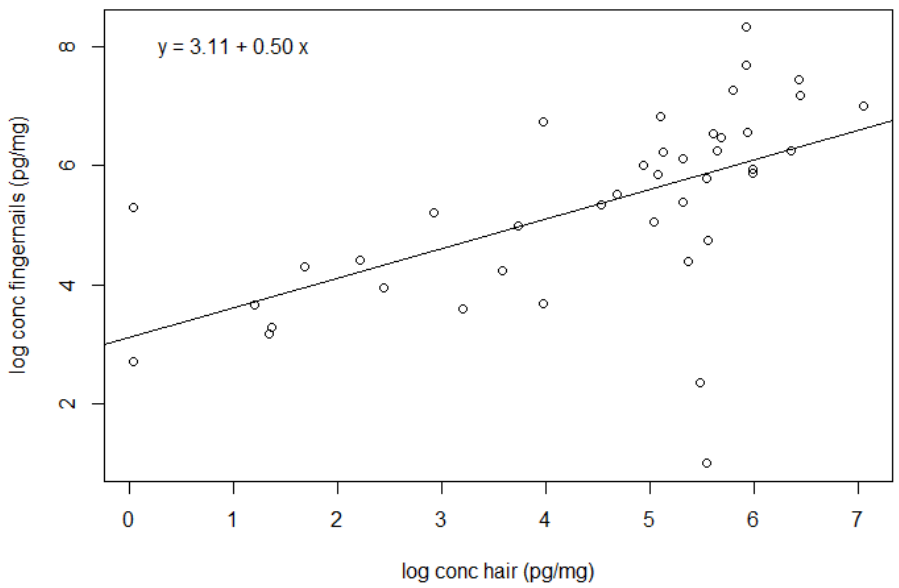


Figure 6.2: Correlation between EtG concentrations in hair and fingernail samples. The values for both variables were log-transformed.

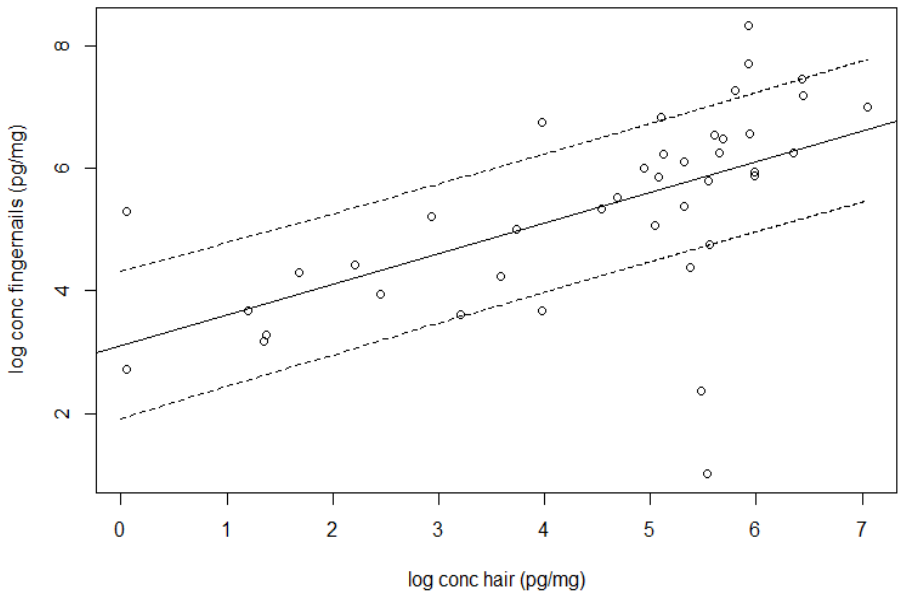


Figure 6.3: Plot of the log-transformed hair concentrations versus log-transformed fingernail concentrations. The solid line shows the linear regression line. The dashed lines show the 80% prediction interval, which is the interval around the regression line containing 80% of the individuals' observations.

Table 6.1: Preliminary predictions of cut-off values for fingernail analysis based on those that exist for hair analysis.

	Cut-off value in hair	Predicted cut-off value in fingernails
Excessive alcohol consumption	> 30 pg/mg	> 123 pg/mg
Moderate alcohol consumption	7-30 pg/mg	59-123 pg/mg
Alcohol abstinence	< 7 pg/mg	< 59 pg/mg

6.2.4. Discussion

Previous research has indicated that there is a medium to strong correlation between alcohol consumption and the concentration of EtG in hair (Crunelle et al. 2014a, Crunelle et al. 2016a, Crunelle et al. 2016b). EtG concentrations in the proximal 3 cm of head hair reflect the alcohol consumption over the past 3 months. Therefore, EtG in hair is used as a long-term marker for alcohol consumption and cut-off values have been proposed by the SoHT to discriminate

between abstinence, social, and excessive drinking. This section investigates the correlation of EtG concentrations in two keratinous matrices: hair and nails. Our results confirm that there is a significant and positive correlation between EtG concentrations in hair and in nails (both finger- and toenails). In addition, median concentrations are significantly different between hair and nails (both finger- and toenails). These findings are in agreement with those reported in earlier studies by Jones et al. and Berger et al. (Jones et al. 2012, Berger et al. 2014) and suggest that nails can be used as an additional and/or alternative matrix for long-term alcohol consumption monitoring.

Concentrations in nails were higher compared to hair, which might be attributed to the slower growth rate of nails (Pragst et al. 2006, Cappelle et al. 2015c)), resulting in higher accumulation of EtG in nails. This observation has two important implications: (i) analysis of nails can be interesting when low concentrations of EtG are expected, i.e. in the case of discrimination between teetotallers and social drinkers; and (ii) cut-off values as established for hair analysis by the SoHT are not valid for nail analysis and future studies should focus on establishing cut-off values for EtG which are relevant for nails. Based on the calculated 80% prediction interval, we already propose preliminary cut-off values for EtG concentrations in fingernails. An EtG concentration of > 123 pg/mg fingernail would suggest chronic excessive alcohol consumption, a concentration between 59 and 123 pg/mg fingernail would point towards moderate alcohol consumption, and an EtG concentration of < 59 pg/mg fingernail would indicate alcohol abstinence. Due to the fact that some participants were already engaged in treatment for alcohol use disorders for a longer period of time, we could include some participants that had been abstinent for several days to weeks. This is reflected in the lower concentrations of EtG detected in the hair of some participants (Table 6.2.). Therefore, although the participants were collected in a group of alcohol abusers, we had a relatively good spreading of the amounts of alcohol consumed.

Table 6.2: Overview of the ethyl glucuronide concentrations obtained in the paired hair, finger- and toenail samples from patients engaged in treatment for alcohol use disorders.

Subject number	Concentration in hair (pg/mg)	Concentration in fingernails (pg/mg)	Concentration in toenails (pg/mg)
1	11.6	52.1	n.a.
2	295	645	n.a.
3	1.05	15.2	n.a.
4	204	449	n.a.
5	375	4090	1700
6	164	927	n.a.
7	93.3	208	n.a.
8	215	80.3	n.a.
9	203	218	n.a.
10	571	517	n.a.
11	109	250	n.a.
12	271	689	n.a.
13	331	1440	n.a.
14	373	2200	n.a.
15	139	405	n.a.
16	36.0	69.5	n.a.
17	168	508	n.a.
18	3.84	24.1	n.a.
19	283	520	n.a.
20	254	2.74	n.a.
21	258	116	n.a.
22	256	326	n.a.
23	240	10.7	n.a.
24	3.93	26.8	n.a.
25	396	382	n.a.
26	54.7	n.a.	127
27	1150	1090	1370
28	625	1310	1060
29	73.9	n.a.	198
30	53.6	39.6	n.a.
31	41.9	149	379

Abbreviations: n.a., not available

Table 6.2: Continued.

Subject number	Concentration in hair (pg/mg)	Concentration in fingernails (pg/mg)	Concentration in toenails (pg/mg)
32	160	351	448
33	9.13	82.5	n.a.
34	18.7	184	n.a.
35	140	n.a.	687
36	155	159	n.a.
37	1.05	198	177
38	3.33	39.3	n.a.
39	24.7	36.9	n.a.
40	179	n.a.	438
41	396	357	n.a.
42	53.1	847	1950
43	5.39	73.7	n.a.
44	377	707	707
45	618	1710	3790

Abbreviations: n.a., not available

Due to the limited number of paired finger- and toenail samples (N = 9), the difference in median concentrations and the correlation were not statistically evaluated. From the presence of a correlation between hair and nails, and the similarity between finger- and toenails, one can expect a correlation between finger- and toenails will be present. Additionally, due to the slower growth rate of toenails compared to fingernails, higher concentrations can be expected in toenails. The average growth rate of fingernails is 3.0 mm per month, while toenails grow at an average rate of 1.1 mm per month (Cappelle et al. 2015c). To the best of our knowledge, this article is the first to report EtG concentrations in both finger- and toenails. However, the number of toenail samples was rather small (N = 13), and therefore more research is needed to investigate the proposed hypotheses.

In comparison to the two articles that previously investigated EtG concentrations in hair and fingernail samples from the same subject (Jones et al. 2012, Berger et al. 2014), the current article encompasses a wider range of EtG concentrations (not only low to moderate alcohol

consumption), a wider range of ages (not only college-aged students) and the collection of toenails. This study also includes the registration and exclusion of cosmetic treatments.

6.2.5. Conclusions

Today, the detection of EtG in hair samples is used in routine laboratories to evaluate long-term alcohol consumption. This study shows that EtG concentrations in hair and nails (both finger- and toenails) are significantly and positively correlated. In addition, higher concentrations were observed in nails compared to hair. The results indicate that nails can be a suitable alternative for hair. In view of this, we propose some preliminary cut-off values for fingernail analysis, but future studies are needed to confirm these.

6.3. Keratinous matrices for the assessment of drugs of abuse consumption: a correlation study between hair and nails

6.3.1. Preface and aims

As introduced in Chapter 2, amphetamine-like stimulants, cocaine, and opioids continue to be in high demand and supply, with major impacts on public health (European Monitoring Centre for Drugs and Drug Addiction 2017). Often, several drugs of abuse are used in combination, i.e. polysubstance abuse (Connor et al. 2014). Due to drug-drug interactions, these combinations of several drugs of abuse can significantly increase the risks of already harmful individual drugs. The monitoring of polysubstance abuse patients within a therapeutic or forensic/legal framework, represents a particular challenge that requires multi-analyte methods capable of detecting several compounds in a single run.

In recent years, the importance of *hair* as a matrix for illicit drug testing has substantially increased, e.g., in driver's license regranting procedures and in investigations related to causes of death (Kronstrand et al. 2010, Dufaux et al. 2012, Tassoni et al. 2014, Wille et al. 2014). In this context, scientific organizations, such as the Society of Hair Testing (SoHT) and Substance Abuse and Mental Health Services Administration (SAMHSA), have published guidelines for hair analysis and recommendations for confirmatory cut-offs to identify use (Table 6.3) (Helander et al. 2009, Cooper et al. 2012). On the other hand, studies on *nail* analysis for drugs of abuse are limited. Neither guidelines nor cut-off values are available for nail analysis, and therefore interpretation of results is often complicated. However, nail analysis can offer multiple advantages over hair analysis (Baumgartner 2014, Cappelle et al. 2015c). First, the average growth rate of nails is substantially lower compared to hair (3 and 1 mm/month for finger- and toenails, respectively, compared to 1 cm/month for head hair); which would allow a more significant accumulation of substances (Gupchup et al. 1999, Barbosa et al. 2013). This can be relevant in situations where low concentrations are expected (e.g. low dosages). Secondly, while hair is characterized by a cyclic growth rate with different stages, nails grow at a constant rate, which facilitates the interpretation of results (Palmeri et al. 2000). Hair strands that have been in a resting stage might result in the detection of more remoted consumption. Third, in contrast to hair, nails do not contain melanin. Since drug incorporation might be influenced by melanin concentrations, hair

pigmentation becomes an important source of bias when interpreting detected drug concentrations (Kronstrand et al. 1999, Kronstrand et al. 2001). Fourth, nails can provide an alternative option in cases where hair is not (sufficiently) available (e.g., alopecia, newborns). Fifth, cosmetic hair treatments have been proven to reduce drug content in hair, which can be overcome through nail analysis (Martins et al. 2008, Ettlinger et al. 2016). Possible disadvantages linked to the use of nails as matrix can be an insufficient sample amount in cases of (finger)nail biting, presence of nail diseases (e.g. bacterial or fungal infections), and effects of nail polishing or cleaning. Also, drugs are incorporated into hair via the blood flow in the hair follicle, while incorporation into nails occurs by a dual mechanism of deposition into the root via blood flow in the nail matrix and the nail bed (Cappelle et al. 2015c). The vertical and horizontal incorporation into nails might complicate the interpretation of results.

Studies comparing concentrations of drugs of abuse in hair and nails are very scarce. Most include either a limited number of samples ($N < 9$; (Ropero-Miller et al. 2000, Lin et al. 2004, Krumbiegel et al. 2016)) or encompass a limited number of compounds ($N \leq 3$; (Cingolani et al. 2004, Madry et al. 2016)), and do not distinguish between finger- and toenails. To the best of our knowledge, only Shen et al. compared concentrations of opiates in hair and fingernails of 12 positive samples (Shen et al. 2014). Except for morphine, they found significantly higher concentrations in hair than in nails and reported lower 6-monoacetylmorphine to morphine ratios in nails than in hair.

In this section, we evaluate drug of abuse concentrations in paired hair and nail samples (both finger- and toenails) from individuals with a dependency to one or more drugs of abuse, and investigate the differences and correlations between both matrices. The investigated drugs of abuse and their metabolites, together with the recommended cut-off concentrations in hair, are presented in Table 6.3. The comparison between hair and nails can provide more information about the usefulness of nail analysis in forensic and clinical toxicology, which is especially relevant for those cases where hair is not available or not reliable (e.g. cosmetic treatment). Furthermore, by analysing both nails and hair, additional evidence can be gathered, and erroneous results and interpretations can be diminished.

Table 6.3: Overview of the nine drugs of abuse (indicated in bold), their metabolites, and the corresponding cut-off concentrations.

Compound	Abbreviation	Recommended cut-off concentrations in hair (pg/mg)		Notes
		SoHT	SAMHSA	
Amphetamine-type stimulants				
Amphetamine	AMP	200	300	
Methamphetamine	mAMP	200	300	
3,4-Methylenedioxy-methamphetamine	MDMA	200	300	
3,4-Methylenedioxy-ethylamphetamine	MDEA	200	300	
Opioids				
Morphine	MOR	200	200	Parent compound, but also metabolite of **heroin** and codeine
Codeine	COD	200	200	
6-Monoacetylmorphine	6-MAM	200	200	Minor but exclusive metabolite of heroin
Methadone	MTD	200	/	
2-Ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine	EDDP	50	/	Metabolite of methadone
Cocaine				
Cocaine	COC	500	500	
Benzoylecgonine	BE	50	> 5% COC level	Metabolite of cocaine
Ecgonine methyl ester	EME	50	/	Metabolite of cocaine

Abbreviations: SAMHSA, Substance Abuse and Mental Health Administration; SoHT, Society of Hair Testing

6.3.2. Materials and methods

6.3.2.1. Reagents, standard solutions and materials

Reagents and materials were as described under 4.1.2.1 and 4.1.2.2.

6.3.2.2. Sample collection

Hair and nail samples were collected from inpatients engaged in a treatment program for substance use disorders at the Psychiatric Centre Multiversum together with an informed consent. All patients met DSM-5 criteria for substance use disorders (American Psychiatric Association 2013). The Ethical Committee of the University Hospital of Antwerp approved the study (Belgian registration number B30020169233). Hair samples were collected from the vertex posterior region of the head and cut as closely to the scalp as possible. The first 3 cm segment from the proximal end was used for further analysis. Nail samples were obtained by clipping approximately 1-2 mm of the distal edges of all ten finger- and toenails. Finger- and toenail samples were collected and analysed separately. Samples were stored in aluminium foil at room temperature until analysis.

6.3.2.3. Data on alcohol consumption and cosmetic treatment

Together with hair and nail samples, a detailed anamnesis of past drug consumption was taken. Patients were asked about their drug consumption in the past year using an adapted version of the TLFB method (Sobell et al. 1992). Per subject, the amount of drug consumed in the past year was used to calculate an average weekly consumption.

Patients were asked whether they had bleached, dyed, permed or thermally straightened their hair in the past year, as cosmetic hair treatment may lead to a degradation or removal of drugs in hair (Martins et al. 2008, Ettlinger et al. 2016). Nail polishing, the use of acetone and other nail treatments were also recorded. Patients reporting any cosmetic treatment of the hair or nails were excluded from the study.

6.3.2.4. Sample preparation and instrumental analysis

The sample preparation and the liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) analysis were performed according to a previously validated method (see 4.1.2.4 and 4.1.2.5) (Cappelle et al. 2017). Briefly, hair and nail samples were subsequently decontaminated, pulverized, extracted and filtered. Finally, the extracts were analysed on a LC-system (Agilent Infinity 1200, Diegem, Belgium) coupled to a triple quadrupole mass spectrometer (Agilent 6410 MS).

The accuracy and the analytical performance of the method were assessed and controlled by means of successful participation to proficiency tests organized by Arvecon GmbH and the SoHT. Furthermore, each batch included the analysis of a certified authentic hair sample containing common drugs of abuse as well as the verification of the retention time and the qualifier/quantifier ratios.

6.3.2.5. Data analysis

Statistical analysis was performed using R (version 3.3.1., The R Foundation for Statistical Computing). Due to non-normality of the data (tested through histograms and QQ-plots of the raw data), further analyses were performed with non-parametric statistics. Differences between matrix concentrations and metabolite to parent compound ratios between matrices were assessed using Wilcoxon rank sum test. Associations between hair and nails (finger- and toenails) were expressed using Spearman correlation. Using the regression coefficients $\beta 0$ and $\beta 1$, the existing cut-off concentrations for COC, BE, EME and AMP in hair (Cooper et al. 2012) were translated to preliminary cut-off concentrations in finger- and toenails. For all statistical tests, a p-value < 0.05 was considered statistically significant. Results are presented as mean ± standard deviation (SD) or as median [interquartile range (IQR)].

6.3.3. Results

A total number of 26 patients engaged in treatment for substance use disorders were included in the study. Four participants were female and 22 participants were male, aged 22 to 59 with a mean age of 36 ± 8.5 years. Sixteen patients provided hair, finger- and toenail samples, 8 patients provided only hair and fingernail samples, and 2 patients provided only hair and toenail samples.

Table 6.4 shows the number of positive samples, the concentration ranges and the median [IQR] concentration for each analyte. Differences in concentrations between matrices and correlations were statistically assessed for COC, BE, EME, AMP, MDMA (Table 6.5), whilst for MOR, COD, 6-MAM, MTD, and EDDP, only visual examinations were performed due to the limited number of positive samples available. No significant difference in concentrations measured in the various matrices collected from the same individuals could be observed. Only for COC, concentrations in toenails were significantly lower compared to the other two matrices. Although the differences in concentrations between matrices were not statistically significant, some trends could be observed. Except for COC, compound concentrations were higher in nails (finger- or toenails) compared to hair. COC concentrations were higher in hair, followed by finger- and then toenails. For BE, EME, MOR, 6-MAM and EDDP, concentrations were highest in fingernails, whilst for AMP, MDMA, COD and MTD, concentrations were highest in toenails.

Table 6.4: The number of positive samples, the concentration ranges and the median [interquartile range (IQR)] concentration for each drug of abuse. Results of methamphetamine are not shown since there was only one positive sample.

Compound	Matrix	Nr. of positive cases	Conc. range (pg/mg)[a]	Median [IQR] (pg/mg)
COC	Hair	26	25 - 163420	2011 [298; 22304]
	Fingernails	24	25 - 224085	942 [284; 10246]
	Toenails	18	25 - 30802	260 [25; 1423]
BE	Hair	26	25 - 76924	566 [123; 3621]
	Fingernails	24	25 - 242124	1311 [499; 21645]
	Toenails	18	25 - 35985	676 [109; 2024]
EME	Hair	19	25 - 3837	178 [25; 607]
	Fingernails	18	25 - 5059	179 [32; 697]
	Toenails	12	25 - 325	63 [25; 129]
AMP	Hair	26	25 - 25999	189 [25; 1540]
	Fingernails	23	25 - 87131	244 [61; 8604]
	Toenails	19	25 - 25726	552 [45; 2194]
MDMA	Hair	9	25 - 8298	119 [25; 1325]
	Fingernails	8	25 - 95411	95 [45; 1875]
	Toenails	6	68 - 6461	368 [156; 868]
MDEA	Hair	2	25 - 50	38 [31; 44]
	Fingernails	2	72 - 258	165 [119; 212]
	Toenails	2	25 - 50	38 [31; 44]
MOR	Hair	4	25 - 4446	485 [166; 1679]
	Fingernails	4	25 - 1253	562 [379; 784]
	Toenails	3	91 - 604	456 [274; 530]
COD	Hair	7	25 - 2467	82 [47; 355]
	Fingernails	6	25 - 189	99 [58; 129]
	Toenails	4	64 - 346	156 [110; 251]
6-MAM	Hair	4	25 - 6916	202 [25; 855]
	Fingernails	4	25 - 34665	1966 [25; 11344]
	Toenails	3	25 - 557	244 [104; 406]
MTD	Hair	5	25 - 25518	389 [617; 13900]
	Fingernails	5	80 - 2835	2092 [1668; 2112]
	Toenails	4	80 - 4353	4073 [2663; 4213]
EDDP	Hair	5	25 - 1420	274 [25; 883]
	Fingernails	5	68 - 517	308 [77; 358]
	Toenails	4	25 - 433	279 [152; 356]

[a] Positive samples with concentrations lower than the LLOQ (50 pg/mg) were reported as 25 pg/mg (LLOQ/2)

Abbreviations: 6-MAM, 6-monoacetylmorphine; AMP, amphetamine; BE, benzoylecgonine; COC, cocaine; COD, codeine; EDDP, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine; EME, ecgonine methyl ester; LLOQ, lower limit of quantification; MDEA, 3,4-methylenedioxyethylamphetamine; MDMA, 3,4-methylenedioxymethamphetamine; MOR, morphine; MTD, methadone

Table 6.5: Statistical investigation of the correlations and the differences in median concentrations for the following analytes: COC, BE, EME, AMP, MDMA.

Compound	Correlation	Spearman		Regression coefficients		Wilcoxon rank sum
		rho	p-value	β0	β1	p-value
COC	H vs FN	0.79	< 0.001	1.30	0.77	0.5
	H vs TN	0.76	< 0.001	1.08	0.63	0.009
	FN vs TN	0.79	0.002	1.68	0.60	0.05
BE	H vs FN	0.79	< 0.001	1.56	0.92	0.1
	H vs TN	0.79	< 0.001	1.51	0.80	0.9
	FN vs TN	0.76	0.002	2.24	0.61	0.2
EME	H vs FN	0.75	< 0.001	1.10	0.84	0.7
	H vs TN	0.80	0.005	1.94	0.44	0.2
	FN vs TN	0.83	0.005	2.20	0.37	0.2
AMP	H vs FN	0.86	< 0.001	0.57	1.06	0.3
	H vs TN	0.84	< 0.001	2.46	0.71	0.4
	FN vs TN	0.91	< 0.001	1.56	0.74	0.8
MDMA	H vs FN		0.3	1.78	0.76	0.7
	H vs TN		0.4	3.50	0.42	0.3
	FN vs TN	0.97	0.005	3.22	0.48	0.4

Abbreviations: AMP, amphetamine; BE, benzoylecgonine; COC, cocaine; EME, ecgonine methyl ester; FN, fingernails; H, hair; MDMA, 3,4-methylenedioxymethamphetamine; TN, toenails

Between matrices correlations were investigated for each compound. All Spearman correlations were significant and positive with ρ ranging from 0.75 to 0.97, except for MDMA (correlation of hair versus fingernails and hair versus toenails were positive but not significant) (Table 6.5). In Figures 6.4 and 6.5 the different correlations for COC, BE, EME, AMP and MDMA are represented. The existing cut-off concentrations for hair analysis in combination with the regression coefficients β0 and β1 of the hair versus fingernails and hair versus toenails correlations for COC, BE, EME and AMP (Table 6.5) were used to make some preliminary predictions of cut-off concentrations for finger- and toenail analysis (Table 6.6). As visualized in Figure 6.6, there are positive relationships between the different matrices for MOR, COD, 6-MAM, MTD and EDDP.

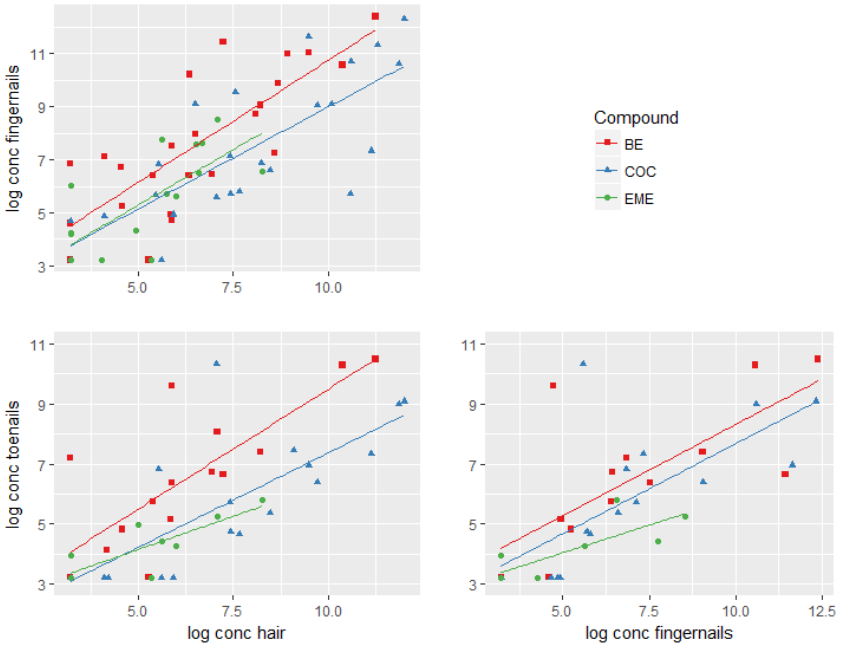


Figure 6.4: Correlations for benzoylecgonine (BE), cocaine (COC) and ecgonine methyl ester (EME) concentrations between hair, finger- and toenail samples. Concentrations were log-transformed.

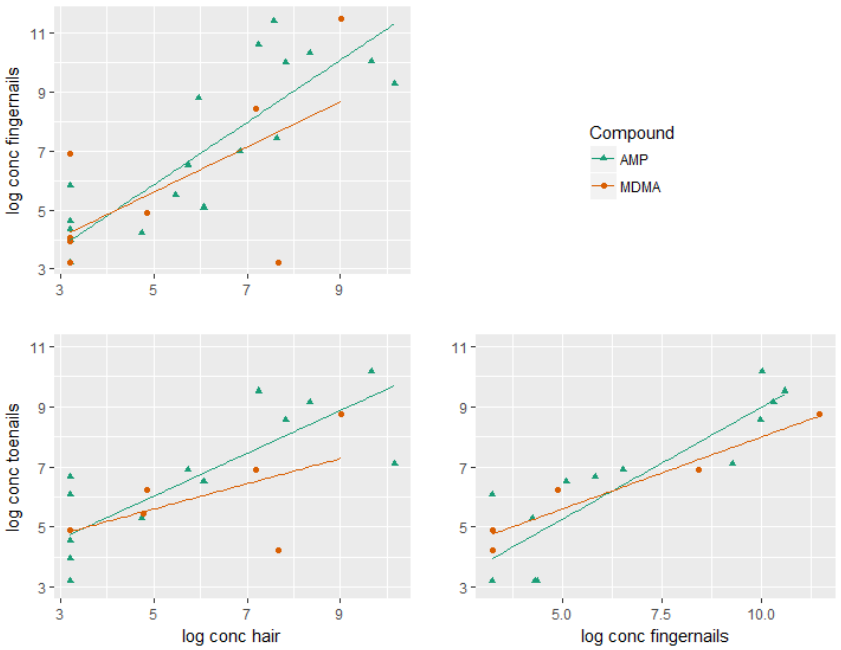


Figure 6.5: Correlations for amphetamine (AMP) and 3,4-methylenedioxymethamphetamine (MDMA) concentrations between hair, finger- and toenail samples. Concentrations were log-transformed.

Table 6.6: Preliminary predictions of cut-off values for finger- and toenail analysis.

Compound	SoHT cut-off value in hair (pg/mg)	Predicted cut-off value in fingernails (pg/mg)	Predicted cut-off value in toenails (pg/mg)
COC	500	440	150
BE	50	175	105
EME	50	80	40
AMP	200	485	505

Abbreviations: AMP, amphetamine; BE, benzoylecgonine; COC, cocaine; EME, ecgonine methyl ester; SoHT, Society of Hair Testing

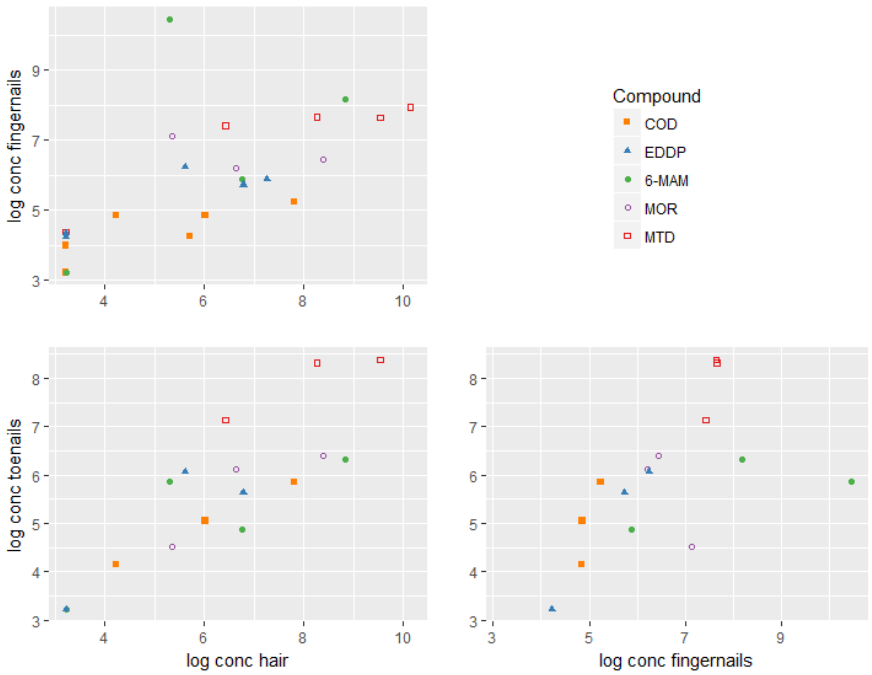


Figure 6.6: Scatterplots for codeine (COD), ethylidene dimethyldiphenyl pyrrolidine (EDDP), 6-monoacetylmorphine (6-MAM), morphine (MOR) and methadone (MTD) concentrations between hair, finger- and toenail samples. Concentrations were log-transformed.

In addition, the ratios of metabolite versus parent compound were calculated for 6-MAM/MOR, EDDP/MTD, BE/COC and EME/COC in all matrices. Due to the limited number of samples for 6-MAM/MOR and EDDP/MTD, only the ratios BE/COC and EME/COC were assessed statistically. Median ratios for 6-MAM/MOR were 1.06, 3.35 and 0.92 in hair, finger-

and toenails, respectively. For EDDP/MTD, median ratios were 0.063, 0.19 and 0.063 in hair, finger- and toenails, respectively. Median ratios for BE/COC were 0.21, 0.90 and 1.87 in hair, finger- and toenails, respectively. Differences in BE/COC ratios between matrices were significant (p-value < 0.001 for hair versus fingernails and hair versus toenails, p-value = 0.006 for finger- versus toenails). Figure 6.7 displays the boxplots of BE/COC ratios in the different matrices. For EME/COC median ratios were 0.019, 0.033 and 0.068 in hair, finger- and toenails, respectively. Differences in EME/COC ratios between matrices were significant for hair versus fingernails and hair versus toenails (p-value = 0.02 and p-value = 0.003, respectively). For finger- versus toenails the difference in EME/COC ratios was not significant (p-value = 0.2). The boxplots of EME/COC ratios in the different matrices are visualized in Figure 6.8.

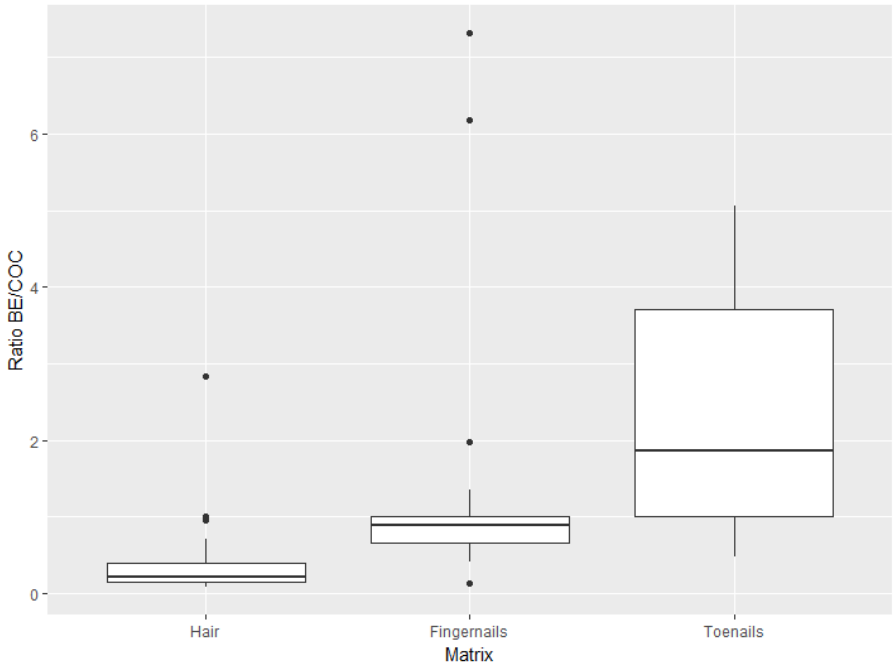


Figure 6.7: Boxplots comparing the benzoylecgonine (BE)/cocaine (COC) ratios in hair, finger- and toenails.

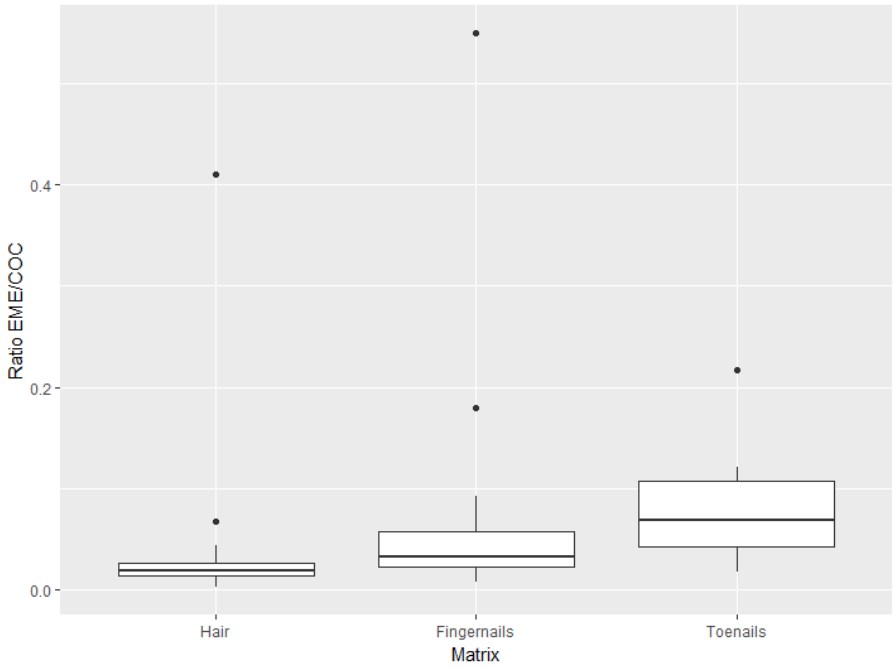


Figure 6.8: Boxplots comparing the ecgonine methyl ester (EME)/cocaine (COC) ratios in hair, finger- and toenails.

Tables 6.7, 6.8, 6.9 and 6.10, show a comparison between the average weekly consumption as reported using the TLFB method and the concentrations in hair, finger- and toenails for each subject. In terms of reported substances versus detected substances, there was a good agreement between the TLFB method and hair and nails with regard to declared use of substances and detection in the keratinous matrices (positive or negative). The agreement between the TLFB method and hair and nails in terms of amounts reported and concentrations measured in hair and nails was not that clear. In general, high reported use corresponded to high hair, finger- and toenail concentrations. However, some patients reporting limited use still had high concentrations in both hair and nails.

Table 6.7: Overview of the reported cocaine (COC) consumption per subject compared to the COC, benzoylecgonine (BE) and ecgonine methyl ester (EME) concentrations in hair, finger- and toenails.

COC			BE			EME			COC consumption (g/week)	Remarks
Conc. in hair (pg/mg)	Conc. in fingernails (pg/mg)	Conc. in toenails (pg/mg)	Conc. in hair (pg/mg)	Conc. in fingernails (pg/mg)	Conc. in toenails (pg/mg)	Conc. in hair (pg/mg)	Conc. in fingernails (pg/mg)	Conc. in toenails (pg/mg)		
16594	8576	597	3739	8554	1623	398	280	72	31.5	
39587	309	n.a.	7561	59329	n.a.	799	2101	n.a.	17.5	Weekend use
1899	13818	n.a.	566	27293	n.a.	25	410	n.a.	14	
274	25	25	196	25	25	n.a.	n.a.	n.a.	14	Smoking
40516	44811	n.a.	5972	19762	n.a.	728	677	n.a.	10.5	Smoking
8906	n.a.	1729	1187	n.a.	3227	147	n.a.	144	10	Nasal insufflation
235	289	n.a.	665	2896	n.a.	n.a.	n.a.	n.a.	8.75	Nasal insufflation
3805	952	n.a.	565	615	n.a.	56	25	n.a.	7	Last use 1 year ago, nasal insufflation
1157	270	30802	361	113	14559	25	25	537	7	Last use 6 years ago
4803	741	218	1028	634	828	208	25	25	7	Last use 1 month ago, smoking
142681	40485	8160	32252	39073	29404	3837	703	325	7	Nasal insufflation
80656	83450	n.a.	13194	62302	n.a.	676	1948	n.a.	6.5	Nasal insufflation
163420	224085	8846	76924	242124	35985	1189	5059	190	4.5	
13243	114773	1055	1392	93880	771	273	2348	84	4	Nasal insufflation

Abbreviation: n.a., not available

Table 6.7: Continued.

COC			BE			EME			COC consumption (g/week)	Remarks
Conc. in hair (pg/mg)	Conc. in fingernails (pg/mg)	Conc. in toenails (pg/mg)	Conc. in hair (pg/mg)	Conc. in fingernails (pg/mg)	Conc. in toenails (pg/mg)	Conc. in hair (pg/mg)	Conc. in fingernails (pg/mg)	Conc. in toenails (pg/mg)		
24207	8854	n.a.	3267	6112	n.a.	311	301	n.a.	3	Nasal insufflation
252	931	n.a.	94	821	821	n.a.	53	53	3	Nasal insufflation or smoking
1673	1242	302	221	602	311	n.a.	n.a.	n.a.	2	Weekend use, nasal insufflation
68221	1545	n.a.	5428	1398	1398	138	77	77	2	Nasal insufflation
61	129	25	25	944	1332	25	71	25	1.5	Weekend use, nasal insufflation or smoking
663	9055	n.a.	61	1223	n.a.	25	67	n.a.	Depending on availability	Nasal insufflation
68	n.a.	25	65	n.a.	62	n.a.	n.a.	n.a.	Sporadic use	Nasal insufflation
25	25	25	25	25	25	n.a.	n.a.	n.a.	Sporadic use	

Abbreviation: n.a., not available

Table 6.8: Overview of the reported amphetamine (AMP) consumption per subject compared to the AMP concentrations in hair, finger- and toenails.

AMP				Remarks
Conc. in hair (pg/mg)	Conc. in fingernails (pg/mg)	Conc. in toenails (pg/mg)	Consumption (g/week)	
1942	87131	n.a.	35	Stopped 1 month ago, nasal insufflation
2497	21459	5170	28	Nasal insufflation
15834	22577	25726	17.5	Nasal insufflation
25999	10653	1202	14	Nasal insufflation
1406	39504	13639	10.5	Injections
4199	29851	9279	7	
n.a.	74	25	7	Last use 3 years ago, nasal insufflation
139	n.a.	n.a.	7	Last use 12 years ago
2082	1694	1694	2	Nasal insufflation
25	338	788	1.5	Nasal insufflation
391	6555	n.a.	1	
25	25	25	Unspecified quantities	

Abbreviation: n.a., not available

Table 6.9: Overview of the reported 3,4-methylenedioxymethamphetamine (MDMA) consumption per subject compared to the MDMA concentrations in hair, finger- and toenails.

MDMA				Remarks
Conc. in hair (pg/mg)	Conc. in fingernails (pg/mg)	Conc. in toenails (pg/mg)	Consumption (pills/week)	
119	n.a.	228	1	
1325	4528	988	0.6	
25	990	n.a.	0.5	
129	132	508	Unspecified quantities	
8298	95411	6461	Unspecified quantities	Crystals
2138	25	68	Sporadic	
25	57	57	Sporadic	

Abbreviation: n.a., not available

Table 6.10: Overview of the reported heroin consumption per subject compared to the morphine (MOR), codeine (COD) and 6-monoacetylmorphine (6-MAM) concentrations in hair, finger- and toenails.

MOR			**COD**			**6-MAM**			**Heroin consumption (g/week)**	**Remarks**
Conc. in hair (pg/mg)	Conc. in fingernails (pg/mg)	Conc. in toenails (pg/mg)	Conc. in hair (pg/mg)	Conc. in fingernails (pg/mg)	Conc. in toenails (pg/mg)	Conc. in hair (pg/mg)	Conc. in fingernails (pg/mg)	Conc. in toenails (pg/mg)		
757	497	456	411	129	156	855	361	131	28	Smoking, injections
213	1253	91	68	127	64	202	34665	356	14	Injections
4446	627	604	2467	189	346	6916	3570	557	7	User for 26 years, smoking

6.3.4. Discussion

Significant positive correlations between hair and nails (both finger- and toenails) and between finger- and toenails were present for COC, BE, EME and AMP (Figures 6.4 and 6.5). For MDMA, correlations were positive, but only the correlation between finger- and toenails was significant (Figure 6.5), probably due to the limited number of positive MDMA samples (N = 9). For MOR, COD, 6-MAM, MTD, and EDDP, positive relationships could be observed but these could not be investigated statistically due to the limited number of samples (N < 9, Figure 6.6). The presence of these positive trends indicates that finger- or toenails can be used as alternative matrices to hair.

Compound concentrations were generally higher in nails (finger- and toenails) than in hair, except for COC. Observing high concentrations in nails could be linked to the slower growth rate of finger- and toenails compared to hair (3 and 1 mm/month versus 1 cm/month, respectively) which might result in a higher accumulation of compounds in nails (Gupchup et al. 1999). The slower growth rate could explain why for AMP, COD, and MTD, highest concentrations were measured in toenails, followed by fingernails and then by hair. However, other factors must be taken into consideration when comparing concentrations between matrices. First, the different mechanisms of incorporation into hair or nails may influence the observed concentrations. In fact, blood supply through the hair follicle is the main route for hair (Pragst et al. 2006), while for nails compounds incorporate through a dual mechanism of deposition into the root via blood circulation in the nail matrix and bed (Cappelle et al. 2015c). Although the influence of the vertical incorporation is limited (approximately 20%), this vertical and horizontal incorporation into nails might complicate the interpretation of results. Second, unlike hair which is characterized by a cyclic growth rate with different stages (Barbosa et al. 2013), nails are permanently formed and do not undergo resting growth stages (Palmeri et al. 2000). The cyclic growth rate of hair can sometimes complicate the interpretation of the obtained results, as it might result in the detection of past use in hair strands that are in resting stage and thus have not been growing. However, as the number of hair stands in resting stage is rather small (approximately 15%), especially at the vertex posterior region where hair samples are taken, the impact of this factor is likely limited (Barbosa et al. 2013). Third, the physicochemical properties of drugs might influence the extent of incorporation into hair and nails. In fact, molecules have to diffuse from the

systemic blood circulation across the cell membrane of the growing cells at the hair follicle or the nail matrix and the nail bed. The rate of diffusion is related to the lipid solubility of the compound, and the pH gradient between the plasma and the cell. In hair, it has been shown that the pH gradient from plasma (pH = 7.3) to more acidic conditions, within the melanocytes/ keratinocytes (pH 3-6) favours the incorporation of basic drugs over acidic drugs (Pragst et al. 2006, Barbosa et al. 2013). This was also observed by Kuwayama and colleagues, who reported higher concentrations of basic compounds in hair compared to toenails, whereas concentrations of weakly acidic and neutral compounds tended to be higher in toenails than in hair (Kuwayama et al. 2016). The physicochemical properties of a drug are also likely to influence the amount of incorporation through sweat and sebum and will be more important for neutral and lipophilic drugs.

The higher concentrations of COC present in hair compared to nails might be explained by the absence of melanin in nails in contrast to hair. Melanin is known to bind several compounds in hair and the affinity of a compound to melanin depends on the physicochemical properties of the compound. Incorporation of more basic drugs into hair has shown a positive relationship to melanin (Kronstrand et al. 1999, Kronstrand et al. 2001). The possible influence of melanin was also acknowledged by Shen et al., which attributed the detection of higher acetylcodeine, COD and 6-MAM in hair to the black hair of subjects (Shen et al. 2014). For some compounds, the higher affinity to melanin might counterbalance or even surpass the higher accumulation due to the slower growth rate of nails, and thus result in higher concentrations in hair than in nails as observed for COC. The influence of melanin might also explain why for EME, MOR and MDMA, the second highest concentrations are found in hair.

An important consequence of the observed differences in concentrations between hair, finger- and toenails is that cut-off concentrations established for hair are not valid to interpret concentrations measured in nails. Therefore, for the compounds COC, BE, EME and AMP, some preliminary cut-off concentrations for finger- and toenails are proposed (Table 6.6). To determine these preliminary cut-off concentrations, the regression line was calculated using nail concentrations as predicted variable and hair concentrations as predictor. The cut-off concentrations in hair were then fed to the equation to estimate the corresponding y-values (predicted cut-off concentrations in nails). As COC concentrations are

higher in hair compared to finger- and toenails, the preliminary cut-off concentrations are lower in finger- and toenails. For AMP, the concentrations are lower in hair compared to finger- and toenails, and thus the preliminary cut-off concentrations are higher in finger- and toenails. There is an absolute need to update or confirm these proposed cut-offs in future studies with a larger population.

The amount and type of external contamination varies between hair, finger- and toenails. Fingernails can be easily externally contaminated with drug residues, while external contamination from sweat is more probable for toenails. Hair is more likely to be contaminated by vapors from smoked drugs. The influence of external contamination on the compound concentration should be minimal as several strategies are used to reduce it. First, a decontamination step is performed before analysis in order to eliminate external contaminants and leave only the embedded substances. A second strategy is the detection of metabolites as indicators of human metabolism in addition to the parent compounds. For example, the presence of 6-MAM is required if MOR is present, and the presence of BE is required if COC is present (Helander et al. 2009, Cooper et al. 2012). Third, the determination of metabolite to parent drug ratios is also recommended for discrimination between drug intake and external contamination. In hair, the SAMHSA recommends a ratio of BE/COC greater than 5%. Ratios between parent compounds and their respective metabolites also give information on the differences in accumulation profiles between hair and nails. Results obtained showed that the ratios were different between hair, finger- and toenails. In hair, median COC concentrations were higher than median BE concentrations, while in nails median BE concentrations were higher than median COC concentrations. As a result, BE/COC ratios were significantly higher in finger- and toenails than in hair, with the highest ratio present in toenails (Figure 6.7). This finding was also reported by Krumbiegel et al. in three post-mortem cases where they found higher COC than BE concentrations in hair, but higher BE than COC concentrations in nails (Krumbiegel et al. 2016). EME/COC ratios were also highest in toenails followed by fingernails and then hair (Figure 6.8). 6-MAM/MOR and EDDP/MTD ratios were highest in fingernails and similar in hair and toenails. However, as only limited amount of positive samples were available for 6-MAM, MOR, EDDP and MTD, these findings need to be confirmed. Results obtained so far indicate that the ratios are

different in hair, finger- and toenails and this should be taken into consideration when using the ratios to discriminate between external contamination and drug intake.

There was a good agreement between patients reporting the consumption of a drug of abuse, and the presence of this substance in the hair, finger- and toenails of the patient. In addition, patients reporting high consumption of drugs, also had high concentrations in their hair and nails. Still, some patients reporting relatively low consumption showed relatively high concentrations in both hair and nails. In these cases, one might question the reliability of the answers given by the patients. Although patients were encouraged to report their true consumption, some of them might have reported an underestimation of their effective consumption.

Hair grows at a rate of 1 cm per month (Barbosa et al. 2013), therefore the 3 cm proximal segment corresponds to 3 months of growth and consumption of drugs in the last 3 months. On the other hand, finger- and toenails grow at a rate of 1 mm and 0.3 mm per month, respectively . Moreover, as the 1-2 mm clippings were taken from the distal edges of the nails, the regeneration time (i.e., the time to grow from the nail matrix to the nail's free edge) of the nail must be taken into account. The regeneration time is 3-5 and 8-16 months for finger- and toenails, respectively (Palmeri et al. 2000). Thus, the 1-2 mm fingernail clippings correspond to 1-2 months of growth 3-5 months ago, while the 1-2 mm toenail clippings correspond to 6-9 months of growth 8-16 months ago. As a result, the collected hair, finger- and toenail segments did not represent the same period of time. However, the samples in this study were collected from patients with a diagnosed and long-lasting addiction problem, which reported stable drug consumption patterns. This could be deduced from the TLFBs that were collected from these patients; in these questionnaires patients reported a stable consumption for a period of one to several years prior to sample collection.

To the best of our knowledge, this is the first study that systematically compares and statistically investigates concentrations of drugs of abuse in hair, finger and toenails obtained from the same subjects. Future studies with a higher number of positive samples, especially for MDMA, MDEA, mAMP, MOR, COD, 6-MAM, MTD and EDDP, are needed to confirm and to elaborate on the current results. However, results obtained so far show that nails are a valuable alternative or complement to hair as positive correlations and relationships are

present between them. At the same time, this study underlines the need for specific cut-off concentrations for nail analysis as they exist for hair.

6.3.5. Conclusions

Today, the detection of drugs of abuse in hair samples is used in routine laboratories to evaluate long-term drug consumption. This study shows that COC, BE, EME, AMP and MDMA concentrations in hair and nails (both finger- and toenails) are significantly and positively correlated. For MOR, COD, 6-MAM, MTD, and EDDP, positive relationships are present between hair and nails. The differences in parent compound to metabolite ratios (BE/COC, EME/COC, 6-MAM/MOR and EDDP/MTD) can indicate important variances in accumulation of compounds between hair, finger- and toenails. In summary, nails are a useful alternative to hair for monitoring long-term drugs of abuse consumption. However, care should be taken regarding the variability in the accumulation of compounds between the matrices. Some preliminary cut-off values for analysis of finger- and toenail are proposed, but future studies are needed confirm these.

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CHAPTER 7:

GENERAL DISCUSSION AND FUTURE PERSPECTIVES

The main objective of this PhD was to investigate the use of hair and nails as matrices for the long-term follow-up of alcohol and drug consumption, thereby expanding the detection window compared to traditional matrices such as blood and urine. This main objective was subdivided into two aims. The first aim was to develop and validate bioanalytical assays for the detection of biomarkers of alcohol and drugs in hair and nails, while the second aim was to use these methods to evaluate the correlations between both matrices and between the consumption of drugs and alcohol. This section will critically discuss the achievement of these predefined aims and formulate recommendations for future research.

7.1. Method development and validation for biomarkers of alcohol and drugs in hair and nails

The complexity of keratinized matrices and the presence of low concentrations of compounds require an optimized sample preparation (i.e., decontamination, pulverisation, extraction and clean-up), and a sensitive and specific quantification method. In Part I of this thesis, sensitive analytical methods for biomarkers of alcohol and drugs of abuse in hair and nails were optimized and validated.

7.1.1. EtG in hair and sensitivity of methods for hair (and nail) analysis

First, a sensitive GC-NICI-MS/MS method for the detection of the alcohol biomarker EtG in hair was developed and validated (Part I, section 3.1.2). The GC-NICI-MS/MS method showed linearity over a range from 2 to 400 pg/mg hair, with a LOD of 0.05 pg/mg hair and a LLOQ of 0.2 pg/mg hair. Through the use of tandem mass spectrometry, a higher analytical sensitivity and an improved signal-to-noise ratio were achieved compared to single quadrupole mass spectrometry. The use of such a sensitive method is important to accurately differentiate between teetotallers and moderate alcohol consumers according to the current cut-off as recommended by the SoHT (i.e., 7 pg/mg hair).

Moreover, sensitive methods such as the one developed in this thesis will be of importance for future research. The concentration of 7 pg/mg in hair was proposed by the German

Society of Toxicological and Forensic Chemistry (GTFCh) as first tentative cut-off to verify abstinence (Albermann et al. 2010, Boscolo-Berto et al. 2013). This cut-off value was fixed on the basis of analytical method's detection capability, rather than the assessment of endogenous levels of EtG in hair. Several studies have shown that subjects consuming moderate amounts of alcohol had EtG concentrations lower than 7 pg/mg. In one study, subjects consuming 16 g ethanol per day for 3 months had EtG concentrations in hair below 7 pg/mg (Kronstrand et al. 2012). In another study, EtG concentrations below 8 pg/mg were reported after an average consumption of 3.1 g alcohol/day (Stewart et al. 2013). Also in the study of this thesis performed in non-excessive alcohol consumers, concentrations below 7 pg/mg were detected in alcohol-drinking subjects. According to these results, much lower concentrations of EtG in hair are likely to be expected for real abstinent people. As a result, some studies proposed to lower the cut-off for abstinence (Pirro et al. 2013, Crunelle et al. 2017). On the other hand, a basal EtG concentration in hair, even in abstinent subjects, is not to be excluded. Pirro et al. estimated basal EtG concentrations in hair at 0.8 ± 0.4 pg/mg (Pirro et al. 2013). To re-assess the cut-off between moderate alcohol consumption and alcohol abstinence, more studies performed on large populations are required. These studies should also investigate factors that can lead to basal EtG concentrations such as alcohol-containing food, medication, cosmetics and body-care products. The methods used for this purpose should have LLOQs lower than 1 pg/mg to allow quantification of basal EtG concentrations below the current cut-off of 7 pg/mg. Methods used for the quantification of EtG according to the current cut-off of 7 pg/mg should have LLOQs below 7 pg/mg to allow reliable assessment of alcohol consumption.

7.1.2. EtG in nails and sample preparation for hair and nail analysis

In the next section, a sensitive GC-NICI-MS/MS method for the detection of the alcohol biomarker EtG in nails was developed and validated (Part I, section 3.1.3). The calibration curve was linear from 2 to 100 pg/mg, and the LLOQ was 2 pg/mg. So far, only three studies have reported on EtG concentrations in nails (Jones 2012, Morini et al. 2012, Keten et al. 2013, Berger et al. 2014). The method presented in this thesis is the first GC-based method, and the first to report on EtG concentrations in toenails. The method was similar to the one

developed for hair, in order to allow simultaneous analysis of hair and nail samples in future applications (as was done in Part II, section 6.2).

The sample preparation of keratinized matrices is of critical importance to obtain reliable results and has only been scarcely investigated in nails. Therefore, the influence of sample preparation (i.e., decontamination and pulverisation) on EtG concentrations in nails was evaluated. Decontamination should remove external contaminants, such as residues of daily care products, sweat, sebum, dust, and analytes from the environment that may have deposited on the nail surface, without extracting incorporated substances. The three investigated washing procedures were effective in removing external contamination, and none of the procedures removed significantly more EtG from the nails. Pulverisation allows the achievement of small particle size and a homogeneous powder, thereby enhancing extraction efficiency, reducing extraction time from the matrix, and improving accuracy in the determination of EtG concentrations in nails. More intense pulverisation of the nail samples increased EtG concentrations, in parallel with a decrease in particle size, but reaching a plateau. However, future research is needed to confirm and elaborate upon these findings, and to establish optimal decontamination and homogenization procedures in nails.

7.1.2.1. Decontamination of hair and nails

Guidelines for sampling, sample treatment and analysis of nail samples are not available yet. An interesting feature of nails in this context is that sampling can be performed in two ways: vertical segmentation achieved by nail clipping, or horizontal segmentation by nail scraping. Scraping off the upper nail layer can reduce external contamination, and thus increase the reliability of the obtained results. When the entire nail is available, scraping off the underside of the nail can further eliminate external contamination. Finally, contamination resulting from manipulation of the substance (especially important for powdered substances like cocaine) will mainly be found in fingernails, while contamination from sweat will mainly be observed in toenails. Consequently, comparison of the results obtained in finger- and toenails gives an indication about the extent and source of external contamination.

In hair, the general recommendation of the SoHT is to include a decontamination strategy that consists of an initial organic solvent wash, followed by aqueous washes. However, there

is no consensus on the actual procedure to be followed to effectively remove any trace of external contamination without actively removing the drugs incorporated into the hair, and there is a lot of variation in the procedures used. Therefore, the laboratory should investigate to what extent their wash procedure removes surface contamination. Several approaches have been described to discriminate between external contamination and drugs incorporated through consumption. Usually, the effectiveness of a decontamination procedure is assessed through the evaluation of drug concentrations in the wash fractions but it remains difficult to know if what is found in the wash fractions comes from inside or outside of the hair.

Mass spectrometric imaging provides an interesting way to gain more information about the influence of decontamination procedures on incorporated substances. Cuypers et al. studied the consequences of decontamination protocols on the spatial distribution of cocaine in hair (Cuypers et al. 2016). For the first time, the authors were able to directly show the difference between cocaine-contaminated and -user hair without any prior washing procedure. In another study, mass spectrometric imaging was used to study the effect of hydrogen peroxide treatment on cocaine concentration in hair (Cuypers et al. 2014). Images revealed a decreased detectability of cocaine in hair, most likely through breaking of melanin–cocaine bonds, and degradation of cocaine into reaction products. Unbound cocaine as well as the more hydrophilic reaction products, including benzoylecgonine, were easily washed out, thus removing any evidence of cocaine use.

7.1.2.2. Pulverisation of hair and nails

Studies in nails, including the one in this thesis, have shown that (cryogenic) pulverisation leads to a higher surface area and smaller particles compared to cut samples (Takaichi 2004, Shen et al. 2014, Cappelle et al. 2016). In hair, mechanical pulverisation increased extraction efficiency compared to scissors-cut samples (Albermann et al. 2012, Mönch et al. 2013). Recently, the SoHT recognized the importance of pulverisation. The guidelines of 2012 stated that hair samples should be cut into smaller pieces or milled to a powder (Cooper et al. 2012), while the revised consensus of 2016 states that powdering hair for the extraction of EtG is best practice (Society of Hair Testing 2016). Since 2016, the proficiency tests organized by

the SoHT make a distinction between laboratories that pulverize the hair samples and laboratories that directly use the finely pre-cut hair samples. Significantly higher concentrations are found by laboratories performing pulverisation. Thus, cutting of hair samples without further pulverisation may underestimate actual EtG concentrations and, consequently, influence the interpretation of results (as was also demonstrated in this thesis for EtG concentrations in nails, Part I, section 3.1.3).

7.1.3. Biomarkers for alcohol in hair (and nails)

The main advantage of direct markers compared to indirect markers (e.g. ASAT, ALAT, GGT) is that they are formed upon the consumption of alcohol, and therefore are specific for alcohol consumption. Examples of direct alcohol markers are EtG, phosphatitdylethanol, FAEE and EtS. So far, only EtG and FAEE have been reported in hair. EtS is currently used in urine and blood as a biomarker for alcohol consumption, but had never been detected in hair. In this thesis the presence of EtS in hair is reported for the first time (Part I, section 3.2). For the quantification of EtS in hair, an assay using LC-MS/MS was successfully developed and validated. Linearity ranged from 5 to 500 pg/mg and the LLOQ was achieved at 5 pg/mg. The novel method was successfully applied to hair samples from patients treated for alcohol use disorders. Hair concentrations of EtS and EtG were compared to assess the relationship between both biomarkers. There was a significant and positive correlation between EtS and EtG in hair, primarily due to alcohol consumption. Compared to what has been reported for EtG, relatively high basal levels of EtS were observed in alcohol-abstinent persons. EtS concentrations in hair were higher than EtG concentrations, which can indicate a more efficient incorporation of EtS in hair. The determination of EtS in hair provides an additional option to assess alcohol consumption. The concurrent determination of EtS and EtG in hair may improve the sensitivity of the detection, enhance the reliability of the results, and facilitate the data interpretation. EtS might also react differently to external factors such as cosmetic treatment. However, more research is needed to gain more information on the concentrations and the characteristics of EtS in hair.

7.1.4. Spiking of hair and nails

Typically, hair and nails are spiked with internal standard(s) to control for losses during the entire analytical procedure (including extraction recovery), or with analyte(s) and internal standard(s) to prepare quality control (QC) samples. QC samples are required to validate a bioanalytical method, and to ensure the performance of a method in daily routine. Due to their solid nature, spiking hair and nails is not as straightforward as spiking blood and urine. When hair or nail samples are spiked, the analyte is placed in contact with the surface of the hair or nail for a limited period of time before its extraction from the sample (Figure 7.1). It is unknown how much of the analyte, if any, is incorporated into the hair or nail matrix, and this procedure is not representative for the incorporation of compounds into hair or nails via the blood stream, sweat and/or sebum. For hair, two alternatives for producing QC samples have been proposed (Figure 7.1): (1) fortification of blank matrix through incubation with analytes for a period of several days to allow their incorporation into the hair matrix; and (2) homogenization of authentic hair specimens containing the analytes of interest (Welch et al. 2003, Lee et al. 2008, Turfus et al. 2013). Authentic hair samples are most representative of case specimens as the analyte has been incorporated into the matrix following consumption. However, it is very difficult to know the absolute concentration of analyte in the hair specimen, and old case samples may not always be available for use as QC samples. Fortified hair provides an alternative to authentic hair but is not frequently used in laboratories or reported in articles (Welch et al. 2003, Lee et al. 2008, Turfus et al. 2013). The use of authentic and fortified hair may necessitate other requirements for validation, as QC samples prepared this way showed higher CVs for repeatability and inter-day precision (Turfus et al. 2013). On the other hand, neither fortified nor authentic hair provides a solution for spiking with internal standard(s).

In this thesis, QC samples were prepared by spiking. As recommended by the SoHT (Cooper et al. 2012), two strategies were employed to ensure the performance of the validated methods. First, hair reference material for internal quality assurance was purchased from Medichem (Steinenborn, Germany) or ACQ science GmbH (Rottenburg-Hailfingen, Germany). Second, regular participation to proficiency tests for EtG and drugs of abuse organized by the SoHT and GTFCh (Proficiency Testing Organization ARVECON GmbH, Walldorf, Germany) was accomplished.

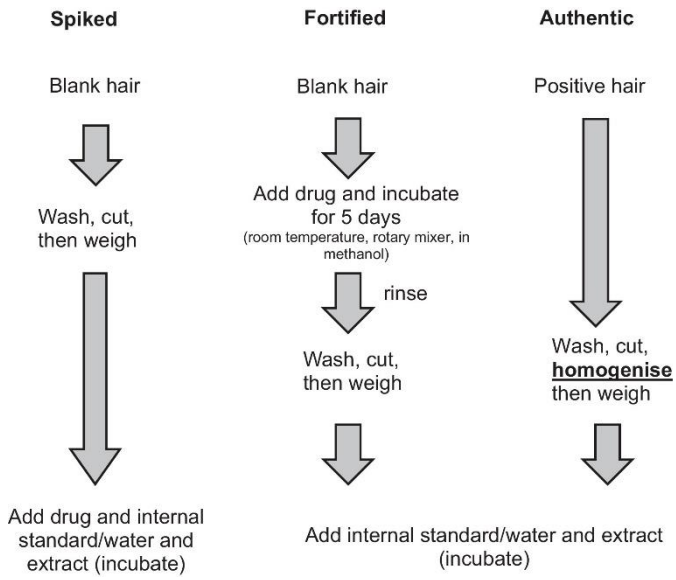

Figure 7.1: Schematic overview of the preparation of spiked, fortified and authentic hair for use as quality controls (Turfus et al. 2013).

7.2. Correlations for biomarkers of alcohol and drugs in hair and nails

7.2.1. Correlations between alcohol consumption and ethyl glucuronide concentrations in hair

Several studies, including the studies presented in this thesis, have reported a positive and linear correlation between alcohol consumption and EtG concentrations in hair. However, in subjects with very high EtG concentrations (e.g., 600 pg/mg) this linear correlation can be questioned. According to the consensus of the SoHT, a subject with a hair EtG concentration of 30 pg/mg should consume 60 g of pure alcohol (6 alcohol units, e.g., 6 glasses (25 cl) of regular beer) daily. If a linear correlation is assumed, a subject with a hair EtG concentration of 600 pg/mg should consume 1200 g of pure alcohol (120 alcohol units, e.g., 120 glasses (25 cl) of regular beer) daily. Even for alcohol dependent persons, consuming such a high amount of alcohol is unlikely. Therefore, the correlation between alcohol consumption and EtG concentrations may not remain linear at excessive alcohol consumption. The glucuronidation to EtG by parallel mechanisms or different isoforms of UDP-glucuronosyltranferase occurring

at high ethanol concentrations has been proposed as an explanation (Jatlow et al. 2014, Huppertz et al. 2015). Controlled trials should investigate the correlation between very high amounts of alcohol consumed and EtG concentrations in hair. However, from an ethical perspective these investigations are not easy to perform.

7.2.2. Correlations between hair and nails

Hair and nails are the sole matrices known to store xenobiotics over relatively large periods of time (months to years), and from which retrospective information on substance use can be retrieved. As a consequence of their keratinized nature, hair and nails have several characteristics in common, but differ from each other in some aspects. Firstly, substances are incorporated into hair via the blood flow in the hair follicle, while incorporation into nails occurs through a dual mechanism of deposition into the root via blood flow in the nail matrix and the nail bed. Secondly, nails grow slower than hair (3 and 1 mm/month for finger- and toenails, respectively, compared to 1 cm/month for head hair), which allows the detection of smaller levels of exposure, because of the higher accumulation, and the investigation of longer periods of time. Thirdly, hair is characterized by a cyclic growth rate with different stages, whereas nails grow at a constant rate, which facilitates the interpretation of results. Finally, melanin concentration in hair is known to influence the extent of incorporation depending on the physicochemical properties of drugs (Green et al. 1996, Reid et al. 1996). Because nails do not contain melanin, the bias due to pigmentation is absent for nails.

As a result of these differences, a variation in the extent of incorporation between both matrices can be expected (depending on the physicochemical properties of a substance). In this context, correlation studies comparing hair and nails are of great interest but are very scarce (Cappelle et al. 2015). In this thesis, the differences between hair and nails were assessed through correlation studies comparing EtG and drugs of abuse concentrations in paired hair, finger- and toenail samples (Part II, sections 6.1 and 6.2).

7.2.2.1. Differences and correlations for biomarkers of alcohol and drugs in hair and nails

For all investigated compounds, positive correlations or relationships were observed between hair and nails, indicating the possible use of nails as alternative to hair. Except for cocaine, compound concentrations were generally higher in nails (finger- and toenails) than in hair. This is probably mainly due to the slower growth rate of nails compared to hair. The higher concentrations of cocaine present in hair compared to nails might be explained by the presence of melanin in hair. It could be that the higher affinity to melanin counterbalances or even surpasses the higher accumulation due to the slower growth rate of nails, and thus results in higher concentrations in hair than nails. As it has been shown that the incorporation of EtG in hair is not influenced by melanin content (Appenzeller et al. 2007), the effect of melanin on the incorporation can be ruled out through the investigation of EtG concentrations. EtG concentrations were significantly higher in nails than in hair probably due to the slower growth rate of nails, as mentioned previously.

An important practical consequence of the observed differences in concentrations between hair, finger- and toenails is that cut-off concentrations established for hair are not valid to interpret concentrations measured in nails. Using the regression coefficients and the available cut-off concentrations in hair, this thesis proposed some preliminary cut-off concentrations for EtG, COC, BE, EME and AMP in finger- and toenails. However, there is an absolute need to confirm these proposed cut-offs in future studies with larger populations.

The ratios of metabolite versus parent compound were calculated for 6-MAM/MOR, EDDP/MTD, BE/COC and EME/COC (Part II, section 6.2). The comparison of these ratios between hair and nails, indicated important differences in the accumulation of compounds between both matrices. For example, BE/COC ratios were significantly higher in finger- and toenails than in hair, with the highest ratio present in toenails (0.21, 0.90 and 1.87 in hair, finger- and toenails, respectively). This follows from the observation that in hair median COC concentrations were higher than median BE concentrations, while in nails median BE concentrations were higher than median COC concentrations. As outlined in the general introduction, research has shown that due to their higher polarity metabolites are less likely

to accumulate in hair than parent compounds. For nails, different factors seem to influence the accumulation but until now these remain unknown.

An important point to keep in mind when comparing concentrations in hair and nails is the time frame investigated. If hair and nail samples are taken at the same moment, the time frames investigated will be different in hair and nails. Hair grows at a rate of 1 cm per month (Barbosa et al. 2013), therefore a 3 cm proximal segment corresponds to 3 months of growth and consumption in the last 3 months. On the other hand, finger- and toenails grow at a rate of 1 mm and 0.3 mm per month, respectively. Moreover, as nail clippings are taken from the distal edges of the nails, the regeneration time (i.e., the time to grow from the nail matrix to the nail's free edge) of the nail must be taken into account. The regeneration time is 3-5 and 8-16 months for finger- and toenails, respectively (Palmeri et al. 2000). Thus, 1-2 mm fingernail clippings correspond to 1-2 months of growth 3-5 months ago, while the 1-2 mm toenail clippings correspond to 6-9 months of growth 8-16 months ago. In this thesis, the problem of differences in the represented time frame was circumvented through the selection of patients with a long-lasting addiction problem and a stable consumption of alcohol or drugs of abuse.

7.2.2.2. Nails as an alternative to hair

Throughout this thesis, nails showed to be a useful and relevant matrix in forensic toxicology and it is clear that nail analysis should be given more attention in the future. Nails could be used instead of hair in cases where hair is not available or not reliable. Nails could also be analysed in combination with hair to achieve information regarding an extended period of time. For some compounds, nails could be the matrix of choice because of the higher accumulation. However, more research is needed to make nails a valuable matrix in forensic toxicology. An advantage is that, due to their similar nature, methods developed for hair are relatively easily transferred to nails.

Mechanisms for substance incorporation into nails have only been scarcely investigated (Cappelle et al. 2015). Thus far, only two studies (Hang et al. 2013, Madry et al. 2014) reported on the incorporation of substances in nails, both after administration of a single dose of zolpidem. The findings indicate that incorporation into nails occurs (i) by sweat-

mediated transport (detectable after 24 h), (ii) through the vertical growth of the nail bed (detectable after 2 weeks), and (iii) through the horizontal growth of the germinal nail matrix (detectable after 10 weeks).

Substance incorporation can be influenced by several factors, including nail-specific, individual-specific and substance-specific characteristics (e.g., growth rate and physical state of the nail, age and gender of the individual, physicochemical properties of the substance). For example, variations in growth rate can lead to differences in incorporation levels of ingested substances. While this effect may be minor due to the relatively slow growth rate of nails per se, it may bias the (retrospective) time frame that is interpreted. So far, not many studies have investigated the factors influencing nail growth, and the majority of such studies, have been published before 1980. Moreover, several authors did not examine whether the differences that they found were significant or not, and not all the influencing factors were confirmed by later studies (Le Gros Clark et al. 1938, Gilchrist 1939, Jones 1942, Sibinga 1959, Orentreich et al. 1979, Geyer et al. 2004, Yaemsiri et al. 2010, Cappelle et al. 2015). Another important factor that might alter substance incorporation into nails is the use of nail polish or, more importantly, acetone when removing the nail polish. Thus far, no studies have investigated the influence of these cosmetic treatments on drug concentrations in nails.

Taken the lack of complete understanding of the mechanisms and factors influencing substance incorporation, results of nail analysis for retrospective detection of substances use should be interpreted with caution. More research is required on this topic, including studies assessing nail-specific, individual-specific and substance-specific characteristics that may alter incorporation of substances into nails.

The lack of standardized sampling techniques, pre-analytical and analytical procedures makes it difficult to compare the results of the conducted studies. Indeed, the relatively large variations in pre-analytical and analytical methods employed can influence the obtained results. Also, proficiency testing programs to verify the quality of the developed methods and their results, and to compare results between laboratories are not available for nails. Although some preliminary cut-off concentrations were proposed in this thesis, no cut-off values exist to aid interpretation. Moreover, the quality of the results in studies is hampered

by the unreliability of self-reports of drug use, the limited number of paired samples and the lack of controlled dosing.

7.3. Future perspectives

Throughout the thesis and within the discussion several recommendations for future research have been pointed out, these are summarized below.

As the growth rate influences the incorporation of compounds into hair and nails, it could be interesting to investigate the differences in growth rate across the scalp and between the finger- and toenails. It is generally accepted that the vertex posterior region of the head has the lowest variability in growth rate, and therefore it is recommended to take hair samples from this region of the head (Cooper et al. 2012). However, very little information is available on how variable the growth rate is across the scalp. Meier et al. recently investigated the distribution patterns of EtG and caffeine concentrations over the scalp in a single subject, and found large variations of the concentration across the scalp, with factors of ca 3.0 and 10.6 for EtG and caffeine, respectively (Meier et al. 2017). Although these results should be confirmed, they indicate that depending on the region from where the sample is taken, variances in the compound concentration can be detected. Nails are usually collected by clipping of the free edge of the nail, and the clippings from the ten finger- or toenails are combined to achieve a sufficient amount of sample. Here, differences in the growth rate of individual fingers or toes might influence the concentrations detected. Some studies have hypothesized that the longer the finger or toe, the faster the growth (e.g., the third digit of the hand grows faster than the first and the fifth digits) (Orentreich et al. 1979, Geyer et al. 2004, Yaemsiri et al. 2010). However, these results should be confirmed and studies should investigate whether or not these differences are significant for the interpretation of compound concentrations.

Mass spectrometric imaging should be used to gain more information about the influence of decontamination procedures on incorporated substances, and to study the effect of cosmetic treatments on substances in hair. Furthermore, this technique can be used to achieve knowledge on the distribution of substances in the keratinized matrix.

In hair, using sensitive methods with LLOQs below 1 pg/mg, studies in large populations of alcohol abstinent and moderate alcohol consumers should be performed to re-assess the cut-off between moderate alcohol consumption and alcohol abstinence. In addition, studies should investigate factors that can lead to basal EtG concentrations such as alcohol containing food, medication, cosmetics and body-care products. These studies should also confirm or adjust the range of basal EtG concentrations that has been reported.

Related to this, factors that can lead to basal EtS concentrations should be assessed. Studies performed in subjects consuming different amount of alcohol should confirm or adapt the EtS concentrations in hair reported in this thesis. It would also be interesting to investigate why EtS concentrations in hair are higher than EtG concentrations, perhaps the incorporation of EtS in hair is more efficient. More research on the characteristics of EtS in hair could indicate some advantages of EtS over EtG (e.g. a different sensitivity to cosmetic treatments). Methods able to simultaneously determine EtG and EtS should be developed to improve the sensitivity of the detection, enhance the reliability of the results, and facilitate the data interpretation.

Large numbers of nail samples should be collected and analysed to establish concentration ranges and distribution patterns. Controlled dosing studies should be performed to establish the pharmacokinetics of the distribution of substances into the nail matrix, their accumulation and elimination, and the effects of cosmetic treatment on the substances within the matrix. Cut-offs proposed in this thesis should be confirmed and elaborated by studies in larger populations. Recommendations concerning sample preparation (including decontamination and pulverisation) should be established. Internal and external quality control samples should be provided to ensure the performance of methods developed for nail analysis.

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SUMMARY

In forensic and clinical toxicology, the determination and monitoring of exposure to and consumption of xenobiotics through the detection of reliable biomarkers in adequate matrices is crucial. Besides the 'traditional' matrices (including blood and urine), keratinized matrices, such as hair and nails, are of particular interest. The most important feature of keratinized matrices is the possibility to retrospectively assess substance use. Once substances are incorporated into hair or nails, they are no longer subject to biotransformation. As a consequence, many substances proved to be stable in keratinized matrices for prolonged periods of time, yielding a detection window of several months or years, depending on the hair or nail segment analysed. This is complementary to the more traditional matrices, which have detection windows of only hours to days. Combined analysis of traditional and keratinized matrices gives additional information about short-term (i.e. acute) and long-term (i.e. chronic) substance use. Furthermore, keratinized matrices can also be used for standalone analyses, when these relate to an event that occurred several weeks or months earlier, or when retrospective information over a longer period of time is required. Other advantages include the easy and non-invasive sample collection, which does not require qualified medical staff nor specific conditions for transport and storage. Moreover, sample collection can be performed under close supervision to avoid adulteration.

Part I of this thesis presents the development and validation of bioanalytical methods to measure biomarkers of alcohol and drugs of abuse consumption in hair and nails. The complexity of keratinized matrices and the low concentrations of compounds present require an optimized sample preparation, including decontamination, pulverisation, extraction and clean-up, as well as a sensitive and specific detection method.

In **Chapter 3**, bioanalytical methods to measure alcohol biomarkers in hair and nails were developed and validated according to international guidelines. Given the widespread use of alcohol and the impact alcohol abuse has on many aspects of health and society, there is a need for accurate markers of alcohol intake. Direct alcohol markers, such as ethyl glucuronide (EtG) and ethyl sulphate (EtS), are formed upon alcohol consumption, and therefore are possible markers to detect alcohol consumption with better specificity compared to indirect alcohol markers. First, a sensitive method using gas chromatography coupled to tandem mass spectrometry (GC-MS/MS) operated in negative ion chemical ionization (NICI) mode for the determination of EtG in hair was developed and validated. The method was applied to hair

samples from teetotallers, moderate and excessive alcohol consumers, and results were compared to a previously validated GC-NICI-MS method. The GC-NICI-MS/MS method showed linearity over a range from 2 to 400 pg/mg hair, with a limit of detection (LOD) of 0.05 pg/mg hair and a lower limit of quantification (LLOQ) of 0.2 pg/mg hair, compared to an LOD of 0.5 pg/mg hair and LLOQ of 1.5 pg/mg hair obtained with GC-NICI-MS. Furthermore, using GC-NICI-MS/MS a lower background noise and higher selectivity at lower concentrations were achieved. Therefore, the GC-NICI-MS/MS method should preferentially be used for the determination of EtG in hair, especially when differentiating between teetotallers and moderate drinkers according to the current cut-off provided by the Society of Hair Testing (i.e., 7 pg/mg hair). Next, a similar sensitive bioanalytical method for the determination of EtG in nails using GC-NICI-MS/MS was developed and validated. Furthermore, the influence of sample preparation on EtG concentrations was evaluated. The calibration curve was linear from 2 to 100 pg/mg and the LLOQ was 2 pg/mg. EtG concentrations in the washing solutions did not differ significantly with the use of different decontamination procedures, including hexane, dichloromethane, methanol, acetone and water as solvents. More intense pulverisation of the nail samples increased EtG concentrations, parallel with a decrease in particle sizes, but coming to a plateau. In the last section of Chapter 3, an analytical assay based on liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) for the quantification of EtS in hair was developed and validated. Sample preparation, chromatographic and mass spectrometric parameters, such as solid-phase extraction, column type, and transitions were optimized. Linearity ranged from 5 to 500 pg/mg and an LLOQ of 5 pg/mg was achieved. The novel method was successfully applied to hair samples from patients treated for alcohol use disorders. Hair concentrations of EtS and EtG were compared to assess the relationship between both biomarkers. There was a significant and positive correlation between EtS and EtG in hair, primarily due to alcohol consumption. Basal EtS levels were observed in alcohol-abstinent persons, comparable to what has been reported for EtG. The developed analytical procedure offers an alternative method to prove alcohol consumption using hair analysis.

The combined consumption of several drugs of abuse is a prevalent pattern of substance use which represents a significant health concern and requires multi-analyte methods capable of detecting several compounds in a single run. **Chapter 4** presents the development and

validation of a method based on LC-MS/MS for the simultaneous detection of nine drugs of abuse and their metabolites in hair and nails, including amphetamine (AMP), methamphetamine, 3,4-methylenedioxymethamphetamine (MDMA), 3,4-methylenedioxyethylamphetamine (MDEA), morphine (MOR), codeine (COD), 6-monoacetylmorphine (6-MAM), methadone (MTD), 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP), cocaine (COC), benzoylecgonine (BE), and ecgonine methyl ester (EME). The emphasis lied on a simple and straightforward sample preparation to reduce costs, and allow application in routine laboratory practice. Chromatographic and mass spectrometric parameters, such as column type, mobile phase, and multiple reaction monitoring transitions were optimized. Linearity ranged from 25 to 20 000 pg/mg hair and from 50 to 20 000 pg/mg nails, and the lowest calibration point met the requirements for the LLOQ (25 pg/mg for hair and 50 pg/mg for nails). The reliability of the method was proven through successful participation in proficiency tests, and by investigation of authentic hair and nail samples from self-reported drug users.

In **Part II**, the developed methods were used to investigate the relationships between the consumption of alcohol and drugs of abuse and the concentrations of their biomarkers in hair and nails on one hand, and between the biomarker concentrations in hair and nails on the other hand.

The first section of **Chapter 5** presents the results of a prospective controlled alcohol-dosing study in 30 healthy volunteers matched on age and gender. Individuals were instructed to drink no alcohol (N = 10), 100 g alcohol per week (N = 10) or 150 g alcohol per week (N = 10) for 12 consecutive weeks, before and after which hair was collected. Participants in the non-drinking group had median EtG concentrations of 0.5 pg/mg hair (range < 0.21 – 4.5 pg/mg). Participants consuming 100 and 150 g alcohol per week showed median EtG concentrations of 5.6 pg/mg hair (range 2.0 – 9.8 pg/mg) and 11.3 pg/mg hair (range 7.7 – 38.9 pg/mg), respectively. The study showed that the amounts of alcohol consumed correlated with the concentrations of EtG in hair, and that EtG concentrations in hair can be used to differentiate between repeated (low-to-moderate) amounts of alcohol consumed over a long time period. In the last section of Chapter 5, the correlation between alcohol consumption and EtG concentrations measured in hair of non-excessive alcohol consumers and alcohol-dependent patients was assessed. In addition, the influence of gender on this correlation was evaluated

in these populations. In both non-excessive alcohol consumers and alcohol-dependent patients, a positive linear correlation between the amounts of alcohol consumed and the concentrations of EtG in hair was observed. Gender had no influence on this correlation, indicating that the same cut-offs can be applied in male and female subjects.

Chapter 6 first investigated EtG concentrations in hair, finger- and toenails from the same individuals in order to evaluate the correlations between the matrices. Hair EtG concentrations ranged from < LLOQ to 1149 pg/mg (median = 164 pg/mg). Fingernail EtG concentrations ranged from < LLOQ to 4090 pg/mg (median = 250 pg/mg). Toenail EtG concentrations ranged from 127 to 3792 pg/mg (median = 687 pg/mg). EtG levels in hair and nails were significantly and positively correlated. Higher concentrations were present in finger- and toenails compared to hair, which might be attributed to the slower growth rate of nails, resulting in increased accumulation of EtG. Hence, nail analysis may be interesting when low concentrations of EtG are expected, e.g. to discriminate between teetotallers and social drinkers. In addition, preliminary cut-off values for EtG concentrations in fingernails were proposed: > 123 pg/mg for chronic excessive alcohol consumption, 59-123 pg/mg for moderate alcohol consumption, and < 59 pg/mg for alcohol abstinence. Next, concentrations of drugs of abuse and their metabolites were assessed in hair, finger- and toenails collected from the same individuals to evaluate differences and correlations between the matrices. Samples were analysed using the LC-MS/MS method validated in Chapter 4. Strong positive correlations between hair, finger- and toenails were present for COC, BE, EME, AMP and MDMA, while positive trends were observed for MOR, COD, 6-MAM, MTD and EDDP. Concentrations were generally higher in nails compared to hair. Ratios between parent compounds and their metabolites were assessed for 6-MAM/MOR, EDDP/MTD, BE/COC and EME/COC. Preliminary cut-off concentrations for COC, BE, EME and AMP in finger- and toenails were proposed. Based on these results, nails can be considered as a useful alternative to hair for monitoring of long-term drug consumption. However, care should be taken regarding the variability in accumulation of compounds between the matrices.

Within this thesis, the use of hair and nails as matrices for the long-term follow-up of alcohol and drug consumption was investigated. Sensitive bioanalytical methods using GC-MS/MS and LC-MS/MS, and including an optimized sample preparation were developed and validated for EtG, EtS and drugs of abuse in hair and/or nails. The results of the correlation

studies showed positive correlations between alcohol consumption and EtG concentrations in hair, and between EtG and drugs of abuse concentrations in hair and nails. So far, research on keratinized matrices has primarily focused on hair analysis, while studies on nails are scarce. The comparison between hair and nails provided evidence that nails are a viable matrix in forensic toxicology which deserves more attention in future research.

SAMENVATTING

Zowel in de forensische als in de klinische toxicologie is de betrouwbare bepaling en opvolging van blootstelling aan en gebruik van toxische stoffen, farmaca en drugs van groot belang. Hiertoe wordt gebruik gemaakt van biomerkers, die als indicatoren voor gebruik en/of blootstelling aan stoffen fungeren. Naast de meer 'traditionele' matrices zoals bloed en urine zijn keratine matrices zoals haar en nagels erg interessant. De belangrijkste eigenschap van keratine matrices is hun mogelijkheid om retrospectieve informatie te verschaffen over ingenomen stoffen. Eens stoffen geïncorporeerd zijn in haar of nagels zijn ze niet meer onderhevig aan biotransformatie. Als gevolg kunnen stoffen gedurende lange tijd stabiel worden opgeslagen in keratine matrices, hetgeen resulteert in een detectievenster van verschillende maanden tot zelfs jaren afhankelijk van de haar- of nagellengte. De verkregen informatie is complementair aan deze uit de traditionele matrices zoals bloed en urine die een detectievenster van uren tot dagen hebben. Gecombineerde analyse van traditionele en keratine matrices geeft bijkomende informatie over gebruik op korte termijn (acuut) en lange termijn (chronisch). Bovendien kunnen keratine matrices ook op alleenstaande wijze gebruikt worden wanneer staalafname pas lange tijd na inname mogelijk is of er nood is aan informatie over een langere tijdsperiode. Keratine matrices bieden daarenboven nog bijkomende voordelen zoals de eenvoudige en niet-invasieve staalafname waarvoor geen medisch opgeleid personeel nodig is en de eenvoudige opslag en transport van stalen. Bovendien kan de staalafname onder toezicht gebeuren zodat staalvervalsing kan vermeden worden.

Deel I van deze thesis beschrijft de ontwikkeling en validatie van bio-analytische methoden voor de bepaling van biomerkers voor alcohol- en druggebruik in keratine matrices. De complexiteit van keratine matrices en de lage concentraties aanwezig in deze matrices vereisen een geoptimaliseerde staalvoorbereiding (nl. decontaminatie, pulverisatie, extractie en opzuivering), evenals een gevoelige en specifieke detectiemethode.

In **Hoofdstuk 3** werden bio-analytische methoden voor de bepaling van alcoholmerkers in haar en nagels ontwikkeld en gevalideerd volgens internationale richtlijnen. Gezien de wereldwijde incidentie en de verstrekkende gevolgen die alcoholgebruik op het individu en de samenleving kan hebben, is een accurate en betrouwbare meting van het alcoholgebruik d.m.v. biomerkers van groot belang. Directe merkers, zoals ethyl glucuronide (EtG) en ethyl sulfaat (EtS), worden enkel in aanwezigheid van alcohol gevormd. Bijgevolg zijn deze merkers

specifieker en kunnen ze, in tegenstelling tot indirecte merkers, een definitief bewijs van alcoholinname leveren. In eerste instantie werd voor de bepaling van EtG in haar een gevoelige methode gebaseerd op gaschromatografie gekoppeld aan tandem massaspectrometrie (GC-MS/MS) in negatieve chemische ionisatie (NCI) modus ontwikkeld en gevalideerd. De methode werd toegepast op haarstalen van geheelonthouders, gematigde en excessieve alcoholgebruikers en de resultaten werden vergeleken met een reeds gevalideerde GC-NCI-MS methode. De GC-NCI-MS/MS methode was lineair van 2 tot 400 pg/mg haar met een detectielimiet van 0.05 pg/mg haar en een kwantificatielimiet van 0.2 pg/mg haar, vergeleken met een detectielimiet van 0.5 pg/mg haar en een kwantificatielimiet van 1.5 pg/mg haar voor de GC-NCI-MS methode. Bovendien werden bij gebruik van de GC-NCI-MS/MS methode een lagere achtergrondruis en een hoger selectiviteit bij lagere concentraties verkregen. Bijgevolg zou bij voorkeur de GC-NCI-MS/MS methode gebruikt moeten worden, zeker wanneer er volgens de huidige cut-off van 7 pg/mg onderscheid gemaakt moet worden tussen geheelonthouders en gematigde alcoholgebruikers. Voor de bepaling van EtG in nagels werd vervolgens een gelijkaardige gevoelige bio-analytische methode gebaseerd op GC-NCI-MS/MS ontwikkeld en gevalideerd. Bovendien werd de invloed van staalvoorbereiding op de EtG concentraties nagegaan. De kalibratiecurve was lineair van 2 tot 100 pg/mg nagels en de kwantificatielimiet was 2 pg/mg nagels. Bij verschillende decontaminatieprocedures, gebruik makend van hexaan, dichloromethaan, methanol, aceton en water als solventen, verschilden de EtG concentraties in de was-oplossingen niet significant van elkaar. Intensere pulverisatie van de nagelstalen resulteerde in hogere EtG concentraties, in parallel met kleinere deeltjesgrootte, maar met bereik van een plateau. In het laatste deel van Hoofdstuk 3 werd voor de bepaling van EtS in haar een methode gebaseerd op vloeistofchromatografie gekoppeld aan tandem massaspectrometrie (LC-MS/MS) ontwikkeld en gevalideerd. Staalvoorbereiding, chromatografische en massaspectrometrische parameters, zoals vaste fase extractie, kolom type en transities werden geoptimaliseerd. De methode was lineair van 5 tot 500 pg/mg en de kwantificatielimiet bedroeg 5 pg/mg haar. De nieuwe methode werd succesvol toegepast op haarstalen van patiënten behandeld voor alcoholmisbruik. Concentraties van EtS en EtG in haar werden vergeleken om het verband tussen beide merkers te evalueren. Er was een significante en positieve correlatie tussen EtS en EtG in haar gerelateerd aan het gebruik van alcohol. Bij geheelonthouders werden basale EtS concentraties vastgesteld, gelijkaardig aan

hetgeen gerapporteerd werd voor EtG. De ontwikkelde analytische procedure levert een alternatieve methode om alcoholgebruik m.b.v. haaranalyse aan te tonen.

Het gemengd gebruik van verschillende drugs is een veelvoorkomend fenomeen dat een aanzienlijk gezondheidsprobleem vormt en multi-analiet methoden vereist die in staat zijn verschillende componenten in één enkele run te detecteren. **Hoofdstuk 4** beschrijft de ontwikkeling en validatie van een methode gebaseerd op LC-MS/MS voor de gelijktijdige detectie van negen drugs en hun metabolieten in haar en nagels, namelijk amfetamine (AMF), methamfetamine, 3,4-methyleendioxymethamfetamine (MDMA), 3,4-methyleendioxyethylamfetamine (MDEA), morfine (MOR), codeïne (COD), 6-monoacetylmorfine (6-MAM), methadon (MTD), 2-ethylideen-1,5-dimethyl-3,3-difenylpyrrolidine (EDDP), cocaïne (COC), benzoylecgonine (BE) en ecgonine methyl ester (EME). De nadruk lag op een eenvoudige en onomwonden staalvoorbereiding teneinde de kosten te drukken en de toepassing in routine laboratoria mogelijk te maken. Chromatografische en massaspectrometrische parameters, zoals kolomtype, mobiele fase en transities, werden op punt gesteld. De methode was lineair van 25 tot 20 000 pg/mg haar en van 50 tot 20 000 pg/mg nagels en het laagste kalibratiepunt voldeed aan de eisen voor de kwantificatielimiet (25 pg/mg voor haar en 50 pg/mg voor nagels). De betrouwbaarheid van de methode werd aangetoond door succesvolle deelname aan ringtesten en door analyse van authentieke haar- en nagelstalen van druggebruikers.

In **Deel II** werden de ontwikkelde methodes gebruikt om enerzijds het verband tussen alcohol- en druggebruik en de concentraties van deze merkers in haar en nagels na te gaan, anderzijds het verband tussen de concentraties van de merkers in haar en nagels te evalueren.

Het eerste deel van **Hoofdstuk 5** beschrijft de resultaten van een prospectieve en gecontroleerde alcohol-doseer studie bij 30 gezonde vrijwilligers. Aan de deelnemers werd gevraagd om gedurende 12 opeenvolgende weken geen alcohol (N = 10), 100 g alcohol per week (N = 10) of 150 g alcohol per week (N = 10) te drinken. Zowel voor als na deze periode van 12 weken werden haarstalen afgenomen. De mediaan EtG concentratie bedroeg 0.5 pg/mg haar (bereik < 0.21 – 4.5 pg/mg) in de groep van geheelonthouders. Bij de deelnemers die 100 en 150 g alcohol per week dronken bedroegen de mediaan EtG concentraties respectievelijk 5.6 pg/mg haar (bereik 2.0 – 9.8 pg/mg) en 11.3 pg/mg haar (bereik 7.7 – 38.9

pg/mg). Met deze studie werd aangetoond dat de hoeveelheid alcoholgebruik correleert met de EtG concentraties in haar en dat EtG concentraties in haar gebruikt kunnen worden om onderscheid te maken tussen herhaald (laag tot gematigd) alcoholgebruik over een lange tijdsperiode. In het laatste deel van Hoofdstuk 5 werden de correlaties tussen alcoholgebruik en EtG concentraties in haar van gematigde alcoholgebruikers en alcohol-afhankelijke patiënten onderzocht. Daarnaast werd in beide populaties de invloed van gender op deze correlaties nagegaan. Bij zowel gematigde alcoholgebruikers als bij alcohol-afhankelijke patiënten was er een positieve lineaire correlatie tussen de hoeveelheid alcoholgebruik en de EtG concentraties in haar. Gender had geen invloed op deze correlatie, hetgeen aantoont dat de cut-offs op gender-onafhankelijke wijze gebruikt kunnen worden.

Hoofdstuk 6 onderzoekt eerst de EtG concentraties in haar, vinger- en teennagels van dezelfde individuen teneinde de correlaties tussen de matrices te evalueren. EtG concentraties in haar reikten van < LLOQ tot 1149 pg/mg (mediaan = 164 pg/mg). EtG concentraties in vingernagels varieerden tussen < LLOQ en 4090 pg/mg (mediaan = 250 pg/mg). EtG concentraties in teennagels reikten van 127 tot 3792 pg/mg (mediaan = 687 pg/mg). EtG concentraties in haar en nagels waren positief en significant gecorreleerd. In vergelijking met haar waren in vinger- en teennagels hogere concentraties aanwezig, hetgeen het gevolg kan zijn van de tragere groei van nagels resulterend in een hogere accumulatie van EtG. Bijgevolg kan nagelanalyse van belang zijn wanneer lage EtG concentraties verwacht worden, bijv. om geheelonthouders en gematigde gebruikers van elkaar te onderscheiden. Bovendien werden preliminaire cut-off concentraties voor EtG in vingernagels voorgesteld: > 123 pg/mg voor chronisch excessief alcoholgebruik, 59-123 pg/mg voor gematigd alcoholgebruik en < 59 pg/mg voor geheelonthouding. Vervolgens werden concentraties van drugs en hun metabolieten in haar, vinger- en teennagels van dezelfde individuen bepaald teneinde de verschillen en correlaties tussen de matrices te evalueren. De stalen werden geanalyseerd m.b.v. de methode die gevalideerd werd in Hoofdstuk 4. Positieve correlaties tussen haar, vinger- en teennagels waren aanwezig voor COC, BE, EME, AMF en MDMA. MOR, COD, 6-MAM, MTD en EDDP vertoonden positieve trends. Over het algemeen waren de concentraties hoger in nagels dan in haar. De verhoudingen tussen de oorspronkelijke en de gemetaboliseerde componenten werden bepaald voor 6-MAM/MOR, EDDP/MTD, BE/COC en EME/COC. Er werden preliminaire cut-off concentraties voorgesteld voor COC, BE, EME en

AMF in vinger- en teennagels. Op basis van deze resultaten kunnen nagels als een waardevol alternatief voor haar beschouwd worden voor de opvolging van druggebruik op de lange termijn. Desondanks is voorzichtigheid geboden met betrekking tot de variabiliteit in accumulatie van componenten tussen de matrices.

Doorheen deze thesis werd het gebruik van haar en nagels als matrices voor de lange termijnsopvolging van alcohol- en druggebruik onderzocht. Voor EtG, EtS en drugs in haar en nagels werden gevoelige bio-analytische methoden gebaseerd op GC-MS/MS en LC-MS/MS met inbegrip van een geoptimaliseerde staalvoorbereiding, ontwikkeld en gevalideerd. De resultaten van de correlatie studies toonden aan dat er positieve correlaties zijn tussen alcoholgebruik en EtG concentraties in haar en tussen EtG- en drugconcentraties in haar en nagels. Tot dusver heeft onderzoek over keratinematrices zich voornamelijk toegelegd op haaranalyse, terwijl studies over nagels beperkt zijn. De vergelijking tussen haar en nagels toont aan dat nagels een beloftevolle matrix zijn in forensische toxicologie en meer aandacht verdienen in toekomstig onderzoek.

CURRICULUM VITAE

Personalia

Name:	Delphine Cappelle
Date of birth:	October 23, 1989
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Education

2014 – 2018	PhD in Pharmaceutical Sciences Toxicological Centre, University of Antwerp, Belgium Title: Hair and nail analysis for the long-term monitoring of alcohol and drug consumption: focus on bioanalytical and correlation aspects Promotors: prof. dr. Hugo Neels, prof. dr. Alexander L.N. van Nuijs
2011 – 2013	Master of Sciences in Drug Development: Pharmacist (great distinction) University of Antwerp, Belgium Thesis title: Quantification of PTH (1-34) by mass spectrometry: pre-analytical considerations Promotors: prof. dr. Ingrid de Meester, prof. dr. Eric Grouzmann
2008 – 2011	Bachelor of Sciences in Pharmaceutical Sciences (distinction) University of Antwerp, Belgium
2002 – 2008	High School degree Latin Sciences Onze-Lieve-Vrouwinstituut Pulhof, Belgium

Work experience

2014 – 2018	PhD student at the Toxicological Centre, Faculty of Pharmaceutical, Biomedical and Veterinary Sciences, University of Antwerp (Belgium)
July – December 2013	Pharmacist at Apotheek Buyst, Antwerp, Belgium

Internships

October 2015	Internship at Retsch Technology GmbH, Haan, Germany
November 2014	Internship at the Laboratoire National de Santé, Dudelange, Luxemburg
February 2014	Internship at the Laboratoire National de Santé, Dudelange, Luxemburg
March - June 2013	Erasmus internship at the Centre Hospitalier Universitaire Vaudois, Laboratoires des Catécholamines et Peptides, Lausanne, Switzerland
August 2012 – January 2013	Pharmacy internship, Apotheek Buyst, Mortsel, Belgium
July - August 2012	Pharmacy internship, AZ Monica, Deurne, Belgium

Scientific curriculum

Hirsch-index (August 2018): 6

Total citations Web of Knowledge (August 2018): 106

Peer-reviewed publications (15)

1) Crunelle CL, **Cappelle D**, Covaci A, van Nuijs ALN, Maudens KE, Sabbe B, Dom G, Michielsen P, Yegles M and Neels H. Hair ethyl glucuronide as a biomarker of alcohol consumption in alcohol-dependent patients: Role of gender differences. *Drug and Alcohol Dependence* 141 (2014) 163-166.

2) **Cappelle D**, Yegles M, Neels H, van Nuijs ALN, De Doncker M, Maudens K, Covaci A and Crunelle CL. Nail analysis for the detection of drugs of abuse and pharmaceuticals: a review. *Forensic Toxicology* 33(1) (2015) 12-36.

3) Crunelle CL, Yegles M, De Doncker M, Dom G, **Cappelle D**, Maudens KE, van Nuijs ALN, Covaci A and Neels H. Influence of repeated permanent coloring and bleaching on ethyl glucuronide concentrations in hair from alcohol-dependent patients. *Forensic Science International* 247 (2015) 18-22.

4) Patteet L, **Cappelle D**, Maudens KE, Crunelle CL, Sabbe B and Neels H. Advances in detection of antipsychotics in biological matrices. *Clinica Chimica Acta* 441 (2015) 11-22.

5) **Cappelle D**, Neels H, Yegles M, Paulus J, van Nuijs ALN, Covaci A and Crunelle CL. Gas chromatographic determination of ethyl glucuronide in hair: comparison between tandem mass spectrometry and single quadrupole mass spectrometry. *Forensic Science International* 249 (2015) 20-24.

6) Crunelle CL, **Cappelle D**, Flamand E, Cox J, Covaci A, De Doncker M, van Nuijs ALN, Michielsen P, Yegles M and Neels H. Ethyl glucuronide in hair of non-excessive alcohol consumers: correlations and gender influence. *Forensic Toxicology* 34(1) (2016) 186-190.

7) **Cappelle D**, Neels H, Yegles M, Fransen E, Dueffels K, Bremenfeld S, Maudens KE, van Nuijs ALN, Covaci A and Crunelle CL. Ethyl glucuronide in nails: method validation, influence of decontamination and pulverization, and particle size evaluation. *Forensic Toxicology* 34(1) (2016) 158-165.

8) Crunelle CL*, **Cappelle D***, Yegles M, De Doncker M, Michielsen P, Dom G, van Nuijs ALN, Maudens KE, Covaci A and Neels H. Ethyl glucuronide concentrations in hair: a controlled alcohol-dosing study in healthy volunteers. *Analytical and Bioanalytical Chemistry* 408(8) (2016) 2019-2025. ***shared first author**

9) Crunelle CL, Verbeek J, Dom G, Covaci A, Yegles M, Michielsen P, De Doncker M, Nevens F, **Cappelle D**, van Nuijs ALN and Neels H. Hair ethyl glucuronide and serum

carbohydrate deficient transferrin for the assessment of relapse in alcohol-dependent patients. *Clinical Biochemistry* 49(7) (2016) 554-559.

10) Crunelle CL, Neels H, Maudens K, De Doncker M, **Cappelle D**, Matthys F, Dom G, Fransen E, Michielsen P, De Keukeleire S, Covaci A and Yegles M. Influence of Body Mass Index on Hair Ethyl Glucuronide Concentrations. *Alcohol and Alcoholism* 52(1) (2017) 19-23.

11) **Cappelle D**, De Doncker M, Gys C, Krysiak K, De Keukeleire S, Maho W, Crunelle CL, Dom G, Covaci A, van Nuijs ALN and Neels H. A straightforward, validated liquid chromatography coupled to tandem mass spectrometry method for the simultaneous detection of nine drugs of abuse and their metabolites in hair and nails. *Analytica Chimica Acta* 960 (2017) 101-109.

12) Crunelle CL, Yegles M, De Doncker M, **Cappelle D**, Covaci A, van Nuijs ALN and Neels H. Hair ethyl glucuronide concentrations in teetotalers: Should we re-evaluate the lower cut-off? *Forensic Science International* 274 (2017) 107-108.

13) **Cappelle D**, Neels H, De Keukeleire S, Fransen E, Dom G, Vermassen A, Covaci A, Crunelle CL and van Nuijs ALN. Ethyl glucuronide in keratinous matrices as biomarker of alcohol use: A correlation study between hair and nails. *Forensic Science International* 279 (2017) 187-191.

14) **Cappelle D,** De Keukeleire S, Neels H, Been F, De Doncker M, Dom G, Crunelle CL, Covaci A and van Nuijs ALN. Keratinous matrices for the assessment of drugs of abuse consumption: A correlation study between hair and nails. *Drug testing and analysis* 10(7) (2018) 1110-1118.

15) **Cappelle D**, Yin Lai F, Covaci A, Vermassen A, Crunelle CL, Neels H and van Nuijs ALN. Assessment of ethyl sulphate in hair as a marker for alcohol consumption using liquid chromatography-tandem mass spectrometry. *Drug Testing and Analysis* (2018) doi: 10.1002/dta.2410.

Oral presentations (5)

1) Analytical, Clinical and Forensic Toxicology Meeting, France, Bordeaux, SFTA, ccCTA, SoHT & BLT, June 10th – 13th 2014. Alcohol consumption vs. hair EtG concentration in alcohol-dependent individuals; role of gender differences. Crunelle CL, **Cappelle D**, Yegles M, van Nuijs ALN, Covaci A, Neels H (presenter).

2) 20th Scientific Meeting of the Society of Hair Testing (SoHT), Brazil, Sao Paulo, SoHT, May 3th – 6th 2015. Preliminary findings of a controlled alcohol dosing study to assess the correlation between hair EtG and alcohol consumption in healthy volunteers. **Cappelle D,** Crunelle CL, Covaci A, van Nuijs ALN, Maudens KE, Yegles M, Neels H (presenter).

3) Belgium and Luxembourg Toxicological Society (BLT) Scientific Meeting, Luxembourg, Echternach, BLT, October 21st – 22nd 2016. A straightforward, validated LC-MS/MS method for the simultaneous detection of nine drugs of abuse and their metabolites in hair and nails. **Cappelle D**, De Doncker M, Gys C, Krysiak K, De Keukeleire S, Maho W, Crunelle CL, Dom G, Covaci A, van Nuijs ALN, Neels H (presenter).

4) 22nd Scientific Meeting of the Society of Hair Testing (SoHT), United Kingdom, Cardiff, SoHT, June 12th –14th 2017. Ethyl glucuronide in keratinous matrices as biomarker of alcohol use: a correlation study between hair and nails. **Cappelle D**, Neels H, De Keukeleire S, Covaci A, Crunelle CL, van Nuijs ALN (presenter).

5) 55th The International Association of Forensic Toxicologists (TIAFT) Meeting, USA, Boca Raton, TIAFT & SOFT, January 6th – 12th 2018. Are nails an alternative to hair for the assessment of drugs of abuse consumption? A correlation study. **Cappelle D**, De Keukeleire S, Neels H, Been F, De Doncker M, Dom G, Crunelle CL, Covaci A, van Nuijs ALN (presenter).

Poster presentations (5)

1) Analytical, Clinical and Forensic Toxicology Meeting, France, Bordeaux, SFTA, ccCTA, SoHT & BLT, June 10th – 13th 2014. GC-MS or GC-MS/MS for the determination of ethyl glucuronide in hair? **Cappelle D**, Crunelle CL, van Nuijs ALN, Yegles M, Covaci A, Neels H (presenter).

2) 20th Scientific Meeting of the Society of Hair Testing (SoHT), Brazil, Sao Paulo, SoHT, May 3th – 6th 2015. Ethyl glucuronide vs fatty acid ethyl esters concentrations in hair of a dialysis patient. **Cappelle D**, Crunelle CL, Süβe S, Couttenye M-M, Michielsen P, Yegles M, van Nuijs ALN, Covaci A, Neels H (presenter).

3) 42nd International Symposium on High Performance Liquid Phase Separations (HPLC), Switzerland, Geneva, HPLC, June 21st – 25th 2015. Immunoassays vs. LC-MS/MS for PTH 1-34 (teriparatide) quantification: which method has the best specificity and sensitivity? Eugster PJ, **Cappelle D**, Dunand M, Herren A, Grouzmann E (co-author).

4) 53rd The International Association of Forensic Toxicologists (TIAFT) Meeting, Italy, Firenze, TIAFT, August 30th – September 4th 2015. A non-fatal self-poisoning attempt with sildenafil. Matheeussen V, Maudens KE, **Cappelle D**, Anseeuw K, Neels H (presenter).

5) XV Scientific meeting of the Spanish society of Chromatography and related techniques (SECyTA), Spain, Castellon de la Plana, SECyTA, October 28th – 30th 2015. Exploring potential of gas chromatography with atmospheric pressure chemical ionization and tandem mass spectrometry for sensitive determination of ethyl glucuronide in hair. Portolés T, Kinyua J, van Nuijs ALN, **Cappelle D**, Sancho JV, Hernández F (1st price for best poster) (co-author).

Supervised master theses (3)

1) Hanssens M, Cappelle D, van Nuijs, ALN, Maudens KE, Covaci A. Analyse van ethyl glucuronide in haar als biomerker van alcoholinname. Biomedical Sciences, M.Sc. thesis, University of Antwerp, Belgium (2014).

2) Gys C, Cappelle D, Covaci A, van Nuijs ALN. Validatie en toepassing van een LC-MS/MS methode voor de simultane kwantificatie van 12 drugs en metabolieten in haar. Pharmaceutical Sciences, M.Sc. thesis, University of Antwerp, Belgium (2016).

3) Krysiak K, Cappelle D, Neels H, Koba M. Validation and application of a LC-MSMS method for simultaneous quantification of 12 drugs of abuse and their metabolites in nails. Pharmaceutical sciences, M.Sc. thesis, Nicolaus Copernicus University, Poland (2016).

Memberships of scientific organizations

The International Association of Forensic Toxicologists (TIAFT)

The Society of Hair Testing (SoHT)

The Toxicological Society of Belgium and Luxembourg (BLT)

Reviewer for

Analytical and Bioanalyical Chemistry

Anaytica Chimica Acta

Journal of Chromatography B

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