Alcohol markers in hair
New detection techniques and evidence interpretation

Lydia Cornelia Adriana Maria Bossers MSc

A submission presented in partial fulfilment of the requirements of the University of Glamorgan/Prifysgol Morgannwg for the degree of Doctor of Philosophy

March 4, 2014

Abstract

It can be useful to discover a person's chronic drinking consumption in child custody cases and to aid in the diagnosis of diseases like fetal alcohol spectrum disorder. When one alcohol marker in hair is analysed to indicate chronic use false negatives and false positives can occur. When two (ethyl glucuronide (EtG) and fatty acid ethyl esters (FAEEs)) are analysed false negatives and false positives can be recognized and provide stronger evidence as is underlined statistically in this work. For a combined method, the sample preparation and analytical procedures were optimized. The effect of the decontamination step was difficult to interpret, which shows that addressing issues with external contamination is challenging. Analytes may be extracted from the hair matrix during decontamination and analytes can diffuse into the hair shaft from external contamination. The last is illustrated by the incorporation via excretions of endogenous EtG and FAEEs. A novel and sensitive analytical procedure was developed and validated which saves time and possibly money compared to analysing of both markers separately. The best overall method had a linear calibration curve ($r^2 > 0.99$) and an intra-day (n=3) and inter-day (n=9) accuracy for the quality control samples at three concentration levels between 84–118% with a coefficient of variation of 3–30% for both EtG and the FAEEs. The Bayesian approach was suggested as a new interpretation framework for hair tests, to account for the uncertainties in these tests in a transparent manner. In this work databases were constructed with EtG and FAEEs hair concentrations linked to the subject's chronic alcohol use, the likelihood ratios were calculated and working examples were provided. This showed that a positive hair test for either EtG or FAEEs may very well be only 'limited' evidence and therefore should only be used with a high prior odds. This means that a hair test result should not be used in isolation. The large confidence interval in this study also underlines the need for more control data.

Declaration

I hereby declare that this thesis is my own work and effort and that it has not been submitted anywhere for any award. Where other sources of information have been used, they have been acknowledged.

Name: .Lydia..Bossers..

Signature: ....

Date: ......04.03-2014...

Ethics assurances

Ethics approval for this work has been granted by the ethics committee of the University of Glamorgan. The subjects were interviewed about their alcohol usage and were asked to complete a questionnaire (see Appendix A). In addition to this a consent form (see Appendix B) was completed by the subject prior to the cutting of the hair. It was pointed out to the subject that at any point in time they could withdraw from this study and thus not donate hair for this study. For the selection of heavy drinkers two local detox clinics (Drugaid Cymru in Caerphilly and Huggard in Cardiff) had given consent to contact their clients.

Acknowledgement

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Glossary as applied in this work

Alcoholic A person who is alcohol dependent or is an alcohol abuser

Anagen Growing phase of hair growth cycle.

Catagen Transitional phase of hair growth cycle.

Chronic heavy drinker Someone who consumes more than 60 grams of alcohol per day over several months.

Confidence interval A summary measure that provide useful information from clinical investigations, especially when comparing data.

Correlation coefficient A measure of the strength and direction of the linear relationship between two variables.

Decontamination The removal of external contamination from the hair sample. It removes external impurities and as such reduces analytical noise from, for instance, hair care products and sweat.

Derivatisation The transformation of a chemical compound into another similar compound, called the derivative, by altering one or more of its functional groups. Here derivatisation is used to alter volatility.

Distal The part of hair at the tip of the hair shaft.

Esterification The formation of an ester.

Full scan In this mode the MS will monitor a range of mass over charge values.

Infrequent drinker A teetotaller or social drinker.

In vitro In a laboratory environment; in a glass, outside a living organism.

In vivo In a living organism.

Likelihood ratio A ratio between the likelihood of finding the evidence given one hypothesis and the likelihood of the same event given another hypothesis. This ratio is a measure of the strength of the evidence.

Matrix effect Change in the analytical signal caused by anything in the sample other than the analyte.

Minor metabolite A metabolite that is produced from less than 25% of the absorbed drug.

Posterior odds or probability The belief of the odds or probability that the hypothesis of the prosecution is correct over the hypothesis of the defendant taking the evidence into account.

Precursor ion Ion that reacts in the collision cell of a mass spectrometer to form particular product ions or undergoes specified neutral losses.

Prior odds or probability The belief of the odds or probability that the hypothesis of the prosecution is correct over the hypothesis of the defendant prior to finding the evidence.

Product ion The product ion is selected in the second mass analyser in tandem MS.

Product ion scan A precursor ion is selected in the first mass analyser, allowed to fragment in the collision cell and then all resultant masses are scanned in the second mass analyser.

Proximal The hair roots are the proximal ends.

Receiver operator curve A graph in which the relation between the sensitivity and specificity at specific cut-off values is displayed.

Sensitivity A measure for the actual positives that are correctly identified (for the evaluation of a test) or a measure of the smallest mass or concentration of analyte that can be accurately and reliably analysed (for the evaluation of an analytical method).

Social drinker Someone who consumes up to 60 grams of alcohol per day over several months.

Specificity A measure for the actual negatives that are correctly identified (for the evaluation of a test) or a measure of the confidence in the identification of the analyte (for the evaluation of an analytical method).

Tandem mass spectrometry This analytical measurement method involves multiple steps of mass spectrometry selection, with some form of fragmentation occurring in between the stages.

Teetotaller Someone who has not consumed alcohol for several months or more.

Telogen Resting phase of hair growth cycle.

Transesterification The process of exchanging the alkoxy group of an ester compound by another alcohol.

Abbreviations

ADH alcohol dehydrogenase

AEAT acyl-CoA:ethanol O-acyltransferase

ALDH aldehyde dehydrogenase

ALT alanine aminotransferase

AST aspartate aminotransferase

AUC area under plasma concentration-time curve

BAC blood alcohol concentration

BSTFA N,O-bis(trimethylsilyl)trifluoro-acetamide

CAL calibrator

CAR carboxen

CDT carbohydrate deficient transferrin

CI chemical ionisation (mass spectrometry), confidence interval (statistics)

CV coefficient of variation

CW carbowax

CYP2E1 cytochrome P450 2E1

DC direct current

DCM dichloromethane

DIS deuterated internal standard

DMDCS dimethyldichlorosilane

DUI driving under the influence

DVB divinylbenzene

E14:0 ethyl myristate

E16:0 ethyl palmitate

E18:0 ethyl stearate

E18:1 ethyl oleate

EE electron energy

EI electron ionisation

EtG ethyl glucuronide

EtG-BSTFA EtG derivatised with BSTFA

EtG-PFPA EtG derivatised with PFPA

FA fatty acid

FAEE fatty acid ethyl ester

FASD fetal alcohol spectrum disorder

GC gas chromatography

GGT gamma-glutamyltransferase

HD heavy drinker(s)

HS head space

LC liquid chromatography

LOD limit of detection

LLOQ lower limit of quantification

LR likelihood ratio

LR+ LR of a positive test

LR- LR of a negative test

LR1 LR in favour of chronic heavy drinking

LR2 LR in favour of drinking

LVI large volume injection

MCV mean corpuscular volume (of erythrocytes)

MeOH methanol

MEOS microsomal ethanol oxidizing system

M_r relative molecular mass

MS mass spectrometry

m/z mass-to-charge ratio

PA polyacrylate

PDMS polydimethylsiloxane

PFPA pentafluoropropionic anhydride

PTV programmable temperature vaporization

QC quality control

RF radio frequency

ROC receiver operating characteristic

ROS radical oxygen species

RT room temperature

SD social drinker(s)

SIM selected ion monitoring

S/N signal-to-noise ratio

SoHT Society of Hair Testing

SPE solid phase extraction

SPME solid phase micro-extraction

SRM selected reaction monitoring

T teetotaller(s)

TMS trimethylsilyl

TMCS trimethylchlorosilane

UGT uridine 5'-diphosphate glucuronosyl-transferase
(or UDP-glucuronosyltransferase)

WHO World Health Organization

Chapter 1

Introduction

1.1 The importance of alcohol testing

One might wonder why research concerning alcohol use is performed. Alcohol can offer a threat to the user and others and its use is therefore regulated by law under certain conditions such as whilst driving [1]. The knowledge of the amount of alcohol consumed over time may be helpful in legal cases like child custody cases [2,3] and in clinical settings to provide extra information to aid in the diagnosis of a disease [4]. For these purposes it is valuable to have an independent laboratory test for long-term alcohol use as self-reports can be unreliable [5–8]. Especially for those who are alcohol dependent, because they may be more inclined to lie about their alcohol consumption [8–10].

The danger posed by alcohol consumption is often underestimated in the Western society; data from the world health organization (WHO) shows it should not be. According to the WHO [11] alcohol is ranked eighth among global risk factors for premature death and the third leading global risk factor for disease and disability. In 2004, 3.8% of all global deaths were attributable to alcohol; these people would not have died without the consumption of alcohol by them or third parties who have inflicted the injury [11]. Driving under the influence (DUI) appears to be more common for chronic heavy drinkers than infrequent drinkers [12]. Therefore,

characterization of early events leading to alcoholic diseases and determination of their markers are important for early preventive or therapeutic measures [13–15]. Furthermore, during alcohol withdrawal therapy it may be helpful to keep track of relapses [16, 17].

Information about the history of alcohol intake may be crucial information for doctors when, for instance, a baby is expected to suffer from withdrawal symptoms after being intoxicated in the uterus [4, 18–20]. Fetal alcohol spectrum disorder (FASD), which is caused by exposure to alcohol during pregnancy, would often remain undiagnosed early in life as maternal self-reports are unreliable. Early in life, however, interventions can be relatively effective [21]. Hence, various diagnoses are developed to determine alcohol exposure in these early stages [22–25].

Heavy drinking and drunkenness are involved in many accidents, suicide and violence and are, therefore, of forensic interest [16, 26]. As a consequence alcohol use may not be forbidden by law in the Western society as such, but is illegal under some circumstances such as whilst driving. At times the establishment of the history of alcohol use provides valuable additional information. In custody cases, someone can be considered to be an unfit parent due to chronic alcohol abuse [2, 3]. In Sweden and Italy to re-grant a driving license after being caught for DUI one must prove a period of abstinence to the best of their abilities [1, 27, 28]. Furthermore, when a dead body is found the alcohol history may provide a useful clue for the identification of a person. Hair is better in identifying that someone was a heavy drinker and possibly thus dependent on drinking (an alcoholic) as the blood alcohol concentration of alcoholics is often low at the time of death [29].

In summary, for clinical and forensic reasons the determination of someone's chronic alcohol consumption can be relevant as is stressed by the work of Kintz [30], Palmer [18], Kaphalia et al. [13], Dennis and Hayes [14], Bhopale et al. [15] and Wurst et al. [16, 17].

1.2   Alcohol consumption tests

The type of test that is selected including the sample matrix and analytes that are detected depends on the information that is required. Testing for alcohol metabolites in hair allows for the characterization of someone's chronic drinking behaviour.

Different sample materials are used in which ethanol (or metabolites) are detected. The breath test for drunk driving is a non-invasive and quick test to establish the blood alcohol concentration (BAC) at that moment in time by measuring the amount of alcohol in the air one breathes out [31]. Alcohol in blood is volatile and is in equilibrium with the air above, which enables the estimation of BAC [31]. Another measurement from which the BAC can be derived is the urine test [20,32]. A more reliable measurement is the determination of BAC directly from blood. Acquiring a blood sample is invasive and it may thus be a better option to analyse breath or urine [31].

BAC is only useful as an indication of intoxication level at that very moment due to a self-cleansing ability of blood or a short-term history based on various samples. The window of detection for breath, urine and blood is in general only a couple of days [33]. When two urine or blood samples are analysed a kinetic model developed by Høiseth et al. [34] may determine whether alcohol is consumed before or after the accident. The story of a drunk driver that claims to have drunk a moderate amount of alcohol after the accident and thus one hour before sample collection, is less probable if high or decreasing levels of the direct marker were found. Høiseth et al. [35] found only one exception to this model when EtG was looked at: someone who had renal disease and therefore a significantly slower elimination rate of EtG. Hence, a history of alcohol consumption can be revealed from self-cleansing sample materials when several samples are taken during that time period. Retrospective information without having samples around that time period is, however, simply impossible and obtaining various samples may be cumbersome. Hair analysis is therefore preferred in most cases where alcohol history is relevant, since with one hair sample a wider window of detection is realized and retrospective information can be obtained (see Table 1.1

and Figure 1.1). Taking into account that an alcoholic may be able to refrain from drinking a couple of days, it is no surprise that of this group more have a positive hair test than urine sample [36]. Therefore, chronic heavy drinking may be better detected with hair as matrix for the analysis than with urine samples.

Table 1.1: The detection window of drugs in various matrices from Wennig [33]. h: hours, d: days, w: weeks, m: months.

Saliva	1–24h
Sweat	3h–2d/1w
Plasma	3h–2d
Serum	3h–2d
Urine	6h–3d
Hair	>3d–m/y
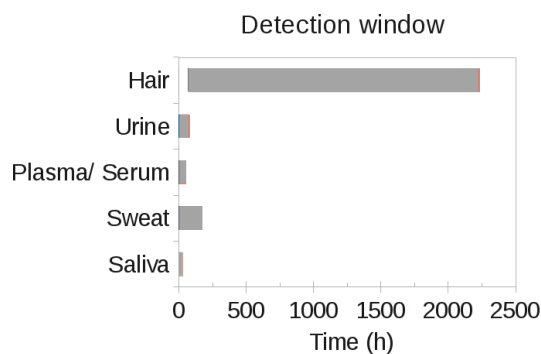
Figure 1.1: The detection window of drugs in different matrices.

Ethanol itself is not the analyte targeted in scalp hair, because ethanol is volatile and can be absorbed by the hair from external sources [37]. Currently, the analytes tested for in hair to determine long-term alcohol use are the alcohol metabolites ethyl glucuronide (EtG) and the fatty acid ethyl esters (FAEEs): ethyl myristate (E14:0), ethyl palmitate (E16:0), ethyl oleate (E18:1) and ethyl stearate (E18:0) [38]. Ethyl sulphate and ethyl phosphate were looked at previously, but are not currently used in hair analysis. Probably because the concentration in hair is even lower than for EtG and that EtG was promising as marker for heavy drinking [39].

When alcohol is ingested, ethanol and its metabolites are quickly absorbed into various matrices of the body [37]. Alcohol metabolites end up in the hair via the

bloodstream, sweat and sebum (see Figure 1.2) [27]. FAEEs and EtG become trapped and bound into the hair structure where they remain static as the hair grows out and can provide evidence of alcohol use by that person [37]. Head hair grows at approximately 1 cm per month [27], so a 1 cm section of hair can give a record of one months alcohol use when analysed. By analysing several 1 cm sections from the root of the hair it may be possible to obtain retrospective information about an individuals alcohol use [40].
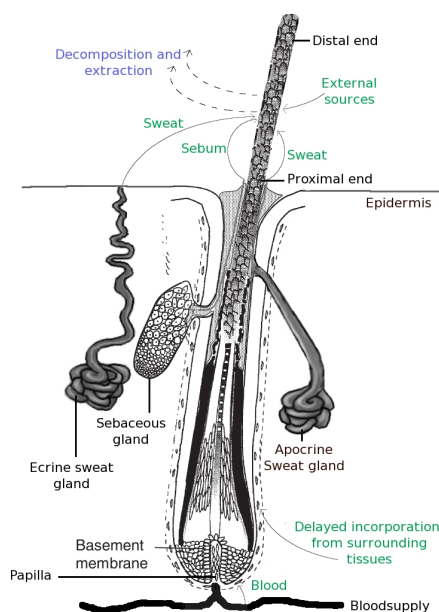
Figure 1.2: The incorporation routes and extraction of analytes in hair. Adjusted from Pragst and Balikova [27].

For long-term (ab)use methods other than the detection of alcohol metabolites in hair can be used. Indirect markers are up-regulated or down-regulated due to pathological and metabolic changes in the body associated with chronic alcohol consumption [37] and can be tested for. Four major indirect markers that are up-regulated by chronic heavy alcohol consumption and are detected or their enzyme activity can be measured in blood or plasma. These are: Gamma-glutamyltransferase (GGT), Mean corpuscular volume of erythrocytes (MCV), Carbohydrate-Deficient transferrin (CDT), Aminotransferases (AST or ALT) [41]. Though these tests may be more accurate than questionnaires for alcohol-dependent patients [42], the indirect markers are likely to show a posi-

tive result for chronic heavy drinking with severe liver damage independent from the cause of liver damage [43] and are therefore not as sensitive for the current state of chronic alcohol consumption as the direct markers in hair as the liver damage may have been caused by previous episodes of heavy drinking while the person does not consume alcohol any more (see Appendix C). When someone has been abstinent for only a short amount of time the indirect markers will still be elevated, since the liver damage is unlikely to disappear overnight [41].

Hair analysis may be seen as advantageous compared to urine and serum analysis for reasons other than the wider time window, retrospective ability and stability of the matrix. First of all, hair is easier to store and transport [44]. Secondly, sampling is less invasive than blood sampling [44] and less private than taking a urine sample [27]. Both are regarded positively by the person undergoing the test [1]. Furthermore, the matrix is more difficult to manipulate as wearing a wigg can be easily spotted when the hair sample is taken and is therefore considered by clients to provide a better proof of their abstinence [1].

Hair analysis is also preferred in some post-mortem cases when not enough tissue is available and hair is at hand [45,46]. In other cases, putrefaction causes ethanol to be produced in body fluids [46] whereas hair is protected against these internal changes [47]. Furthermore, hair retains foreign substances well as is shown by the detection of trace amounts of fatty acid ethyl esters in hair of South American mummies dating back to 1000–1250 AD [23].

As described earlier the type of test that is selected depends on the information that is required. The analysis of matrices other than hair can be important too, especially when current intoxicated state is important to prove (e.g. DUI). Hair analysis can be considered to complement the more recent information about alcohol use from urine and blood tests [48, 49].

1.3 EtG and FAEEs in hair as markers for long-term alcohol consumption

Ethanol is primarily metabolised in the liver. 2–10% of alcohol that is absorbed is eliminated through the kidneys or the lungs, the rest is broken down via an oxidative or non-oxidative pathway. More than 90% of consumed ethanol is metabolised by oxidative pathways [50]. The pathways are shown in Figure 1.3 and in more detail in Figure 1.4, which summarizes the author's literature review focussed on the effect of alcoholism on alcohol metabolism. Three groups of enzymes oxidise alcohol to the more toxic metabolite acetaldehyde: i) alcohol dehydrogenase (ADH), ii) cytochrome P450, chiefly CYP2E1, the microsomal ethanol oxidizing system (MEOS) and iii) catalase [43, 50, 51]. Aldehyde dehydrogenase (ALDH) rapidly converts this intermediate into acetate [43], which is broken down further into carbon dioxide and water via the citric acid cycle [52]. Two examples of metabolites that are produced via non-oxidative pathways are: EtG and FAEEs [50]. Only 0.02–0.06% of ethanol is converted into EtG [53, 54] and also FAEEs are minor metabolites [25, 55]. Relatively high levels of non-oxidative metabolites are reported in alcoholic patients, which indicates an up-regulation of the non-oxidative metabolism during chronic alcohol abuse [13, 56, 57].

As discussed previously, ethanol as well as its metabolites are targeted to test for alcohol use in various human biological materials like blood, urine and hair. The detection of FAEE and EtG in hair is more sensitive than most other markers for chronic heavy drinking [3] and these markers are stable in hair [19, 27, 39, 58, 59]. Both markers are not biased toward hair colour [54, 60] and higher alcohol consumption generally leads to a higher concentration of both metabolites in hair [3].

FAEEs are formed by the esterification of ethanol with free fatty acids, triglyceride, lipoproteins or phospholipids. A reaction catalysed by acyl-CoA:ethanol O-acyltransferase (AEAT) and FAEE synthase (see Figure 1.5) [61, 62]. The metabolite is more lipophilic than ethanol and therefore is more difficult to excrete from the body. Whereas normally the degradation pathways ensure the
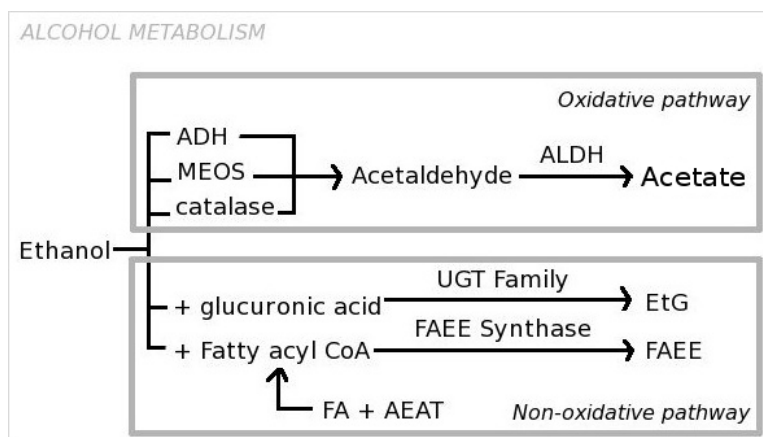
Figure 1.3: A simplified model of ethanol metabolism. ADH: alcohol dehydrogenase, MEOS: microsomal ethanol oxidation system, ALDH: aldehyde dehydrogenase, UGT: uridine 5'-diphosphate–glucuronosyltransferase, EtG: ethyl glucuronide, fatty acyl CoA: coenzyme A attached to the end of a long-chain fatty acid, FAEE: Fatty acid ethyl ester, FA: fatty acid and AEAT: acyl-CoA:ethanol O-acyl transferase.

excretion of toxic compounds from the body [63]. Most of the accumulation of these esters is observed in the liver, pancreas, heart and brain which are commonly damaged by alcohol abuse [56,64–66]. More FAEEs are produced in cases of heavy drinking compared to social drinking, and therefore FAEEs can be used as a marker for heavy drinking.

Ethanol elimination via conjugation with activated glucuronic acid yields EtG (see Figure 1.6) [68]. This reaction is catalysed by the uridine 5'-diphosphate-glucuronosyltransferase (UDP-glucuronosyltransferase or UGT) super-family of enzymes that utilize UDP-glucuronic acid as a cofactor [69]. Of the multiple UGT isoforms that are identified to be responsible for catalysing the addition of glucuronic acid to ethanol, UGT1A1 and UGT2B7 are the two most prevalent isoforms [69]. However, because a combination of UGT isoforms is responsible for the EtG formation pharmacogenetic differences between individuals are likely to be masked [70]. The incorporation of EtG is thought to be mainly via sweat [71,72]. The incorporation of EtG from blood to hair is unfavourable, because the hair has an isoelectric point close to 6 which indicates poor binding of EtG [39]. This in addition to the low conversion rate of alcohol to EtG results in merely

trace amounts of EtG in hair.
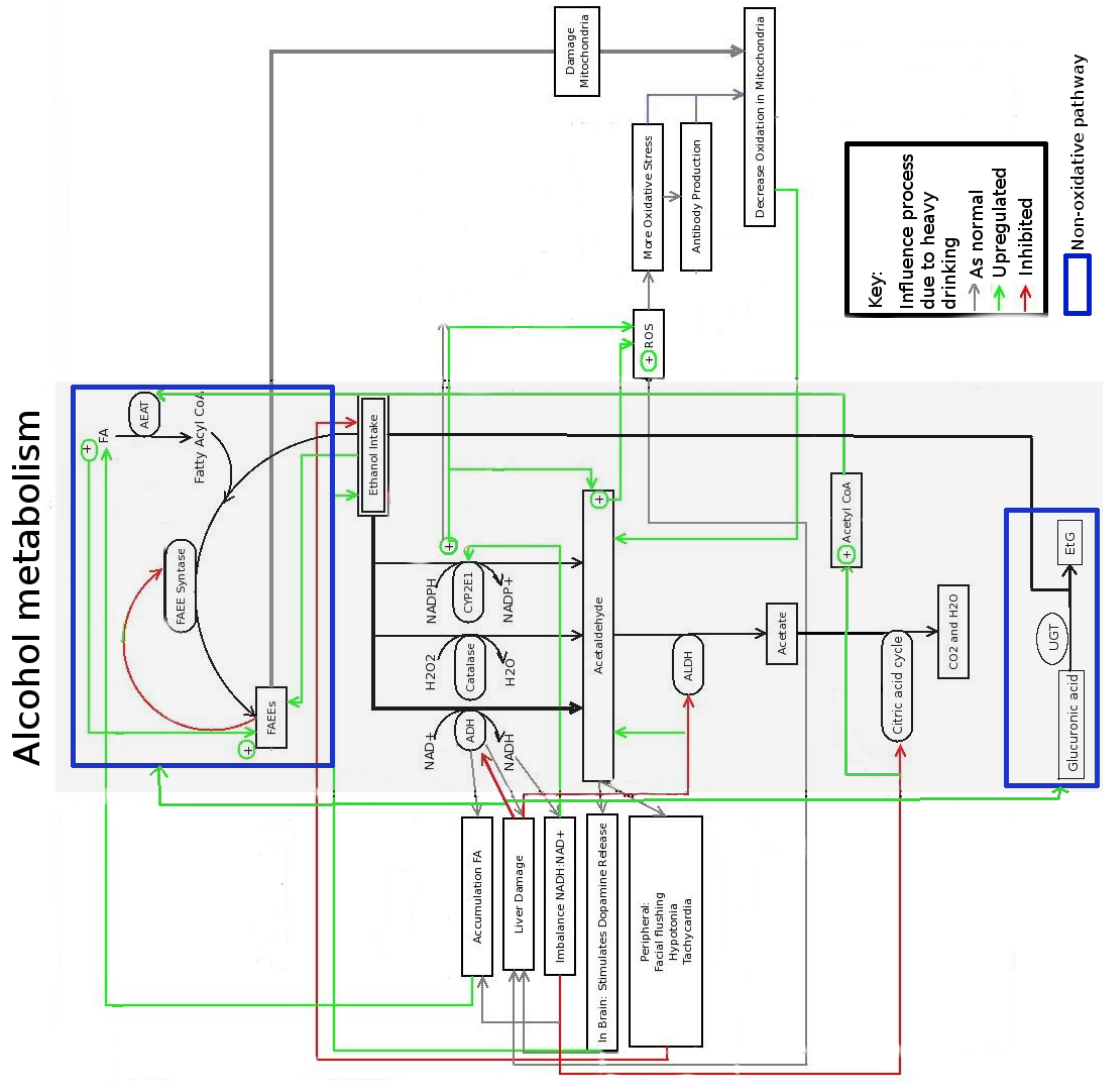
Figure 1.4: A detailed model of ethanol metabolism with the effects of increased alcohol consumption on in particular the non-oxidative pathways. ROS: reactive oxygen species.
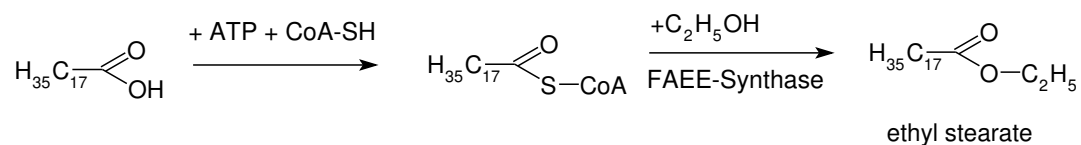
Figure 1.5: The formation of ethyl stearate from a free acid via fatty acyl CoA to ethyl ester. The ethyl group originates from ethanol. Adjusted from Pragst [67].
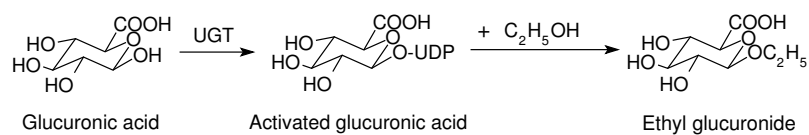
Figure 1.6: The formation of ethyl glucuronide from ethanol and glucuronic acid.

1.4 The aim of this thesis

To solve some of the problems that cause false positives and false negatives, it is suggested by Pragst et al. [3] to test for two alcohol markers (EtG and FAEEs) in hair instead of only one. A statistical analysis may determine whether this is indeed the case. The determination of the dependence of the two markers was therefore one of the goals of this work. If with the analysis of both markers stronger evidence for chronic heavy drinking can be obtained, a combined method in which both markers are tested for in a single run is likely to be more attractive for the industry than performing two different tests. Since currently no combined method for EtG and FAEEs exists it was the aim of this work to optimize and develop a method that tests both alcohol markers in one run. This method would need to detect at least chronic heavy drinking and thus a concentration of 0.5 ng/mg FAEEs and 30 pg/mg EtG in hair.

It is possible that this combined method is not yet developed because of the difficulties for a combined sample preparation and analysis due to the distinct physical properties of the analytes (see Table 1.2) as appendices E and F imply. Extraction is based on these physical properties: the polarity for instance defines where a compound can be found after the extraction. One of the aims of this research is to overcome these issues and develop a combined, simultaneous extraction, sample preparation and analysis that enables the industry to establish the concentration of EtG and FAEEs in hair. A combined method may save time and money compared to the separate tests. Less extraction solvent may be required and fewer man hours for the combined method compared to the two different tests that are currently used. Also, a comparison between the current tests and the developed combined method is useful to determine how the analytical analysis is affected and determine how the feasibility of the developed test.
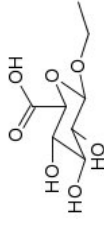
Another goal of this work was to have a closer look at the problems concerning alcohol hair analysis. In particular the possibility of decontamination of exogenous alcohol markers (EtG and FAEEs) and the interpretation issues. Currently experts can provide two different opinions and it is unclear how to combine their evidence as was clear in the case Pragst presented in his article [73]. A new frame

work, the Bayesian theory, may help with this. The aim of this research was to see how this framework can be used, how this improves the interpretation in a scientific way and how evidence from two different hair tests can be combined. The latter is obviously important in the light of this research because this work focusses on the use of both EtG and FAEEs in hair.

Currently, there is no general consensus on the value of hair testing in the scientific community. To get a more objective insight in this, another aim was to calculate the likelihood ratios to show how strong the evidence of alcohol hair tests actually is.

In short, this work aims to evaluate the value of the evidence when both EtG and FAEEs are detected in hair instead of only one. Another aim is the development of a combined analysis that is more attractive for the industry than the use of two different tests to indicate chronic heavy drinking. Thereby looking at the influence of different steps in the sample preparation, in particular the decontamination step with respect to external contamination and the incorporation of endogenous alcohol metabolites. Another goal is to use the Bayesian theory to determine the value of the evidence of the separate tests and illustrate the impact of this manner of reporting. With the Bayesian theory assumptions are easier to recognize and evidence of different tests are also easier to combine compared to the currently used cut-off method where two experts can state the opposite and it is unclear how to combine their views. The use of the Bayesian theory may also shed light on what is required for both the interpretation for casework and the research into alcohol hair tests concerning the controversies.

Table 1.2: Physical properties of the five alcohol metabolites: ethyl myristate, ethyl palmitate, ethyl oleate, ethyl stearate and ethyl glucuronide.

	E14:0	E16:0	E18:1	E18:0	EtG
Structure					
Formula	$C_{16}H_{32}O_2$	$C_{18}H_{36}O_2$	$C_{20}H_{38}O_2$	$C_{20}H_{40}O_2$	$C_8H_{14}O_7$
Molar mass (g/mol)	256	284	310.5	312.5	222.19
Boiling point (°C)	178	192	210	213	N/A
Solubility	Not soluble in water, slightly soluble in diethyl ether, soluble in heptane and supercritical CO_2.				Soluble in water
Polarity	Lipophilic head and non-polar chain				Polar
pKa	25 for esters in general				3.21

Chapter 2

Literature review

2.1 The controversies surrounding drug testing in hair

Hair analysis has been debated over the years and controversies still exist. In 1995, hair was a seldom used sample in most laboratories due to the controversies concerning the sensitivity of the analytical methods. Kintz and Mangin suggested that a group should be formed that focuses on the areas of agreement and what the issues are that require more research [74]. This group was founded in December of that year: the Society of Hair Testing (SoHT) [75]. Scientific and practical meetings were organized each year and various consensuses have been published [75]. Still, controversies exist mainly based on the variation between individuals that affect the concentrations of the analytes in hair with a similar drug intake and the possibility of external contamination and endogenous production.

2.1.1 The problem of external contamination of hair

An issue in hair testing that also occurs with EtG and FAEEs is that the analytes that are tested for exist in many environments and occasional contact cannot be ruled out [37, 74]. This issue is unlikely to be solved by the sample preparation,

Table 2.1: The correlation between growth cycle, rate and external exposure with the site of origin of the hair. Ana: duration of anagen phase, cat: duration of catagen phase, tel: duration of telogen phase, d: days, w: weeks, m: months. From Kerekes et al. [76].

Hair sites	*Growth cycle (ana/cat+tel)*	*Growth rate (cm/month)*	*External exposures*
Scalp	2–6y/15–20w	0.84–1.41	Cosmetic treatments and external contamination
Beard	14–22m/9–12m	0.75–0.87	After shave lotions
Axillary	11–18m/12–17m	0.87–1.00	high exposure to sweat and deodorant
Pubic	10–17m/11–16m	0.60–0.90	Urine and sweat
Other (arm/leg)	1–5m/2–6m	0.66–0.96	Creams, lotions and patches

because as Blank and Kidwell [77] showed for cocaine the wash-out kinetics are very similar for internally introduced cocaine and the externally introduced p-bromococaine. Even extreme extraction conditions, such as employing strong acid for extended periods, did not remove all externally applied drugs. This implies that external contamination could mimic drug use. Blank and Kidwell conclude that only short hair length near the scalp should be tested to limit environmental exposure [77]. When the analyte is not present in the environment and can only be produced within the body due to drug use, external contamination will not be an issue and therefore markers that can only be produced in the body from consumption have an advantage. Unfortunately both FAEEs and EtG can be present in hair products [78, 79]. However, hair from other sites can be used since that hair is likely to not have the same sources of external contamination [76, 80, 81](see Table 2.1) although slower hair growth rates and a higher percentage of hair in catagen/telogen phase do dictate possibly another cut-off value and the analysis of the whole hair length. Also the selection of an 'appropriate' cut-off value as Kintz and Mangin suggested can reduce the chance of a false positive as a result of external contamination [74].

2.1.2 The issue of inter-individual differences

Scalp hair growth varies from 0.7 to 1.5 cm per month [82] and as a rule of thumb 1 cm per month is used in the interpretation of hair analysis. This affects the time line and possibly the concentration of the drugs and metabolites in hair. The inaccuracy is increased with increasing distance from the scalp. Hence, for alcohol testing to re-grant a driving license abstinence for 6 months needs to be proven and is performed on a 3 cm long hair sample [1], which seems acceptable as far as the time line is concerned for those subjects with a growth rate below 1 cm per month.

In 1995, the issue was stressed by Dupont and Baumgartner [48] of racial bias as a result of easier incorporation of drugs in African versus Caucasian hair. Racial bias in hair analysis means a significant difference between races in incorporation of drugs from internal usage and external contamination [37]. At first the racial bias was explained by a different morphology of scalp hair. Africans have a tightly interwoven mat of hair shafts and more damaged appearance of hair than Caucasians and Asians because each twist is a stress point prone to damage [83]. These damaged hair sites allow access for drug binding. However, a definitive biological marker for racial identification based on hair has been rejected by nearly all researchers who have examined the issue, because it is observed that the variance in hair morphology between groups are equal or even exceed intergroup differences [83–85]. Later Kidwell et al. [83] stated that the 'racial bias' is better explained by social and cultural differences [86] in hair surface treatment, less frequent removal and the route of administration. As previously discussed, it is shown that hair treatments such as dyes, bleaches and perms do damage hair so that is holds less drug than it would otherwise [87] and passive uptake is increased by the increase of hair damage [77].

It is far from settled that the data offered in support of the bias hypothesis has demonstrated let alone conclusively shown that such an effect is real. The results of several studies failed to show any racial bias and thereby suggest that hair testing methodology would not create a disparity among applicants [88,89]. The extensive research performed by Kelly [90] indicated that there was little evidence

of a pattern attributable to either hair colour bias or race. They state: "The degree of both inter- and intra-group variance is largely unstudied and appears most often as an assumption in hair assay research." Studies have examined i) a very small number of subjects (as low as nine for Gleixner et al. [91] and Kronstrand et al. [92]), ii) very low drug dosages or iii) fail to utilize a meaningful definition of 'race' [90]. As Mieczkowski and Newel [86] strikingly state: the findings indicate that although blacks test at higher positive rates than whites for both hair and urine assays, these differentials most likely reflect differential rates of use which is apparent from the self-reported data. Ergo unless drug users can be verified in a controlled study absolute bias cannot be calculated [83]. A controlled study was executed by Huestis et al. [89] with 38 middle-aged men that normally use cannabis daily. There was no difference in detection rates between African-American and Caucasian subjects. More statistically relevant, however, were the studies done by Mieczkowski and Kruger [93–95], which again and again did not indicate a racial bias. Briefly, racial bias is not agreed upon, but it may be more appropriate to relate hair findings with hair morphology and hair treatment.

There may be a difference between pigmented hair and non-pigmented hair due to its melanin content as several studies have found [96–100]. Human hair may contain two types of melanin: pheomelanin and eumelanin. The first is responsible for red to blond hair and the second for brown to black. Only eumelanin is important in relation to the incorporation of drugs in hair since this type is capable of binding basic drugs [71]. This hair pigment is a poly-anionic polymer constituted of a large proportion of carboxylic groups. Therefore, basic drugs, positively charged at physiological pH can bind via electrostatic interactions with these anionic carboxylic groups (COO-). Basic cations can thus be expected in higher amounts than acidic compounds. In addition, no difference is noted for the uptake of neutral compounds in pigmented versus non-pigmented hair [37]. Mieczkowski stresses that a significant effect does not mean an effect that has an impact on the clinical interpretation of the information. The overall effect on the phenomenon may be in fact negligible [93].

Figure 2.1 shows that cut-off values can correct for the variation between individuals with the same drug intake. The selection of an "appropriate" cut-off value,
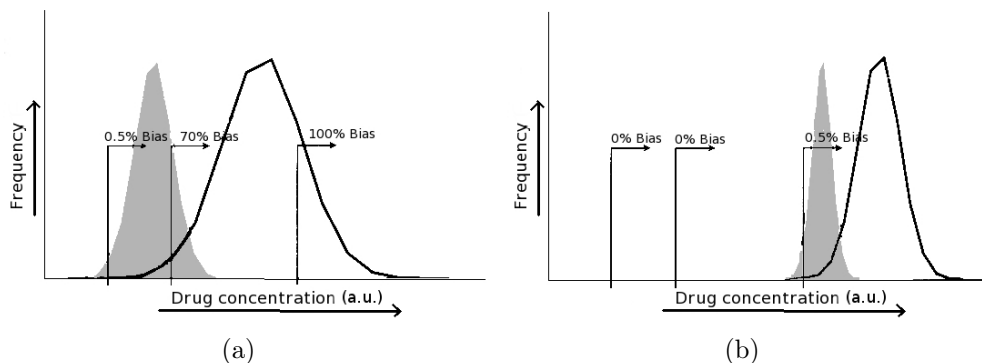
Figure 2.1: Cut-off values play an important role in generating bias if the two populations have a different drug incorporation rate and distinct binding capacities for drugs. The drug users are classified in two different groups based on hair colour (e.g. blond versus brown hair) and are represented by the white and the grey area on the graphs. For a) hair colour bias varies with cut-off value, whereas for b) no bias exist because cut-offs are low enough to detect every user. Adjusted from Kidwell [83].

as Kintz and Mangin referred to it [74], will need to take into account the possible variation between people due to the variation in production with a certain amount of drug consumption, hair growth [33], hair treatment, the incorporation rate in hair, possible hair contamination and possibly the presence in the body without drug consumption. For certain cases a low cut-off value is in favour of the tested subject, for instance when mitigating circumstances would be provided when the person claims to have been using drugs. Other cases would benefit from a higher cut-off value for this prevents false positives in the establishment if the client is an unfit parent [101].

2.2 The current status of hair testing

Since the 1990s hair analysis is offered in evidence in court [102]. It is concluded that hair testing especially when coupled with a confirmatory GCMS test, is now an accepted and reliable scientific methodology for detecting drug exposure [74, 103]. Controversies obviously still exist and the use of hair analysis is not

yet recognized by the International Olympic Committee [45, 104]. The scientific community acknowledges that certain conclusions can be drawn despite of these and other unknown factors [33] like variability of hair growth cycle, influence of cosmetic treatments and hygiene practices, uncertainty of dosages ingested by abusers, typical under-estimation of self-reported doses, unknown purity of compounds, and considerable variation in uptake of drug from blood to hair, rate of sweating and amount of apocrine and sebaceous gland secretions between individuals [45]. Some correlation is possible between hair segments and time periods. It is clear that precise estimation of time of drug consumption is not possible, but estimation can be made in intervals of a month. Despite the impossibility to deduce the exact amount of consumed drugs from the concentration in hair, cut-offs may help to indicate someone's level of usage [33, 105]: i) none to low, ii) medium and iii) high [106].

2.3 The analysis of EtG and FAEEs in scalp hair to monitor alcohol consumption

In 2000, Skopp et al. [39] recognized that EtG was a promising candidate to monitor alcohol consumption and that the short time window in urine and blood could be lengthened when EtG was analysed in hair. On the basis of eleven human hair samples they concluded that i) EtG may not be detectable from alcohol-drinking subjects and ii) the detection of EtG is always associated with recent alcohol consumption. Janda [107] developed in 2002 a more sensitive method for the detection of EtG in hair due to the use of solid phase extraction [37] and successfully applied this to 97 human hair samples. Even though the detection limit was lower, their research still demonstrated that heavy alcohol consumption may not necessarily lead to detectable levels of EtG in hair [107]. In 2009 the performance of the EtG marker in hair was reviewed and seen to outperform other methods as marker of chronic heavy drinking (the consumption of over 60 grams of alcohol per day according to WHO [11]) [108]. At the moment EtG in hair is an accepted method to monitor alcohol consumption [38, 109] and can

be used as a tool prior to organ transplantation [110], to test for abstinence for re-granting one's driving license [1], work placement testing [111] and in child custody cases [58].

It was known for decades that various fatty acid ethyl esters are formed in the human body when alcohol is consumed [56, 112, 113], but it was not until the last decade that FAEEs were recognized as a promising tool for the analysis of alcohol consumption. Pragst [67] discovered FAEEs in a methanol extract from hair of alcoholics and examined a year later whether FAEEs were suitable hair markers. Whereas fifteen esters can be detected in hair, a sum of four (ethyl myristate, ethyl palmitate, ethyl oleate and ethyl stearate) as suggested by Pragst [114] proved to be sufficiently characteristic [101]. Auwärter et al. [115] found that levels in excess of 0.2 ng/mg are not consistent with abstinence and may indicate social or moderate alcohol use. They also observed high concentrations of ethyl oleate in hair in comparison with the other ethyl esters. Kaphalia et al. [13] observed higher concentrations of ethyl oleate compared to ethyl palmitate. They ascribed this due to oleic acid being a better substrate for the formation of FAEEs. Also they obtained higher occurrence of ethyl stearate for alcohol dependent persons than for acute alcohol abusers. They suggested that these fatty acid ethyl esters were therefore especially useful as markers. FAEEs could not be used to distinguish between teetotallers and social drinkers (an alcohol consumption below 60 g per day) according to Agius et al. [72] since FAEEs are observed in teetotallers. This point is debated and the SoHT has come to the consensus that it is possible to differentiate teetotallers from social drinkers based on the level of FAEEs in hair [109]. In addition, FAEEs in hair are seen to outperform other markers of chronic heavy drinking (see Appendix C) and also FAEEs in hair is an accepted method to monitor alcohol consumption [38].

Before the SoHT described the guidelines for EtG and FAEEs detection in hair to confirm chronic drinking, Appenzeller et al. [60] suggested a cut-off of 23 pg/mg and Morini et al. [116] of 27 pg/mg. 50 pg/mg was used as conservative threshold by Kintz et al. [117], which was in the years before a detection limit [27, 107] or quantification limit [58] was introduced. As stated before, the cut-off value determines when a value is considered to be positive or negative and therefore

has an impact on the sensitivity (i.e. the percentage of chronic heavy drinkers who are correctly identified as being a heavy drinker) and specificity (i.e. the percentage of infrequent drinkers who are correctly identified as such) of the test. Infrequent drinkers here are a combination of teetotallers and social drinkers. For the generally accepted cut-off values (30 pg/mg for EtG and 0.5 or 1 ng/mg for the FAEEs), the sensitivity of the tests for FAEEs and EtG is considerably higher as compared to other alcohol markers [16, 108].

The analysis of the markers EtG and FAEEs in hair is suggested to be used to demonstrate alcoholism [38, 118] and abstinence [109]. Süße et al. [101] stressed that the cut-off values currently used are high enough to avoid false positives and that the cut-off should not systematically be applied, but should be seen in the context with all other information about the case. This may sound vague and shows that the interpretation of the analytical data is complicated, and presentation of such evidence in court has the potential to be misleading as Pragst demonstrated [73]. The current consensus of the SoHT relies on the application of cut-off values to the concentrations of alcohol markers in hair, and above these concentrations an analytical result is taken as indicative of alcohol abuse.

As the tables in Appendix D demonstrate the cut-off values to establish whether a test is positive or negative for chronic heavy drinking [38] or abstinence [119] are often determined based on the observation of the different ranges of concentrations of alcohol metabolites in hair found for the different drinking groups. Some researchers used statistical tests (like the Mann Whitney test) to determine whether there is a significant difference between drinking groups. Others looked at the correlation between self-reported drinking amounts based on correlation coefficients. Also, the sensitivity and specificity of cut-off values were evaluated with a receiver operating characteristic (ROC) curve where the trade-off between the sensitivity and specificity is investigated: a decrease in sensitivity and increase in specificity can be found for increasing cut-off values [120, 121] (see Figure 2.2). As is clear from Figure 2.2 for EtG to confirm chronic heavy drinking a cut-off is chosen (30 pg/mg) for a high specificity and not a high sensitivity. Primarily to reduce the possible false positive results, which is often in favour of the tested person.
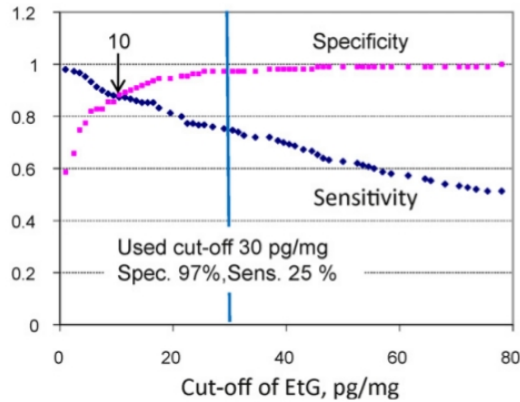
Figure 2.2: The receiver operating characteristic (ROC) curve of EtG from Süße et al. [118]. Note that the sensitivity with the cut-off value selected should be 75% not 25%.

The tables in Appendix D show that the focus of the research of EtG and FAEEs in hair has investigated mainly the differentiation between heavy drinkers and others and not so much between teetotallers and drinkers. From the tables it is also clear that the databases used are in general relatively small and with larger databases the alcohol consumption behaviour is unknown or relevant information is excluded. For the evaluation of EtG in hair as marker for chronic drinking behaviour sample group sizes were often below and around a hundred, and for the larger sample sizes described in the work of Pragst et al. [3], Albermann et al. [122] the alcohol consumption was unknown. For the work of Süße et al. [118] with a total sample size of 1872, information was used in addition to the EtG test to exclude certain samples under the assumption that self-reports are often unreliable. The social drinkers were divided into two groups: the low moderate drinkers who drink only on rare occasions but are not strictly abstinent and the moderate social drinkers who consume up to 60 gram of alcohol per day. The moderate drinking group was excluded for the analysis. When the self-reports would have been correct this means that a differentiation between teetotallers/ low moderate drinkers and heavy drinkers was established and that those who drink more are not taken into account for this analysis. Some other samples were excluded based on the lack of reported drinking data, use of hair products and hair length, which seems appropriate. The sample size for research into

the FAEEs concentration in hair for distinct alcohol consumption groups was up to 124 excluding the previously discussed work of Pragst et al. [3] and Süße et al. [118].

It seems that the cut-off values have not been evaluated in a statistically sound manner as was also previously raised by Tagliaro et al. [123]. Larger data sets are more commonly used in recent research and a more statistically sound analysis of EtG and FAEEs in hair in relation to drinking behaviour are required. Also, more information may be available than the published articles that are reviewed here as was explained in the reaction to the letter of Tagliaro et al. by Kintz [124]. In conclusion, the basis on which the cut-off values are determined is not be transparent and does not seem to be statistically sound.

In this section influences on five levels of the outcome of a test result are discussed with the interpretation of the EtG and FAEEs hair tests in mind. This obviously includes the influence of the cut-off value.

2.3.1 The influence on blood alcohol concentration over time

The current consensus of the SoHT is based on both published research data and according to Kintz on "practical experience gained from thousands of cases in multiple laboratories where alcohol marker detection and reporting have been successfully applied for many years" [124]. Therefore the ground on which the consensus is based is not transparent. This thesis reviews the work that has been published in this field and cannot assess the influence of practical experience. That Kintz [124] states that the techniques have been "successfully applied", may be a stretch for in most casework the expected drinking behaviour is unknown even though certain hair samples are used for quality control purposes.

Pragst et al. [3] postulated that the difference in blood alcohol concentration (BAC) over time (i.e. AUC, see Figure 2.3) is affected by dose and drinking habit due to the zero-order elimination of alcohol. They also showed that the discrimination of social drinking and alcohol abuse is favoured by the over-proportional

increase of the AUC between 60 and 120 grams of alcohol intake per day. Also note that AUC is a better representation than the BAC as an impact on the storage of the alcohol metabolites in hair over time.

The AUC depends on the history of alcohol absorption [73], which may be intentional or accidental exposure [27, 125]. Examples of possible accidental exposure are alcohol intake from: body spray, perfume, cologne, water-less hand soap [126], insect repellent, cough syrup, mouth wash [127] or consumption of food products such as tiramisu, risotto but also ethanol fermentation in the gut. Accidental exposure is particularly a problem when proof of abstinence is required and to a lesser extent for proof of heavy drinking [25, 128]. The possibility of the absorption of alcohol from accidental exposure resulting in higher levels of EtG and FAEEs in hair than the cut-off value must be taken into account. It would be interesting to see what level of absorption can be expected in some professions, such as a distillery worker, a nurse who frequently uses alcohol containing soaps [126] or someone that is exposed to sprays such as a hairdresser. Hence, before one jumps to conclusions specific background information is required about for instance yeast infections and accidental exposure.
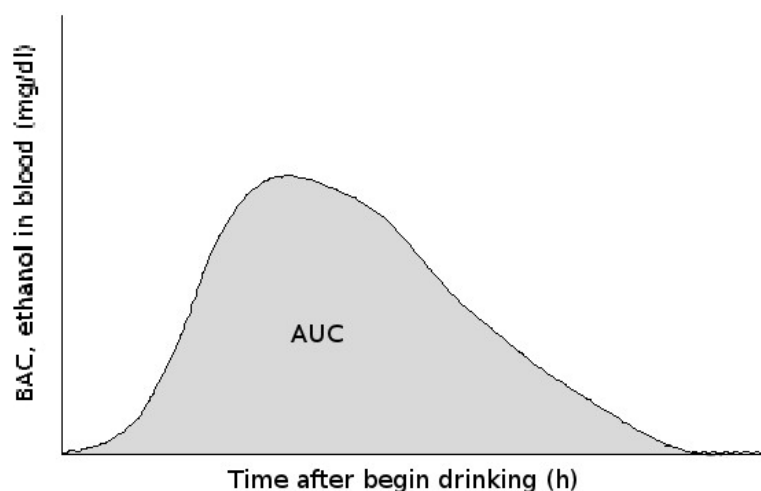
Figure 2.3: The correlation between blood alcohol concentration (BAC) and the grey coloured area under curve (AUC): the AUC provides insight of the BAC over time. Adjusted from Pragst et al. [3].

2.3.2 The influences on EtG and FAEEs in blood

EtG and FAEEs concentrations in blood is affected by alcohol concentrations in blood and individual variation in absorption, distribution, metabolism and elimination. Politi et al. [69] describe an experiment where they used a weight adjusted ethanol intake, FAEEs in blood for men were twice as high than for women. This may be explained by: i) the distribution of the FAEEs in body fat that is in general higher for women [129] or ii) the chronic drinking behaviour of the male subjects in this test for the pathway to FAEEs is up-regulated for chronic heavy drinkers [13, 56, 57].

The formation of EtG is influenced by different factors than FAEEs formation. The alcohol in blood is converted to EtG by the uridine diphosphate glucurono-syltransferase (UGT) super family. As a result of the many UGT isoforms that are involved in this process, any functional differences in the enzymology underlying ethyl glucuronide formation would most likely be masked by a combination of other enzymatic pathways according to Foti and Fisher [70]. According to Pragst et al. [3] AUC alcohol and AUC EtG are directly proportional with a proportionality factor that is the same for heavy drinkers and those who consume less. The conversion of ethanol to FAEEs, on the other hand, is influenced by chronic alcohol consumption. Relatively more FAEEs are produced when someone is a heavy drinker [13, 56, 57]. Briefly, whereas AUC of EtG in blood is nearly proportional to the AUC of alcohol, this is not the case for the FAEEs.

Tagliaro et al. [123] raised their concerns for the use of EtG and FAEEs as markers, because these are only minor metabolites. They reason that biological variation may therefore have a major impact on the concentrations of these metabolites. Kintz [124] explains that the high degree of biological variability between dose and measured concentration in hair for the alcohol markers is widely published and accepted and that this has not prevented the acceptance of alcohol analysis in hair in legal proceedings.

2.3.3 Factors involved in the concentration of EtG and FAEEs in scalp hair

No exact correlation was found when self-reports of estimated amounts of alcohol consumption were compared with the measured concentration of alcohol metabolites in hair [6, 115, 128, 130]. This could be due to the unreliability of the self-reports, the time line in which the amounts are consumed or physiological or environmental differences between people. This section will focus on the inter-individual differences that may cause the lack of such a correlation.

Pragst et al. [3] described that EtG concentration in blood was correlated with the EtG concentration in hair. However, for the FAEEs they described that the FAEEs concentration in hair was likely more related to the blood alcohol concentration. FAEEs are metabolised in blood and are attached to a protein (e.g. albumin) for its diffusion in this medium. Both have a negative impact on the incorporation in hair. The FAEEs in hair are thought to be mainly produced in the sebum glands from alcohol and may therefore be affected more by the blood alcohol concentration than the blood concentration of FAEEs.

2.3.3.1 Variation in the incorporation of alcohol metabolites in hair

Various mechanisms are described for the incorporation of analytes in hair. One medium of the incorporation and transportation of EtG and FAEEs to hair is via the blood supply at the hair root for which FAEEs are most likely attached to albumin [131] due to their hydrophobic nature. EtG is thought to be incorporated via diffusion from sweat too [37] and FAEEs are likely to be distributed on the hair shaft via sebum from underlying tissues [115] due to its hydrophilic nature and the increased concentration towards the distal end of the hair shaft observed with constant drinking [101]. For humans the correlation between the marker in blood and in hair is not straightforward, because the incorporation route is unlike those of rat models [71] not only via blood. It is suggested that variations in sweat and sebum production impact respectively EtG and FAEEs concentration in hair [115, 123].

2.3.3.2   The contamination and removal of alcohol metabolites in hair

The concentrations of the markers in hair can also be affected by the use of hair treatment products that contain the same markers or alcohol. Alcohol-containing products can lead to the formation of FAEEs on the scalp [61, 132]. Auwärter [115] thought this would explain some of their results and pointed out that Laposata [66] found FAEEs synthase activity in almost all human tissue. Gareri et al. [4] have shown that hair products that contain only 10% alcohol can affect levels of FAEEs in hair. FAEEs in hair can originate from alcohol-containing and FAEEs containing hair products. The EtG concentration in hair, however, is not influenced by alcohol containing cosmetics and was affected by cosmetics containing EtG [133].

EtG concentration can be significantly decreased by the use of hair dye and bleaching [60, 118, 134] and Süße et al. [118] has suggested that this may be due to oxidation. Perms are also seen to decrease the concentration of EtG in hair which may be caused by the increase of porosity of hair that allows the contents of the hair, including alcohol markers, to leak out [77, 135].

Removal due to normal hair care routines is not expected; a study where hair was washed twelve times did not show a decrease in levels [132]. A decrease with constant drinking is observed and was previously suggested to be caused by a wash-out effect during normal hair hygiene [20] due to the high solubility in water [38]. The removal of alcohol markers from hair can result in false negative test results.

The effects of external contamination and removal are expected to be more pronounced with increased distance from the scalp. For the FAEEs a higher cut-off value is used for longer hair segments (segments longer than 3 cm) to allow for the normal build up of FAEEs in hair. No other significant difference is observed for the concentration of FAEEs up to 6 cm proximal long hair segments. The decrease in EtG towards the distal end of the hair shaft is taken into account in the Society of Hair Testing (SoHT) consensus by only allowing segments up to 3 cm for EtG [38]. Agius et al. [72] also found a decrease for proximal hair segments 3 cm and those that are shorter than 3 cm. They, however, did not observe a

wash-out effect for segments up to 12 cm. They therefore suggest that longer hair segments can be used. That more external contamination of both markers and removal of EtG may be more pronounced in the more distal hair segments and therefore only measuring hair segments up to 3 cm might thus be overly cautious.

2.3.3.3 The stability of the analytes in hair

Besides removal, the degradation of a molecule itself may also result in a reduction of the concentration of alcohol metabolites in hair. In tests for EtG and FAEEs it is clear that the molecules are stable in hair [39, 59]. However, in special circumstances of post-mortem cases, this may not be the case. Bendroth et al. [47] described a case where an assumed alcoholic was submersed in a river for a long time and no EtG could be detected in the subject's hair. Note that in most post-mortem cases hair is thought to be well protected against internal changes in a decaying body and can therefore be preferred over other bodily matrices [47].

2.3.3.4 The hair properties

Hair colour does not affect EtG and FAEEs concentrations in hair [54, 71], but other hair properties may influence the metabolite variability between individuals like hair growth and hair porosity. It is thought that a higher porosity, found in children [136] and people who use harsh hair treatment [27, 135], leads to an increased incorporation from external sources [137]. Note that when external contamination is determined, positives should be interpreted with caution independent from hair porosity. However, porous hair may also increase the removal [77]. This is one of the reasons negative tests are not seen as conclusive proof of abstinence or social drinking (depending on what cut-off is used). Also hair growth may impact the amount of EtG and FAEEs that are incorporated in hair. When hair grows twice as fast, it is likely that the same hair segment length is exposed to alcohol markers from sweat, sebum and blood for a shorter time and therefore exhibits a lower metabolite concentration. Hair properties are often referred to when bias is discussed, in particular racial bias. It is, however, better to directly relate this to actual hair properties rather than relate it

to something that may have an impact on hair properties such as ethnicity as race cannot be determined by the microscopic examination of hair [138, 139] and increased inter-racial mixing occurs [140].

2.3.4   The factors involved in reporting the concentration of EtG and FAEEs in hair

Pragst and Balikova [27] state that hair analysis needs substantial guidelines throughout the testing process from collection to interpretation. The detected metabolite concentrations must be measured and depending on the precision and accuracy of this measurement a certain deviation from the true value can be expected. Validation studies are performed [59,119,141] and different laboratories are involved to examine their differences. The laboratories that are involved in these tests show an acceptable analytical variation [36]. In addition, Cooper et al. [142] had 52 responses from hair testing laboratories on amount and type of hair tests. 83% follow guidelines and a large group was developing quality systems and aimed to have accreditation within 2–3 years.

2.3.5   Influences of the reported negative or positive test result

Often laboratories interpret the test results with a cut-off value. The threshold for EtG is suggested to be 30 pg/mg [38, 47, 134] and for the FAEEs the cut-off values for the sum of the 4 FAEEs are set to 0.5 ng/mg for hair shorter than 3 cm and 1 ng/mg for longer [38]. Measurements above this value may indicate alcoholism. As stated before, the cut-off value determines when a value is considered to be positive or negative and therefore has an impact on the sensitivity and specificity of the test. For the previously mentioned cut-off values, the sensitivity of the tests for FAEEs and EtG is high compared to other alcohol markers [16, 108].

2.3.6 A summary of the factors involved of EtG and FAEEs hair testing

The factors that are explained previously are visualized in a model in Figure 2.4(a) for EtG and in Figure 2.4(b) for FAEEs. In the latter model the difference with the factors that play a role for EtG are highlighted in grey. This model can be simplified by only taking into account the factors that are agreed to probably have the highest impact (see Figure 2.5) and also the factors that influence both EtG and FAEEs in hair are depicted. It is apparent too that hair treatment (removal and external contamination) is a problem for EtG and FAEEs hair tests.

(a)   (b)
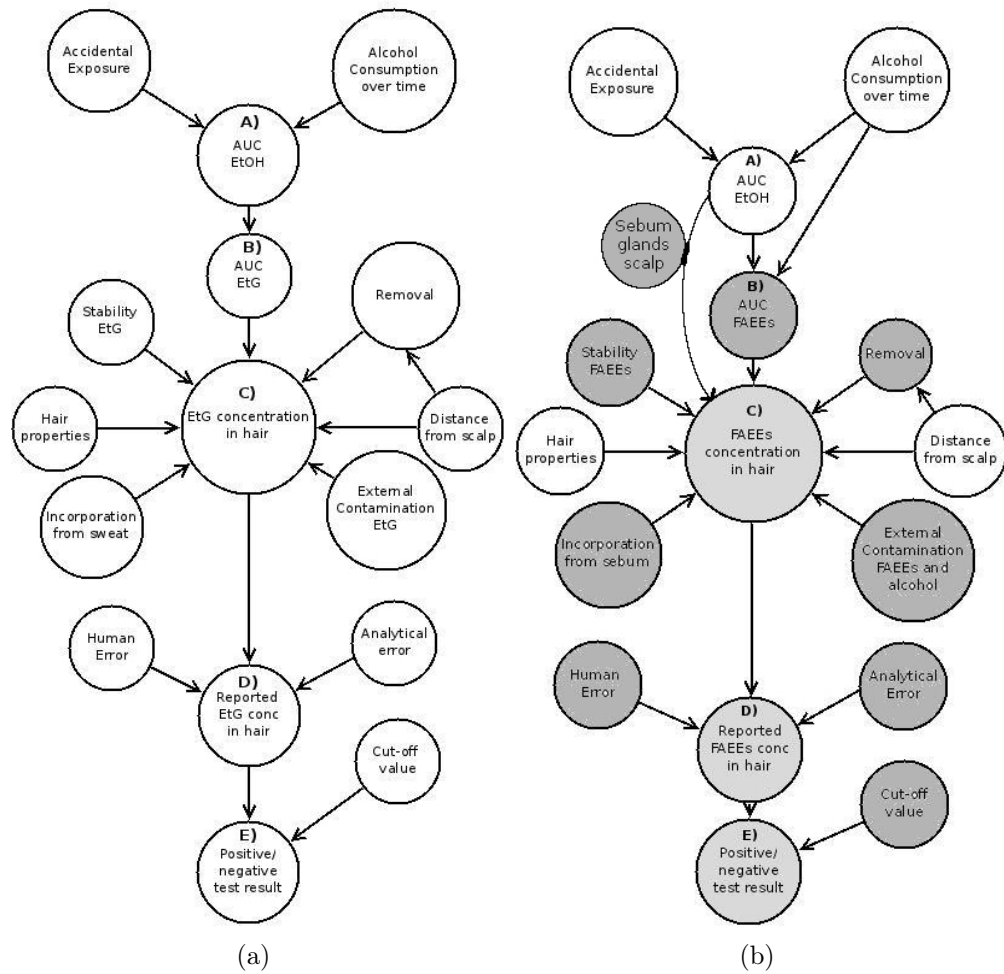
Figure 2.4: Models of the factors that influence a) EtG and b) FAEEs concentrations in hair. The differences with EtG are indicated in grey. A to E are indicated in the factors in the middle column of each of the models and correspond with Sections 2.2.1 to 2.2.5 respectively where these factors are discussed.
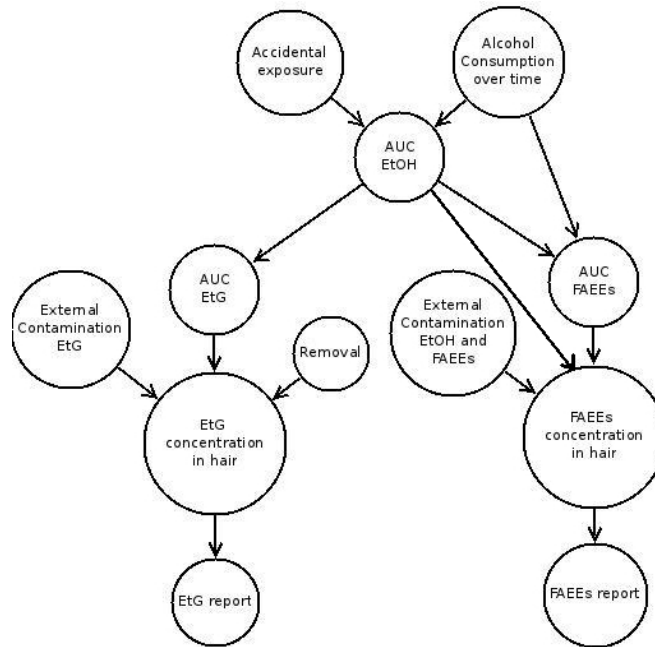
(a)
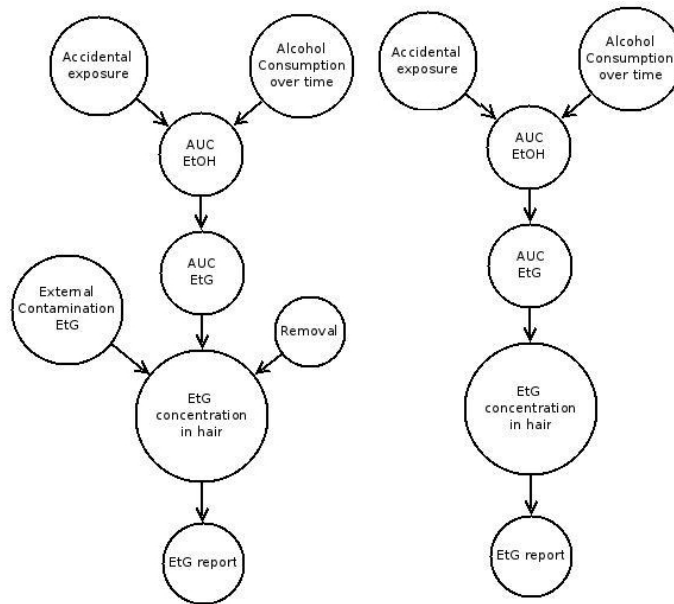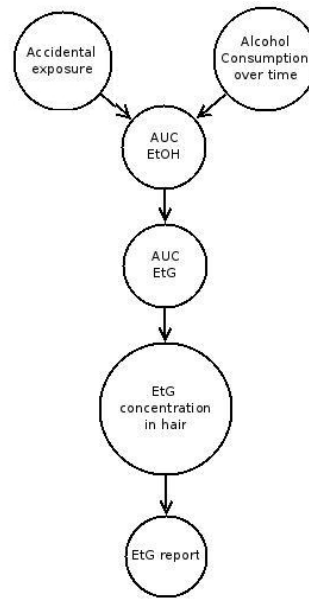
(b)   (c)

Figure 2.5: Simplified models of a) a combined test, b) an EtG test and c) an EtG test for which further simplification was used because the assumption that no hair treatment that may affect the EtG outcome could be used.

Chapter 3

Theory

In this research several techniques were used. The principles on which these techniques are based on are explained in this chapter.

3.1 Solid phase extraction

Kintz et al. [37] claims solid phase extraction (SPE) has decreased the detection limit of EtG in hair significantly (from ng/mg to pg/mg). When the methods to detect EtG in hair are compared over time (see Appendix E) it is clear that SPE may not be the only factor involved in lowering the detection limit; an increasing amount is injected in the GC, less liquid is used to reconstitute the sample before the injection, different derivatisation agents are used and subsequently another mass spectrometry detection method was used. However, background interference from the matrix can be reduced with SPE and in this manner can cause an increase in sensitivity. Moreover, many factors may play a small part in the reduction of the detection limit (e.g. injecting two instead of one microlitre can at most result in a twice as low detection limit), and derivatisation agent is changed over time without an impact on the detection limit. Hence, in agreement with Kintz et al. [37] it is concluded that SPE is likely to have a high impact on the decrease in detection limit.

SPE is a separation process in which various components in a liquid are separated from other compounds in the mixture based on their physical and chemical properties [143, 144] and is particularly useful for hydrophilic compounds that are difficult to extract by liquid liquid extraction (LLE) [145]. The liquid solution (mobile phase) is passed through the stationary phase. Analytes with a high affinity for the sorbent on the stationary phase are retained, while other compounds will remain in solution and pass through. Vacuum, centrifugation or positive pressure is used to force the sample through the solid phase. Before the sample is loaded the sorbent is conditioned to remove any impurities from the cartridge and wet the stationary phase to generate a sorbent environment that efficiently retains certain compounds [146]. After the sample is loaded, the sorbent is washed to remove unwanted compounds that are often co-retained with compounds of interest during sample load. These are removed because they may interfere with the subsequent analysis. In most cases the compound of interest is more tightly bound than other compounds. Elution is performed by disruption of the interaction between the analyte and the stationary phase (see Figure 3.1). When dealing with ionizable compounds, pH manipulation may play an important role [144].

1. conditioning   2. sample application   3. washing   4. elution
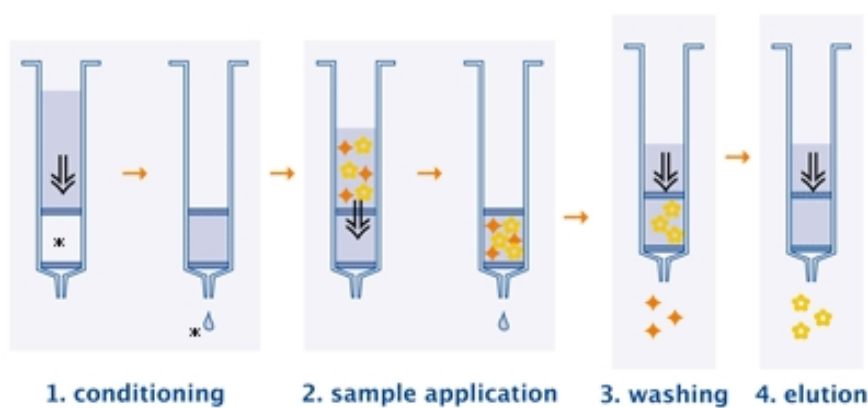
Figure 3.1: The general SPE procedure: 1) conditioning column to remove contaminants and solvate the surface for proper phase interaction, 2) the sample is loaded on the column, 3) the interferences are washed away and 4) the analyte is eluted. Adjusted from [147].

Separation with SPE based on polarity can be done in a normal and reversed

phase [144]. Reversed phase is useful for a semi-polar to non-polar analyte. The stationary phase is non-polar and the solvent is typically polar (e.g. water) to semi-polar. When the sample is loaded, the solvent is not retained on the sorbent and the analyte binds, due to Van der Waals forces, the attractive forces between the carbon-hydrogen bonds of the analyte and the functionality groups of the stationary phase (see Figure 3.2). Then the weakly retained components are eluted with less polar solvent and the more tightly bound components are eluted with more non-polar solutions. Normal phase extraction has a stationary phase that is polar and works therefore in exactly the opposite manner as reversed phase retaining the analyte with hydrogen bonding, pi-pi interactions, dipole-dipole interactions and dipole-induced dipole interactions. Therefore, normal phase extraction may be suitable for polar to semi-polar analytes.
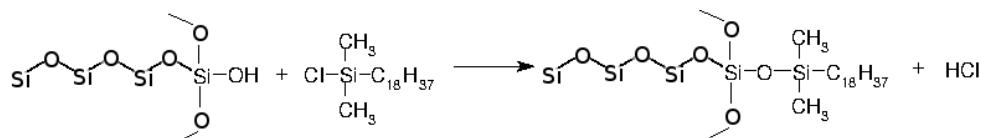
Figure 3.2: The modification of hydrophilic silanol groups of the raw silica packing with a hydrophobic alkyl functional group to obtain a reversed phase stationary phase. Adjusted from [144].

Molecules can also be retained based on electrostatic attraction [144]. For this it is key to have a pH during loading that ensures the analyte and the stationary phase are charged. When either one is neutralized, the electrostatic force is disrupted and the analyte eluted. Also a solution with a high ionic strength can be used that displaces the analyte and results in the elution of the analyte. Two types of ion exchange exist: anion and cation of which the first is suitable for anionic compounds and the second for cationic species. An anion exchange SPE has an aliphatic quaternary amine group that is bonded to the silica surface. For loading the pH must be 2 pH units above the pKa of the acidic (then anionic) analyte. For elution, the pH is then reduced to neutralize and elute the analyte (see Figure 3.3 for the correlation between pKa and pH).

Due to some impurities, besides the main interaction also secondary interaction can occur [144]. A reversed phase can also have some polar secondary interaction with residual silanols (see Figure 3.2) and Si-OH (with a low pH) or SiO- (for a
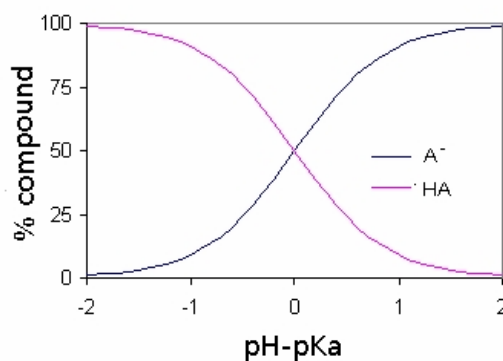
Figure 3.3: The charge of a compound in relation with the pH and its pKa. When the pH equals the pKa similar amounts are found of the charged and uncharged species. When the pH is two units below the pKa most of the compound is present as A- and two pH units above the pKa mostly AH is present. In this example the basic form is negatively charged, however, for other compounds the acidic form can be a cation and its basic form can be neutral. Adjusted from [148].

pH above 4) can result in cation exchange interactions. A solution for the first is that methanol can be used to break up any hydrogen bonding and for the second the pH can be adjusted: a low pH to neutralize the silanol group or high pH to neutralize the basic analyte. The first will often not be a problem since the analyte that is suitable for reversed phase is non-polar and more non-polar groups than residual silanols will be present on the stationary phase.

3.2   Derivatisation

EtG can be derivatised with N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) (see Figure 3.4) as well as pentafluoropropionic anhydride (PFPA). EtG has four hydroxyl groups that may react and replaced by a trimethylsilyl (TMS) group when derivatised with BSTFA and pentafluoroethyl group when derivatised with PFPA (see Figure 3.5). The derivatisation product that is obtained with BSTFA is referred to in this work as EtG-BSTFA and the derivative obtained with PFPA is referred to as EtG-PFPA. Different molecular structures are suggested for the

derivatisation product obtained with PFPA. Agius et al. [44] suggested a structure that may be subjected to more steric interference and is thus less favourable than the structure that is suggested by Kharbouche et al. [59] (cf. Figure 3.5(b) and 3.5(c)). Hence, probably the EtG-PFPA is displayed in Figure 3.5(c) as was suggested by Kharbouche et al. [59].
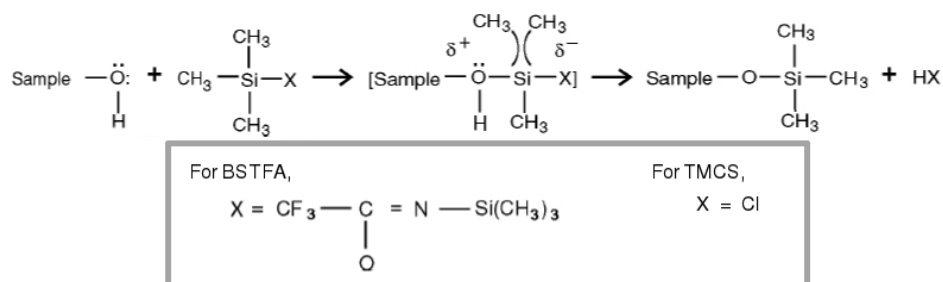
Figure 3.4: The general reaction with BSTFA and TMCS for the formation of a trialkylsilyl derivative by the displacement of the active proton in the OH-group. Obtained from [149].

3.3 Head space solid phase micro-extraction

For the FAEEs head space solid phase micro-extraction (HS-SPME) is used (see [114]). HS-SPME discriminates between compounds based on volatility and polarity [150]. An equilibrium forms between the compound in the solvent (the sample matrix) and its head space (the air directly above the sample) and the latter is sampled with a fibre that contains material that can absorb or adsorb the analytes [151].

Several steps are applied for HS-SPME (see Figure 3.6) [152]. First the septum of the (heated) sample head space (HS) vial is pierced while the fibre is protected by a hollow metal needle and then the plunger is pressed down to expose the fibre to the head space of the sample material. The fibre is exposed for a certain time: the extraction time. The analytes are now on the fibre. Then, the fibre is retracted and the needle is removed from the sample vial. To desorb the analytes, the same procedure is used as for sampling, but on the heated GC injection port.

(a)

(b)   (c)
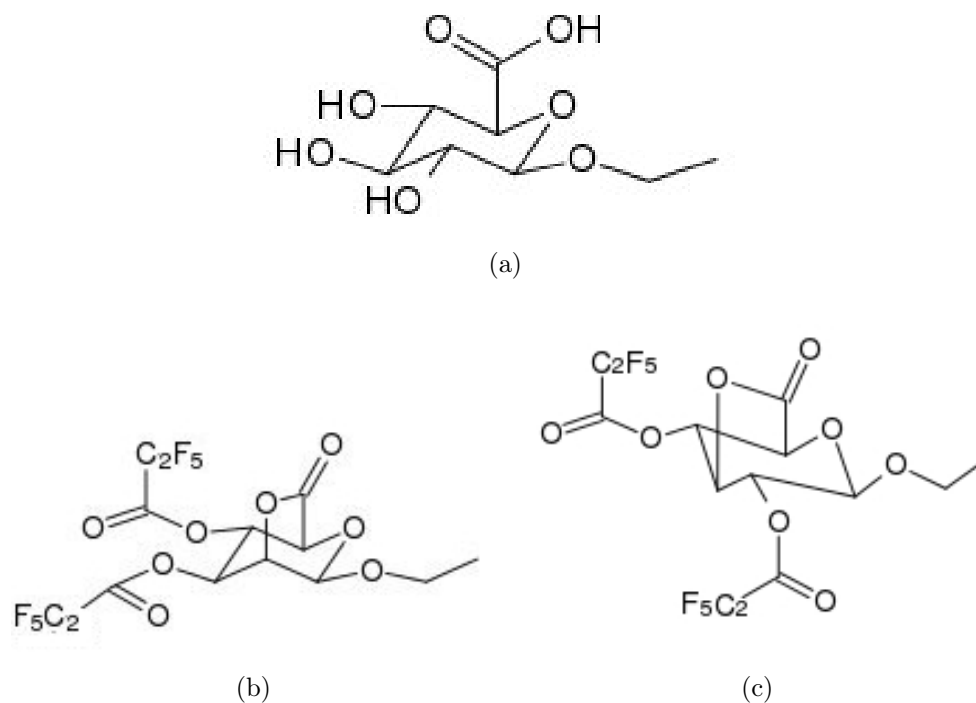
Figure 3.5: The molecular structures of a) EtG, b) EtG-PFPA published by Agius et al. [44] and c) EtG-PFPA published by Kharbouche et al. [59].

SPME is based on the equilibrium between the sample, head space and fibre coating [152]. When molecules from the head space are absorbed or adsorbed to the coating, more will diffuse from the sample to the head space, until the equilibrium sets in. Therefore, a longer extraction time is required for less volatile compounds. However, for porous materials that are based on adsorption, the limited capacity due to the limited available active sites that trap compounds may result in competition of the molecules and possibly displacement of the analyte by a less volatile compound for too long extraction times [154]. The extraction times of various organic compounds in hair extracts are discussed by Sporkert [155].
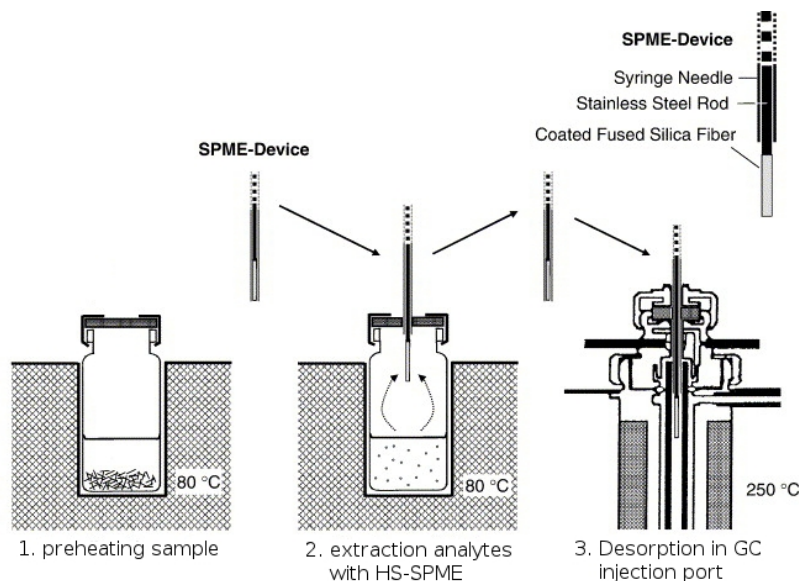
Figure 3.6: The general HS SPME procedure. 1) The sample is prepared for HS SPME, which is often done by preheating the sample to the extraction temperature. 2) The fibre is exposed to the HS of the sample by piercing through the septum of the HS vial with the fibre retracted in the protective needle and subsequently exposing the fibre by depressing the plunger of the SPME device. After the extraction time, the plunger is moved upwards and the fibre is retracted before the removal of the SPME device. 3) The last step is the exposure of the fibre to the GC injection port for desorption. Also here the fibre needs to be retracted for insertion and removal to prevent damaging the coating. Adjusted from Pragst [153].

3.4   Gas chromatography

Gas chromatography (GC) separates compounds based on their affinity to the stationary phase in the column by sweeping vaporized compounds through the column with a carrier gas (e.g. Helium, Hydrogen or Nitrogen) [156]. The stationary phase is often a liquid in which compounds can dissolve or solid particles on to which compounds can adhere. The stronger the affinity of the compound to the stationary phase the longer it takes to elute from the column. This elution time, or retention time is specific to a particular compound, though several compounds can have similar retention times and therefore analysis based solely on GC retention times is often not sufficient for the confirmation of a compound [156].

To enable the compounds to be transported by the carrier gas, the compounds must be vaporized. Some molecules such as EtG are not volatile and therefore need to be derivatised [157, 158].

The sample can be transferred to the GC in several ways. SPME is one example, liquid injection another. For liquid injection a portion of the sample solution is taken and injected in the injection port or straight on the column. On column injection ensures no loss of the analytes but it also means that the majority of the less volatiles are introduced on the GC system, which may lead to contamination of the column, especially when dealing with dirty extracts. Transfer via the injector port, on the other hand, exposes the sample to heat thereby vaporizing compounds on to the column. This can cause thermal degradation of the analytes. For dirty samples this method is highly recommended as the low to non volatiles components are trapped on the injector liner and do not contaminate or block the column [156]. Normally for liquid injection small volumes in the range of a few microlitres are injected. Attempts to increase the injection volume lead to broadened and distorted analyte peaks, large and long solvent peak tails and saturated or damaged detectors [159]. Large volume injection (LVI) has been developed to increase the sensitivity of a method by enabling the introduction of larger volumes: up to millilitres of the sample [160]. The sample solvent is selectively evaporated and removed from the inlet system before the sample is transferred on the column in splitless mode. This requires a temperature programmable injector and is called a programmable temperature vaporization (PTV) [160].

The retention time of a compound can be affected by the temperature of the column in the GC oven [161]. If the oven temperature is kept constant (isothermal) later eluting compounds will tend to have variable retention times; due to the fact that the same molecules do not travel the same path in the GC system as a result of dispersion. This can also cause peak broadening (see Figure 3.7). A temperature gradient can be applied to maintain sharp peaks and avoid the loss in sensitivity (measured via signal to noise ratio) for the compounds with longer retention times (see Figure 3.7). With gradient GC the oven starts at low temperature, below the boiling point of the analytes. This traps the analytes and refocus them at the beginning of the column. Using a temperature below the boil-

ing point of the solvent will trap the solvent and everything that is dissolved in it. This approach is called cold trapping and solvent trapping respectively [156]. Once the sample has been injected the temperature of the oven is increased, this reduces the elution time and peak broadening of the analytes. To assist the elution of possible column contaminants, the oven temperature can be increased or maintained at a high temperature before the next sample is injected [161].
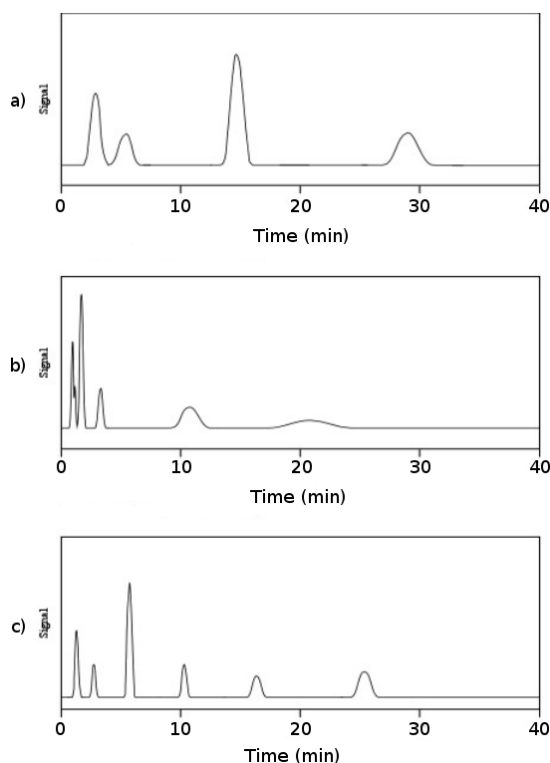
Figure 3.7: The effect of the GC oven temperature on eluting peaks is illustrated with one sample that is analysed with a) an isothermal temperature at 40 ℃, b) an isothermal temperature at 200 ℃ and c) an oven temperature programme from 40 ℃ to 200 ℃. From Dunnivant and Ginsbach [162].

3.5 Mass spectrometry

The mass spectrometer (MS) measures the mass-to-charge ratio (m/z) of charged particles. A mass spectrum can show a fragmentation pattern that enables the

identification of a compound or it may be used to monitor certain characteristic ions to look for specific analytes [163]. Several steps are involved to enable these measurements. First the molecules need to be charged; this is achieved by bombarding them with electrons, i.e. electron ionisation (EI). In addition to simply losing (+ve) or gaining (-ve) one electron to produce a molecular ion which is indicative of molar mass, molecules can fragment, providing structural information. A 'milder' ionisation method is known as chemical ionisation (CI). Here a gas (e.g. methane) is present during the ionisation process. The gas is charged first and subsequently a charged CI gas molecule or charged part of this molecule (e.g. a proton) is transferred to the sample molecule [164]. This provides a mass spectrum containing an enhanced pseudo molecular ion. Ions produced by either the EI or CI process are then focussed with lenses and repelled to a mass analyser like a quadrupole. In a quadrupole only one specific m/z value has a stable trajectory at that moment in time, other values will be deflected and absorbed by the walls of the quadrupole (see Figure 3.8). This is because the quadrupole consists of four rods with a direct current (DC) and radio frequency (RF) and setting the DC and RF selects an ion with a certain m/z to have a stable trajectory. Other m/z will be unstable and collide with one of the rods. The ions with the m/z that is selected are then guided to the photomultiplier detector in which the signal is initially enhanced with a series of dynodes. When an ion hits the photomultiplier, electrons are produced and are directed towards the electron multiplier. The electron arrives at the first dynode with more energy than the original ion because of the acceleration due to the electrical field and upon collision with the dynode several low energy electrons are generated. The emitted electrons are in turn accelerated towards the second dynode producing even more electrons upon impact. The chain of dynodes is constructed such that an increasing number of electrons is produced at each dynode. At the anode, the accumulation of charge is picked up as a current pulse indicating the arrival of an ion at the photo-cathode which will be translated into a intensity for the measured m/z, depending on the amount of ions that hit the photomultiplier. In this way a mass spectrum is produced [163].

As described by Hoffmann and Stroobant [164] different measurement modes
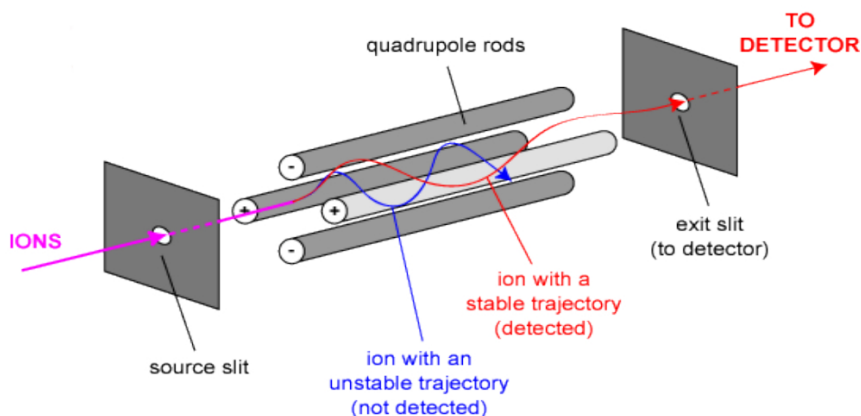
Figure 3.8: The quadrupole consists of four parallel metal rods. Opposing rods have similar signs and adjacent rods have a different sign. The DC and RF voltages ensure that only a certain ions trajectory is stable that of the selected ion. Other ions have a unstable ion trajectories and are absorbed by the rods after collision. From [165].

exist. The quadrupole can scan for a range of m/z values over time which is referred to as a full scan (FS), but can also select certain ions referred to as selected ion monitoring (SIM). In SIM more time can be devoted to an ion, resulting in a higher amount of ions that hit the photomultiplier and therefore is a more sensitive mode compared to FS. A triple quadrupole mass spectrometer consists of two mass analysers (Q1 and Q3) that can measure in either FS or SIM separated by a collision cell (Q2) contained with an inert collision gas that causes further fragmentation (see Figure 3.9). Several combinations or modes can be used with a triple quad (see Figure 3.10). In product ion scan mode one ion is selected in Q1, the ion is fragmented in Q2 and a FS is done in Q3. Structural information is obtained about the ion selected in Q1. In the selected reaction monitoring (SRM) mode one ion can be selected in Q1, but this is then followed by the selection of another single ion. Multiple transitions can be measured in this manner. For a precursor ion scan and neutral loss Q1 is set to scan over a range. For the precursor ion scan after fragmentation in Q1 one ion is selected. This can be useful to detect molecules with a certain characteristic group, that is produced and charged during the fragmentation in Q2. When, however, a characteristic group is lost that is not charged, another scan in Q3 may be able

to reveal this neutral loss.
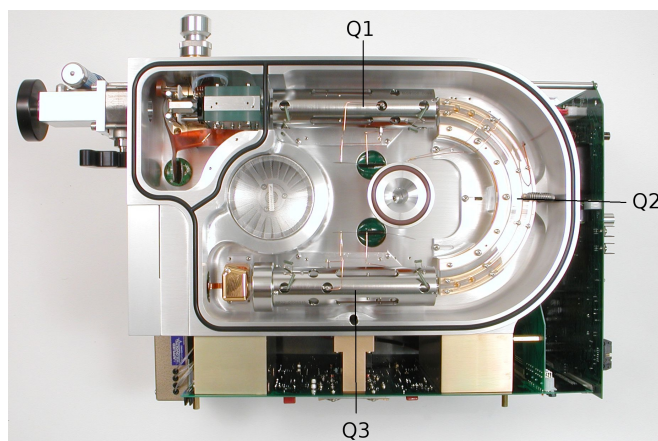
Figure 3.9: A picture of a mass spectrometer with three quadrupoles: Q1, Q2 and Q3. From [166].

For quantitative analysis with GCMS deuterated internal standards can be used [156]. Currently, this is done for EtG and FAEEs analysis from hair extracts (see [3, 119]). Internal standards are affected by the sample preparation and analysis in a similar way as the analytes and are therefore an essential component of a robust analytical method. Deuterated internal standards are isotopically labelled versions of the analytes. For the five metabolites that are measured in hair (EtG, ethyl myristate, ethyl palmitate, ethyl oleate and ethyl stearate) 5 hydrogens in the analytes are replaced by deuterium to obtain the deuterated internal standard and have therefore a mass 5 difference than the analytes that need to be quantified.
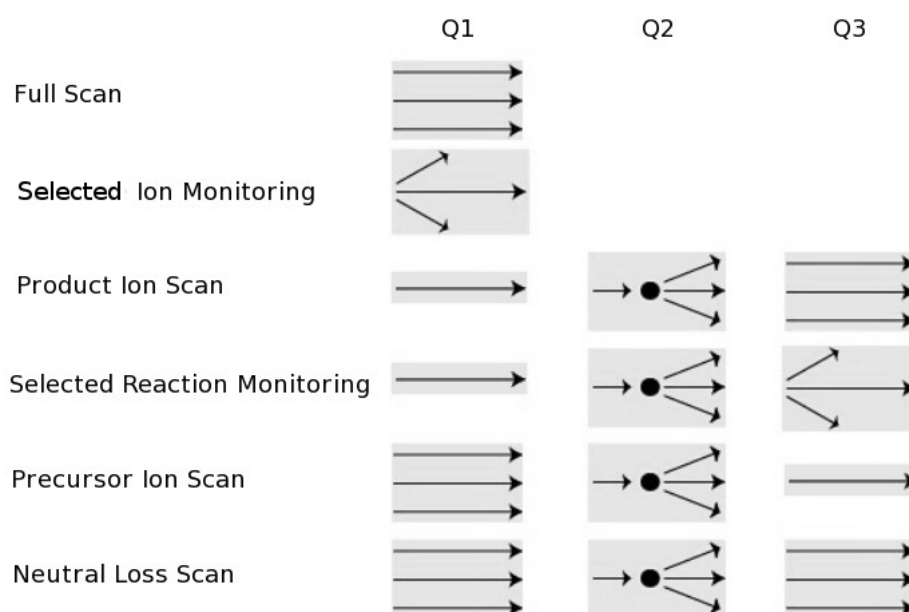
Figure 3.10: The MS modes that can be used with a triple quad. The scanning mode or selective mode can be used in both the first and third quadrupole and the second may or may not be contained with collision gas. Adjusted from [166].

Chapter 4

Material and methods

This chapter explains the development and protocols used for EtG and FAEEs hair analyses in this project, including a discussion of the evaluation of the analyses.

4.1 Classification of drinking behaviour

The terminology used for the classification of alcohol users into three groups (teetotallers, social drinkers and heavy drinkers) is similar in different publications. In the field of alcohol hair testing the consumption of more than 60 grams of alcohol per day [3, 30, 101] is often referred to as a measure for alcoholism or chronic heavy drinking. Someone who consumes alcohol but less than 60 gram per day is referred to as social drinker and someone who does not consume any alcohol as a teetotaller.

In this research two different drinking groups were combined to enable the categorization of the three drinking groups into two mutually exclusive groups (see Equation 4.1) which was required for the Bayesian theory. Teetotallers and social drinkers were summed up to form the group of 'infrequent drinkers' (this is everyone excluding the heavy drinkers). A weighted summation was also applied in which the amount of social drinkers was multiplied to obtain a ratio of either

social drinkers to heavy drinkers or social drinkers to teetotallers that represents the UK population (see Equation 4.2).

$$\text{Infrequent drinkers} = \text{Teetotallers} + \text{Social drinkers}$$
$$\text{Drinkers} = \text{Social drinkers} + \text{Heavy drinkers}$$
(4.1)

$$
\begin{aligned}
\text{Weighted infrequent drinkers} = \quad & \text{Teetotallers} + \\
& \text{Social drinkers} \times \frac{\text{total T}}{\text{total SD}} \times \frac{\%\ \text{SD}}{\%\ \text{T}} \\
\text{Weighted drinkers} = \quad & \text{Heavy drinkers} + \\
& \text{Social drinkers} \times \frac{\text{total HD}}{\text{total SD}} \times \frac{\%\ \text{SD}}{\%\ \text{HD}}
\end{aligned}
$$
(4.2)

Where SD = social drinkers, HD = heavy drinkers, T = teetotallers and the percentage of teetotallers, social drinkers and heavy drinkers are those of the population it needs to represent and thus correct for, which is in this work the UK population. The numbers for the UK population are obtained from the office for national statistics (ONS) [167]. When a database that does not discriminate against gender was used, the distribution between teetotaller, social drinker and alcoholics was described as follows: 14% teetotallers, 82% social drinkers and 4% alcoholics.

4.2   Chemicals, preparation and storage

FAEEs were purchased from Sigma-Aldrich (Gillingham, UK). EtG, FAEEs-D5 and EtG-D5 were purchased from LGC Standards (Teddington, UK). N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) derivatisation grade with a purity >99% was purchased from Stratlab Ltd (Macherey-Nagel, Germany). Pentafluoropropionic anhydride (PFPA) for GC derivatisation >99.0% and BSTFA (derivatisation grade, purity of 99% excluding trimethylchlorosilane (TMCS)) with 1% TMCS were purchased from Sigma-Aldrich (Switzerland). Laboratory reagent grade dimethyl sulfoxide (DMSO), acetone, ethyl acetate (EA), anhydrous magnesium sulphate, 35% ammonium hydroxide and dichloromethane (DCM); tech-

nical grade reagent sodium chloride; analytical grade glacial acetic acid and diethyl ether; HPLC grade heptane, hexane were purchased from Fisher. HPLC grade methanol and toluene were from Sigma-Aldrich (Gillingham, UK). 5% dimethyldichlorosilane (DMDCS) in toluene (Sylon CT) from Supelco (Bellefonte, USA). Methane 5.5, argon 6.0 and helium 5.0 were supplied by Air Liquide (Birmingham, UK). De-ionized water was prepared with a Purite Neptune system with NCP 8 cartridges.

A working standard solution was prepared by dissolving EtG and EtG-D5 in methanol which was then stored at -30 ℃. Sub-dilutions of the working solutions were prepared on a daily basis. These solutions were then used to prepare a five point calibration range and three quality control (QC) samples (for the concentrations see Table 4.1). The extraction was performed in the presence of a blank 30 mg hair sample to obtain similar interferences from the hair matrix as with an actual test of a hair sample. For the FAEEs and FAEEs-D5 the daily preparation of sub-dilutions was not required due to the stability of these analytes. Solutions in methanol and heptane were made for the five point calibration and QC samples and these were stored at -30 ℃. All solutions were prepared and used at room temperature (RT). After opening the bottles in which the derivatisation agents were stored, the agents were transferred to another bottle and put at 8 ℃ in a desiccator with 2–5 mm silica gel orange moisture indicator to reduce the presence of moisture.

Glassware was silanized by the deposition of one drop of 5% DMDCS in toluene, which was left for 30 min while a cap was placed on the glassware. Thereafter the glassware was rinsed with toluene, methanol and subsequently de-ionised water. For glassware without a cap, all surfaces were covered by the solution by tilting the glassware.

Table 4.1: The concentrations of the EtG and FAEEs solutions used for the validation study. Where CAL: calibrator, QC: quality control and DIS: deuterated internal standard. In method 1 described in Section 7.2.1 twice the concentration of DIS was added prior to the extraction stage since only half of the sample was analysed and the concentration of FAEEs in 2 mL heptane was twice as low and the concentration of EtG in the 0.5 mL of DMSO was twice as high (see Section 7.2.3) to account for the difference in extraction volume.

| | **Concentration in ng/mL** | | | | |
	EtG	E18:1	E14:0	E16:0	E18:0
CAL 1	0.3	6.0	3.0	3.0	3.0
CAL 2	0.7	15.0	7.5	7.5	7.5
CAL 3	1.5	30.0	15.0	15.0	15.0
CAL 4	3.0	60.0	30.0	30.0	30.0
CAL 5	5.0	100.0	50.0	50.0	50.0
QC 1	0.5	10.0	5.0	5.0	5.0
QC 2	1.7	35.0	17.5	17.5	17.5
QC 3	3.7	75.0	37.5	37.5	37.5
DIS	1.0	20.0	10.0	10.0	10.0

4.3 Stability tests of the FAEEs solutions stored at -30 °C

The stability of the FAEEs at -30 °C was examined since the (stock) solutions were stored at this temperature during this project. A sample of 25 mL in methanol was made in which the concentration of the FAEEs was between 3 and 7 ng/mL and a sample of 25 mL between 5 and 12 ng/mL of each of the FAEEs was made in heptane. Both samples were stored in two amber vials of which one was used for the freeze-thaw cycles and one was used to establish the stability of the FAEEs at -30 °C for up to 30 days. Amber glassware was used for the FAEEs, because these compounds are light sensitive.

One mL of the solution in methanol and one mL of heptane were compared to a freshly prepared calibration curve of the FAEEs, resulting in estimated concentrations before freezing the sample. Then the samples were stored at -30 °C for at least two hours, left at RT until the solutions were at this temperature and 1 mL of each sample was again measured and compared to the freshly prepared calibration curve. This was repeated until 9 freeze-thaw cycles were completed.

For the samples stored in methanol, two samples were analysed before the sample was stored at -30 °C and two samples were analysed at the ninth freeze-thaw cycle. For the heptane samples two samples were analysed for the second cycle. This was done to indicate the variation that can be expected without the difference in the amount of freeze-thaw cycles.

With the other solutions the vials were stored for 26 days at -30 °C and removed from the freezer at day 0, 7, 19 and 26. When the samples were removed from the freezer these were left at RT until the solutions were at this temperature and one millilitre of each sample was measured and compared to the freshly prepared calibration curve. Two measurements were done on day 7 and day 19 to indicate the variation that can be expected without the difference in the time that the samples are stored at -30 °C.

4.4 Hair collection, preparation and analysis of EtG and FAEEs

The different steps for the analysis of EtG and FAEEs in hair are discussed in this section. Prior to the gas chromatography mass spectrometry (GCMS) measurement of EtG and FAEEs, hair collection, decontamination, extraction of the analytes in hair, and a clean-up step are required (see Figure 4.1).

4.4.1 Producing and collecting hair samples

In this work three different types of hair samples were used: those that were collected from volunteers of all three drinking groups, a combined pool of hair from the hair of social drinkers and heavy drinkers and a hair reference made from blank hair to mimic hair from a user.
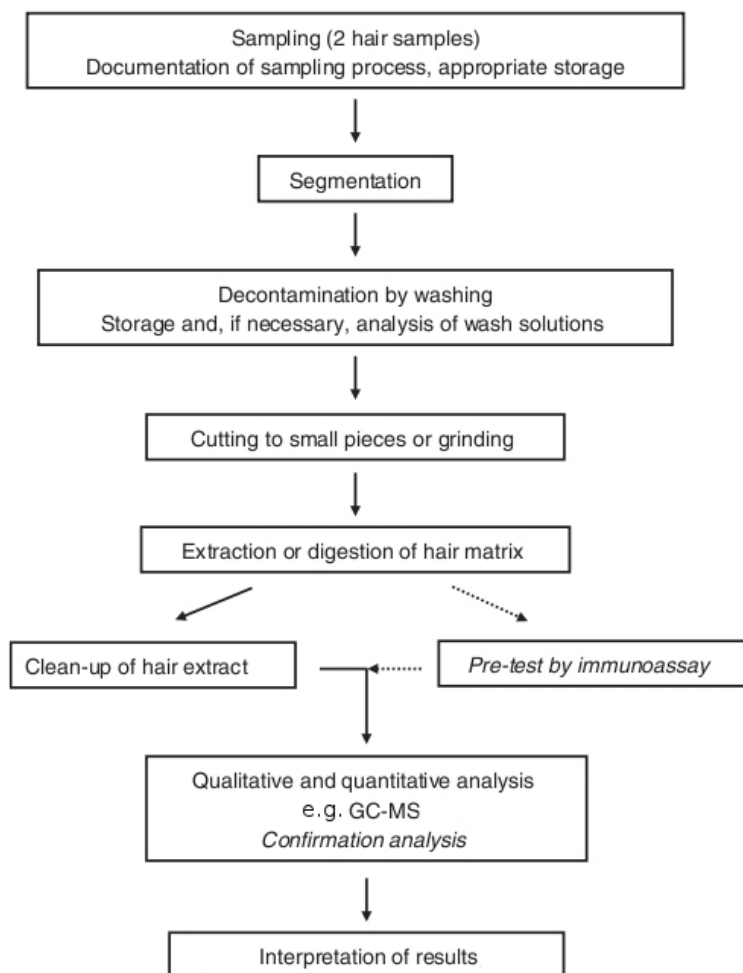
Figure 4.1: General procedure for drug analysis in hair samples. From Pragst and Balikova [27].

4.4.1.1   Collection and storage of the hair samples from volunteers

The collection and storage of hair is important [145]. Detailed guidelines are published by Agius and Kintz [111] and Cooper et al. [168]. Where applicable the standard operating procedures that are described in the guidelines by Agius and Kintz were used.

The hair sample was cut close to the skin from the posterior vertex region of the head, because the growth cycle is constant at this site [105]. When possible, a lock of hair with the thickness of a pencil (with a diameter of around 6-7 mm)

was obtained as was also described by [37, 169]. The proximal and distal part were clearly indicated and information on colour, cosmetic treatment of the hair and the subject's alcohol consumptions were collected via a questionnaire (see Appendix A) or an interview was conducted in order to obtain this information. After collection the hair samples were stored in aluminium foil at RT in line with guidelines set by the SoHT [75]. When segmental analysis was performed, a lock of hair was fixed before cutting and the hair sample was cut in segments prior to the decontamination step to prevent cross-contamination during this stage.

To test the extraction and decontamination steps hair was obtained from chronic heavy drinkers via collaboration with Huggard Cardiff, a local clinic. For calibration and repeatability studies hair from volunteer teetotallers and limited social drinkers was used and to this the analytes were added (this is referred to as 'spiked hair'). The researcher of this project was familiar with these volunteers and could in this manner confirm their alcohol consumption, including an abstinent 11 year old boy. To compare the created method with the currently used ones, hair from social and heavy drinkers was used from the local clinic and volunteers from Drug Aid Caerphilly. Hair samples from social drinkers and chronic heavy drinkers is in this thesis referred to as 'hair samples from users'.

4.4.1.2   Preparation and use of the concentrated hair pool

A major challenge in the development of a hair analysis method for alcohol metabolites is the actual extraction process. A real hair sample is a solid tissue to which most of the analyte is bonded; thus the use of spiked hair samples does not completely mimic or represent the hair from a user. Considering this, the efficiency of the extraction stage must be investigated utilizing hair samples of users. For investigations into the variation of the overall method a 'concentrated hair pool' was used and was prepared by adding hair from social drinkers and chronic heavy drinkers to each other. This hair pool was decontaminated with DCM and methanol prior to being cut into small millimetre pieces and mixed. 30 mg samples were then used to establish the variation of the method for hair samples from users.

4.4.1.3 The preparation of a hair reference

A reference hair standard was prepared to facilitate comparison studies of different decontamination solutions and extraction methods. For this two millilitre of a methanolic solution containing EtG and FAEEs were added to a 50 mg hair sample of a teetotaller. A total of 20 or 400 ng EtG was added in the solution and 160 or 400 ng of each of the FAEEs. The hair samples were left in the solution for 96 h under dark conditions and at RT. For this two batches for each solution were made. The methanol is likely to penetrate the hair fibre and may facilitate the incorporation of the ethanol metabolites.

For the analysis the hair was dried under nitrogen, decontaminated with deionized water and acetone and extracted with 1 mL MeOH under sonication for one hour. The extract was evaporated, derivatised with BSTFA and tested with direct injection. The soaking solution was tested in a similar way.

4.4.2 The decontamination and weighing procedure of a hair sample

After segmentation, more than 30 mg of hair was weighed on the A&D instruments GR-202-EC scale (e=1mg, d=0.01/0.1mg). Thereafter hair samples were decontaminated to diminish the chance of incorrect test results.

Decontamination removes external impurities and as such reduces analytical noise from, for instance, hair care products and sweat. No general consensus has yet been reached regarding the hair decontamination procedure and therefore various decontamination methods and solvents were examined and presented in Chapter 5. The most suitable method for both analytes was: the addition of 1 mL dichloromethane to a hair sample, quickly mixing and removing the solvent, followed by the same procedure with 1 mL methanol. After decontamination, the hair was left to air dry.

4.4.3 The extraction procedure for alcohol metabolites from hair

This was done by dissolving the analytes in a solution and not by digesting the hair sample for this is too harsh for the FAEEs [37]. To facilitate the extraction, the hair was cut in mm pieces with a pair of scissors and weighed again to obtain an accurate weight (around 30 mg) for the hair used during extraction. Also sonication with the Ultrawave U500 or the newer version a Fisherbrand FB11022 was used during the extraction.

4.4.4 Clean-up step prior to the analysis of EtG and FAEEs

After the extraction clean-up can be used to reduce contaminants and to concentrate the analyte. Two approaches are examined in this thesis: solid phase extraction (SPE) and head space solid phase micro-extraction (HS SPME).

4.4.4.1 SPE as clean-up step and the calculation of the recovery

The solid phase extraction procedure described by Paul et al. [170] was used as clean-up step for EtG (see Table 4.2). For this an Oasis MAX 1cc (from Waters corporation Milford, Massachusetts USA) column was used. For the EtG extract in DMSO 0.5 mL de-ionized water was added to the 0.5 mL DMSO extract and loaded as sample instead.

Table 4.2: SPE method for EtG from hair extract.

Function	Action
Precondition and remove impurities	1 mL methanol
	1 mL de-ionised water
Load sample	0.5 mL of the aqueous sample
Remove interfering compounds	1 mL 5% ammonium hydroxide in de-ionized water
	1 mL de-ionised water
	1 mL methanol
Remove liquid	5 minutes full vacuum
Elute EtG	1 mL 5% acetic acid in methanol

When EtG and the FAEEs were extracted with methanol and separated from each other with SPE, the SPE procedure from Table 4.3 was used.

Table 4.3: SPE method A for the separation of EtG and FAEEs when these are extracted from hair with methanol.

	Solvents used
Precondition	1 mL methanol
Load sample	1 mL of sample in MeOH and 20 µL formic acid
Remove interference	1 mL hexane
Remove liquid	5 min full vacuum
Elute EtG	1 mL 5% ammonia in water

The recovery of a SPE method was calculated by comparing i) the response of a spiked sample where SPE was applied to with ii) the response of a sample that is spiked after the SPE procedure at the same concentration as the one mentioned under i) (see Equation 4.3). This method was explained in a presentation by Waters [171].

$$\text{Recovery (\%)} = \frac{\text{Peak area spiked sample extracted through SPE}}{\text{Peak area post-SPE spiked sample}} \times 100\% \quad (4.3)$$

4.4.4.2 HS SPME as clean-up step for the analysis of FAEEs from hair

For the detection of FAEEs in hair HS SPME was performed on an extraction from a hair sample that was evaporated to dryness in a HS vial (for details see Table 4.4). The SPME fibre was placed in the centre of the hot injector zone for a reproducible and efficient method. All HS SPME experiments were carried out with a SPME liner that has a straight narrow ID (0.75 mm) to ensure that the analytes desorbed quickly and focused onto the GC column.

For HS SPME two different HS vials were used:

- certified flat bottom head space crimp top glass vials, 10 mL, 23×75 mm, with a magnetic crimp cap for the CTC auto-sampler (from Agilent, Berkshire, UK) and

Table 4.4: The specifics for the HS SPME method used in this work for the detection of the FAEEs in hair.

Function	Action
Preheat sample	5 minutes at 90 °C
Extraction with SPME	30 minutes at 90 °C while agitating at 250 rpm (alternatively clockwise and anti-clockwise for one second)
Desorption and cleaning fibre in GC injection port	10 minutes at 250 °C

- screw vials 18 mm, 10 mL CTC compatible with a magnetic screw cap and silicone/red PTFE septum (from Kinesis, Cambs, UK).

The screw cap vials were preferred and used unless stated otherwise.

4.4.5 Evaporation prior to the derivatisation or reconstitution of the sample

To reconstitute a sample in another liquid or remove a solvent, nitrogen evaporation was performed at RT with the TurboVap from Caliper LifeSciences. This was required prior to derivatisation, because the derivatisation agents BSTFA and PFPA preferentially react with water and alcohols and these should be removed [149]. Nitrogen was used in order to minimize oxidation of the analytes. In general, evaporation took place in GC vials for EtG samples. For the FAEEs the evaporation was performed by hand in HS vials or in 10 mL test tubes. Special care was taken to ensure the analytes were not exposed to evaporation for too long, by checking the status of the evaporation process every 15 minutes. The sample was removed when the solution was evaporated to dryness.

4.4.6 Derivatisation of EtG for GC analysis

EtG is not volatile and therefore needs treatment prior to GC analysis. The derivatisation of EtG with BSTFA and PFPA were compared. The derivatisation time and temperature was varied on the Grant Scientific QBD2 block heater with

adjusted blocks to fit 48 GC vials when the sample was in a GC vial or in the agitator unit fitted to the CombiPAL auto-sampler for the sample in the HS vials. Also the influence of the addition of the solvent ethyl acetate and hexane were evaluated in this work. The most suitable method was: 10 µL BSTFA and 10 µL EA added to the sample in a GC vial and then heated for at least 2 h at 80 °C as was determined in Section5.1.2.

4.4.7 Different injection methods to introduce the sample on the GC system

For direct injection de-activated split-splitless liners with an ID of 4 mm were used to accommodate the expansion of the evaporated solvent. An injection of 2 µL was used on the 1177 injector of the GC system. The injection was performed with the automated system (CombiPAL) and two different solvent washes (methanol and the same solvent as in which the sample is dissolved in) were used prior and after the injection of a sample. These solvent washes were replaced daily and blanks were measured to observe if the system was contaminated.

For large volume injection (LVI) larger volumes of up to 40 µL were injected manually on the 1079 injector. A temperature programme was used to ensure that the solvent would not be transferred onto the GC column. During the injection the injector was set at 38 °C and after one minute (when no more of the sample was introduced to the injector) the temperature was rapidly increased with 96 °C/min to 260 °C and held at this temperature. After 1.5 min the split was closed. The oven temperature was held at 50 °C for one minute longer than for other types of injection and thus held at this low start temperature for two minutes. Various volumes and time programmes were used for the injection from quickly injecting 20 µL to injecting 4 times 5 µL with 15 s time intervals.

Also HS SPME was used as an injection method (for details see Section 4.4.4.2).

Table 4.5: The finalized GC settings that are developed and used in this work. For the m/z values and oven temperature programme is referred to Tables 4.6 and 4.7. CI: chemical ionisation.

GCMS settings	
Injector temperature	250 °C
Transfer line temperature	200 °C
Ion source temperature	175 °C
Helium carrier gas flow	1 mL/min
Ionisation method	CI
CI gas	methane
Gas pressure	1 Torr
Electron energy	70 eV
Dwell time	0.02 s for fragments and 0.04 s for molecular ions
Scan width	0.7 Da
Detector voltage	optimised via auto tune, around 1000 V

4.4.8   The analysis of EtG and FAEEs from hair with GCMS

The GCMS system used was: a Varian CP3800 gas chromatograph equipped with a 320 series triple quadrupole mass spectrometer. The column used was a Varian Factor IV 5-MS (30 m x 0.25 mm x 0.25 µm film thickness). The data were acquired and analysed using MS workstation 6.9.2. In this thesis the optimisation and final settings of the GCMS system are discussed in Chapter 5 and the final optimised method can be found in Tables 4.5, 4.6 and 4.7. Briefly, for the development of the MS methods: a SIM method was developed based on a full scan and a product ion scan revealed the possible transitions that could be used for a tandem MS method. This was done in the negative mode, positive mode and with both electron ionization (EI) and chemical ionization (CI).

Table 4.6: The m/z values used in CI SIM for EtG-PFPA, EtG-BSTFA, the 4 FAEEs and their deuterated forms.

Compound	m/z values
EtG-PFPA	-347, -496
EtG-PFPA-D5	-352, -501
EtG-BSTFA	160, 261, 405
EtG-BSTFA-D5	165, 266, 410
E14:0	88, 101, 257
E14:0-D5	93, 106, 262
E16:0	88, 101, 285
E16:0-D5	93, 106, 290
E18:1	88, 101, 311.5
E18:1-D5	93, 106, 316.5
E18:0	88, 101, 313.5
E18:0-D5	93, 106, 318.5

Table 4.7: The finalized oven temperature programme for the combined method.

Temperature (℃)	Gradient (℃/min)	hold (min)
50	0	1
140	20	0
220	5	0
300	30	1
Total run time: 25.17 min		

To confirm the analytes and the compounds that were analysed the fragmentation pattern under a peak was compared in EI with the full scan in the NIST library.

For the development of a MS method various parameters were considered. Deuterated standards were used and these eluted at a similar time as the analytes. Therefore, it was important to specify different m/z values for the deuterated standards than for the analytes these correspond to. For a SIM method three ions were suggested to be analysed according to the GCMS guidelines for analyses involved in the forensic field [172]. Also for the SIM methods that are published in the last decade for the analysis of FAEEs and EtG in hair use three positive ions. Hence, for the development of a SIM method three ions should be measured. Tandem MS is more specific and therefore one or two transitions are accepted in this field (see Appendices E and F).

4.4.9   The calibration of EtG and FAEEs with spiked hair

The calibration curve was aimed to have concentrations above and below the cut-off value as described for chronic heavy drinking. When 30 mg was used, the calibration curve would represent the values per mg hair as represented in Table 4.8. For ethyl oleate a twice as concentrated calibrator was used compared to the other FAEEs, because the signal for this ethyl ester was low compared to that of the other FAEEs. Since no joint injection was accomplished the solution was divided in two equal aliquots after the extraction from hair. The cut-off value for EtG lies between calibrators (CAL) 2 and 3. For the FAEEs it is more difficult to establish what concentrations are required, because the sum of the four FAEEs have a cut-off value of 0.5 ng/mg or 1.0 ng/mg (depending on the length of the hair segment). When a longer hair segment with an equal distribution of the FAEEs,

the concentration of each of the FAEEs would be at least 0.2 ng/mg. Hence, the cut-off value for FAEEs lies between CAL 1 and 2. It is more likely that the FAEEs are not distributed equally and that one of the FAEEs has a significantly higher concentration as was mentioned by Khapalia et al. [13]. When only ethyl oleate would be detected, this requires a concentration of 1 ng/mg to determine heavy drinking, which is equal to CAL 3. If the same scenario is used for the other FAEEs, then the values would be equal to CAL 4. In addition, CAL 1 were above the LOQ value to ensure that a calibrator curve can also be obtained when the system is slightly contaminated due to the analysis of hair extracts, which makes the method more robust. It was possible to use a higher concentration of the highest calibrator and obtain a linear curve, but in this research it was chosen to mainly look at how the method performed around the cut-off value for chronic heavy drinking. As stated previously, for a combined method it is interesting to detect the exact concentration of one metabolite when the value of the other metabolite is above the cut-off value to make an interpretation table for the combined detection of two alcohol markers as was previously done by Pragst et al. [3].

All calibration curves were linear, were set to ignore the origin point with a regression weighting of $1/x$. Dolan [173] explains that the simplest model that adequately describes the concentration-response relationship is best. Hence, a regression weighting of $1/x$ was appropriate since this obtained correlation coefficients above 0.99.

Table 4.8: Concentrations of the EtG and FAEEs calibrators used for the validation study converted to an equivalent per mg hair when 30 mg is used. CAL: calibrator.

	Concentrations of the EtG and FAEEs solutions (ng/mg)				
	EtG	E18:1	E14:0	E16:0	E18:0
CAL 1	0.01	0.2	0.1	0.1	0.1
CAL 2	0.02	0.5	0.25	0.25	0.25
CAL 3	0.05	1.0	0.5	0.5	0.5
CAL 4	0.10	2.0	1.0	1.0	1.0
CAL 5	0.17	3.3	1.7	1.7	1.7

4.5 Evaluation of the steps in the sample preparation and analysis

The peak area was measured and compared to establish the sensitivity of a method and the effect of certain factors of the sample preparation and analysis. For measurements with the overall method a concentration in hair could be given instead. In general, for one specific condition two or three samples were prepared and measured of which the average was displayed in a graph. The standard deviation was in these cases shown as an error bar in the scatter graphs and bar charts. For other graphs the average was displayed as a red horizontal line and the average plus-minus the standard deviation of all these measurements were shown as yellow horizontal lines.

4.6 Evaluation of the overall analytical methods developed

The aim was to develop a method that is suitable for the detection of EtG and FAEEs in hair to distinguish between social drinkers and heavy drinkers. Therefore, the lower limit of quantification (LLOQ) was established. The linearity of calibration curves was measured by the determination of the correlation coefficient. The calibration curve was chosen to be in the range below and above the cut-off values (30 pg/mg for EtG and 0.5 ng/mg for the FAEEs) to enable the classification of the metabolites in the hair of social and heavy drinkers. One blank sample was analysed with each validation run of which neither EtG nor FAEEs should be found in the range of the calibration curve to test for carryover. Besides spiked hair samples the validation also included hair samples of social and heavy drinkers.

Different newly developed methods were compared based on: the chemicals used, specificity, limit of detection, limit of quantification, intra-day and inter-day variation. Methods were favoured with a lower impact on health, safety and the

environment. Also the less expensive chemicals and methods were preferred. The specificity of the signal was tested by spiking different hair samples from teetotallers and social drinkers with EtG, FAEEs and their deuterated forms before the extraction and then go through the whole procedure of the method. The sensitivity of a method was obtained by testing a range of concentrations and establishing the limit of detection (LOD) based on the visibility of the peak at the corresponding retention time that was not visible in the blank sample as described by Underwood et al. [174]. For this the hair of an eleven year old teetotaller was spiked, which had a relatively low matrix interference and all the peaks that were observed were well above the signal-to-noise ratio (S/N) of 3:1. The intra-day variability was determined by the analysis of three different concentrations of spiked hair samples from teetotallers and limited drinkers run in triplicate. The control samples were spiked at 5.0, 17.5 and 37.5 ng/mL for the three FAEEs and twice the concentration was used for ethyl oleate. For EtG the concentrations prepared were: 0.75, 1.75 and 3.75 ng/mL. These control samples were run on a single day. The inter-day variability was determined using the same concentrations in triplicate over three separate days during a period of three weeks. The precision determined at each concentration level was aimed not to exceed 15% of the coefficient of variation (CV) except for the LLOQ, where it should not exceed 20% of the CV [175] (see Equation 4.4). In addition, the accuracy was calculated with Equation 4.5 and was aimed to be in the range of $100 \pm 10\%$ described by the "Guidance for Industry, bioanalytical method validation" [175].

$$CV = \frac{\text{standard deviation}}{\text{average}} \times 100\% \qquad (4.4)$$

$$Accuracy = \frac{\text{actual value}}{\text{average}} \times 100\% \qquad (4.5)$$

The newly developed fully validated methods were also compared with the currently available methods. The newly developed combined method should have an advantage over the use of an EtG and a FAEEs hair test and this was examined in Chapter 7. Reduction in costs, time, specificity and sensitivity were considered. Also was examined whether the recovery of the alcohol metabolites from

hair that is linked to the cut-off value was comparable for the newly developed method to enable rapid implementation of this method in the industry.

4.7 Quantitative analysis of the strength of the evidence of EtG and FAEEs hair tests

The Bayesian theory can be used to determine the strength of the evidence. In this section the Bayesian theory and the equations that were used to apply this theory are explained.

4.7.1 The use of the Bayesian theory to interpret hair test results

It is not possible to directly address someone's alcohol use based on scientific evidence. The calculation of a likelihood ratio (LR) makes it possible for experts to make statements about the strength of the evidence under two hypotheses: one being the prosecution hypothesis that the defendant is a heavy drinker (H_{p1}) and the other is the defence hypothesis, that the defendant is not a heavy drinker (H_{d1}). Alternatively, in the case of re-granting one's driving license other hypotheses can be used; the prosecution hypothesis is then drinking H_{p2} and the defence hypothesis is that the defendant has not consumed alcohol (H_{d2}). It is common practice to calculate the ratio by dividing the probability of evidence found under H_p (i.e. $Pr(E \mid H_p)$) by the probability of this evidence found under H_d (i.e. $Pr(E \mid H_d)$). LR is then a measure of how much more likely it is to have observed the evidence if H_p is true than if H_d is true. The LR thus indicates how well the evidence discriminates between pairs of hypothesis and is therefore seen as the value of the evidence [176]. LR is not only used as a measure of the strength of the evidence, but also as a multiplicative factor by which the ratio of probabilities of two propositions (H_d and H_p) changes with observation of the evidence (see Equation 4.6).

$$\underbrace{\frac{\Pr(H_p \mid E)}{\Pr(H_d \mid E)}}_{\text{Posterior Odds}} = \underbrace{\frac{\Pr(H_p)}{\Pr(H_d)}}_{\text{Prior Odds}} \times \underbrace{\frac{\Pr(E \mid H_p)}{\Pr(E \mid H_d)}}_{\text{Likelihood Ratio}} \qquad (4.6)$$

In this thesis hypothesis H_{p1} and H_{d1} were used to calculate LR1. In this thesis this likelihood ratio was converted to the posterior odds in specific examples with the estimation of the prior odds (with Equation 4.6). The prior odds are the degrees of belief in favour of the defendant being a heavy drinker before the evidence is provided ($\Pr(H_{p1})/\Pr(H_{d1})$). In these examples the chance of selecting a heavy drinker or not a heavy drinker in the UK population was used. The outcome was the posterior odds which is the chance the defendant is a heavy drinker given the evidence ($Pr(H_{p1} \mid E)$) divided by the chance the defendant is not a heavy drinker given the evidence ($Pr(H_{d1} \mid E)$). This information is likely to be asked for in court. Once the posterior odds (x) were calculated, the posterior probability ($Pr(H_{p1} \mid E)$) was determined with mutually exclusive hypotheses (Equation 4.7). This was also done for LR2 with hypotheses H_{p2} and H_{d2}.

$$\Pr(H_p \mid E) = \frac{x}{1+x} \qquad (4.7)$$

Where x is the posterior odds.

4.7.2 The strength of a hair test expressed in the verbal equivalent of the likelihood ratio

The following scale was used to determine the strength of the evidence verbally:

- When the LR is below 1, the evidence is in favour of H_d.

- For a LR of 1 equal chances are given for the evidence when someone is a heavy drinker as opposed to given someone is not a heavy drinker: the evidence is thus inconclusive.

- When the LR is higher than 1 the evidence is in favour of H_p. How well the

evidence supports H$_p$ for values above 1 is indicated in Table 4.9 [177, 178].

Table 4.9: The verbal equivalent of the LRs for values above 1 [177, 178].

Likelihood Ratio	Verbal equivalent
$1 < LR < 10$	limited evidence to support
$10 \leq LR < 100$	moderate evidence to support
$100 \leq LR < 1000$	moderate strong evidence to support
$1000 \leq LR < 10000$	strong evidence to support
$LR \geq 10000$	very strong evidence to support

4.7.3   The calculation of the strength of the evidence of a hair test from sensitivity and specificity

From the sensitivity and specificity the LR was calculated for a positive test (LR+) and for a negative outcome (LR-) both in favour of Hp with Equation 4.8 [179, 180].

$$LR+ = sensitivity/(1 - specificity)$$
$$LR- = (1 - sensitivity)/specificity \tag{4.8}$$

4.7.4   The calculation of the strength of the evidence of two independent hair tests

To combine the LR+ of the EtG (x) and FAEEs (y) hair tests Equation 4.9 was used, because the tests were determined to be independent (see Section 4.7.5) for the currently used cut-off values for chronic heavy drinking and this equation can be used for independent tests [177].

$$LR_{x,y_{indep.}} = \frac{\Pr(y \mid H_p, I)}{\Pr(y \mid H_p, I)} \times \frac{\Pr(x \mid H_p, I)}{\Pr(x \mid H_p, I)} \tag{4.9}$$

For which x is the test result for test 1: EtG concentration found in hair,
y is the test result for test 2: FAEEs concentration detected in hair,
I background information (e.g. the use of hair products),
H_p the hypothesis of the prosecution (e.g. the tested person is a chronic heavy

drinker) and

H_d the hypothesis of the defence (e.g. the tested person is not a heavy drinker).

4.7.5   The calculation of the dependencies of two tests

The methodology of Gardner et al. [181] was used to calculate dependencies. For this, information of both tests is required under a group that has the condition and a group that has not got the condition. In this research a group of heavy drinkers and infrequent drinkers was used. The first determined the dependency in sensitivity and the data from the second group revealed the dependency in specificity. When the specificity and sensitivity covariances are close to zero the tests are considered independent.

The difference in specificity with the theoretical values and the group of infrequent drinkers was calculated (y_{Sp}) with the Equations 4.10.

$$
\begin{aligned}
\Pr(x + \cap y+) &= (1 - sp_x) \times (1 - Sp_y) + \gamma_{Sp} \\
\Pr(x + \cap y-) &= (1 - Sp_x) \times (Sp_y) - \gamma_{Sp} \\
\Pr(x - \cap y+) &= Sp_x \times (1 - Sp_y) - \gamma_{Sp} \\
\Pr(x - \cap y-) &= Sp_x \times Sp_y + \gamma_{Sp}
\end{aligned}
\tag{4.10}
$$

Where Sp_x is the specificity EtG test,
Sp_y is the specificity FAEEs test and
γ_{Sp} is the specificity covariance.

From the chronic heavy drinking group the sensitivity variance was calculated with the Equations 4.11.

$$
\begin{aligned}
\Pr(x + \cap y+) &= Se_x \times Se_y + \gamma_{Se} \\
\Pr(x + \cap y-) &= Se_x \times (1 - Se_y) - \gamma_{Se} \\
\Pr(x - \cap y+) &= (1 - Se_x) \times (Se_y) - \gamma_{Se} \\
\Pr(x - \cap y-) &= (1 - se_x) \times (1 - Se_y) + \gamma_{Se}
\end{aligned}
\tag{4.11}
$$

Where Se_x is the sensitivity EtG test, Se_y is the sensitivity FAEEs test and γ_{Se} is the sensitivity covariance.

4.7.6 The calculation of the likelihood ratio and its 95% confidence level on an ordinal scale

From the frequency database for multilevel test results (see Table 4.10) LR1 was derived for each concentration level for a known group of heavy drinkers and infrequent drinkers. Imagine, in A number of heavy drinkers a concentration is detected that falls within level 1 and B infrequent drinkers have a value in that same concentration range. C heavy drinkers and D infrequent drinkers have a concentration at level 2 and so on. Then with four different concentration levels, as is displayed in Table 4.10, LR1 for level 1 was calculated with Equation 4.12, where n_1 is the total number of heavy drinkers and n_2 the total number of infrequent drinkers.

Table 4.10: The r $\times$ 2 matrix for multilevel test results adjusted from Simel et al. [182].

	Chronic heavy drinking	
	Present	Absent
Level 1	A	B
Level 2	C	D
Level 3	E	F
Level 4	G	H
	n_1	n_2

Test result $\{$

disease

$$LR_{Level1} = \frac{A/(A + C + E + G)}{B/(B + D + F + H)} = \frac{A/n_1}{B/n_2} \tag{4.12}$$

The 95% confidence interval (CI) was calculated as is described by Simel et al. [182] (see Equation 4.13 for concentration in level 1). As Simel et al. stated: "Confidence intervals are important summary measures that provide useful information from clinical investigations, especially when comparing data from different

population or sites." [182]. The CI provided in this research viable information about the robustness of the LR approach with the data that were used.

$$LR_{Level1,95\%} = \exp\left[\ln\frac{A/n_1}{B/n_2} \pm 1.96 \times \sqrt{\frac{1-A/n_1}{A} + \frac{1-B/n_2}{B}}\right] \quad (4.13)$$

Where n_1 is the total number of heavy drinkers and
n_2 the total number of infrequent drinkers,
A number of heavy drinkers a concentration is detected that falls within level 1 and
B infrequent drinkers have a value in that same concentration range.

$$LR_x = \exp\left[\ln\frac{p_1}{p_2} \pm 1.96 \times \sqrt{\frac{1-p_1}{p_1n_1} + \frac{1-p_2}{p_2n_2}}\right] \quad (4.14)$$

Where for $LR_x = LR+$
$p_1 = $ sensitivity,
$p_2 = $ 1-specificity,
$p_1n_1 = A$ and
$p_2n_2 = B$.

The general formula to calculate the CI for any value x instead of a concentration in level 1 (as was with Equation 4.13) is shown in Equation 4.14. This general formula was used to achieve a defined lower limit of the CI with the sensitivity and specificity from Süße et al. [101] and in this manner determined the number of subjects that would be required to achieve this defined lower limit.

$$LR+_{\text{min 95\% CI}} = exp\left[\ln(\frac{se}{1-sp}) - 1.96\sqrt{\frac{1-se}{n_1 \times se} + \frac{sp}{n_2 \times (1-sp)}}\right] \quad (4.15)$$

4.8   Qualitative analysis of the strength of the evidence

When the LR is calculated from another population, this must be corrected for. A qualitative analysis was performed to visualize how an influence on the test result can be depicted in case no quantitative analysis can be performed, because for instance the LR is obtained from a population where hair treatment is not an issue. Biedermann et al. [183] show there are three types of qualitative inference: predictive, diagnostic and inter-causal (see Figure 4.2). In the first step of the qualitative analysis the result is analysed with diagnostic inferences, indicating what influences can be derived from the result. Then, when all factors are set to zero, the predictive and inter-causal relations show the correlation between the factors involved and the result, assuming one of the factors to be correct. These qualitative inferences were displayed in a model and used to visualize the effect of background information of a specific case.
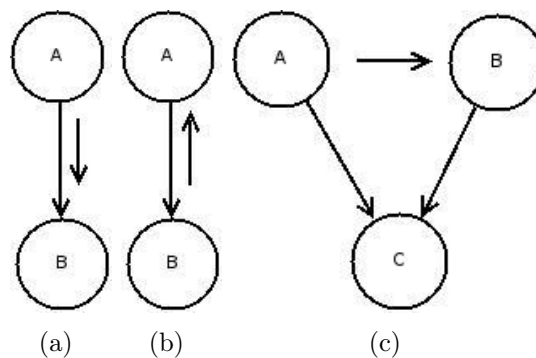
Figure 4.2: Basic types of qualitative inference: a) predictive b) diagnostic and c) inter-causal. From Biedermann et al. [183].

Chapter 5

Sample preparation for the detection of EtG and FAEEs in hair

Hair analysis consists of several steps; each can influence the sensitivity and specificity of the overall analysis. The significant difference in the chemical and physical characteristics of EtG and FAEEs potentially presents issues in sensitivity within a combined method. To address this it is important to fully optimize each step during sample preparation (see Figure 5.1 for the steps in the sample preparation and Appendices E and F). Work of this nature has not been published in detail to date. Here comparisons are made with the methods that are currently used in the literature and industry.

5.1 Preliminary research into chemicals and materials

Throughout this work various procedures have been used. Here, preliminary studies focusing on the influence of the use of different chemicals and materials is reported.
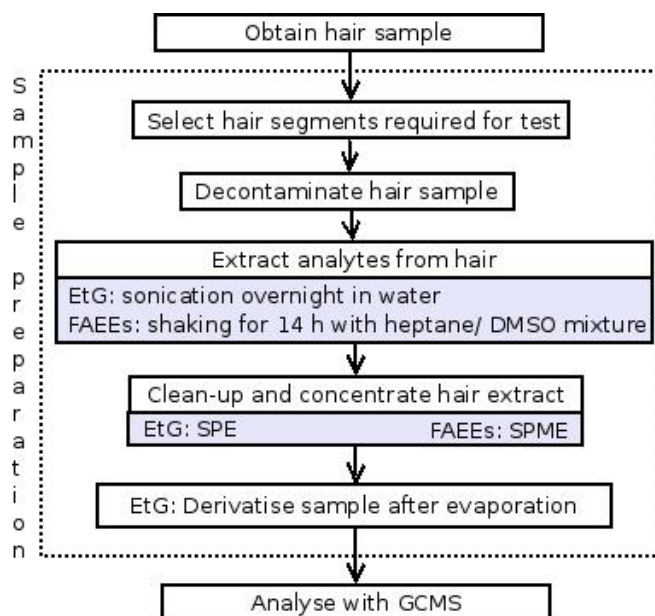
Figure 5.1: A schematic representation of the steps used in hair sample preparation for the analysis of EtG and FAEEs.

5.1.1 Comparison of different glassware

Glassware was silanized to ensure that none of the analytes were lost due to adsorption to the glass surface. It was apparent that during the evaporation step, the removal of the solvents like methanol, dichloromethane and heptane was prolonged. Whilst only 10 minutes was required for the complete evaporation of 1 mL of methanol in non-silanized vials, 30 minutes was necessary using silanized glassware. Additionally, the removal of PFPA after derivatisation took more than 30 minutes from a silanized GC vial compared to 2 minutes from a non-silanized vial. When the glass was silanized mainly the last drop(s) took long to evaporate due to surface tension. As a result of this effect, higher quantities of FAEEs and EtG-PFPA were lost due to inadvertent evaporation from silanized vials (see Section 5.5). No significant difference was observed when the standard calibrators were stored in silanized glassware for up to a month. So to lose as little as possible of the trace analytes it was decided to use silanized glassware store solutions and non-silanized glassware for the optimum sample preparation.

Two different makes of GC vial were compared to establish potential adsorptive variability. These were the short thread 9 mm vial of which the inside of the base had a conical shape (Kinesis) and blue screw cap clear vials (Agilent). For the screw cap clear vials the signal for a concentration varied largely and seemed to depend on the amount of solution that was present in the vial. For the short thread vials the analysis was more repeatable and did not depend on the amount that was present in the vial as long as enough solution was present to be picked up by the autosampler. Therefore, the short thread vials were used in this work. An additional advantage with these vials was that less solution was required for the syringe in the autosampler to pick an aliquot up.

At times no analytes were observed when crimp cap vials were used for HS SPME. This phenomenon was not observed with the screw cap variant. It is reasonable to assume that not every cap is crimped on in a similar way: too much force results in an uneven surface of the cap, leaving less material for complete closure of the vial at the rim. Completely air tight vials are important when HS SPME is performed. In addition, the samples in a screw cap vial, can be recapped quickly and remeasured, whereas this was more difficult for the crimp caps. Therefore, it was decided to use screw cap vials in this work.

The use of plastic tubes for the extraction stage produced high background noise. This was due to presence of plasticizers, one of which was identified as 1,2-benzenedicarboxylic acid, diisooctyl ester also called diisooctyl phthalate. These background peaks were not observed when glass containers were used. Hence, for the storage and sample preparation of the analytes glass containers were used.

5.1.2 The comparison of BSTFA agents for the derivatisation of EtG

Different BSTFA agents were used to derivatise EtG. When BSTFA with 1% trimethylchlorosilane (TMCS, Sigma-Aldrich) was used, two peaks were observed when the transition m/z 261 $\rightarrow$ 143 was used (see Figure 5.2). According to Sigma-Aldrich the addition of TMCS to BSTFA enhances the donor strength

of BSTFA [184]. However, the presence of two peaks in this work may be explained by the incomplete derivatisation of EtG. This resulted in a loss in response compared to the use of pure BSTFA where only 1 peak was observed (at 8.14 min and thus the bigger peak of the two observed with the derivatisation with BSTFA+TMCS). Hence, for the rest of this work BSTFA from Stratlab was used.
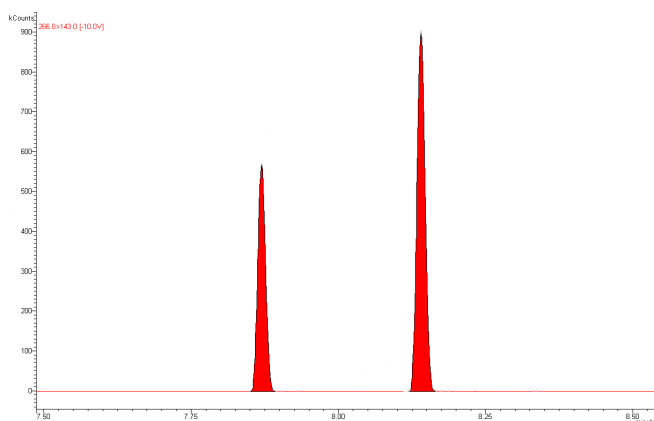
Figure 5.2: Chromatogram with two peaks for EtG-BSTFA using BSTFA + 1% TMCS (Sigma-Aldrich) measured with the transition m/z 261 → 143.

5.1.3 The stability of the FAEEs solutions

Initially, FAEE standards of equal concentration were used. However, ethyl oleate was not always detectable at CAL 1 level, therefore a standard with twice the concentration was used for this analyte compared to the other FAEEs. A higher quantification limit for ethyl oleate is reasonable, because both in this work and by Khapalia et al. [13] it was observed that this ester often has a relatively high concentration for chronic heavy drinkers.

The stock, quality control (QC) and calibration solutions were made using either methanol or heptane. The stability was tested for the stored QC samples at a low, medium and high concentration (5, 17.5 and 37.5 ng/mL respectively) for two months without the presence of a hair sample. When the samples were stored at -30 °C all analytes were detectable with a standard deviation below 10% when compared with a freshly prepared solution of the deuterated standards that were

stored at a concentration of 10 mg/mL. This was also the case for the calibration samples that were stored at 8 °C for a month and that were used for the calibration curves of the spiked hair samples used in the validation studies (see Chapter 7). Hence, no concentration effect was observed for the possible degradation or sample loss of the FAEEs. The stability of the spiked hair extracts after evaporation, on the other hand, was poor. When the samples were measured a day after preparation and stored at 8 °C, FAEEs were no longer quantifiable for the lower concentrations (CAL 1 and CAL 2). Hence, in the experiments that followed samples that were evaporated after the extraction were analysed on the same day.

5.1.4 The stability of the EtG solutions

The stock solution was made in methanol and the QCs and calibrators were made in de-ionised water. The stability was tested for the stored QC samples at a low, medium and high level (0.5, 1.7 and 3.7 ng/mL respectively) without the presence of a hair sample. After storage at -30 °C none were detectable after one day. This is in agreement with the observations of Paul et al. [170]. Therefore fresh sub-dilutions were made every day for the preparation of the calibration curve and QCs of 100 ng/mL EtG in methanol. The stock solution in methanol appeared stable for at least two years when stored at -30 °C because the lowest QCs and calibrators that were prepared with this solution were still quantifiable after this time period.

5.1.5 The amount of hair that can be used for the analysis of both EtG and FAEEs

When EtG and FAEEs were extracted from hair, a background was observed (Figure 5.3). The background may interfere with the analysis of the FAEEs in the positive CI mode and therefore the matrix effect was evaluated.

The calibration curve of FAEEs was similar when 40 mg instead of 20 mg hair was
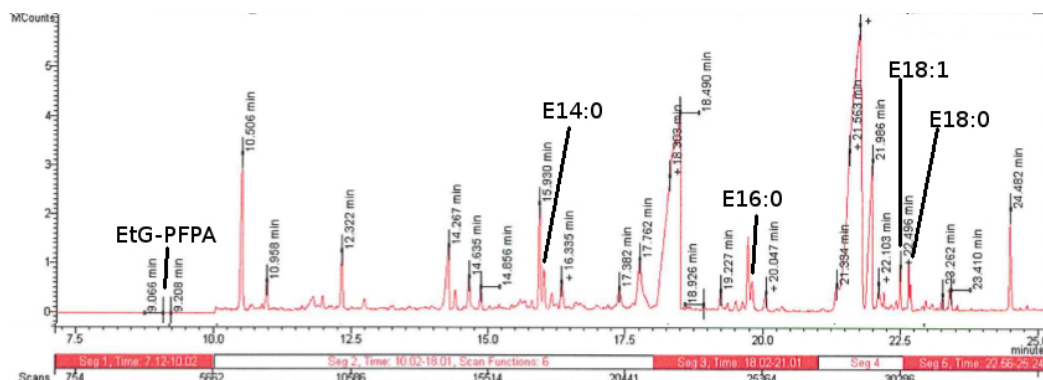
Figure 5.3: CI SIM chromatogram of analytes showing co-extractant from the hair matrix. In segment 1: EtG-PFPA, 2: ethyl myristate, 3: ethyl palmitate, 4: ethyl oleate and 5: ethyl stearate. For the ions used see Table 6.4 on page 137.
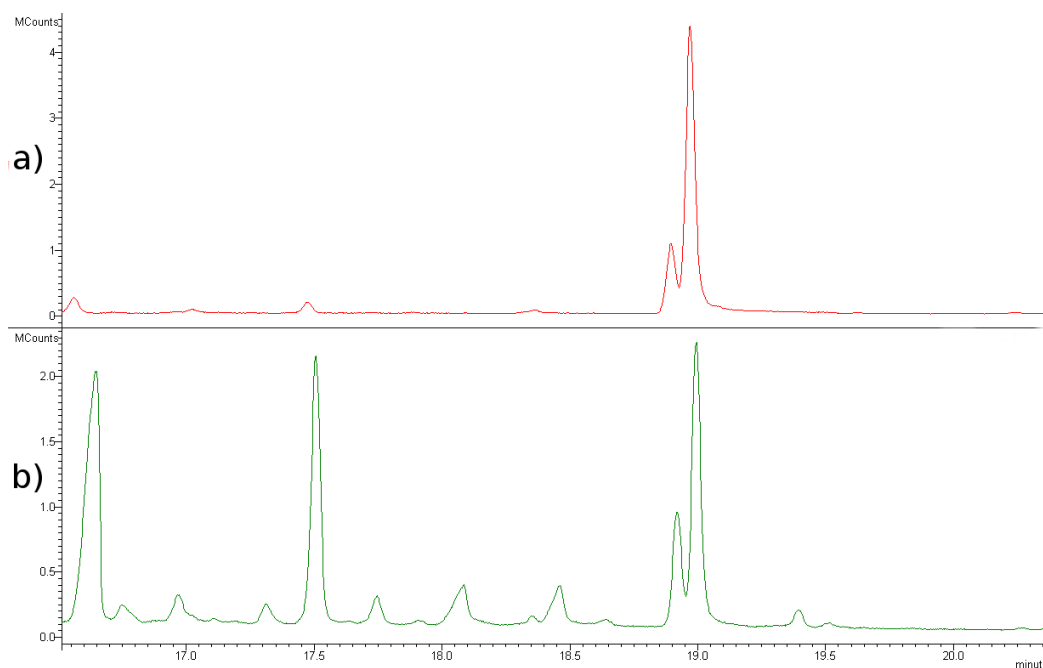
Figure 5.4: Chromatogram of ethyl palmitate and its deuterated form in CI SIM, a) without and b) with the extraction of a blank hair sample. For the m/z ions used see Table 6.4 on page 137.

used. Interfering peaks were observed when the amount of hair was increased and an increase in the amount of hair analysed may not be in favour of the FAEEs, especially not for ethyl myristate and ethyl palmitate. Due to the interfering peaks (see Figures 5.4 and 5.5), a decrease in accuracy was observed leading to

a calibration curve with a correlation coefficient below 0.99 (0.96), which was not solved by using twice the concentration of the calibrators. In addition, Süße [185] showed a decrease in extraction yield with more hair and argued that the saturation of the SPME fibre by matrix constituents may have caused this. When 30 mg hair was used, interfering peaks were observed for CAL 1 and CAL 2 in this work, but not in the other calibrators and a correlation coefficient above 0.99 could be obtained. Therefore, the size of the hair sample was set at 30 mg. This is in agreement with findings by Süße [185] where the difference between 20 and 50 mg was relatively big. The slight increase in weight of the hair sample used increased the sensitivity of the overall method, while the matrix interference was kept to a minimum.
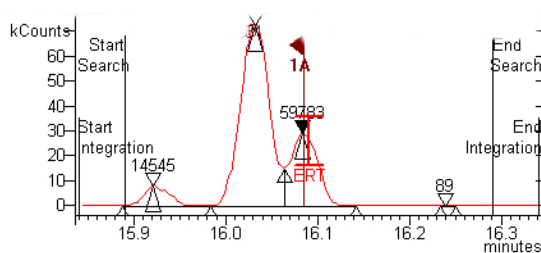
Figure 5.5: CI SIM chromatogram of ethyl myristate and matrix interference for 40 mg hair spiked with FAEEs on the level of CAL 4. For the ions used see Table 6.4 on page 137.

5.1.6 The development of a hair reference sample with EtG and FAEEs

In this work different hair matrices were seen to provide different interferences. To compare different decontamination solutions and extraction methods an attempt was made to incorporate FAEEs and EtG in a blank hair sample, which would provide a reference hair standard on which to work.

A reference sample was made by soaking the hair in a methanolic solution with EtG and FAEEs. After 96 h soaking, both the soaking solution and the extract of the previously soaked reference hair were examined. After the decontamination procedure, the hair was extracted with sonication for one hour in the presence of

methanol. In all the solutions obtained from the reference hair EtG and FAEEs were detected. An overload was observed in the extract when a spike of 400 ng was used. This indicates that incorporation of the ethanol metabolites in hair is feasible (see Figure 5.6). It is interesting that a relatively high concentration of ethyl oleate was found in the reference hair whereas the concentration of FAEEs in the soaking solution was the same. The repeatability was unfortunately poor and the produced reference hair samples could therefore not be used as a standard control sample in this study.
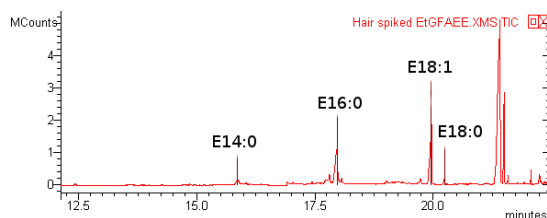
Figure 5.6: Chromatogram of the reference hair with the 4 FAEEs detected in CI SIM (see Table 6.4 on page 137 for the m/z used).

5.2 Decontamination procedure of hair

Several decontamination procedures and solutions are currently used for the decontamination of hair prior to analysis. Most FAEEs methods include a non-polar solvent as was suggested by Politi et al. [69] to remove the greasy layer from hair. Decontamination procedures for EtG are more variable and no general consensus seems to have been reached concerning i) the polarity of the solvents used, ii) the volume of the solvents, iii) the amount of washes and iv) the application of sonication. In this study solvents were selected offering a range of polarities and distinct hair swelling properties as protic solvents are said to have the ability to swell hair [37]. In this way the influence of these characteristics on the decontamination and extraction efficiency was investigated, which has not yet been presented in another study. Moreover, the decontamination step is an important step in hair analysis since this may affect the amount of analytes that is detected during the extraction. It may also influence the differentiation between analytes

that are deposited on the hair via external contamination and internal incorporation. In addition, various different decontamination procedures are published for EtG and FAEEs detection but not yet compared.

5.2.1 Overview of decontamination procedures and the experimental set-up

For this study hair from alcohol abusers was obtained via collaboration with a local clinic. 1 mL of a decontamination solvent was applied to approximately 30 mg of hair. The mixture was shaken briefly after which the solvent was removed. 5 µL of 200 ng/mL EtG-D5 and 20 µL of a mixture of 2000 ng/mL ethyl oleate-D5 and 1000 ng/mL of the other three FAEEs-D5 were then added to the decontamination solvent which were subsequently analysed. 1 mL methanol and the same amounts of deuterated standards were added to each of the decontaminated hair samples after these were cut in mm pieces. Subsequently the extraction was performed with 30 min sonication prior to analysis. All experiments were performed in duplicate.

The decontamination procedures that were investigated are listed in Table 5.1. One millilitre of each decontamination solvent was used and when more than one decontamination solvent was used in a decontamination procedure the solvents were used one after the other. The decontamination solvents were selected based on the variation in polarity and have been used by several laboratories according to the literature (see Table 5.1).

To assess the decontamination efficiency of the solvents, analyte concentrations in both the extract and corresponding decontamination solvent were compared. When at least twice as much analyte was found in the decontamination solvent compared to the extraction solution, the decontamination solvent was categorized as 'high' wash-out efficiency for EtG. For similar amounts, the solvent was categorized as 'medium', and for amounts three times lower as 'low' wash-out efficiency. The results were categorized in the same groups for the FAEEs analysis: low, medium and high. The values in the range of the average plus-minus

Table 5.1: Summary of decontamination solvents used in this work.

Decontamination solvents	Analytes	Used by
1. de-ionised water, dichloromethane	FAEEs	[67]
2. heptane	FAEEs	[21, 79, 101, 115, 119, 132, 186]
3. de-ionised water, heptane	EtG and FAEEs	[25, 186]
4. 2 × dichloromethane	EtG	[117]
5. diethyl ether, acetone	EtG	[39] but with ether
6. de-ionised water, acetone, methanol	EtG	[187]
7. methanol	EtG and FAEEs	[170, 172]
8. methanol, acetone	EtG	[107, 158]
9. dichloromethane, methanol	EtG	[3, 19, 47, 69, 119, 134, 188]
10. de-ionised water, acetone	EtG	[25, 36, 58–60, 76, 78, 79, 111, 133, 189]
11. de-ionised water	EtG and FAEEs	[25, 36, 58–60, 67, 76, 78, 79, 111, 114, 133, 186, 187, 189, 190] as part of a decontamination protocol

the standard deviation obtained from all solutions tested was used as 'medium' wash-out efficiency. Low and high efficiencies were below and above these values respectively.

5.2.2 Comparison of decontamination solvents

Most solvents removed roughly half of the EtG during the decontamination step and half during the 30 minutes of sonication with methanol. This is in contrast to Morini et al. [19] where it was shown that the loss of EtG was negligible. This may be related to the hair samples that were used in this work. For this study, hair was analysed from people who claimed to regularly wash their hair (i.e. from once a week up to once a day) and more importantly also claimed to consume alcohol nearly constantly. High concentrations of EtG may be expected on the surface of hair if the main route of incorporation is via sweat [37, 71]. This incorporation mechanism and possibly the difference in the concentration of EtG in sweat due to the high alcohol consumption of the subjects used may be the reason why more EtG was washed-out in these experiments compared to what

was observed by Morini et al. [19]. Unfortunately, Morini et al. did not describe the washing habits of the subjects.

Table 5.2: The decontamination efficiency of various decontamination solvents for EtG analysis from hair.

Efficiency decontamination of EtG	Decontamination procedures
high	heptane 2 × dichloromethane
medium	de-ionised water, dichloromethane diethyl ether, acetone de-ionised water, acetone, methanol methanol methanol, acetone dichloromethane, methanol de-ionised water, acetone de-ionised water
low	de-ionised water, heptane methanol

Most decontamination procedures were thought to be comparable for EtG. Of all the solvents tested dichloromethane and heptane showed superior decontamination efficiency and a lower than average efficiency was demonstrated by pure methanol as well as a combination of water and heptane (see Table 5.2). An explanation for this is that dichloromethane and heptane may remove EtG with a superior efficiency compared to solvents similar in polarity to EtG due to better hair swelling property or a better grease capturing action. It may be possible that grease captures or encapsulates EtG, and therefore a non-polar solvent needs to be used first to liberate it. In addition, Politi et al. [69] state that a dichloromethane wash improved the recovery of EtG during the extraction. When water is used prior to a non-polar solvent, the water may form a hydrophilic layer which may hinder the hydrophobic solvent from dissolving the greasy layer on the hair shaft and is thereby unable to liberate the encapsulated EtG. This may explain why heptane alone decontaminates better than water plus heptane. This also suggests that the hair sample should be dry before another decontamination solution is applied when the second decontamination solution is immiscible with the previous decontamination solution.

Water alone was as effective as most other solvents as a decontamination solvent but lower than dichloromethane. Water is protic and thus has hair swelling

properties. It is also an excellent solvent for EtG. However, as suggested earlier, the greasy layer may need to be removed first to liberate EtG. Normal hair hygiene including shampoo that removes this greasy layer is thought to explain the significant decrease of EtG in the more distal segments of hair from chronic heavy drinkers [191].

Another observation was that the results for pure methanol were variable; two samples indicated methanol as 'medium' and two others as a 'low' efficiency decontamination solution. This is likely to indicate that decontamination efficiencies were difficult to determine.

The efficiency of the washes for FAEEs was relatively low compared to what was observed for EtG. From the ratio between the response for the washes and extraction amount it was established that approximately 10% was removed, whereas for EtG this was about 50%. This observation is in agreement with the published hypothesis that normal hair hygiene may remove EtG and has a relatively low impact on the concentration of FAEEs in hair [132]. It is also in line with the implication that EtG binds poorly to a hair fibre as the keratinized hair is acidic in nature with an isoelectric point close to 6 and as a result facilitates its removal by routine washing and hair care products [39].

Table 5.3 shows that two decontamination protocols removed more than 20% of the total FAEEs measured in the decontamination and extraction solutions; the use of methanol+acetone or methanol+dichloromethane are therefore considered to be highly efficient as decontamination protocol. This figure also shows that diethyl ether+acetone, water+heptane and water remove less than 7% of the detected FAEEs in hair, which is low compared to the other decontamination protocols. Both methanol and heptane show an overlap with this group due to their large performance variation.

A wash with a solvent that swells the hair fibre and is immiscible with heptane (e.g. water) prior to the heptane wash worsened the efficiency of the heptane wash (see Table 5.3). This was also observed for EtG. Water may form a layer and thereby hinders the non-polar heptane wash to remove FAEEs. Since this was not observed for the combination of water and dichloromethane, some other

Table 5.3: The decontamination efficiency of various decontamination solvents for FAEEs analysis from hair.

Efficiency decontamination of FAEEs	Decontamination procedures
high	methanol, acetone dichloromethane, methanol
medium	de-ionised water, dichloromethane $2 \times$ dichloromethane de-ionised water, acetone, methanol methanol de-ionised water, acetone de-ionised water
low	heptane de-ionised water, heptane diethyl ether, acetone

factors such as drying time between the decontamination washes may be crucial.

Dichloromethane and dichloromethane+methanol were very similar in decontamination efficiency of FAEEs when the washing solutions were compared. However, from the extraction after the decontamination procedure it is clear that more was washed-out with the combination of dichloromethane and methanol. Furthermore, the analysis of the decontamination solvent, extraction solvent and the ratio between these two consistently showed that the use of both dichloromethane and methanol had a relatively high wash-out effect compared to other decontamination procedures (see Table 5.3). Morini et al. [19] showed that decontamination with only methanol resulted in interference of the analytes whereas the combination of methanol and dichloromethane did not. Hence, a combination of dichloromethane and methanol is preferred.

The use of methanol and dichloromethane for both EtG and FAEEs seems to be a good compromise, since with a medium decontamination efficiency 50% of the EtG content was removed and a decontamination efficiency at high efficiency for FAEEs was lower than 50%. For FAEEs a higher decontamination efficiency compared to the other decontamination procedures was appropriate, but a high decontamination efficiency for EtG was probably due to the removal of EtG from within the hair shaft. EtG may be more loosely bound to hair than FAEEs [191] and therefore a medium decontamination efficiency is likely to be more suitable. On the other hand there is no data to support this hypothesis other than a

decreasing concentration of EtG along the hair shaft of one subject that may have also been caused by an increase in alcohol consumption over time. Hence it may also be that the due to the incorporation route, a relatively high concentration of EtG is still on the outside of the hair shaft. Especially since EtG is more difficult to incorporate in the hair matrix than the FAEEs due to its polar, acidic and charged state and the difficulty crossing lipophilic membranes in this state. This combined with a high level of alcohol consumption may be the reason why a relatively high amount of EtG was removed during the decontamination process compared to the extraction. From the experiments done here it is unclear what lies at the basis of the phenomenon that is observed and how exactly to chose the optimum decontamination procedure. Due to the relatively high decontamination efficiency, it was thought that an average washing-out level may be appropriate for EtG.

5.3 Extraction of EtG and FAEEs from hair

In the current consensus of the SoHT [38] the extraction procedure that should be used as standard has not yet been discussed or agreed upon. A standard procedure is not necessarily the best procedure but one that ensures a similar approach in different laboratories. Currently, most researchers use water for the extraction of EtG and a mixture of dimethyl sulfoxide/heptane for the extraction of FAEEs in hair (see Table 5.4).

5.3.1 Experimental set-up for the extraction of EtG and FAEEs from hair

To establish the optimum extraction solvent for the extraction of both analyte targets various solvents were examined. 30 mg hair was decontaminated with 1 mL dichloromethane and subsequently with 1 mL methanol. Deuterated standards were added to the 1 mL extraction solvent. For extraction, each of the samples were treated with one of the following solvents: acetone, water,

methanol, methanol+water (1:1 v/v), methanol+dichloromethane (1:1 v/v), heptane+dimethyl sulfoxide (4:1 v/v) and hexane+dimethyl sulfoxide (4:1 v/v). The extraction was facilitated by 30 minutes or 4 hours of sonication. All experiments were performed in duplicate. It should be noted that these extraction solvents have been previously discussed in the literature (see Table 5.4) and provide a range of properties from protic polar to non-polar. The combination methanol and dichloromethane was used too because methanol was used with a more polar solvent (i.e. water) and was therefore also used in combination with a more non-polar solvent (i.e. dichloromethane). Moreover, dichloromethane was used in the decontamination step in which the analytes may also be (partially) extracted.

Table 5.4: Summary of solvents used for the extraction of EtG and FAEEs from hair. Ratios are expressed in volume.

Extraction solution	Analytes	Used by
1. methanol	EtG and FAEEs	[58, 192]
2. acetone	FAEEs	[192]
3. heptane : DMSO (4:1)	FAEEs	[3, 21, 25, 79, 101, 115, 119, 132, 186]
4. n-hexane : DMSO (4:1)	FAEEs	[114]
5. de-ionised water	EtG	[3, 19, 36, 47, 58–60, 69, 76, 78, 79, 107, 111, 117, 119, 128, 133, 134, 158, 170, 186, 188]
6. methanol : de-ionised water (1:1)	EtG	[58]
7. methanol : DCM (1:1)	-	-

For the extraction of FAEEs in methanol by sonication it is known that transesterification can take place [114]. To investigate the extent of transesterification, 30 and 500 ng/mL FAEEs in methanol were sonicated for 15, 30, 60, 120, 240 minutes up to sonication overnight. The methyl esters were detected using GCMS in full scan and SIM (m/z: 74, 87 and M+1) and the ratio of methyl to ethyl esters was calculated. Also, the amount of transesterification was compared when two different sonication baths were used: of which the Fisherbrand FB11022 creates a higher temperature of the water in the sonication bath and thus of the sample during sonication.

5.3.2 The influence of the extraction time on the extract

Two different extraction times were investigated: 30 minutes and 4 hours. This study showed that an extended period of 4 hours did not result in an increase in the signal for EtG nor in a higher response (ratio area analyte to area corresponding deuterated internal standard (DIS)). SPE was not performed on samples prior to final analysis using GCMS to avoid the introduction of other potential variables. Not using SPE resulted in green colouration and solid materials after derivatisation of the samples. The solid materials could block the syringe when direct injection was used and therefore SPE was used in other experiments. With methanol EtG was detected after 30 minutes of extraction by sonication, but was not detected after 4 hours. Therefore in this work 30 minutes was used as a guideline for the extraction time. A negative effect of a longer extraction time was also observed by Zimmerman and Jackson [192]. Their comparison study showed that lower and higher sonication times than 30 minutes with methanol resulted in a lower extraction efficiency. This is in line with the statement that was made by Süße [185] about the degradation of FAEEs in the ultrasonicator.
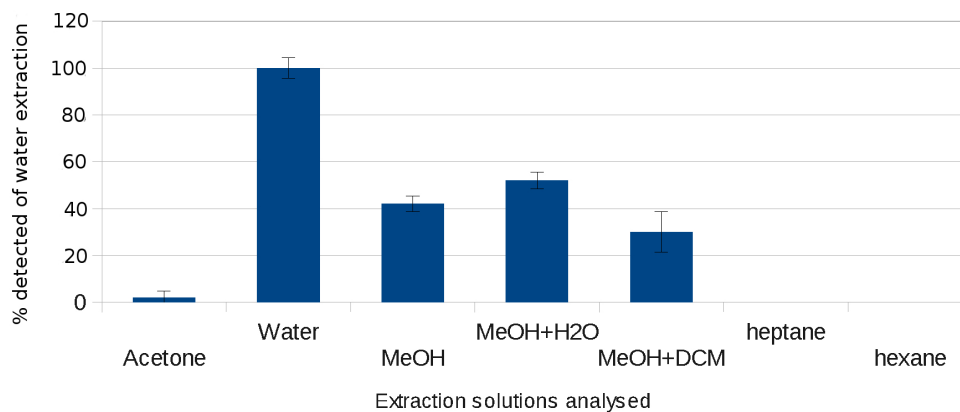
Figure 5.7: EtG extraction from hair with different solutions.

5.3.3 The evaluation of the solvents used to extract EtG and FAEEs from hair

It is clear that water was the best solvent for the extraction of EtG from hair (see Figure 5.7). The second-best extraction solvents for EtG were those containing methanol. This is supported by the work of Jurado [58] in which a lower recovery of EtG for methanol was found than water. For the alkane/dimethyl sulfoxide mixtures EtG was not detected in heptane or hexane probably due to the higher solubility of EtG in dimethyl sulfoxide. EtG was not measured in dimethyl sulfoxide because the evaporation process after extraction takes too long even when the sample was heated at 100 °C. Note that evaporation was required for the subsequent derivatisation of EtG. The EtG level in dimethyl sulfoxide could possibly be tested when another method would be deployed such as SPE to redissolve EtG in another solvent.

In conclusion, in the current sample preparation process the second-best extraction solvents for EtG were those containing methanol. However, two disadvantages of the use of methanol have been articulated by Jurado [58]. Methanol would show more variation and a lower recovery of EtG than water or a water/methanol mixture. Jurado does not use SPE after the extraction and these disadvantages could possibly be solved with the use of this clean-up step.

FAEEs were normalized based on the highest peak area found for ethyl myristate (E14:0), ethyl palmitate (E16:0), ethyl oleate (E18:1) and ethyl stearate (E18:0) (see Figure 5.8). This makes it easier to interpret all of FAEEs in one figure since for these hair samples the ethyl oleate concentration in hair was much higher than the concentration of the other FAEEs (see Figure 5.8(a)). The relatively high concentration of ethyl oleate compared to the other FAEEs was also observed by Khapalia et al. [13]. Also, hardly any ethyl myristate was detected for the methanol/water mixture (see Figure 5.8(b)). Ethyl myristate recovery is thought to be low for the extraction with water and methanol due to the increased evaporation time after the extraction. Note the evaporation was performed after the extraction to perform HS SPME GCMS and FAEEs are semi-volatiles of which ethyl myristate has the lowest boiling point of the four. That the low signal

(a)

(b)
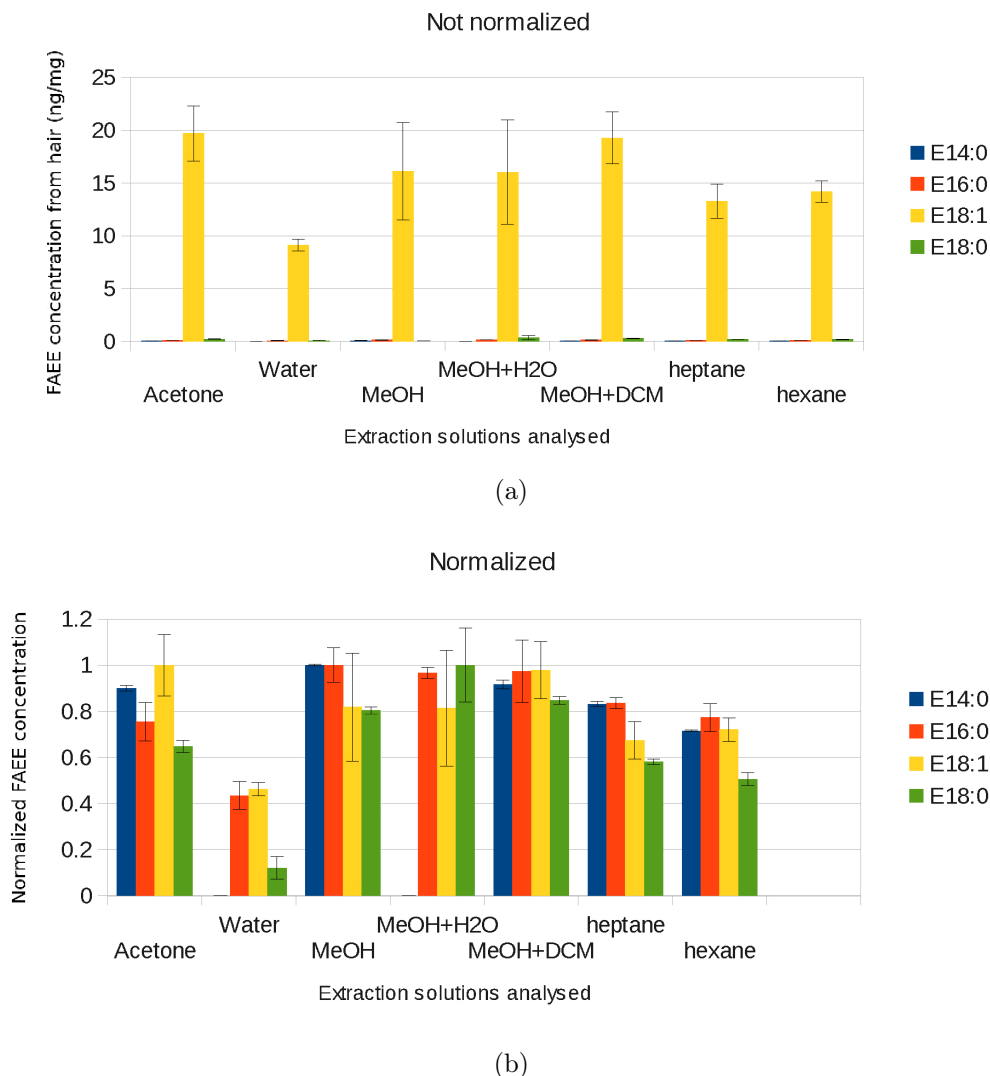
Figure 5.8: FAEEs extraction from hair with different solutions a) not normalized and b) normalized.

for ethyl myristate may have been caused by evaporation is supported by the undetected deuterated form of ethyl myristate. This phenomenon was also observed for the extraction with water. Moreover, water has the lowest extraction efficiency. This may be explained by the low solubility of FAEEs in water and the relatively long evaporation step after the extraction. The first is supported by Pragst [67] who states that the extraction by aqueous buffer is inefficient for these highly lipophilic substances.

The difference between the use of heptane and hexane was negligible (see Figure 5.8(b)). Pragst [114] and Auwärter et al. [115] also observed this and explained that for the extraction of FAEEs in hair the more toxic n-hexane should be replaced for heptane.

Methanol (with and without dichloromethane) is an interesting candidate for extraction (see Figure 5.8(b)). Methanol has been used in the past for both EtG and FAEEs extraction from hair. Moreover, Zimmerman and Jackson [192] clearly show that methanol outperforms acetone, hexane and dimethyl sulfoxide/hexane when sonication is used, in particular with an extraction time of 30 minutes. Methanol is also a reasonable candidate for EtG since it was the second-best extraction solvent of those that were tested. However, since water had a superior extraction efficiency this would be preferred for tests that solely analyse EtG.

Methanol has the perceived disadvantage that the ethyl esters may transform into methyl esters, as was described by Pragst [67]. In this PhD study no methyl esters were detected at the lower concentration (30 ng/mL), more in the range of values that can be detected in real hair samples. With the higher concentration sample (500 ng/mL), only two markers were observed to convert to methyl esters: ethyl stearate and ethyl palmitate. These markers showed 2% conversion from ethyl to methyl at sonication times ranging from 15 minutes to several hours. Up to 10% of the ethyl esters were converted to methyl esters with overnight sonication and therefore long periods of sonication should be avoided. Which of the two sonicators was used did not matter.

5.3.4 Practical implications from the extraction experiments

The previous sections showed that with an extraction of 30 minutes i) the intensity of the signal was higher than for several hours and ii) only up to 2% transesterification occurred for 500 ng/mL FAEEs. With 30 minutes sonication of spiked hair samples in methanol linear calibration curves with a correlation coefficient above 0.99 were obtained. FAEEs could thus be quantified despite transesterification. Hence, an extraction with methanol by sonication is likely to be feasible for 30 minutes.

Some practical implications arose from the extraction experiments. Methanol seemed to be a good common ground for which both EtG and FAEEs were extracted, although to a lesser extent than the extraction solvents currently used for the single methods (water for EtG and a dimethyl sulfoxide/heptane mixture for FAEEs). It may be necessary to establish a new cut-off value, due to the difference in extraction efficiency for in particular EtG compared to the industry standard (the extraction of EtG from hair with water). If for instance only 40% is extracted with methanol of what would be extracted with the currently used industry standard (the extraction of EtG with water), the results need to be converted to enable a comparison of the extracted amounts obtained with the combined method and the currently used method. Possibly the percentage that is extracted with methanol may depend on the concentration of the alcohol metabolite present. Hence receiver operating characteristic curves need to be processed and analysed for this combined method to determine the new cut-off values. If instead of a cut-off value the Bayesian theory is applied for different ranges, other statistical work is required (see Chapter 8).

5.4 Optimisation of the derivatisation of EtG

EtG is not volatile and needs treatment prior to GC analysis. The derivatisation methods were evaluated with 100 ng EtG and an excess (20 µL) of PFPA or BSTFA. Jurado [58] reported a high EtG concentration of 5 ng/mg, which is similar to the 100 ng EtG that is used here when 20 mg hair is analysed. The derivatisation agents were also used by Agius et al. [44], Kharbouche et al. [59] and Paul et al. [170] (see Appendix E).

The BSTFA derivatisation of EtG was optimised. For this 10 µL ethyl acetate (EA) was added to 10 µL BSTFA and heated at 80 ℃, as this was the temperature used by other researchers in the field like Paul et al. [170]. A faster formation of the derivatised product was observed when ethyl acetate was present. In addition, a longer derivatisation time resulted in a higher response for EtG-BSTFA and the difference between 3 h and overnight derivatisation was relatively small (see

Figure 5.9). Hence, the sample needs to be heated for at least 2 h to obtain a relatively high response, which is long compared to the protocols used by other researchers (like Paul et al. [170]) who derivatise the samples for 20 min. Also the derivatisation will be performed in the presence of ethyl acetate as a higher response is obtained.
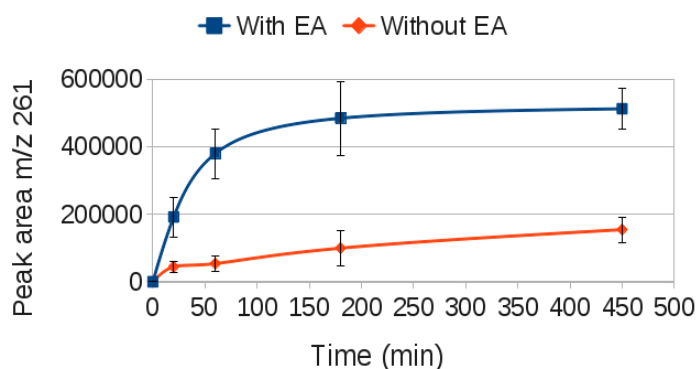
Figure 5.9: Effects of reaction times and the presence of ethyl acetate on the derivatisation of EtG with BSTFA at 80 °C. Error bars indicate the standard deviation for three measurements from the mean value.

The derivatisation of EtG with PFPA was optimised. Performing the derivatisation at room temperature resulted in a large variation in response when different days were compared, possibly due to the variation in temperature in the laboratory. After 30 minutes of derivatisation with PFPA the response was higher at 90 °C than at 60 °C or RT (see Figure 5.10). A longer derivatisation time may not be preferred when using this agent (see Figure 5.11). This is in line with the correlation that Agius et al. found [44]. Therefore, 20 minutes of heating the sample at 90 °C in the presence of PFPA was used as derivatisation protocol for EtG, which is close to the 30 minutes that was used by Kharbouche et al. [59] and the 10 minutes used by Agius at al. [44].

After the derivatisation with PFPA, the acid anhydride needs to be removed and evaporation may result in the loss of the volatile FAEEs in a combined method where both markers are extracted and analysed. In most published research the removal of PFPA involves an evaporation step [44, 193–195] or a liquid liquid extraction [196–199]. A simpler and possibly effective method to stop the derivati-
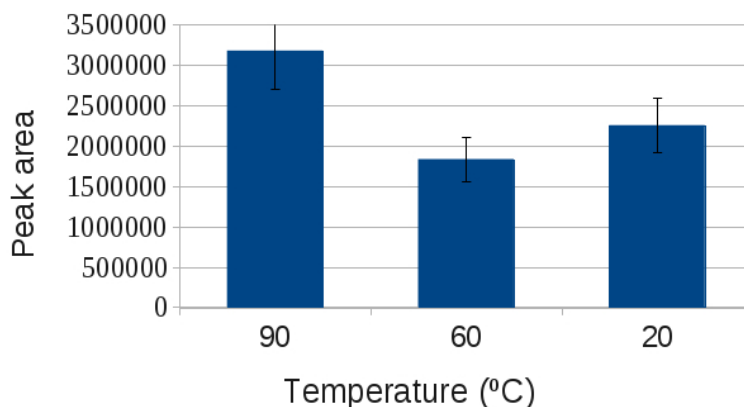
Figure 5.10: The effect of different reaction temperatures on the derivatisation of EtG with PFPA for 30 min.
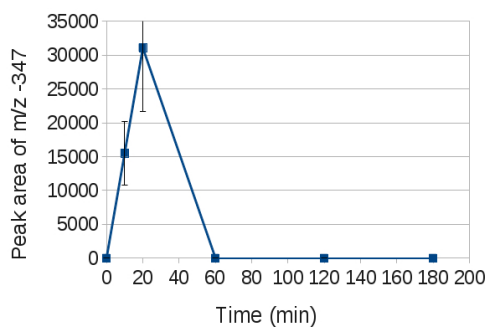
Figure 5.11: The effect of different reaction times on the derivatisation of EtG with PFPA at 90 °C. Error bars indicate the standard deviation for three measurements from the mean value.

sation reaction and remove excess PFPA is the addition of methanol [200]. At first the concentration of 500 ng EtG was used (5 times more than previously to ensure a similar concentration as the previous experiments) and after the derivatisation with 20 µL PFPA also 80 µL of methanol was added. No EtG-PFPA was detected. This may be due to the degradation of EtG-PFPA in favour of the derivatisation of methanol. When only 20 µL methanol was used instead of 80 µL and the head space of the mixture was exposed to a PDMS-DVB SPME fibre, the fibre coating was stripped off and the fibre turned black after desorption in the injection port. The material on this fibre is closely related with the GC column; 95% of the liquid layer inside the column (the stationary phase) that is used

in this project consists of dimethylpolysiloxane which is the same as the PDMS layer on the SPME fibre. It was therefore concluded that the addition of too little methanol may damage the column and evaporation may be more suitable than the addition of methanol. Other researchers have pre-spiked the SPME fibre with PFPA (Hong et al. [201]) or used other procedures where derivatives were produced and no evaporation or washing procedure was used prior to the injection of the sample on the GC column (Warzecha et al. [202]). This may work for their research but not for this study because of the requirements of the detection limit: the analysis of EtG in hair is trace analysis. Hence, it is suggested to use evaporation after the PFPA derivatisation of EtG.

When a combined method is developed, it may be possible to derivatise EtG in the presence of FAEEs and heptane. The addition of FAEEs at CAL 3 level and 80 µL heptane did not significantly affect the derivatisation of 50 ng/mL EtG with BSTFA or PFPA. Neither did the derivatisation affect the detection of FAEEs (see Figure 5.12). However, no EtG was detected when the heptane layer of the hair extract (extracted in the presence of 0.5 mL dimethyl sulfoxide) was added to EtG, FAEEs and 50 µL BSTFA or PFPA. It is likely that the hair contains water and other compounds that can be derivatised too. Therefore, anhydrous magnesium sulphate was added to the hair extract to remove the water. The sample was then filtered and derivatised with either PFPA or BSTFA. Still, no EtG was detected. Hence, the hair extract needed to be evaporated to dryness before derivatisation even though this means that the FAEEs concentration would be reduced due to this sample preparation step.

When PFPA was used followed by evaporation and HS SPME the response for EtG decreased in the timespan of 5 to 10 samples (see Table 5.5 and Figure 5.13 for the chromatography of two consecutive days). The derivatisation with BSTFA was more robust and therefore BSTFA was preferred for this work. Hence, samples were derivatised with 10 µL BSTFA and 10 µL ethyl acetate at 80 ℃ for at least 2 h.
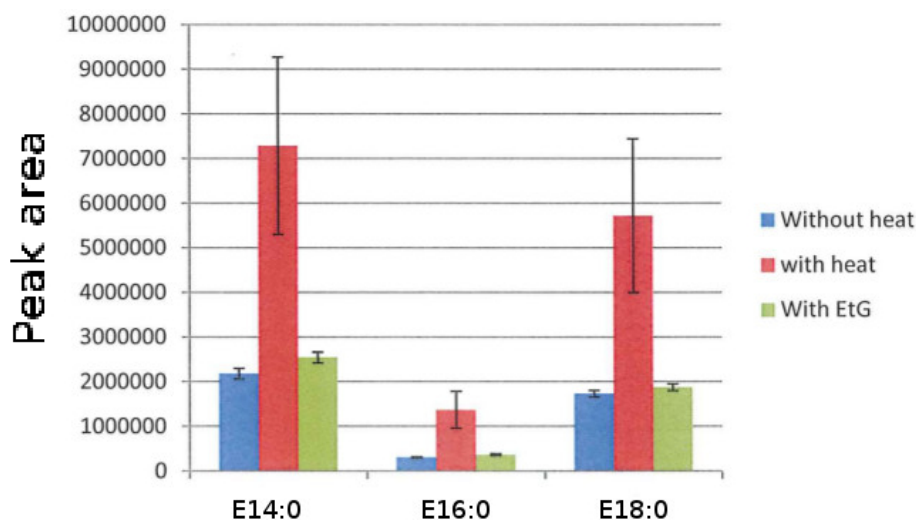
Figure 5.12: Effect of heat (30 min at 80 °C) and the presence of 5 ng EtG on ethyl myristate, ethyl palmitate and ethyl stearate.

Table 5.5: The signal of EtG-PFPA-D5 for four consecutive injections with HS SPME (m/z 501 in negative chemical ionisation mode was used).

Measurement	Peak area
1	1,741,355
2	844,271
3	298,327
4	116,213

5.5   The influence of evaporation on the detection of EtG and FAEEs

FAEEs were evaporated under nitrogen to prevent oxidation of the analytes [114]. Evaporation under nitrogen affected mainly ethyl myristate as this compound was reduced more than the other three FAEEs when the evaporation time was increased and the ratio between the FAEEs changed for increasing evaporation times (see Figure 5.14). Typically a relatively high signal for ethyl myristate was observed with little or no evaporation. Lower responses were obtained with SPE and longer evaporation times due to silanized glassware or in experiments to ensure PFPA was removed. The other FAEEs were affected less and EtG-PFPA was not affected by evaporation times up to an hour. Ethyl myristate is more

(a)

(b)
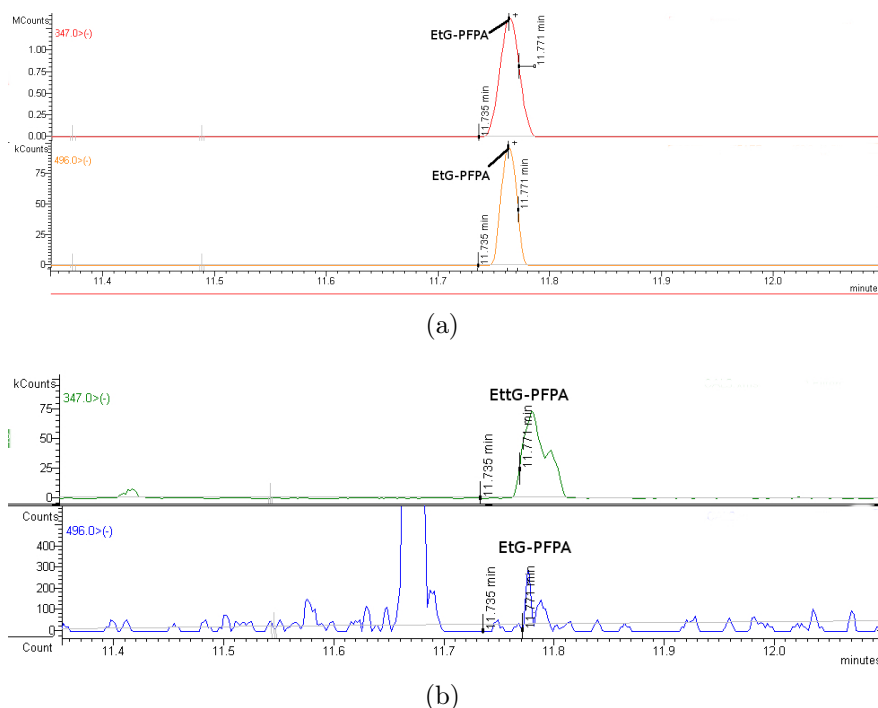
Figure 5.13: Chromatogram of 1.5 ng/mL EtG-PFPA with HS SPME at two consecutive days: a) at day 1 and b) at day 2. The m/z values used were -347 and -496.

volatile than the other FAEEs, which explains why this analyte was affected most by evaporation time. In conclusion, ethyl myristate is likely to be at the highest risk of being affected by the evaporation. As little as possible evaporation steps should be used in the overall method and the evaporation should not take longer than necessary.

5.6 The development and evaluation of SPE procedures for the overall combined method for EtG and FAEEs analysis

The SPE method will depend on the overall method that is used for the extraction and detection of the analytes. The solvent in which the analytes are extracted
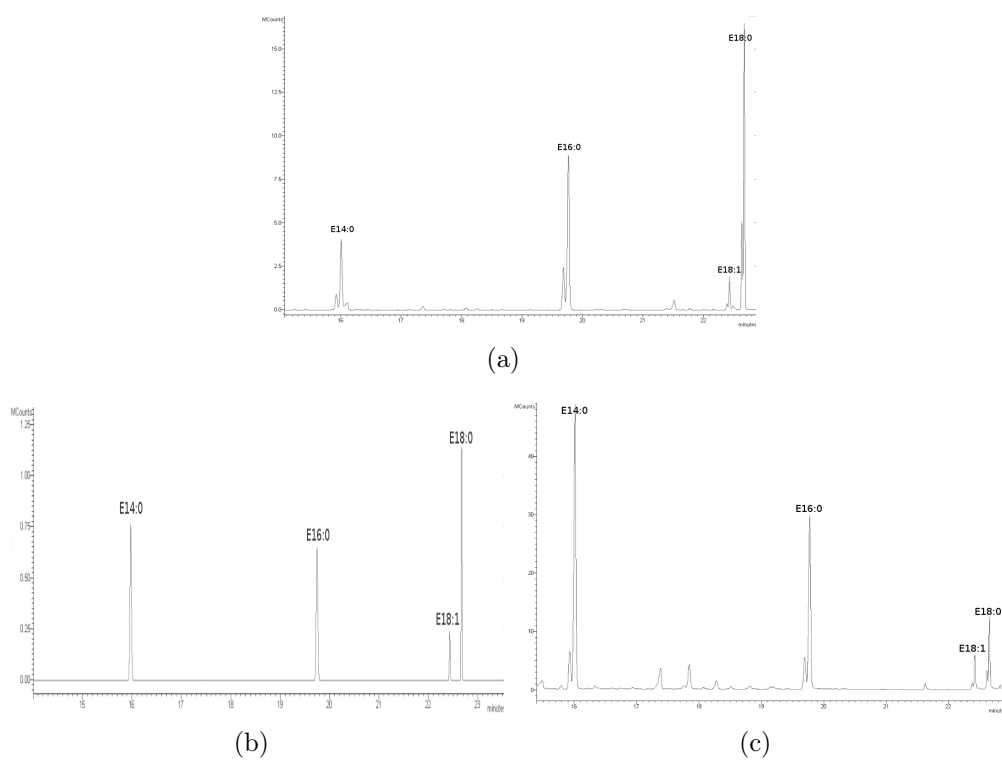
Figure 5.14: Chromatogram of the four FAEEs with a decreasing evaporation time from a to c. FAEEs were present at the same concentration (CAL 5 level of ethyl myristate). a) and c) also contained a 15 mg hair extract and deuterated compounds (for the concentration see Table 4.1 on page 50).

plays an important role as well as the analytes that are targeted. In Section 5.6.1 the aim of the SPE procedure is to separate the FAEEs from EtG. In the overall method both markers were extracted with methanol. In Section 5.6.2 a SPE procedure was developed for EtG dissolved in dimethyl sulfoxide. In the overall method EtG was extracted and dissolved in dimethyl sulfoxide whereas FAEEs were extracted in heptane. Since dimethyl sulfoxide would be derivatised when a derivatisation agent is added [157] and dimethyl sulfoxide was not easy to evaporate, SPE was used to redissolve EtG in another solvent that can be evaporated easily prior to the derivatisation step that is required for the detection of EtG with GCMS. In both sections four SPE procedures were used and compared.

5.6.1 The development and evaluation of SPE procedures where FAEEs are separated from EtG

In this method the EtG and FAEEs were separated during the SPE procedure. An aminopropyl column was used, because this can be used in reversed and normal phase and can be used for aqueous ionizable and non-ionizable analytes [146]. In addition Bernhardt et al. [203] and Battistutta et al. [204] used SPE for the purification of FAEEs with aminopropyl silica SPE columns and Janda [107] used this column to clean up an EtG hair extract. Carcia-Algar et al. [205], Kwak et al. [206] and Pichini et al. [207] also used this type of column for FAEEs and conditioned the column with hexane.

The procedures that were used to separate FAEEs from EtG with SPE are depicted in Tables 5.6, 5.7, 5.8 and 5.9. Two completely different SPE procedures were performed. Method A and B were similar to the SPE procedure used for EtG (see Tables 5.6 and 5.7) and method C and D were similar to the procedures used for FAEEs (see Tables 5.8 and 5.9).

When the four SPE procedures (referred to as method A-D) were compared several observations were made. The addition of a base or an acid to the non-aqueous samples and elution step was irrelevant in the SPE procedure as no difference was observed between method A and method B (for details about the protocols see Tables 5.6 and 5.7). In both procedures FAEEs were detected in the sample loading phase and the first wash with a non-polar solvent (hexane). When the recovery was calculated when both the wash and loading phase were combined, FAEEs-D5 were added, evaporated to dryness and measured with HS SPME. The recovery of ethyl myristate was approximately 10%. Hence, FAEEs were not retained with these procedures and for ethyl myristate in particular evaporation may have been an issue. EtG was detected in the loading step and in the elution step in these procedures. FAEEs were detected in the same steps when the sample was (partially) evaporated and dissolved in hexane (see method C and D described in Tables 5.8 and 5.9). A relatively low recovery was seen for ethyl myristate: approximately 23%. In these procedures EtG was not detected, probably because EtG does not dissolve well in hexane. In conclusion, a mixture

of EtG and FAEEs redissolved in hexane as loading step is not appropriate for a combined method since EtG is not detected in any of the SPE steps. The SPE procedures where the methanolic hair extract was loaded on the column was more suitable for a combined method that focusses on both EtG and FAEEs. In addition, method B described in Table 5.7 is most suitable, since EtG is eluted in methanol and not in water (as is the case in method A shown in Table 5.6). The evaporation of methanol is quicker than that of water and the sample needs to be evaporated to dryness for the derivatisation step that follows this procedure in the overall method. Hence of methods A to D method B was preferred.

Table 5.6: SPE method A for the separation of EtG and FAEEs when these are extracted from hair with methanol.

	Solvents used	**Analytes Detected**
Precondition	1 mL methanol	N/A
Load sample	1 mL of sample in MeOH and 20 µL formic acid	FAEEs and EtG
Remove interference	1 mL hexane	FAEEs
Remove liquid	5 min full vacuum	N/A
Elute EtG	1 mL 5% ammonia in water	EtG

Table 5.7: SPE method B for the separation of EtG and FAEEs when these are extracted from hair with methanol.

	Solvents used	**Analytes Detected**
Precondition	1 mL methanol	N/A
Load sample	1 mL of sample in MeOH and 20 µL 35% ammonia in water	FAEEs and EtG
Remove interference	1 mL hexane	FAEEs
Remove liquid	5 min full vacuum	N/A
Elute EtG	1 mL 2% formic acid in methanol	EtG

Table 5.8: SPE method C for the separation of EtG and FAEEs when these are extracted from hair with methanol.

	Solvents used	**Analytes Detected**
Precondition	1 mL methanol	N/A
	1 mL DCM	N/A
	1 mL hexane	N/A
Load sample	1 mL of sample in hexane	FAEEs
Remove interference	1 mL hexane	FAEEs
	1 mL DCM	None
Remove liquid	5 min full vacuum	N/A
Elute EtG	1 mL MeOH	None

Table 5.9: SPE method D for the separation of EtG and FAEEs when these are extracted from hair with methanol.

	Solvents used	Analytes Detected
Precondition	1 mL methanol	N/A
	1 mL DCM	N/A
	1 mL hexane	N/A
Load sample	1 mL of sample in hexane and 100 µL MeOH (sample only partially evaporated)	FAEEs
Remove interference	1 mL hexane	FAEEs
	1 mL DCM	None
Remove liquid	5 min full vacuum	N/A
Elute EtG	1 mL MeOH	None

5.6.2   The development and evaluation of SPE procedures for EtG in dimethyl sulfoxide

Table 5.10: SPE methods E and F for EtG in DMSO.

	Method E	Method F
SPE cartridge	Oasis MAX	Oasis MAX
Condition	1 mL methanol	1 mL methanol
	1 mL de-ionised water	1 mL de-ionised water
Load sample	0.5 mL of sample in DMSO and	1 mL aqueous sample
	0.5 mL water	(re-substituted after evaporation)
Remove interference	1 mL 5% ammonium hydroxide in water	1 mL 5% ammonium hydroxide in water
	1 mL de-ionised water	1 mL de-ionised water
	1 mL methanol	1 mL methanol
Remove liquid	5 min full vacuum	5 min full vacuum
Elute EtG	1 mL 5% acetic acid in methanol	1 mL 5% acetic acid in methanol

Table 5.11: SPE methods G and H for EtG in DMSO.

	Method G	Method H
SPE cartridge	NH2 SPE	Oasis MAX
Condition	1 mL water	1 mL water
	1 mL MeOH	1 mL MeOH
Load sample	sample in DMSO + 0.5 mL MeOH	sample in DMSO + 0.5 mL MeOH
Remove interference	1 mL water	1 mL water
	1 mL MeOH	1 mL MeOH
Remove liquid	5 min full vacuum	5 min full vacuum
Elute EtG	1 mL hexane	1 mL hexane

Tables 5.10 and 5.11 show the methods that were tested in this work. The first two were very similar to the method that is described by Paul et al. [170] and were based on ion-exchange interaction [144]. The other two were based on normal phase SPE that retain polar compounds with polar interactions and possibly also FAEEs due to the polar heads of these compounds. The EtG concentration in dimethyl sulfoxide was 0.5 ng/mL and 0.5 mL was used, because this is the volume that was used for the dimethyl sulfoxide/heptane mixture for the extraction of FAEEs from hair.

For these experiments Oasis MAX (Mixed-mode Anion eXchange) was used. Sporkert explained in the 12th annual meeting of SoHT [208] that this SPE cartridge outperforms aminopropyl because Oasis MAX is a stronger anion exchanger and the articles that were published thereafter that describe SPE use for EtG detection also use Oasis MAX (see [59, 76, 170]). Oasis MAX is a stronger anion

exchange and is more resistant to pH extremes [209, 210]. In addition, biological fluids have both neutral and ionised interferences and therefore a mixed mode non-polar/ anion exchange may be suitable for the hair extracts [146].

Better results were obtained for the recovery with SPE when water was added to the EtG dissolved in dimethyl sulfoxide (see Figure 5.15). The ionic interaction for EtG is probably essential for the recovery of this compound in SPE. In addition, when normal phase SPE was applied (see Table 5.11), NH2 was a better cartridge than Oasis MAX. The stronger anion exchange and pH resistance of Oasis MAX explained by Sporkert [208] do not play a role with this method. Of the SPE methods, method F yielded the highest recovery and method E was second best (see Figure 5.15). Method E, however, is much more practical because a simple addition of water to the dimethyl sulfoxide mixture is less time consuming than evaporating dimethyl sulfoxide (see Table 5.10). Hence, for a method in which EtG is extracted from hair with dimethyl sulfoxide, method E was the most appropriate SPE method.
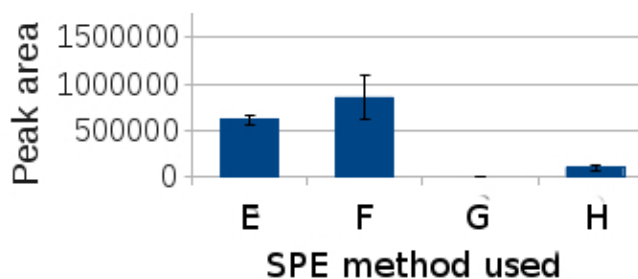
Figure 5.15: Recovery EtG in DMSO with SPE methods E to H described in Tables 5.10 and 5.11.

5.7 SPME fibre selection and optimisation of the procedure for the combined method of EtG and FAEEs from hair

5.7.1 Comparison of SPME fibres for EtG and FAEEs

SPME is a potential sample preparation tool for the analysis of both EtG and FAEEs. Also, when a suitable combined method is made with the use of HS SPME, SPE may no longer be required which may save money and time. SPME is used for the detection of FAEEs from hair extracts (see Appendix F). In this research, the LOD was approximately 10 times as low for the injection of FAEEs via HS SPME after evaporation compared to the direct injection of 2 µL sample dissolved in 20 µL hexane (LOD was between 0.05 µg/mL for ethyl myristate and ethyl palmitate and 0.1 µg/mL for ethyl oleate and ethyl stearate with direct injection). In addition, the peak heights were also about 10 times higher in HS SPME (of 1 mL of the sample evaporated to dryness) than with direct injection (1 µL injected of the 1 mL sample) (see Figure 5.16). For a combined method it is therefore worth investigating the use of HS SPME for the detection of both EtG and FAEEs.

5.7.1.1 Experimental set-up for the comparison of SPME fibres

Whereas a selection of SPME fibres can be made based on suitability of the analytes, ultimately the best fibre for a particular application should be determined by testing [211]. Therefore in this work various SPME fibres were evaluated: 85 µm polyacrylate (PA), 65 µm carbowax-divinylbenzene (CW-DVB), 65 µm polydimethylsiloxane-DVB (PDMS-DVB), 75 µm carboxen-PDMS (CAR-PDMS), 55/30 µm DVB-CAR-PDMS, 7 µm, 30 µm and 100 µm PDMS. According to Pragst [114] the most suitable fibre for the FAEEs is PDMS-DVB and according to Agius et al. [44] the most suitable fibre for EtG-PFPA is CAR-PDMS. Other work where FAEEs were analysed in meconium as opposed to hair
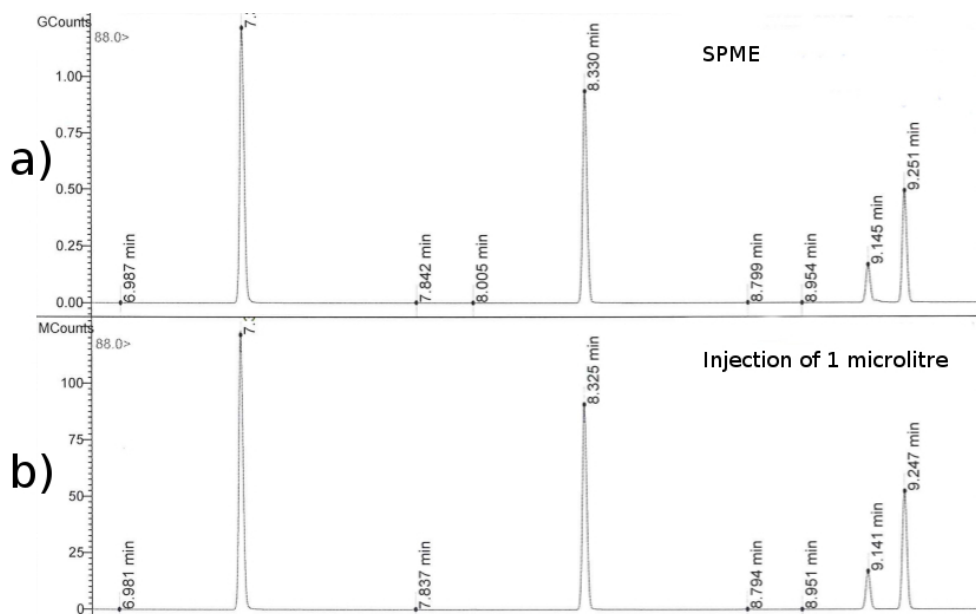
Figure 5.16: Chromatogram of the 4 FAEEs with a) SPME where the sample is evaporated to dryness in a HS vial and b) direct injection of 1 µL of the 20 µL sample in EA.

uses the 100 µm PDMS fibre [212]. These fibres were thus included in the evaluation of fibres. For a combined method, the fibre needs to be suitable for both EtG and FAEEs, which means: i) a low detection limit to enable the detection of the analytes below the cut-off value established for chronic heavy drinking, ii) quantifiable trace amounts and thus a good fit for the calibration curve and iii) the analysis of EtG and FAEEs should not influence each other. A high concentration of one marker should not block the detection of the other marker due to competition. In the light of the latter fibre coatings of one material were preferred. Since the SPME fibres with more than one type of material contain pores to trap the analytes in where the analytes may compete for the analysis of two markers may not be independent.

For the comparison of the different fibres the following concentrations were used for ethyl myristate, ethyl palmitate, ethyl stearate and EtG: CAL 1, CAL 2 and twice the concentration of CAL 5 (for exact concentrations see Table 4.1 on page 50). The concentration of ethyl oleate was equal to those of the other three FAEEs. In addition, a calibration was performed for the PDMS based fibres.

Also, for the highest concentration ($2 \times$ CAL 5) comparisons were made between the intensity of the signal (peak area) with the different fibres. To test the SPME fibres 1 mL of the analytes dissolved in methanol was evaporated, derivatised with 50 µL BSTFA or PFPA and evaporated to dryness. Then, the sample was heated for 5 min at 90 °C, extracted for 30 min at that temperature and desorbed for 15 min at 260 °C of which the first 3 min the injector was in splitless mode. This is similar to the extraction and desorption procedure described by Pragst [114], and is not much different from the procedure for the detection of EtG-PFPA described by Agius et al. [44].

5.7.1.2 The results and discussion of the comparison of SPME fibres

The detection limit for EtG derivatised with PFPA was lower than that of EtG derivatised with BSTFA (see Table 5.12). In addition, calibration curves of the four FAEEs and EtG-PFPA revealed a difference in the performance of the fibres and suitability of the fibres for the combined method (see Table 5.12). A new PDMS-DVB fibre detected EtG-PFPA even at 0.03 ng per sample, which is 10 times lower than the lowest calibrator used in this study. However, the fibre and the GC column degraded fairly quickly and replacement of the fibre and cutting the first bit of the GC column was required after 5 to 10 samples. PFPA is a highly corrosive compound that is known to cause damage to the stationary phase on the GC column. Therefore EtG-BSTFA may be better in a commercial setting even though a lower detection limit was observed for EtG-PFPA.

The response for the PDMS fibres was directly proportional to the thickness of the fibre coating as is in line with the statements of Shirey and Mindrup [154]. A thicker coating absorbed more FAEEs than the thinner coatings with an extraction time of 30 min. Normally for larger analytes thinner coatings are recommended due to the shorter equilibrium time that is required [150]. 30 min of HS extraction with SPME is commonly used for FAEEs extracted from hair (see Appendix F), hence this equilibrium time for the thicker coating is acceptable. A 100 µm PDMS fibre is thus preferred over the fibres with thinner coatings.

PDMS based fibres were compared. Based on the signals obtained of FAEEs when

Table 5.12: Summary of the lowest concentration that is detected for the analytes EtG, E14:0, E16:0, E18:1 and E18:0 with different SPME fibres. Only the concentrations: CAL 1, CAL 2 and twice the concentration of CAL 5 were used. For ethyl oleate the concentration that was used does not correspond with the concentrations of the calibrators used for ethyl oleate but correspond with the calibrators used for the other three FAEEs. ND: not detected.

Compound	Fibre: lowest concentration detected
EtG-PFPA	PA: CAL 2
	CW-DVB: 2 × CAL 5
	DVB-CAR-PDMS: CAL 1
	CAR-PDMS: CAL 1
	PDMS-DVB: CAL 1
	PDMS: CAL 1
EtG-BSTFA	PA: ND
	CW-DVB: ND
	DVB-CAR-PDMS: 2 × CAL 5
	CAR-PDMS: 2 × CAL 5
	PDMS-DVB: 2 × CAL 5
	PDMS: 2 × CAL 5
E14:0, E16:0, E18:1	PA: 2 × CAL 5
	CW-DVB: ND
	DVB-CAR-PDMS: CAL 1
	CAR-PDMS: CAL 1
	PDMS-DVB: CAL 1
	PDMS: CAL 2
E18:1	PA: ND
	CW-DVB: ND
	DVB-CAR-PDMS: ND
	CAR-PDMS: 2 × CAL 5
	PDMS-DVB: CAL 2
	PDMS: 2 × CAL 5

these were evaporated to dryness it was clear that ethyl oleate was more difficult to detect and not detected by DVB-CAR-PDMS (Figure 5.17). In general, the difference observed between the different fibre types was not larger than the difference for the same fibre type (PDMS-DVB) (see Figure 5.17 where two different PDMS-DVB fibres were used and of one was used on different days). A larger difference between the PDMS based fibres was observed when a calibration curve was made. Fibres with carboxen (CAR-PDMS and DVB-CAR-PDMS) showed a linearity of the calibration curve with a lower correlation coefficient for ethyl oleate and ethyl stearate in particular (see Figure 5.18). This may be due to i) the double bond in ethyl oleate and carboxen fibres are designed for molecules without pi-bonds [211] and ii) the FAEEs are relatively large compared with the size of the molecules that is recommended for these fibres (DVB-CAR-PDMS is ideal for M_r 40–275 and CAR-PDMS for low molecular mass compounds with M_r
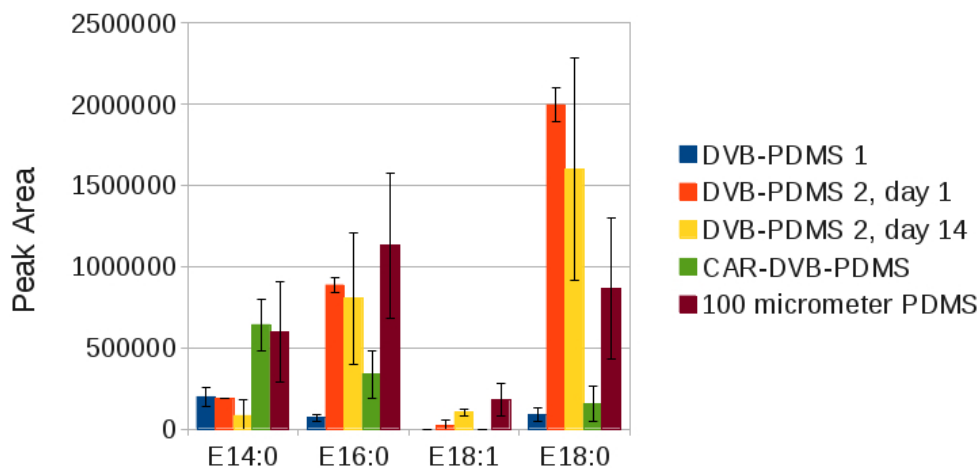
Figure 5.17: The signal of the four FAEEs with different SPME fibres: 100 µm PDMS, PDMS-DVB and DVB-CAR-PDMS. Two different fibres were used for PDMS-DVB and one of them was used at two different points in time. This experiment was measured in duplicate and the error bars indicate the standard deviation of the average that was measured.

30–225 [213]). Briefly, the fibres with carboxen are not suitable for the combined method.

PDMS-DVB had the best detection limit when the carboxen fibres were not taken into account. For 100 µm PDMS only the four higher calibrators were observed in the calibration curve (see Figure 5.18). PA and CW-DVB fibres have a higher detection limit for FAEEs than the 100 µm PDMS fibre, as can be expected due to the polar nature of these fibres versus the non-polarity of the PDMS fibre (see Table 5.12). Also EtG-PFPA was not detected at the concentration for which it was observed with the PDMS fibre. PA did detect EtG-PFPA at a lower concentration than CW-DVB. In general polar fibres are considered to be more useful for polar analytes due to their selective nature [154]. This means that they do not absorb or adsorb more polar molecules but extract a lower amount of non-polar molecules. The latter may have been true in this experiment and the reduced extraction of the more polar EtG-PFPA may also be contributed by the age of these fibres. The CW-DVB fibre is no longer produced and was at least five year old. When a polar PA fibre and a non-polar 100 µm PDMS fibre were both put in the HS vial for the extraction of the analytes, a lower peak area

was obtained for EtG-PFPA and FAEEs on the polar fibre that was analysed first then on the non-polar fibre. Hence, the older PA and CW-DVB fibres that were used in this research performed worse than the 100 µm PDMS fibre and was therefore less suitable for a combined method. The best fibre that showed a limit of detection below CAL 1 and a linear calibration curve was PDMS-DVB and was selected for a combined method.
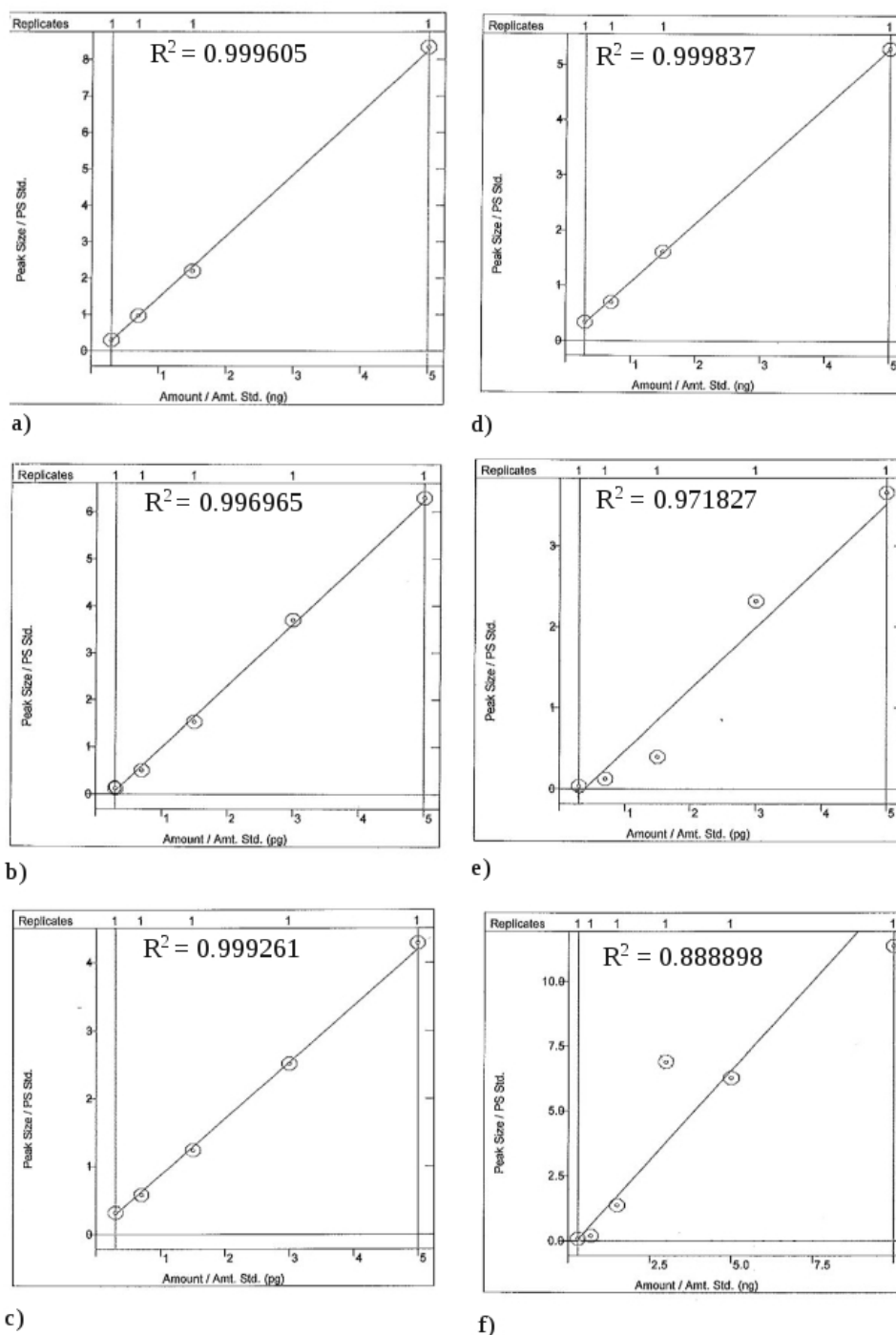
Figure 5.18: The calibration curves of ethyl myristate obtained with a) 100 µm PDMS, b) PDMS-DVB and c) DVB-CAR-PDMS. And the calibration curves of ethyl stearate obtained with d) 100 µm PDMS, e) PDMS-DVB and f) DVB-CAR-PDMS.

5.7.2 Optimisation of HS SPME for the detection of EtG and FAEEs

The same experimental set-up was used as for the comparison of the fibres (see Section 5.7.1). Only a concentration of CAL 5 (see Table 4.1 on page 50) was used for ethyl myristate, ethyl palmitate, ethyl stearate and EtG and the same concentration was used for ethyl oleate as the other FAEEs.

HS SPME was performed in HS vials with the analytes dissolved in several solvents: methanol, phosphate buffer, heptane, ethyl acetate and without solvent. Also samples were analysed with and without prior evaporation of the hair extract before the addition of these solvents. When no solvents were added after sample evaporation a higher signal for FAEEs was obtained with HS SPME compared to the addition of a solvent. For EtG-BSTFA a thousand times higher signal and a higher S/N was obtained for the dry sample compared to leaving the derivatisation agents present during HS SPME (50 µL BSTFA and 50 to 1000 µL EA: a larger amount of BSTFA was used here to compensate for the larger size of the HS vial compared to the GC vial). Methanol and aqueous liquids such as phosphate buffer hydrolysed the derivatised EtG product in such a way that no EtG-PFPA or EtG-BSTFA was observed for even the highest calibration levels (a total amount of 5 ng in the HS vial). The non-polar solvent heptane swelled the fibre and subsequently damaged it when the fibre was retracted in the protective needle [214]. This was evident from the change in fibre appearance: a decreasing amount of fibre material was observed when these non-polar solvents were used. Furthermore, with heptane, methanol and ethyl acetate carry-over was observed. Condensation occurred on the protective needle, which may have led to contamination that was not removed by the heat of the injector port. To avoid this lower extraction temperatures were used as low as 50 °C, but this did not avoid carry-over. Hence, the best sampling preparation for HS SPME was evaporating the sample with nitrogen without the addition of a solvent or phosphate buffer. All analytes were observed with this and no carry-over was observed. In addition, this procedure saves time and has also been suggested by Zimmermann and Jackson [215].

The time the solution was preheated before HS SPME took place did not affect the signal of the analytes. For this solutions without hair extracts were used and the sample was preheated for 5 minutes to obtain an equilibrium between the matrix constituents and the analytes in the HS, which may be useful especially for the reproducibility. Therefore, the preheating time of 5 min was used in this research as was previously suggested by Pragst [114]. In addition, this is less time than the time set to clean the SPME fibre (desorption time) and therefore when the samples were measured in sequence no time was lost (see Figure 5.19).
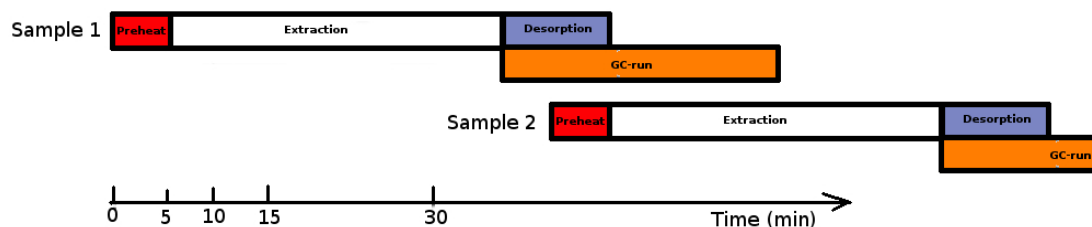
Figure 5.19: A schematic representation of the time required for HS SPME as it was manually performed in sequence. This example only shows two samples, but a similar overlap can be shown for the next samples. The red block represents the time allocated to preheating, the white block the extraction, the blue block the desorption and the orange block the GC-run.

Both the extraction time and temperature for HS SPME with PDMS-DVB were adjusted for samples at the CAL 5 level (with the exception of ethyl oleate that was present at the same concentration as the other FAEEs). At 60 °C and 80 °C a lower signal was obtained for EtG-PFPA and FAEEs compared with the extraction at 90 °C (see Figure 5.20). When the extraction time was varied from 5, 10 to 30 min, 30 minutes offered the best response for the analytes (see Figure 5.21). This is the time that is commonly used for FAEEs (see Appendix F).

Even though the sample prior to HS SPME contained no solvents, the use of the agitator increased the signal obtained with HS SPME compared to the use of heating block that does neither rotate nor surround the HS vial completely. The agitator was used at a speed of 250 rpm for 2 s clockwise, and interval of 1 s, and the next 2 s anticlockwise. The increased signal observed for the extraction with an agitation block compared to a heating block was probably due to the more efficient heating as it was also observed that higher heating temperatures
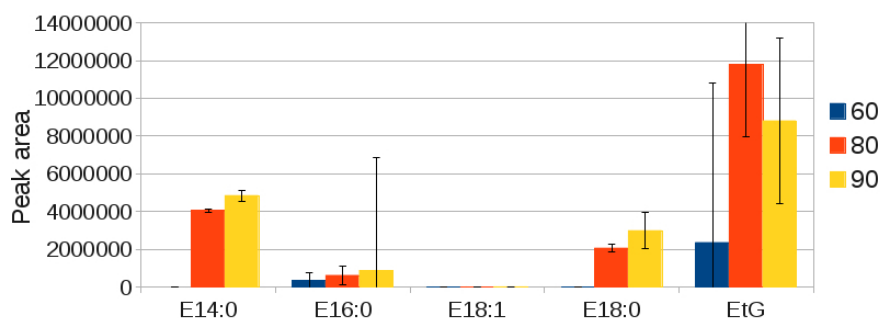
Figure 5.20: Effect of extraction temperature (60, 80 and 90 °C) for 30 minutes of HS extraction with a PDMS-DVB fibre on the detection of EtG-PFPA and FAEEs.
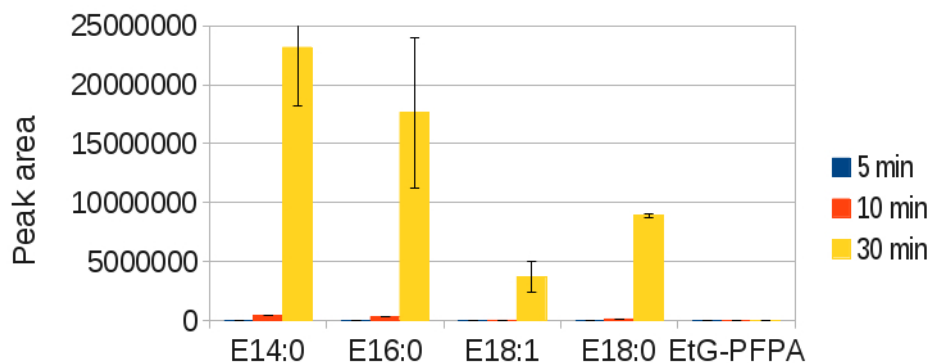
Figure 5.21: Effect of extraction time of HS SPME with a PDMS-DVB fibre at 90 °C on the detection of EtG-PFPA and FAEEs.

resulted in a higher extraction yield.

After desorption for 10 minutes in the injection port at 250 °C no carry-over was observed when amounts in the sample as high as 100 ng EtG and 1 µg of each FAEEs was used when the sample was evaporated to dryness and HS SPME was performed on the dry sample. The EtG and FAEEs concentrations for this experiment were relatively high, but were in the range of the values of EtG and FAEEs that were measured for chronic heavy drinkers previously; Pragst and Yegles [25] detected values for EtG from 4 to 3,380 pg/mg and for FAEEs from 200 to 42,000 pg/mg for people that submitted for withdrawal therapy and others that were known to have been alcoholics.

The optimal temperature for the desorption of both EtG and FAEEs was investi-

gated. The temperature of the injection port was varied between 230 and 260 °C, with increments of 10 °C. These temperatures were used because the boiling point of the FAEEs ranges from 178 to 213 °C (the highest boiling point belongs to ethyl stearate) and the injector temperature is required to be higher than the boiling point of the analytes since the analytes need to be volatile when introduced to the GC system and at 213 °C ethyl stearate is present in both liquid and gaseous form. Also the maximum temperature of this fibre is 270 °C. The temperature differences did not significantly affect the signal for FAEEs or EtG. When spiked hair was used, 240 °C did result in less interfering peaks than 260 °C and was similar to 250 °C. For the SPME procedure the injector was set at 250 °C, which appeared to be crucial for obtaining a calibration curve with a correlation coefficient above 0.99. This is the same temperature as the one used by Agius et al. [44] for the detection of EtG-PFPA (250 °C) and slightly lower than what was used for the detection of FAEEs in the work of Pragst and Balikova [27] (260 °C).

5.8 Final conclusions for optimisation of the sample preparation protocol

From the experiments described in this chapter it was concluded that:

- Silanized glassware should be used for storage, but not for the sample preparation due to the increase in evaporation time and the negative effect of this on the detection of the (semi-)volatile analytes.

- Plastic containers should not be used, due to the introduction of possible contamination to the GC system.

- GC vials with a conical shaped base from Kinesis should be used to allow for a small sample amount and the introduction of the concentrated sample onto the GC system. In addition, the response for different concentrations was repeatable and similar for similar concentrations with these vials.

- Screw cap HS vials were more practical (especially when a sample was recapped) and consistently showed good results and should therefore be

used instead of the crimp cap versions.

- BSTFA without TMCS should be used, because this resulted in only one peak for the derivatised product (EtG-BSTFA) and when one peak was found instead of multiple peaks, the peak area was larger than either one of the peaks that was otherwise detected.

- Ethyl oleate detection was problematic when the same concentrations were used for this compound as for the other FAEEs, therefore a twice as high concentration of this FAEE should be used compared to the other FAEEs for the calibrators and QCs.

- FAEEs should be stored at -30 °C, since the lowest calibrators and QCs could still be detected after two years.

- After the evaporation step of FAEEs hair extract the sample should be measured on that day, because FAEEs were not stable enough to enable their accurate measurement the next day.

- EtG should be stored at -30 °C, since the lowest calibrators and QCs could still be detected after two years.

- The EtG calibrators in de-ionised water were not stable longer than one day and sub-dilutions should thus be made daily.

- 30 mg in mm pieces cut hair should be used for the combined method, because the amount of hair affected the detection of FAEEs.

- For the quantification deuterated compounds should be used of all the analytes as the difference in ratio for different evaporation times indicates. E17:0 (ethyl heptadecanoate) as IS as was used by Best [216] and Moore et al. [217] should be avoided in this research especially because evaporation steps can vary between different samples and a longer evaporation mainly affected ethyl myristate.

- The use of methanol and dichloromethane as decontamination solvents for both EtG and FAEEs seems to be a good compromise, since with medium decontamination efficiency approximately 50% of the EtG content was re-

moved and the decontamination efficiency at high efficiency for FAEEs was between 20 and 50%. A decontamination procedure that would remove more than 50% of EtG may possibly wash away EtG that is incorporated in hair too. This is an important difficulty in determining the most appropriate decontamination procedure. In this work methanol and dichloromethane were used as decontamination solvents to have a relatively average washing out effect for EtG.

- The evaporation time should be carefully monitored. When the evaporation is too long, the analytes are evaporated too increasing the LOQ. When the evaporation is too short acid anhydride is still present and may cause GC column degradation and SPME fibre when PFPA is used as derivatisation agent.

- The most suitable HS SPME method for a combined detection of EtG and FAEEs is shown in Table 5.13 and should be used in the rest of this research project.

- When introducing EtG and FAEEs to the GCMS system, either EtG or FAEEs were affected negatively.

  - When EtG-PFPA was detected using HS SPME, the fibre was damaged by PFPA and only 5–10 samples could be measured before maintenance was required for the GCMS system.

  - When direct injection was performed the detection limit for FAEEs was not low enough.

  Hence, both direct injection and HS SPME should not be used for the introduction of both EtG and FAEEs on the GCMS system.

- PFPA derivatisation should be followed by evaporation to remove the excess of PFPA.

- The optimised derivatisation process of EtG with PFPA was derivatisation of 20 min at 90℃.

- BSTFA is preferred over the use of PFPA, due to the detrimental effect of

PFPA to the column and SPME fibre.

- For the BSTFA derivatisation ethyl acetate should be added and at least 2 h was allowed for the derivatisation at 80 °C.

- Hair extracts need to be evaporated prior to derivatisation even when it concerns solvents that are not derivatised (as was seen for heptane), because hair may contain compounds that will be derivatised and the excess of derivatisation agent may not be enough to overcome this.

- The extraction with methanol when sonication is used should be no longer than 30 min. Longer extraction times increased the percentage of FAEEs that was affected by transesterification.

- A promising extraction method for a combined method was the extraction with methanol. Methanol and dichloromethane is also promising, but dichloromethane is more toxic, more expensive to dispose of and more difficult to accurately pipette with plastic tips. Therefore methanol is preferred as extraction solvent and should be looked at in this project.

- Another interesting extraction method may be an extraction with a mixture of dimethyl sulfoxide and heptane, where the dimethyl sulfoxide layer is subjected to SPE and analysed for EtG and the heptane layer is analysed for FAEEs with HS SPME GCMS.

- When the analytes are extracted from hair with methanol, SPE method A (see Table 5.6) should be used.

- When the extraction of the analytes is performed with a mixture of dimethyl sulfoxide and heptane, the EtG in the dimethyl sulfoxide layer should be redissolved in another solvent with SPE method E described in Table 5.10.

- To evaluate an overall method real hair samples need to be used to establish the effect of the different extraction methods and sample preparation on the analysis of the analytes.

Different variables need to be taken into account for the combined method that needs to be developed. The previously mentioned conclusions are seen as rec-

Table 5.13: The optimal optimal sample preparation procedure for the detection of EtG and FAEEs in this research.

Silanized glassware	for storage not with the evaporation process
Material containers	glass not plastic
GC vial	conical shaped base from Kinesis
HS vial	10 mL and screw cap
BSTFA	pure without TMCS
Concentration ethyl oleate	twice as high as other FAEEs for calibration
Storage EtG and FAEEs solutions	at -30 ℃
Prepare EtG solutions for calibration	daily
Analysis	perform at the same day as evaporation sample
Weight hair sample used for extraction	30 mg
Internal standard	DIS for each analyte
Decontamination procedure	briefly wash with methanol and dichloromethane
Prior derivatisation	complete evaporation of hair extract
Derivatisation	with BSTFA and EA for at least 2 h at 80 ℃
Extraction	30 min sonication in MeOH OR DMSO+heptane
SPE	SPE method A when extracted with MeOH
	SPE method E with DMSO+heptane
SPME fibre	PDMS-DVB
Prior HS SPME	evaporation to 'dryness'
	and preheat sample for 5 min
Agitation HS SPME	250 rpm
Extraction temperature HS SPME	90 ℃
Extraction time HS SPME	30 min
Desorption time SPME	10 min
Desorption temperature SPME	250 ℃

ommendations for the development of a combined method as this was one of the aims for this PhD project.

Chapter 6

Optimisation of GCMS method

As stated in Chapter 5, optimisation of sensitivity of a new analytical method is important so that it competes with currently available methods. This chapter describes optimisation of the GCMS method used for detection of EtG and FAEEs in hair.

6.1   Optimisation of the GC method

6.1.1   Sample introduction

Transfer of the sample to the GC system may be performed in different ways, including SPME, liquid injection and large volume injection (LVI). These various techniques were evaluated for the combined injection of FAEEs and EtG-BSTFA or EtG-PFPA to obtain optimum sensitivity for the combined method of both markers.

As discussed previously the use of HS SPME was investigated. FAEEs had a detection limit which was not sensitive enough when direct injection was used. The detection limit was lower with the use of SPME. This is likely, because the FAEEs are concentrated on the fibre. For the combined extraction however, SPME proved to be difficult because the use of PFPA damaged the SPME fibres

and the GC-column more rapidly than expected as was discussed in Chapter 5. In addition, EtG-BSTFA was difficult to detect with HS-SPME, and thereby a method with SPME and EtG-BSTFA was considered not to be suitable.

An increased injection volume for direct injection (2 versus 1 µL) of the FAEEs and EtG-BSTFA dissolved in EA and BSTFA resulted in increased peak height and area (see Figures 6.1 and 6.2). For direct injection of EtG-BSTFA other researchers have reported the use of 2 µL to increase the sensitivity of the overall method (see [170]). An increase in volume from 1 to 2 µL resulted in a detection limit of the FAEEs that was still higher than CAL 1 used in the calibration curve (see Section 4.2).

(a)

(b)
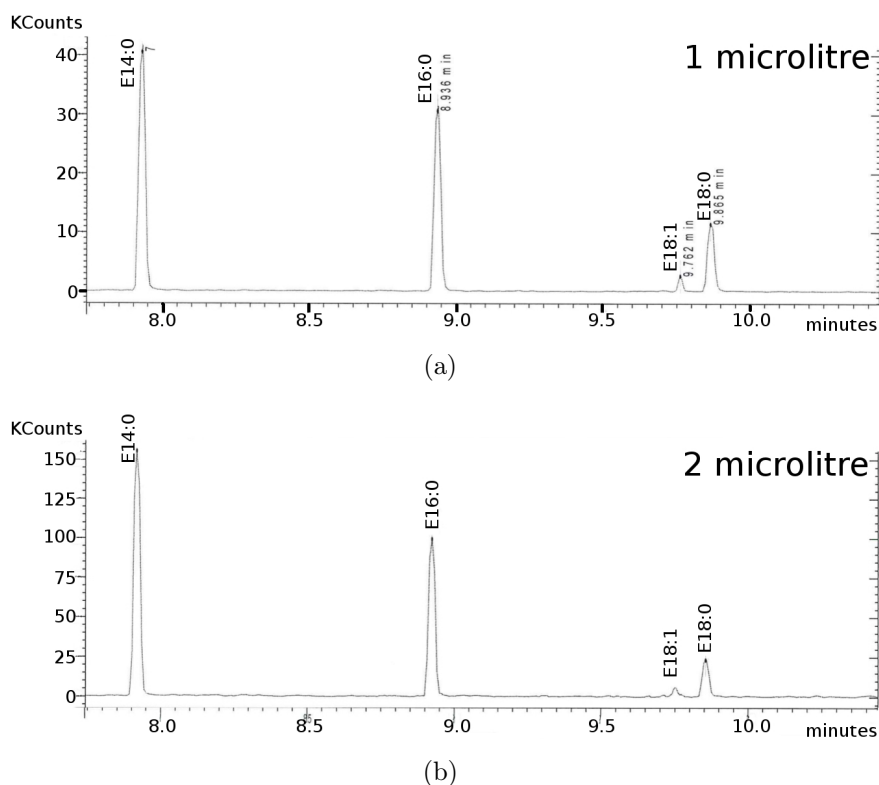
Figure 6.1: Chromatogram when a) 1 and b) 2 µL of FAEEs in EA was injected.

The effect of large volume injection (LVI) was explored with manual injection on the LVI injector to counter the high detection limit of the FAEEs. Shi et al. [218] have showed that LVI was possible for EtG-BSTFA. Hence, a LVI method for
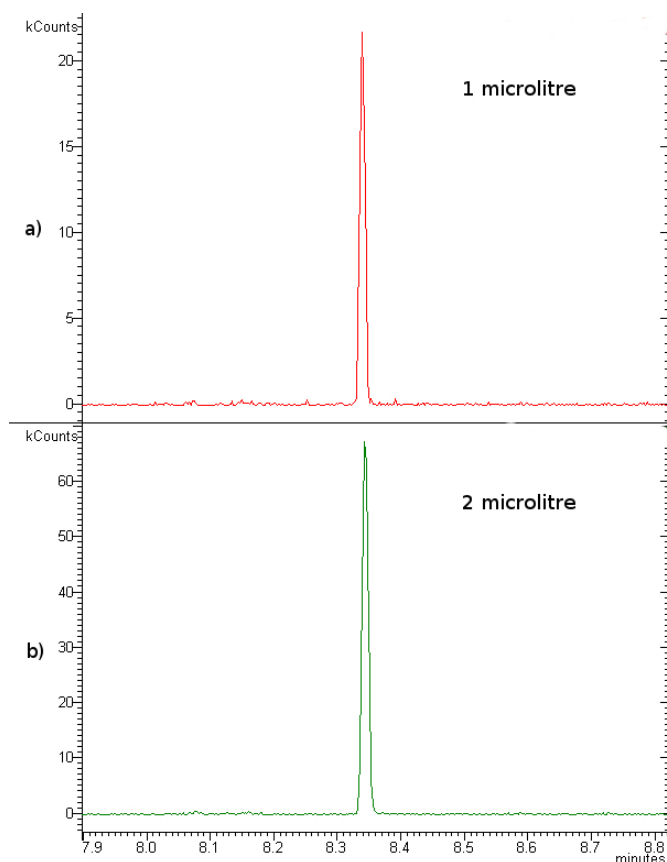
Figure 6.2: Chromatogram when a) 1 and b) 2 µL of EtG derivatised with BSTFA in a mixture of BSTFA and EA was injected. EI SIM was used as detection method.

the combined analysis of EtG-BSTFA and the FAEEs was investigated. For the FAEEs the liquid that needs to be injected should be approximately 10 times the volume that was used for direct injection in order to obtain a sufficiently high sensitivity assuming a linear response in this region to the amount of FAEEs that is injected with DI and LVI (for the details see Section 4.4.7). For the direct injection of 20 and 40 µL a solvent peak was observed but the analytes were not (see Figure 6.3(a)). The expansion of these volumes was probably too large for the liner and part of the sample may have been lost. A slow injection of 20 µL to avoid the overflow due to the liquid expansion and corresponding loss of analytes was unsuccessful (see Figure 6.3(b)). In addition, it was difficult to manually adjust the speed of the injection. When 20 µL was introduced with 4 injections
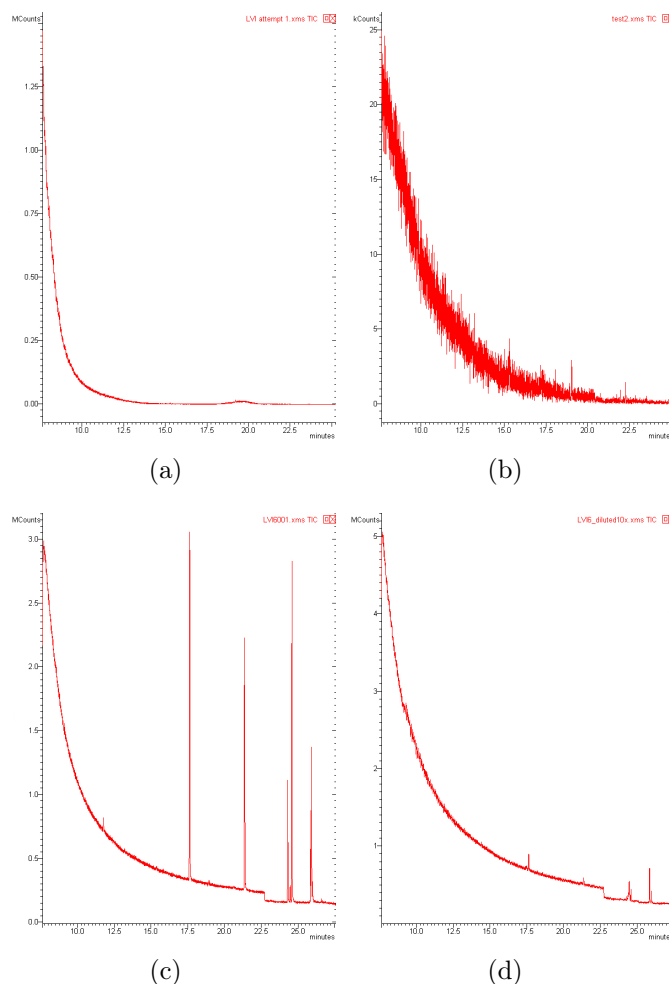
Figure 6.3: Chromatogram of LVI of 10 × CAL 5 level in 20 µL where a) 20 µL was injected at once, b) 20 µL was injected slowly within 30 seconds, c) 4 times 5 µL was injected with a 15 second time interval and d) was similar to what was used in c but with a 10 times lower concentration.

of 5 µL with a 15 s time interval, 10 times the concentration of CAL 5 was visible, but CAL 5 was not (see Figures 6.3(c) and 6.3(d)). Since none of the injection methods were successful for both markers it was decided to divide the extraction solution in two aliquots where one was directly injected for EtG analysis and the other was introduced on the system with HS SPME for the detection of the FAEEs.

6.1.2 Oven temperature programme
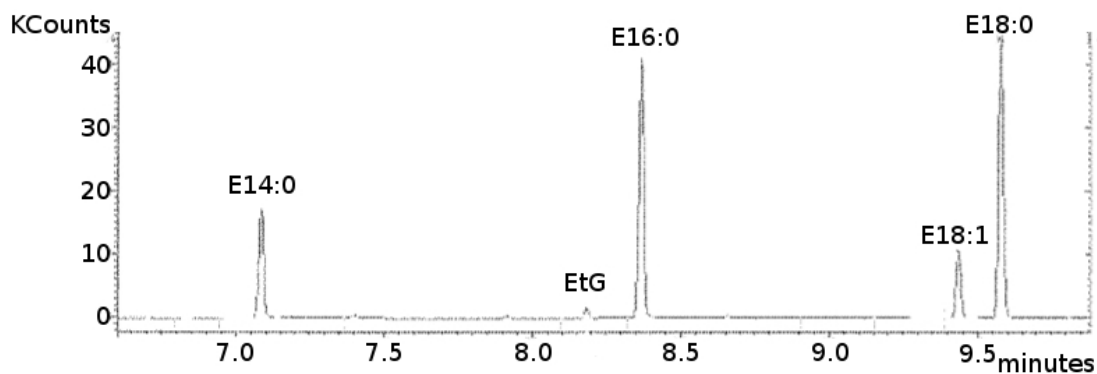
Figure 6.4: Chromatogram of SIM measurement of the 4 FAEEs and EtG-BSTFA. Direct injection of a pure sample with 1 µg/mL of each of the FAEEs and 1.6 µg/mL EtG derivatised with BSTFA.

solved

The detection of EtG-BSTFA benefited from a temperature gradient that was higher, because a sharper peak was obtained with 20 °C/min than with 5 °C/min. Therefore, a lower detection limit could be obtained with the higher temperature gradient. For the FAEEs, on the other hand, 20 °C/min resulted in a poor resolution for the FAEEs when the hair matrix interference was taken into account (see Figure 6.4 for the separation of the analytes). When a calibration was performed, the lower calibrators (CAL 1 and CAL 2) were therefore not quantified correctly which resulted in a calibration curve that was not linear with a correlation coefficient (r^2) lower than 0.9. As a compromise two different temperature gradients were used where a higher temperature gradient was used first followed by a lower temperature gradient (see Table 4.7 on page 60). With this temperature programme the five analytes were resolved, interfering peaks were at least partially resolved and the peaks for the five analytes obtained enough data points to form a smooth peak with reproducible retention times (see Table 6.1 for an example of the retention times).

From the retention times it was clear that EtG-PFPA and the FAEEs were separated in time with a larger time interval than EtG-BSTFA and the FAEEs. If EtG-PFPA was measured and not EtG-BSTFA more time would be available to

Table 6.1: The retention times of the finalized GC method that was developed and used in this work.

Compound	Retention times (min)
EtG-PFPA (DIS)	9.07 (9.03)
E14:0 (DIS)	16.01 (15.94)
EtG-BSTFA (DIS)	19.38 (19.30)
E16:0 (DIS)	19.77 (19.69)
E18:1 (DIS)	22.43 (22.39)
E18:0 (DIS)	22.66 (22.63)

change the MS settings for the different group of markers. However, EtG-PFPA had disadvantage over EtG-BSTFA; PFPA is corrosive and caused degradation of the column (as was observed in Chapter 5).

6.2   The development of a combined MS method

Currently, different MS methods are used for the detection of the analytes. For FAEEs chemical ionisation (CI) is recommended providing less fragmentation and more specificity by way of protonated molecular ions [217]. Moore et al. [217] have developed an analytical method with CI for the detection of the FAEEs in meconium. For EtG-BSTFA a tandem MS method in EI was used by Paul et al. [170] where a fragment is selected at m/z 261 to be fragmented again with collision gas to m/z 143. None of the published measurements were in full scan, due to background. SIM and tandem MS methods were used instead, which reduced the background and are more specific for the analytes.

The aim of this work was to develop an MS method in SIM or tandem MS offering improved sensitivity over the currently used methods for the analysis of EtG and FAEEs in hair. A loss in sensitivity was expected due to the combined sample preparation of the two markers. An MS method needs to be sensitive enough to detect the analytes and specific enough to differentiate background from target analyte signal. Therefore various detection methods were developed and evaluated in EI, CI, SIM and tandem MS.

The development of an MS method for EtG-PFPA is not discussed here, because the derivatisation with BSTFA was preferred over that of PFPA. (More information on EtG-PFPA can be found in Appendix G).

6.2.1   The development of an MS method for the FAEEs

An MS method for the FAEEs was developed both in EI and in methane CI mode and will be discussed in detail in the next sections.

6.2.1.1   The development of an MS method for the FAEEs in EI

Ethyl myristate, ethyl palmitate and ethyl stearate had a similar fragmentation pattern in EI (see Figure 6.5). The base peak of the full scan was at m/z 88 and
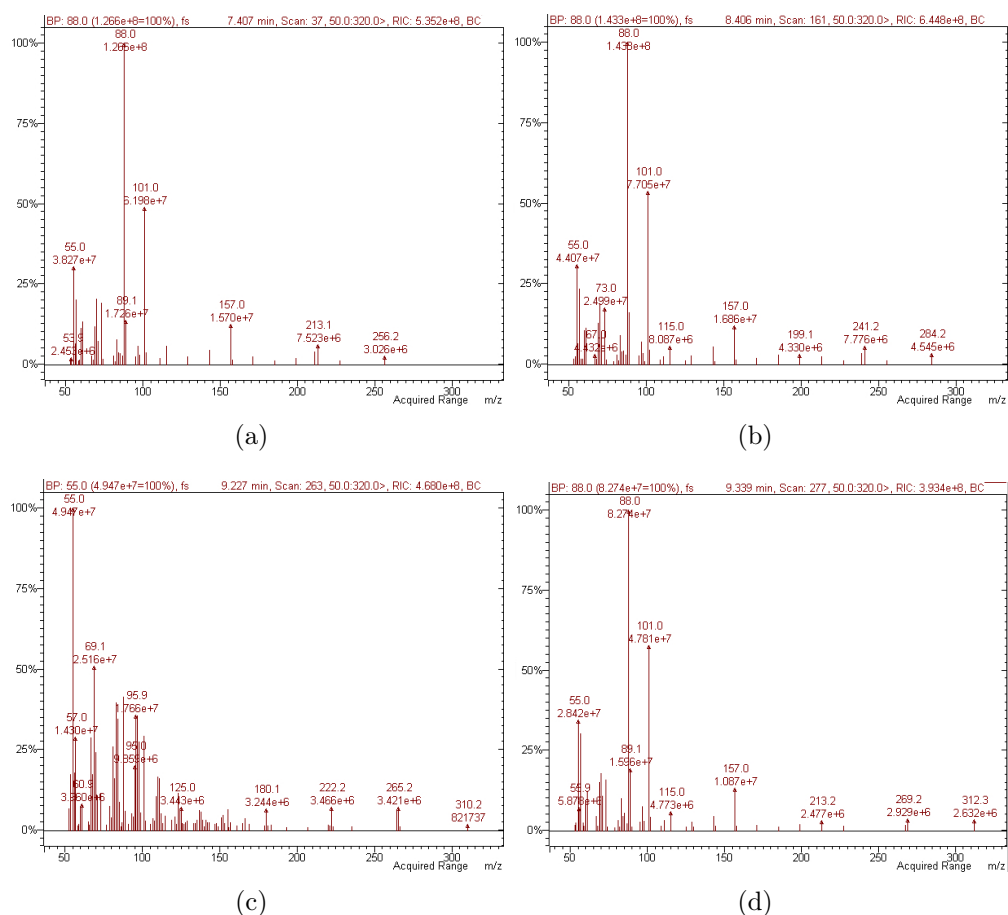
Figure 6.5: Full scan acquired using EI with a scan range from m/z 50 to 320 of a) ethyl myristate, b) ethyl palmitate, c) ethyl oleate and d) ethyl stearate.

is likely a result of McLafferty rearrangement (see Figure 6.6) [37]. McLafferty rearrangement was thus preferred over bond cleavage. In addition, m/z 101 was also highly abundant. This fragment was obtained by the cleavage of the alkyl side chain of the FAEEs (see Figure 6.7). In Chapter 14 of "Analytical and practical aspects of drug testing in hair" [37] fragment m/z 101 is explained as a positive radical produced by β-cleavage. This is not correct because a β-cleavage would result in a positive fragment m/z 87 (see Figure 6.7). β-cleavage is a fission of a bond originating at an atom which is not adjacent to one that bears the charge but one bond further from the charge [219]. Also, a cleavage of the positive radical of the molecular ion in EI can fragment into a positive charged

ion but not a positively charged radical [164]. Another ion that was observed and is very specific to a particular FAEE is the molecular ion (see Figure 6.8). The ions selected for the EI SIM methods were: 88, 101 and the molecular ion (256, 284, 312.5 for ethyl myristate, ethyl palmitate and ethyl stearate respectively). Ethyl oleate fragments more easily, but the same m/z could be selected for the SIM method (with m/z 310.5 for the molecular ion). These are similar to the ions used by other researchers (see Table 6.2).

Figure 6.6: McLafferty rearrangement of a FAEE and the formation of a 88 Da fragment.
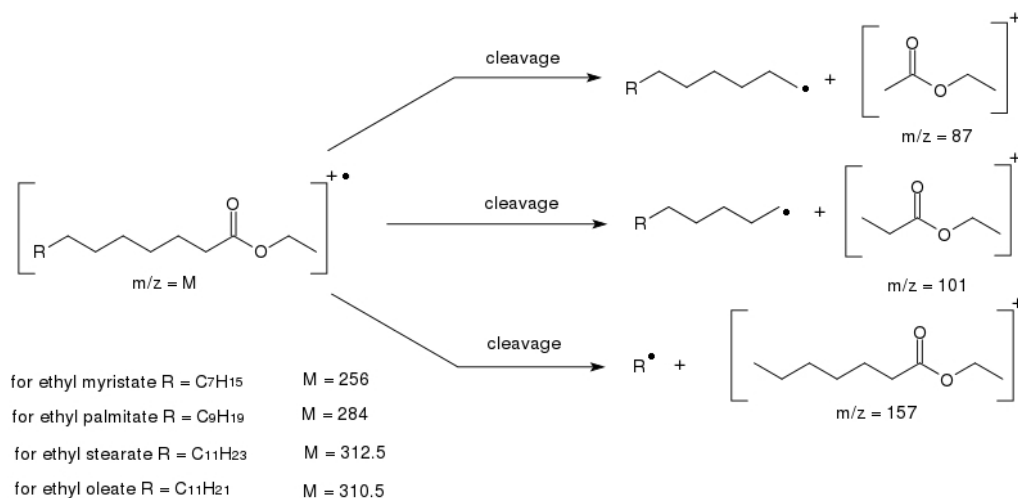
Figure 6.7: Formation fragments due to the cleavage of a FAEE at the chain. Note ethyl oleate has a double bond and the larger ions (of 157 Da) are not abundant.

in

For the development of the tandem MS method for the FAEEs a product ion scan of the molecular ion was performed. The product ion scan of ethyl myristate and ethyl palmitate showed that fragmentation occurred and m/z 87 was high in
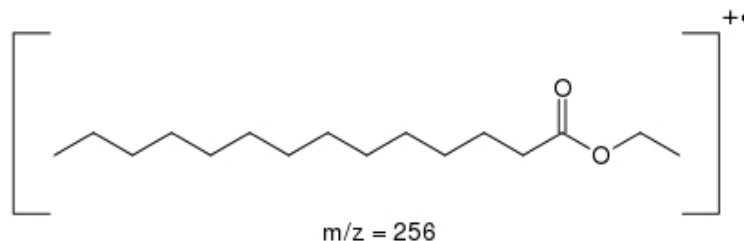
Figure 6.8: Molecular ion of ethyl myristate observed with EI.

Table 6.2: M/z values used in literature for the FAEEs in EI SIM.

Reference	m/z values E14:0	m/z values E16:0	m/z values E18:1	m/z values E18:0
[172]	88, 213, 256	101, 241, 284	101, 265, 310	157, 269, 312
[101]	101, 157, 256	101, 157, 284	88, 101, 310	101, 157, 312
[220]	88, 101, 157	88, 101, 157	88, 101	88, 101, 157
[25]	101, 157, 256	101, 157, 284	101, 310	101, 157, 312
[61]	88, 101, 256	88, 101, 284	88, 101, 310	88, 101, 312
[216]	67, 88, 101	67, 88, 101	67, 88, 101	67, 88, 101
[114][a]	88, 101, 157, 213, 256	88, 101, 157, 241, 284	88, 101, 310	88, 101, 157, 269, 312
[221]	-	55, 88, 101	55, 88, 101	55, 88, 101

[a] Also referred to by: [3, 4, 6, 7, 21, 80, 115, 119, 132, 186, 222–224]

abundance (see Figure 6.9). This ion was probably produced by cleavage and was in tandem MS preferred over the McLafferty rearrangement, which as previously stated has a m/z of 88 (see Figures 6.7 and 6.6). A loss of CH_2 units (m/z of 14) in series was observed (see Figure 6.9). The ion with the highest m/z value that had an intensity above 10% of the base peak had an m/z of 227 for ethyl myristate and 255 for ethyl palmitate and corresponds with a loss of 29. An ethyl group (C_2H_5) was split from the molecular ion. This transition $M^{+\cdot} \rightarrow [M-29]^+$ is thus a possibility for the measurement of the FAEEs in tandem EI. It must be noted that the molecular ion was low in abundance. Other transitions with smaller precursor ions (m/z 101 and 157) were used too for a higher signal and thus a higher sensitivity of the method and the transition $101 \rightarrow 73$ was abundant. This transition is likely not to be specific enough for the detection in the presence of the hair matrix. Currently, the methods in SIM for the FAEEs include the molecular ion whereas older methods would only measure the fragments (see Table 6.2). Since the molecular ions were relatively low in abundance with EI, it was decided to investigate the use of methane CI which could provide less fragmentation and enhanced protonated molecular ion.
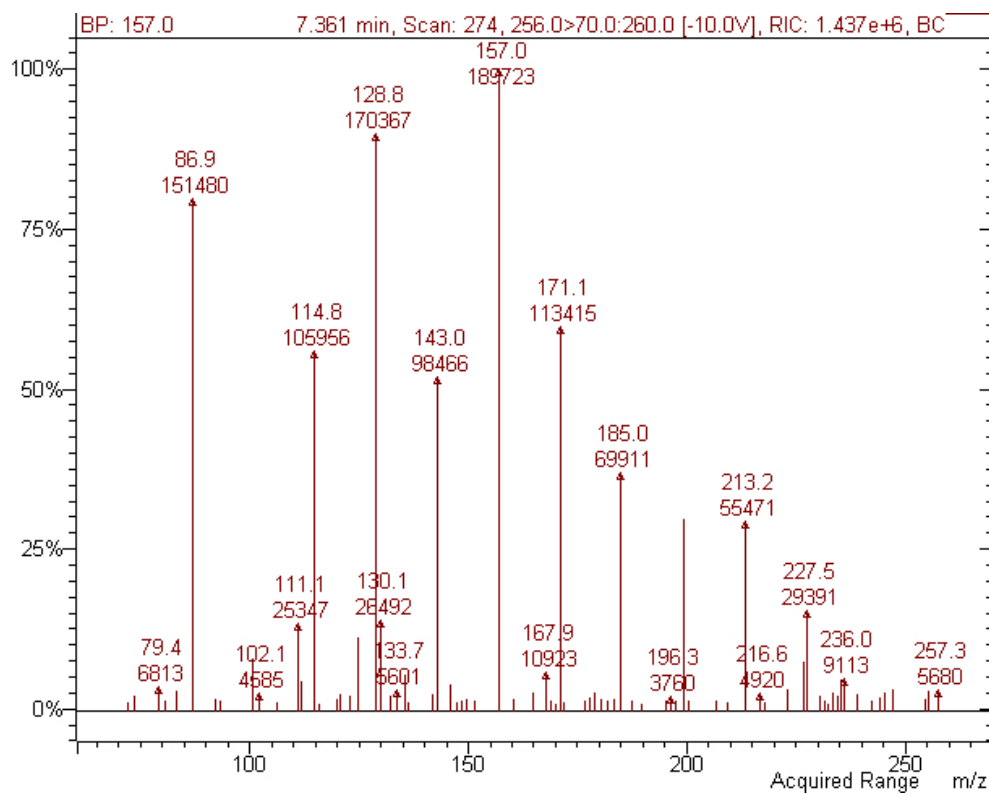
(a)
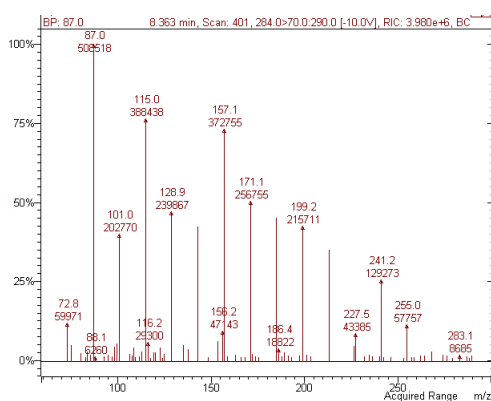
(b)
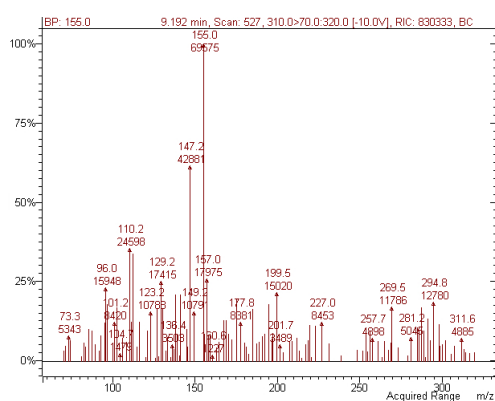
(c)

Figure 6.9: Product ion spectrum of the molecular ion of the FAEEs acquired using EI. The spectrum of a) ethyl myristate, b) ethyl palmitate and c) ethyl oleate.
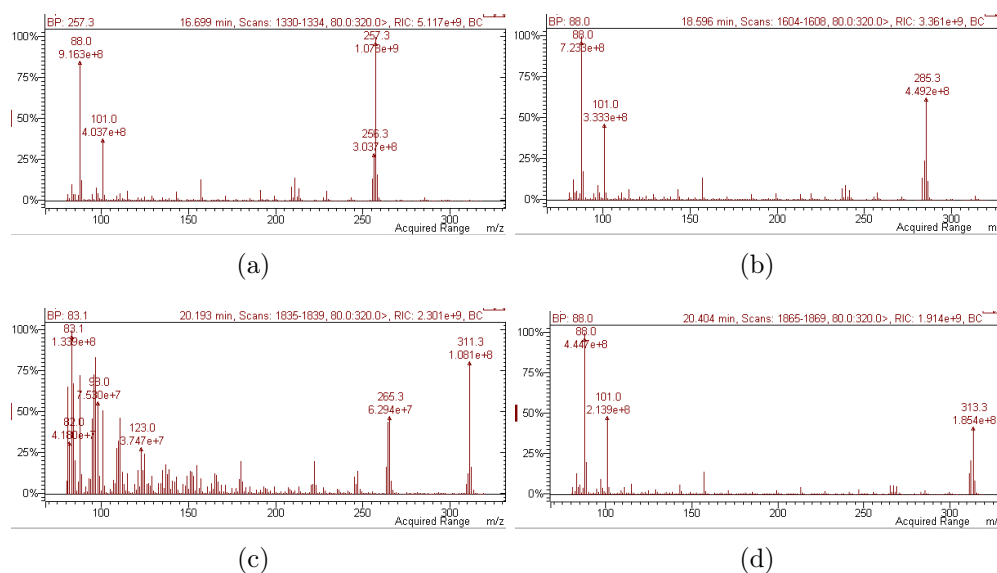
Figure 6.10: Full scan mass spectrum of a) ethyl myristate, b) ethyl palmitate, c) ethyl oleate and d) ethyl stearate acquired using CI.

6.2.1.2 The development of a CI MS method for the FAEEs

In the CI mode an addition of H^+ was observed for the FAEEs resulting in m/z of $M+1$ (see Figure 6.10). This is in line with other research as this is often observed for soft ionisation techniques in the positive ion mode in case of unsaturated compounds or heteroatom containing compounds [164]. The smaller fragments at m/z 88 and 101 were previously discussed in the EI mode. Interesting ions for the detection of the FAEEs in CI SIM are thus: m/z 88, 101 and $M+1$.

For the FAEEs in NCI similar patterns were obtained: mainly one m/z ion was observed: $[M\text{-}H]^-$ (see Figure 6.11). Since the aim with SIM is to detect several ions, a combined method in the negative mode was not achieved for the FAEEs in NCI. In addition, the intensity of the $[M+H]^+$ was much higher than the $[M\text{-}H]^-$ (see Figure 6.12).

As was previously stated the positive ions of the FAEEs after CI were more abundant than the negative ion that was produced in this mode. Therefore a tandem MS method was developed in the positive mode for the FAEEs. In general, CI results in less fragmentation than EI and more of the (protonated)
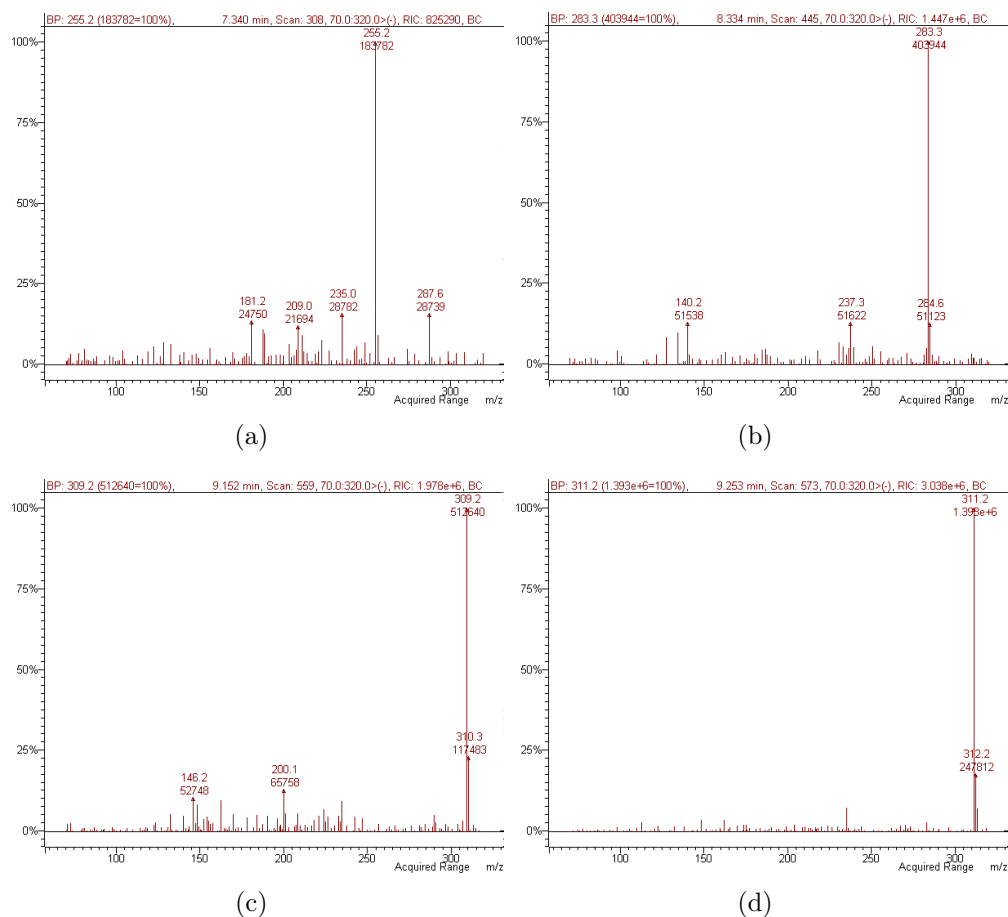
Figure 6.11: Full scan mass spectrum of a) ethyl myristate, b) ethyl palmitate, c) ethyl oleate and d) ethyl stearate acquired using NCI.

molecular ion was detected due to the greater stability of the cation than the radical cation [164]. Therefore, in tandem MS the protonated molecular ion of the FAEEs were used as precursor. A product ion scan of the molecular ions of four of the FAEEs showed that many types of ions were produced after the fragmentation of the protonated molecular ion (see Figure 6.13). For ethyl myristate, ethyl palmitate and ethyl stearate four m/z values had a high abundance and were prominent: 71, 89, 103 and M-27. A lot of fragmentation was also observed in the CI product ion scan of ethyl oleate and the m/z values that were abundant for this compound and relatively high in m/z value was: 265.
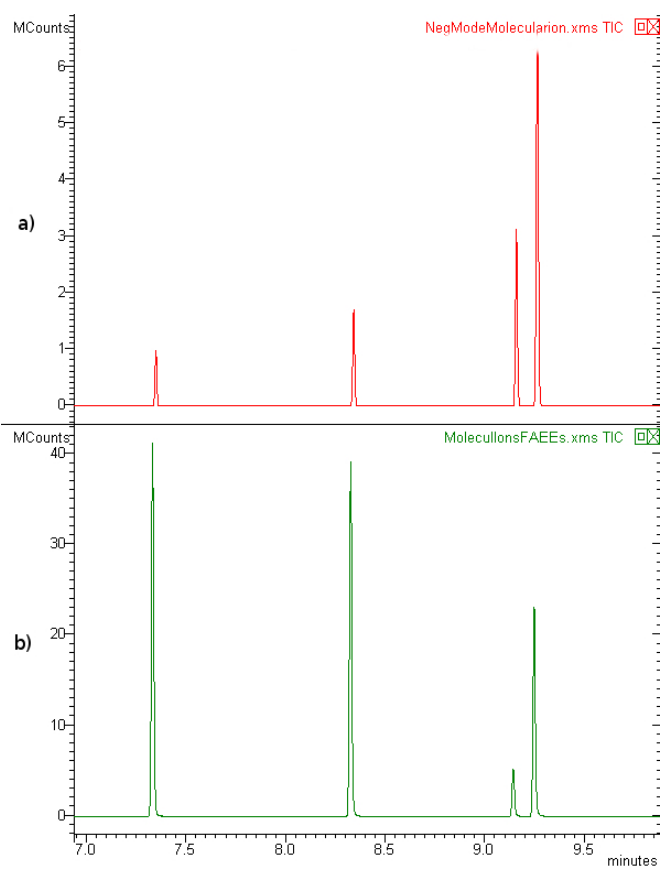
Figure 6.12: Chromatogram of the molecular ions of the FAEEs in a) NCI and b) PCI.
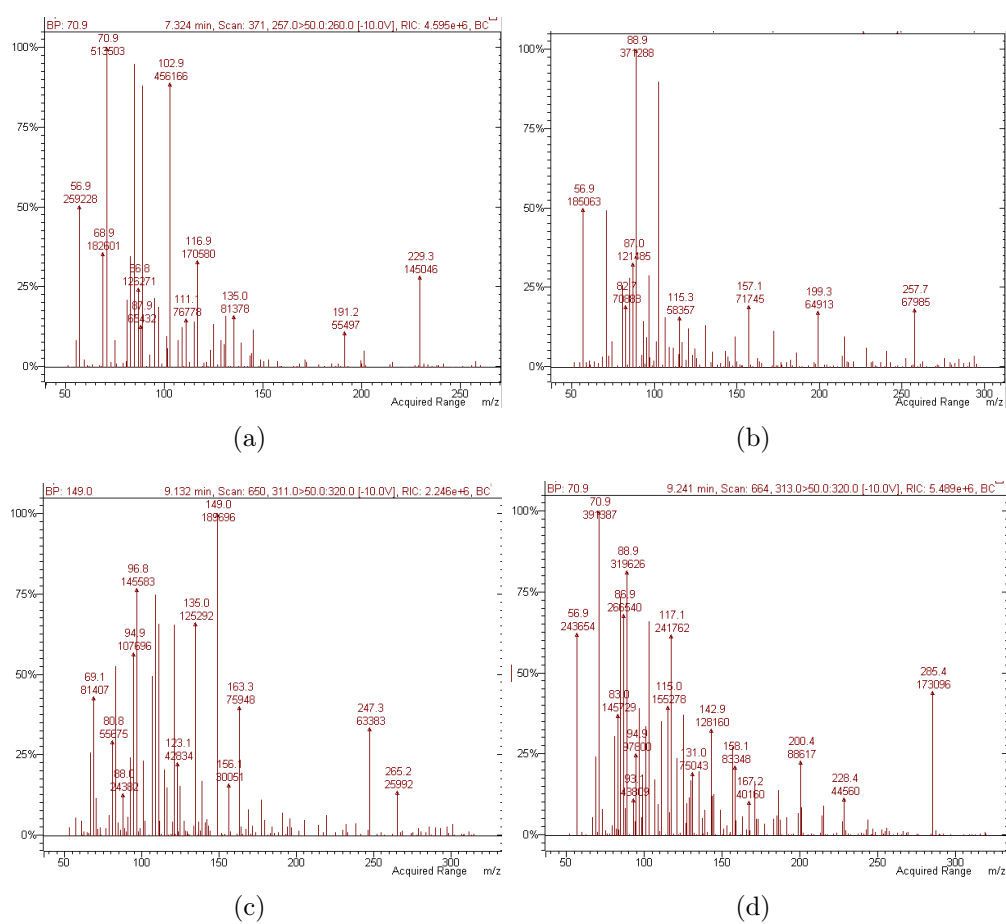
Figure 6.13: Product ion spectrum of the protonated molecular ion of a) ethyl myristate, b) ethyl palmitate, c) ethyl oleate and d) ethyl stearate acquired using PCI.

6.2.2   The development of an MS method for EtG-BSTFA

A full scan spectrum of EtG-BSTFA was obtained with m/z values between 50 and 500. A high intensity was observed for the m/z values: 73, 75, 147 and 217 (see Figure 6.14(a)). These were also observed in high abundancy by Janda and Alt [225] and are not specific for EtG-BSTFA but relate to trimethylsilyl (TMS) derivatives. When a TMS derivative contains a hydroxyl, it fragments and yields $(CH_3)_3Si^+$ at m/z 73 and $[(CH_3)_2Si^+ + OH]$ at m/z 75 according to Hoffmann et al. [164]. This indicates that not all the hydroxyl groups of EtG are derivatised with BSTFA. M/z 147 is systematically observed when a molecule contains more than one $(CH_3)_3SiO$-group [164] (see Figure 6.15 for the structure of this ion). Hence, more than one hydroxyl group was derivatised in EtG with BSTFA. M/z 217 originates probably from a complex with three TMS-groups, making it likely that three hydroxyl groups were derivatised. The molecular mass of the completely derivatised molecule (510 Da) was not observed in the full scan spectrum (Figure 6.14(b)), which is in agreement with the work of Janda and Alt [225] whereas a product with three derivatised groups was observed at m/z 405, again similar to that reported by Janda and Alt as well as Freire et al. [226]. It is noteworthy that the structure of the ion at 405 Da proposed by Freire et al. [226] is incorrect, but an EtG derivative with three TMS-groups is highly likely.

A scan from 250 to 550 was performed to focus on the higher m/z values (see Figure 6.14(b)). The five peaks with the highest intensities had m/z values of: 261, 292, 305, 375 and 405. M/z 292, 305 and 375 are relatively large ions that were observed with a high intensity. M/z 375 and 292 were previously used by Skopp et al. [39] in their EI SIM method for EtG-BSTFA. These fragments lacked the ethyl group that is deuterated with the internal standards that were used in this research, and were therefore not selected for this work. M/z 261 was previously used in the SIM [16,36,158,225] and tandem MS method [128,170,191, 218] for the analysis of EtG-BSTFA. The m/z value for the corresponding ion of the deuterated compound is 266. Hence, 261 is a promising ion for the detection of EtG-BSTFA. To increase the specificity, a larger ion may be suitable like m/z 405 with an intensity of approximately 20% of that of m/z 292. In addition, a
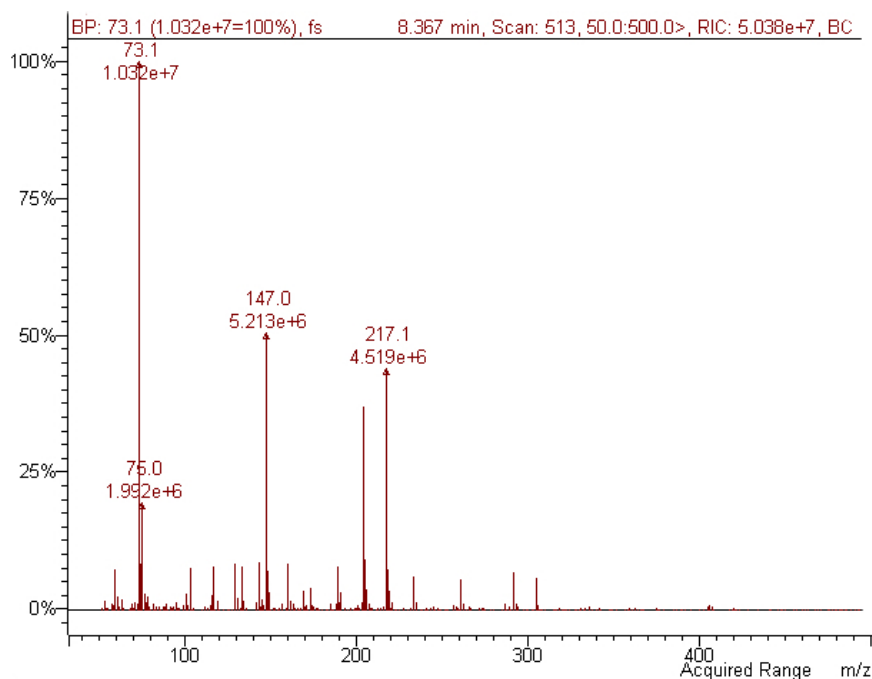
(a)
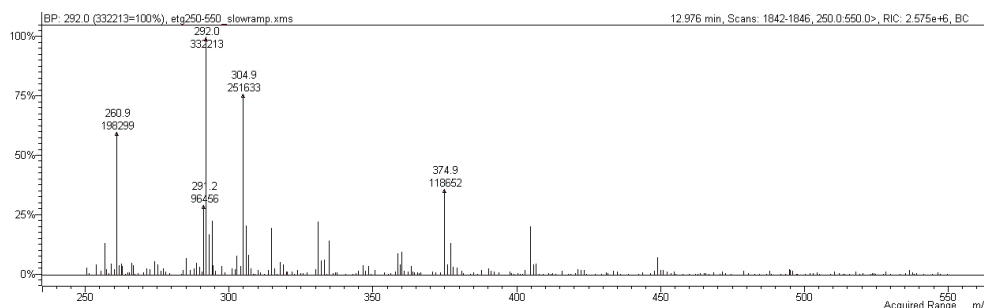
(b)

Figure 6.14: Full scan mass spectrum of EtG-BSTFA acquired using EI with a scan mode a) from m/z 50 to 500 and b) from m/z 250 to 550.

smaller ion like 160, that has a relatively high abundance (see Figure 6.14(a)) may allow for a high sensitivity. The suggested structure of both 261 and 160 Da fragments are shown in Figure 6.16 and are similar to the ions shown by Freire et al. [226]. The three ions that were selected for the detection of EtG-BSTFA in SIM have the m/z value of: 160, 261 and 405. The same ions as used by other researchers (see [16, 36, 158, 225]).
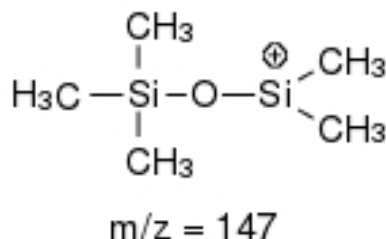
Figure 6.15: Chemical structure of an ion with mass 147 Da. This ion is often observed in the mass spectrum of a molecule that contains more than one trimethylsilyl-group [164].
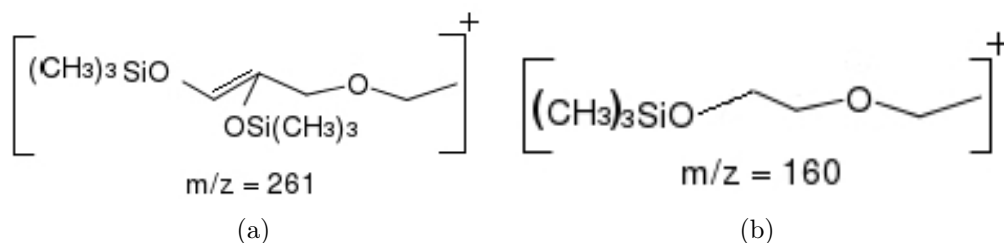
Figure 6.16: Fragments of EtG-BSTFA observed in the full scan mass spectrum acquired using EI at a) m/z 261 and b) m/z 160.

For the development of a tandem MS method, a product ion scan was performed for various ions first to select the transition that can be measured for the detection of EtG-BSTFA. A product ion scan of m/z 261 for EtG-BSTFA showed that mainly one fragment was created with a m/z of 143 (see Figure 6.17). This transition was clearly found with the product ion scans, whereas the product ion scans of m/z 160 and 405 that were selected for the EI SIM method resulted in more noise and not one abundant product ion. The suggested transition of $261 \rightarrow 143$ is shown in Figure 6.18. With this fragmentation a TMS-group and the ethyl group that is deuterated in the internal standard are lost. Therefore the transition for EtG-BSTFA-D5 was: $266 \rightarrow 143$. These transitions were also used by Paul et al. [170], Tsanaclis et al. [191], Shi et al. [218] and Lees et al. [128] for the detection and quantification of EtG in hair.

For EtG-BSTFA in CI mode at a pressure of 1 Torr similar ions were observed as those in the EI mode.
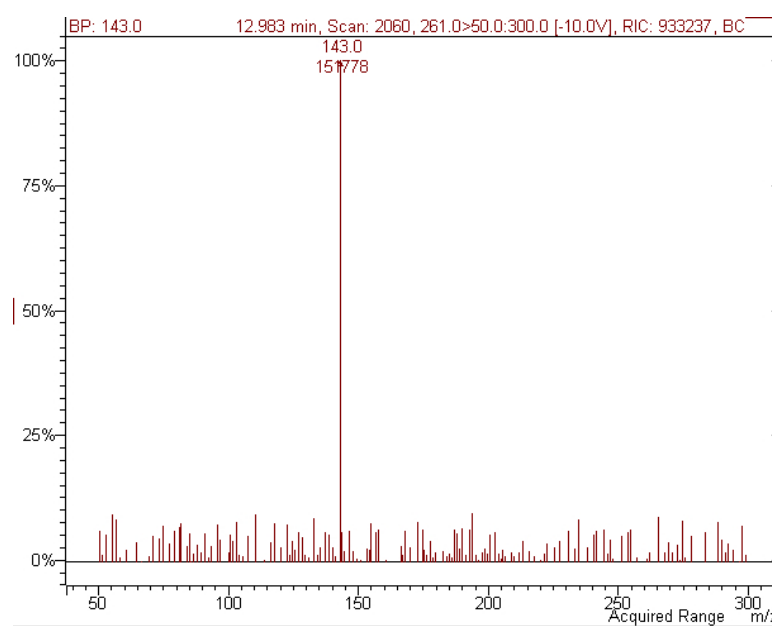
Figure 6.17: Product ion spectrum of the fragment at m/z 261 of EtG-BSTFA.

Figure 6.18: Transition of 261 Da EtG-BSTFA fragment to 143 Da.

6.2.3 The selection of an MS method for EtG-BSTFA and FAEEs

For a pure sample, the sensitivity of the SIM method for EtG was higher than for the tandem MS method. Therefore the SIM method was selected for this work. For a SIM method in EI the ions in Table 6.3 were used for EtG-BSTFA and the 4 FAEEs and for a SIM method in CI the ions in Table 6.4 were used.

Table 6.3: Ions used for data acquisition by EI SIM for EtG-BSTFA, the 4 FAEEs and their deuterated forms.

Compound	M/z values
EtG-BSTFA	160, 261, 405
EtG-BSTFA-D5	165, 266, 410
E14:0	88, 101, 256
E14:0-D5	93, 106, 261
E16:0	88, 101, 284
E16:0-D5	93, 106, 289
E18:1	88, 101, 310.5
E18:1-D5	93, 106, 315.5
E18:0	88, 101, 312.5
E18:0-D5	93, 106, 317.5

When CI was used instead of EI more of the protonated molecular ion was obtained for the FAEEs (see Figure 6.19). A slight reduction was observed in EtG-BSTFA when it was measured in CI compared to EI. Hence, CI did not significantly affect the detection of EtG-BSTFA compared to that in EI and had a big advantage over EI concerning the FAEEs. Note that the (protonated) molecular ion is a very specific ion for that particular compound and comes with relatively little matrix interference [185]. Therefore the measurement of the analytes in CI was preferred.

6.2.4 The optimisation of the methane CI pressure for the detection of EtG and FAEEs

Despite the low percentage of molecular ions of the FAEEs with the CI pressure of 1 Torr compared to 3 Torr (for ethyl palmitate cf. Figure 6.20(a) with 6.20(b)), an approximately two times higher peak area was obtained of ethyl palmitate for
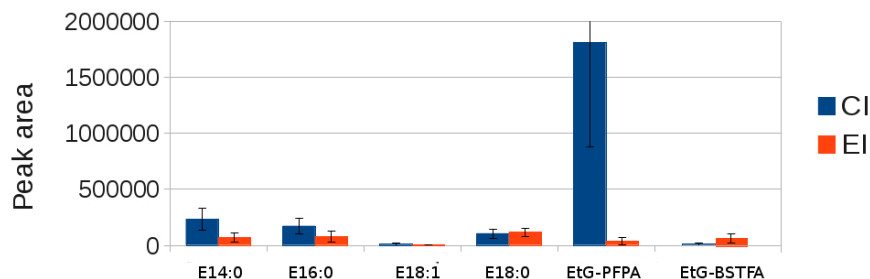
Figure 6.19: The signal of the (protonated) molecular ions of the 4 FAEEs and EtG-PFPA acquired using EI and CI. The error bars indicate the standard deviation that was obtained for this experiment that was performed in duplicate.

Table 6.4: Ions used for data acquisition by CI SIM for EtG-BSTFA, 4 FAEEs and the deuterated forms.

Compound	M/z values
EtG-BSTFA	160, 261, 405
EtG-BSTFA-D5	165, 266, 410
E14:0	88, 101, 257
E14:0-D5	93, 106, 262
E16:0	88, 101, 285
E16:0-D5	93, 106, 290
E18:1	88, 101, 311.5
E18:1-D5	93, 106, 316.5
E18:0	88, 101, 313.5
E18:0-D5	93, 106, 318.5

the protonated molecular ion (m/z 285) with a CI pressure of 1 Torr compared to 3 Torr (431,230,386 versus 179,019,317). In addition, at each of the temperatures for the ion source a higher response was obtained for 1 Torr and at this CI pressure lower ion source temperatures worked best for the FAEEs (see Figure 6.21). An additional experiment was carried out to examine lower pressures and these results indicated that 1 Torr was the best pressure for the detection of the protonated molecular ion when was looked at the response (see Figure 6.22). In addition, the S/N measured at 3 Torr was lower than for those with a CI pressure of 1 Torr at various temperatures (see Figure 6.23). Hence, an optimum result was obtained when the CI pressure was set at 1 Torr. This pressure also allowed for the detection of EtG-BSTFA.
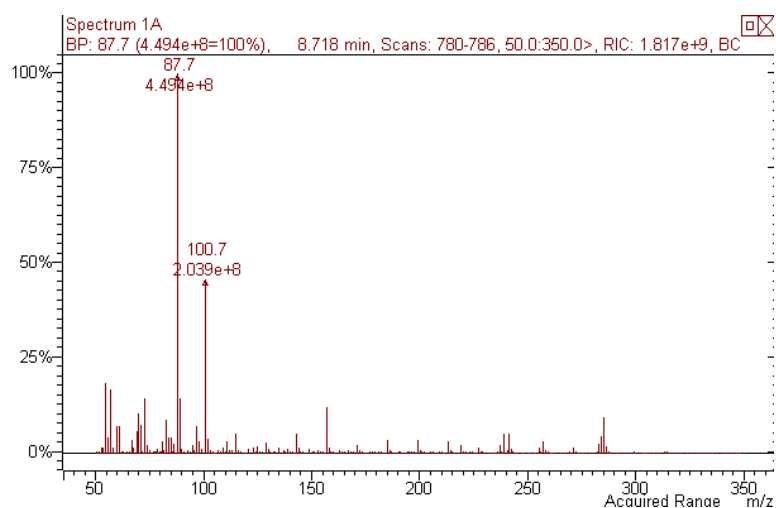
(a)
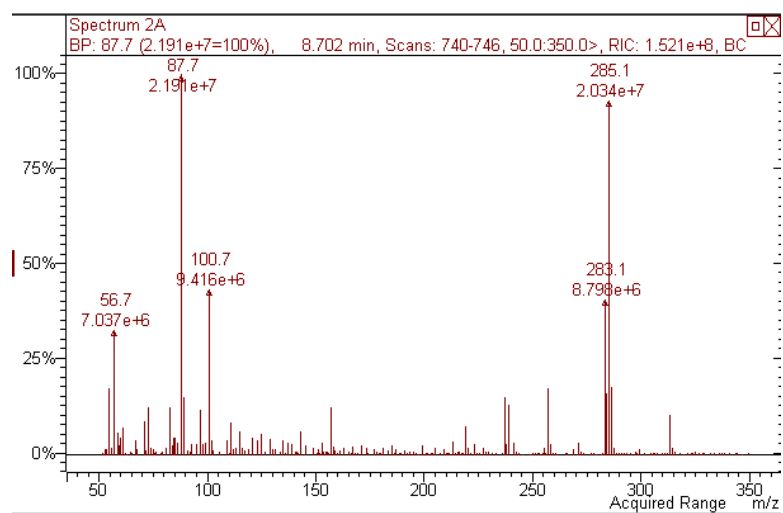
(b)

Figure 6.20: Full scan mass spectrum of ethyl palmitate acquired using a CI pressure of a) 1 and b) 3 Torr.
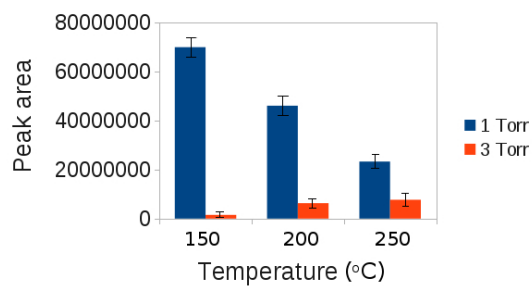
Figure 6.21: Effect ion source temperature and CI pressure on the detection of the protonated molecular ion of ethyl palmitate.
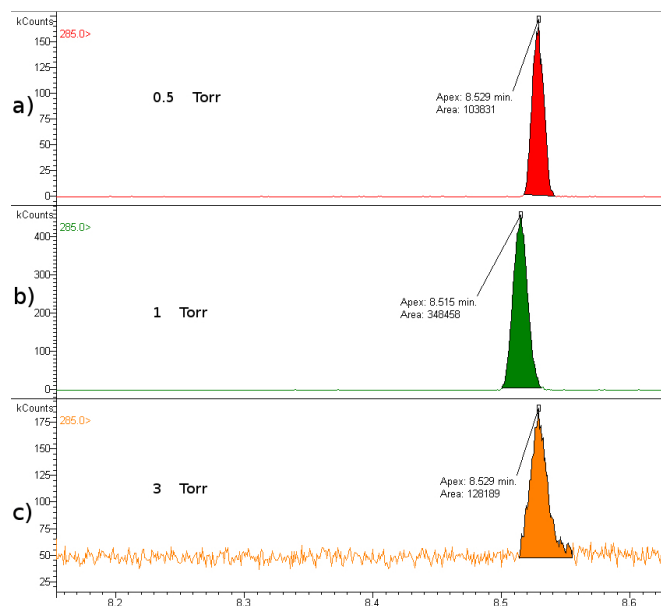
Figure 6.22: Chromatogram of the protonated molecular ion of ethyl palmitate (m/z 285) acquired with a CI pressure of a) 0.5 Torr, b) 1 Torr and c) 3 Torr.
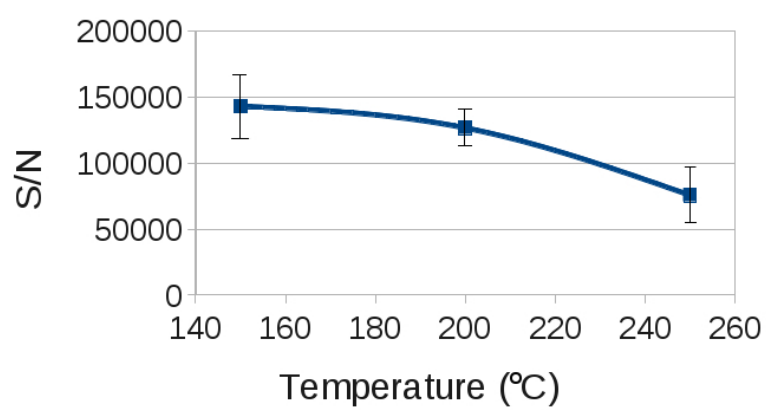
Figure 6.23: Effect of the ion source temperature on the signal to noise ratio of the protonated molecular ion of ethyl palmitate.

6.2.5 The temperature of the transfer line and ion source

The ion source temperature that was best for the FAEEs in CI SIM was the lowest temperature in the tested range (150 °C), since the lowest fragmentation was obtained with this temperature compared to the higher temperatures (200 and 250 °C) (see Figure 6.24). A less well defined peak was obtained in the chromatogram at 150 °C compared to the higher temperatures 175, 200 and 250 °C (see Figure 6.25). Therefore, 175 °C was the best temperature for the combined method for both EtG-BSTFA and the FAEEs. The ion source temperature was relatively low and therefore also a relatively low transfer line temperature was chosen: 200 °C.
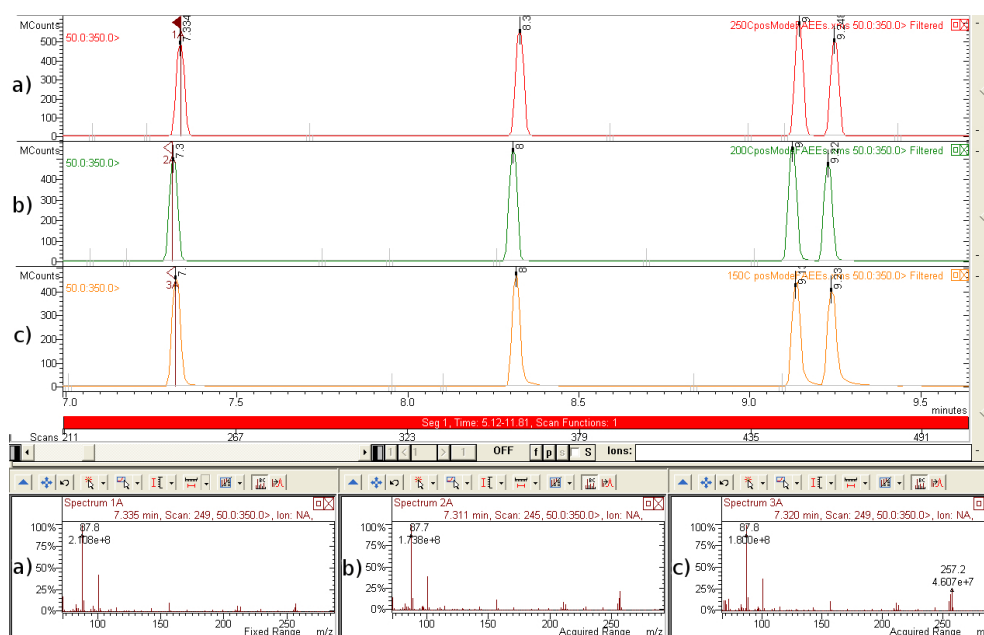
Figure 6.24: Effect ion source temperature on the analysis of the 4 FAEEs. Both the total ion chromatogram of the FAEEs acquired using PCI and the corresponding full scan mass spectrum of ethyl myristate are shown with the ion source temperature set at a) 250 b) 200 and c) 150 °C.
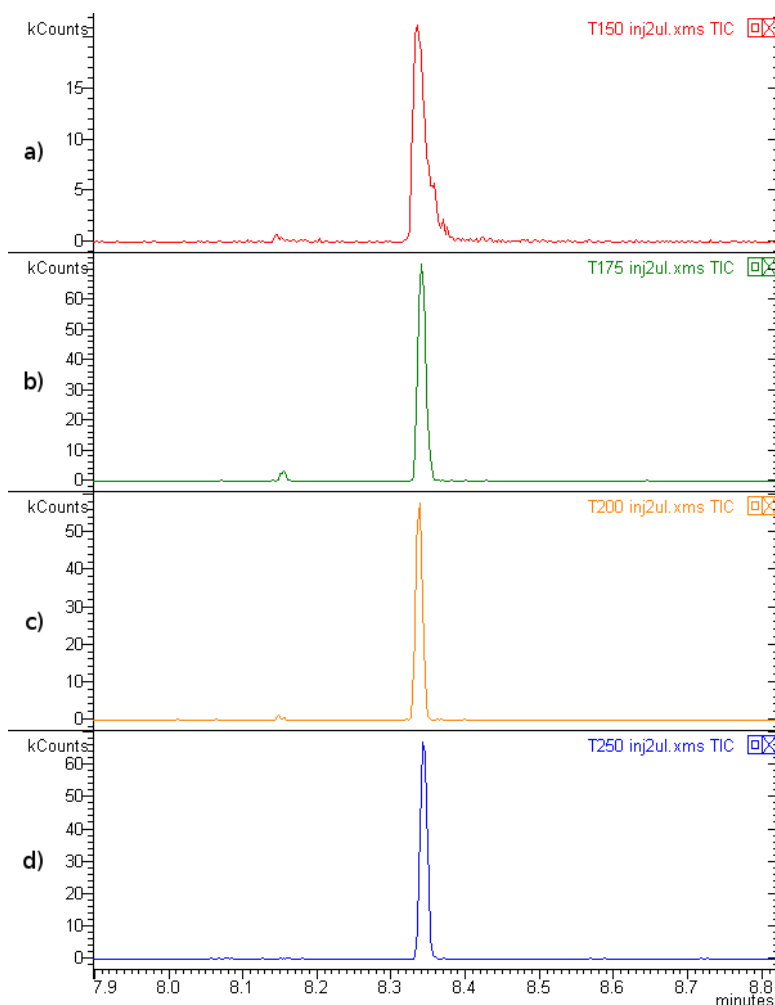
Figure 6.25: Effect ion source temperatures on signal for EtG-BSTFA. Total ion chromatogram of EtG-BSTFA acquired using CI SIM with the ion source temperature at a) 150, b) 175, c) 200 and d) 250 °C.

6.2.6 Dwell time, electron energy and electron multiplier

With a longer scan time of an ion fewer data points are obtained. The shortest dwell time for which the MS was still able to detect the ions was 0.02 s. Furthermore, a longer scan time allowed for the molecular ions to increase the signal. The scan time for these (protonated) molecular ions was set at: 0.04 s.

The electron energy (EE) of the electron beam had three possible settings of which 70 eV was best for the detection of the FAEEs. A lower signal was obtained for

the protonated molecular ions in CI with 20 eV and too much fragmentation was observed when the EE was set to 150 eV.

The detector was turned on after 4.5 minutes to protect the filament against the high intensity peak of the solvent that is a broad peak in the beginning of the run. Moreover, this solvent peak was not of interest in these experiments.

The electron multiplier (or detector voltage) obtained better results when it was set at 1000 V for ethyl myristate and ethyl palmitate. With increasing voltages an increasing peak area was obtained in SIM for ethyl myristate (see Figure 6.26). However, above 1000 V S/N decreased. This may explain why the peak areas of ethyl myristate and ethyl palmitate do not increase when these are measured in FS (see Figure 6.27). For ethyl oleate (and ethyl stearate) an increased voltage of the electron multiplier may aid in the detection of these compounds. Hence, 1000 V was the best optimal setting for the FAEEs except for ethyl oleate, which had a low signal compared to the other FAEEs. 1000 V was the optimum when the system was optimised automatically. Therefore, the electron multiplier was frequently optimised with the auto tune function on the software and set at this voltage for the analysis of the alcohol markers. For the time segment of ethyl oleate the voltage of the electron multiplier was increased to 1400 V.
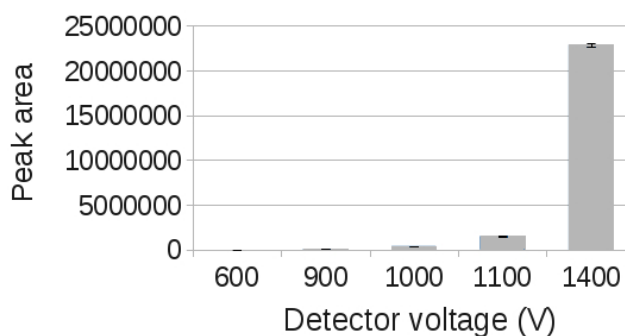
Figure 6.26: Effect of detector voltage on the detection of ethyl myristate acquired using EI SIM.
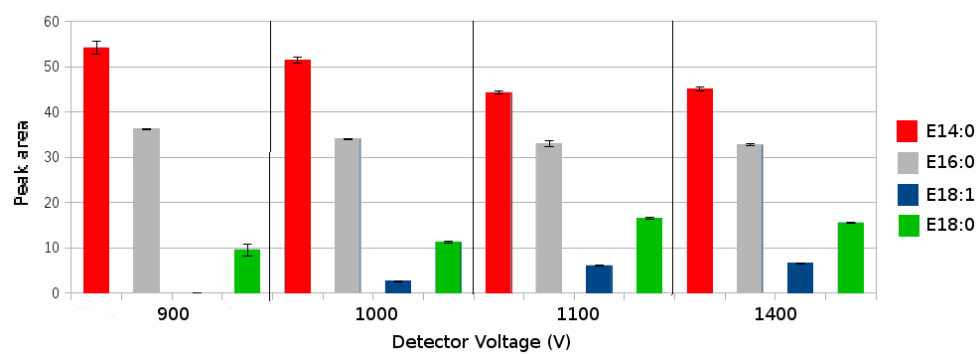
Figure 6.27: Effect of detector voltage on the detection of the FAEEs acquired using EI FS.

6.3   The final optimised GCMS method

Several conclusions were drawn from this work and were used as the basis of the development of a combined method together with the findings of the previous chapter (Chapter 5). In this chapter the following was concluded:

- Attempts of the introduction of both alcohol markers on the GC resulted in a significant loss of either EtG or the FAEEs. It may be more appropriate to divide the sample in two aliquots of which one is analysed for EtG and the other for the FAEEs.

- The oven temperature programme was optimised for both analytes in the presence of the hair matrix obtained with 30 mg hair and extracted for 30 min in MeOH with sonication. The optimised conditions can be found in Table 4.7 on page 60 and were used for the combined methods (in Chapter 7).

- A solvent delay should be used of for example 4.5 min.

- The detection method selected for the two markers was CI SIM and the ions that were detected are shown in Table 6.4.

- A CI pressure of 1 Torr yielded an optimal response.

- An EE of 70 eV yielded an optimal response for the (pseudo) molecular ions.

- The optimum ion source temperature for both the detection of EtG-BSTFA and FAEEs at 175 °C.

- The temperature of the transfer line was set at 200 °C.

- The scan time of 0.02 s was used for all fragment ions and for the (protonated) molecular ions the scan time was set at 0.04 s.

- The electron multiplier was set at the optimum settings according to the findings of auto tune and set at 1400 V when ethyl oleate was measured.

These conditions were used together with the settings obtained from Chapter 5 to obtain an optimum response and develop a sensitive combined method as discussed in Chapter 7.

Chapter 7

Validation study of developed combined methods

As discussed previously the current methods techniques for EtG and FAEEs analysis are very different (see Appendices E and F) reflecting the dissimilarity in the physical properties of the two groups of compound. In this work three methods were developed, validated and then compared based on:

- possible gain in time and cost compared to the two current methods,
- intra-day accuracy and precision,
- inter-day accuracy and precision,
- recovery variation from the concentrated hair pool and
- recovery of EtG and FAEEs from hair samples of drinkers.

7.1   Hair samples used in the validation study

The hair samples of drinkers that were used in this work are referred to with capital letters. The background information of each is summarized in Table 7.1.

In addition to the above, a concentrated hair pool was also used. For this, hair

Table 7.1: Background information of the hair samples of drinkers used in the validation study.

Hair sample	Background information
A	former alcoholic and still drinks. Hair was dyed. Female.
B	Young female who drinks about once a week.
C	Former alcoholic who still drinks. Male.
D	Social drinker. Uses a variety of hair products. Female. A significant amount of hair was available.
E	Chronic heavy drinker who lives on the street. Female.
F	Chronic heavy drinker who lives on the street. Female.
G	Chronic heavy drinker. Female. Hair sample stored in Aluminium foil for 6 months prior to the analysis.
H	Chronic heavy drinker. Female. Hair sample stored in Aluminium foil for 6 months prior to the analysis.

from both social and chronic heavy drinkers were combined, cut in mm pieces and mixed well to obtain a homogeneous blend with detectable values for the analytes (see Section 4.4.1.2). For each of the developed methods the concentration of the analytes was measured for ten samples of the concentrated hair pool for comparison.

Spiked samples were used in the calibration, intra-day and inter-day studies. For this, the hair of an eleven year old boy was used as he was a teetotaller and his hair was very clean in the sense that it showed little matrix interference for the analytes that were measured.

7.2 The development and validation of the combined methods for the extraction and detection of EtG and FAEEs

For each of the three final combined methods (see Figure 7.1), 30 mg hair was used. This was cut into segments prior to the decontamination step and then was cut into smaller pieces prior to extraction. In all cases the hair was decontam-

inated with 1 mL DCM and 1 mL of MeOH. In methods 1 and 2 the analytes were extracted with 30 min sonication in 1 mL of methanol. One sample was divided in two: one for EtG analysis and one for the analysis of FAEEs. In method 1 this was done by dividing the sample in two equal aliquots and in method 2 SPE was performed for this purpose. In method 1, one aliquot was cleaned up with SPE, evaporated, derivatised with BSTFA plus EA at 80 °C for at least two hours and injected on the GCMS system when the sample was cooled down. The other was evaporated to dryness in a HS vial and HS SPME was performed using a PDMS-DVB fibre for detection of FAEEs. For method 2, the SPE procedure described in Section 4.4.4.1 was used. After sequential elution of EtG and FAEEs the samples were treated as previously described with method 1. For method 3 extraction was performed using a mixture of DMSO/heptane. Resulting in the partitioning of EtG in DMSO and the four FAEEs in heptane. The DMSO was frozen by putting the sample in an ice bath. The heptane layer was transferred to a HS vial by decanting the sample, the layer was evaporated and analysed with HS SPME GCMS. The DMSO layer was subjected to the standard SPE procedure for EtG after the addition of 500 µL de-ionized water to the sample. The clean extract was evaporated, derivatised in the same way as described for the other methods and analysed with direct injection GCMS.

The three developed combined methods were compared to each other and to the commercially used method for EtG where EtG is extracted with de-ionized water (see [170]). The SPE procedure, derivatisation and GCMS method were the same as for the developed combined methods and described in Chapter 4. For the comparison hair samples of drinkers, the stability of FAEEs in the extraction solvent (see Section 4.3), the variability in the concentrated hair pool, the intra-day and inter-day variation were measured. The intra-day variability was determined by the analysis of three different concentrations of spiked hair samples from an eleven year old boy (a teetotaller) and were run in triplicate. These control samples were spiked at 5.0, 17.5 and 37.5 ng/mL for three FAEEs and twice the concentration was used for ethyl oleate. For EtG the concentrations prepared were: 0.75, 1.75 and 3.75 ng/mL. Analysis of these samples was performed on a single day. The inter-day variability was determined using the same concentrations in triplicate

over three separate days during a period of three weeks. Five calibrators and one blank sample were analysed with each validation run.

As discussed earlier a comparison was made between the different methods. However, the methods were similar in a couple of aspects. All methods had a linear calibration curve from 0.3 ng/mL to 5 ng/mL for EtG and from 3 ng/mL to 50 ng/mL for the FAEEs with a correlation coefficient (r^2) above 0.99, an LOQ below CAL 1 and no carry-over was observed after the calibrators including CAL 5.
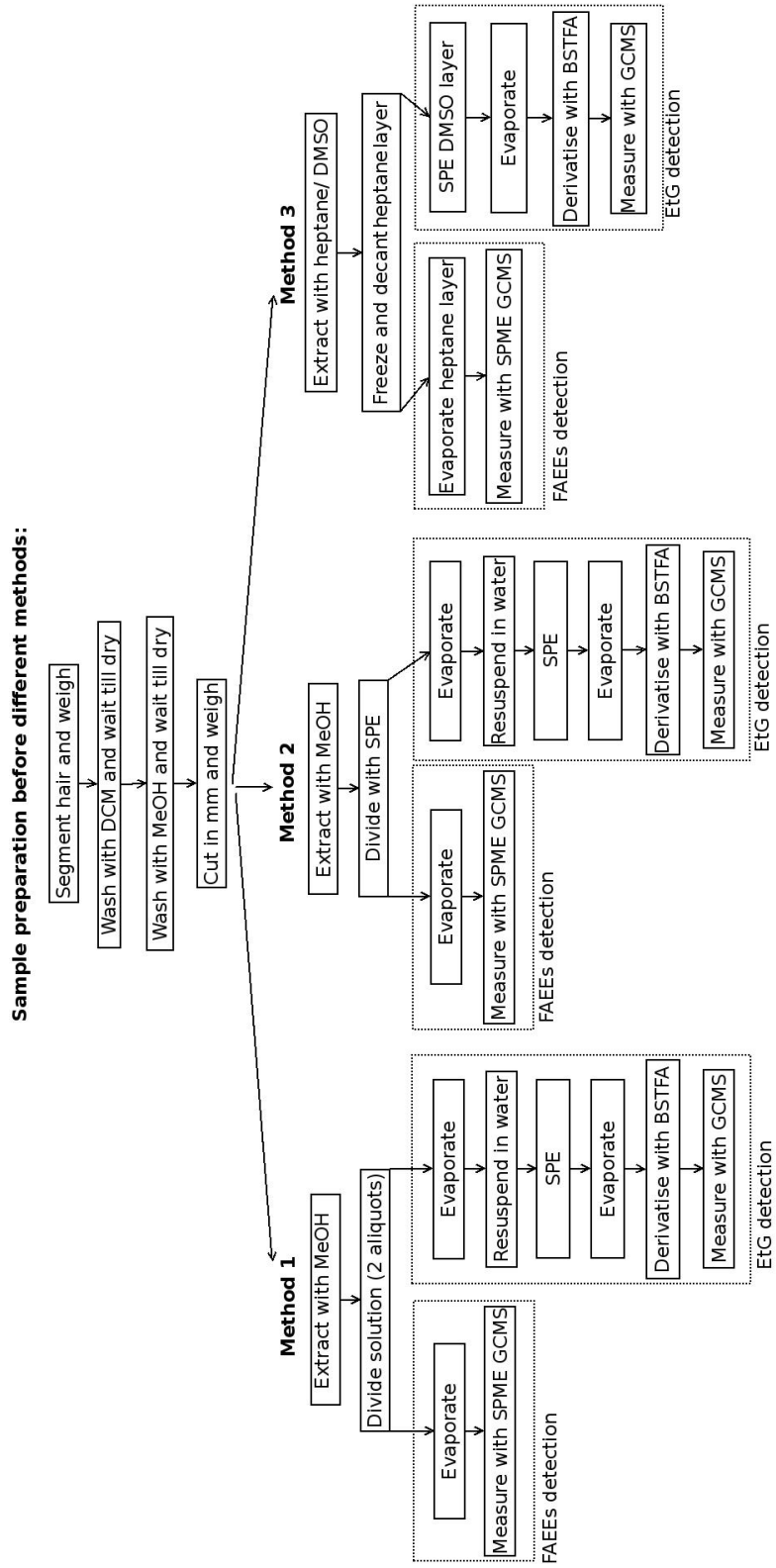
Figure 7.1: Schematic representation of the three methods developed for the combined extraction of EtG and FAEEs.

7.2.1 The validation of method 1: MeOH-two-aliquots method

The first method described in Figure 7.1 yielded the following intra-day and inter-day results (see Tables 7.2, 7.3, 7.4, 7.5 and 7.6). The precision was expressed as coefficient of variation (CV) (see Equation 4.4 on page 63) and the accuracy was calculated with Equation 4.5 on page 63. The figures in the tables are in red when the CV was above 20% and the accuracy below 90% or above 110%. The detection limit of this method was calculated at 0.04 ng/mg for ethyl myristate and ethyl stearate, 0.06 ng/mg for ethyl palmitate, 0.09 ng/mg for ethyl oleate and 5 pg/mg for EtG.

Table 7.2: Summary intra-day and inter-day study of ethyl myristate for MeOH-two-aliquots method.

Actual amount (ng/mL)	Intra-day study (n=3)			Inter-day study (n=9)		
	Average (ng/mL)	CV (%)	Accuracy (%)	Average (ng/mL)	CV (%)	Accuracy (%)
5.0	4.2	29.9	84.1	4.5	14.1	89.9
17.5	16.3	9.5	92.9	16.5	6.8	94.5
37.5	39.7	11.5	105.8	35.1	22.0	93.7

Table 7.3: Summary intra-day and inter-day study of ethyl palmitate for MeOH-two-aliquots method.

Actual amount (ng/mL)	Intra-day study (n=3)			Inter-day study (n=9)		
	Average (ng/mL)	CV (%)	Accuracy (%)	Average (ng/mL)	CV (%)	Accuracy (%)
5.0	5.1	6.7	101.1	5.2	19.1	103.4
17.5	14.7	15.6	83.7	15.8	19.4	90.3
37.5	37.4	2.8	99.7	36.0	8.3	96.1

Table 7.4: Summary intra-day and inter-day study of ethyl oleate for MeOH-two-aliquots method.

Actual amount (ng/mL)	Intra-day study (n=3)			Inter-day study (n=9)		
	Average (ng/mL)	CV (%)	Accuracy (%)	Average (ng/mL)	CV (%)	Accuracy (%)
10.0	11.0	6.6	109.9	10.2	14.3	102.2
35.0	34.3	8.6	97.9	35.0	14.7	100.1
75.0	78.4	11.7	104.5	81.5	11.2	108.7

Table 7.5: Summary intra-day and inter-day study of ethyl stearate for MeOH-two-aliquots method.

Actual amount (ng/mL)	Intra-day study (n=3)			Inter-day study (n=9)		
	Average (ng/mL)	CV (%)	Accuracy (%)	Average (ng/mL)	CV (%)	Accuracy (%)
5.0	5.2	8.9	103.3	5.1	7.9	102.0
17.5	15.6	13.1	88.9	16.2	23.1	92.6
37.5	39.3	10.2	104.7	39.3	12.4	104.8

Table 7.6: Summary intra-day and inter-day study of EtG for MeOH-two-aliquots method.

Actual amount (ng/mL)	Intra-day study (n=3)			Inter-day study (n=9)		
	Average (ng/mL)	CV (%)	Accuracy (%)	Average (ng/mL)	CV (%)	Accuracy (%)
0.75	0.82	13	109	0.89	19	118
1.75	1.91	14	109	1.86	23	106
3.75	4.19	11	112	3.56	18	95

The analyses of the concentrated hair pool showed a CV of 37.2% for the sum of the 4 FAEEs (see Figure 7.2) and EtG was not detected.
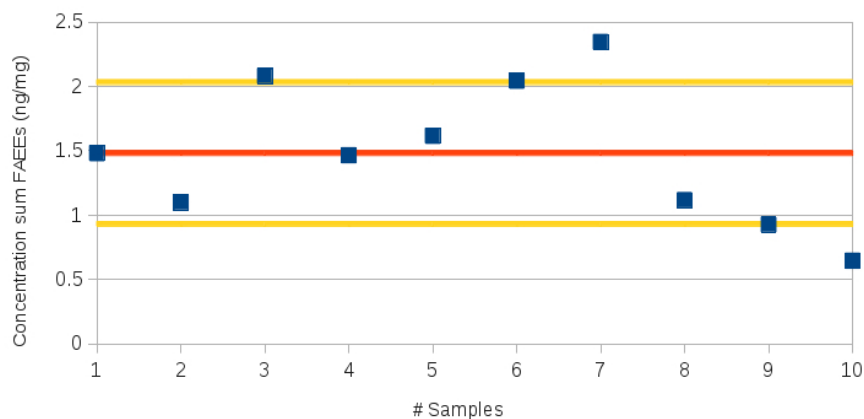
Figure 7.2: The sum of the FAEEs that was detected with MeOH-two-aliquots method in ten different hair samples from the concentrated hair pool.
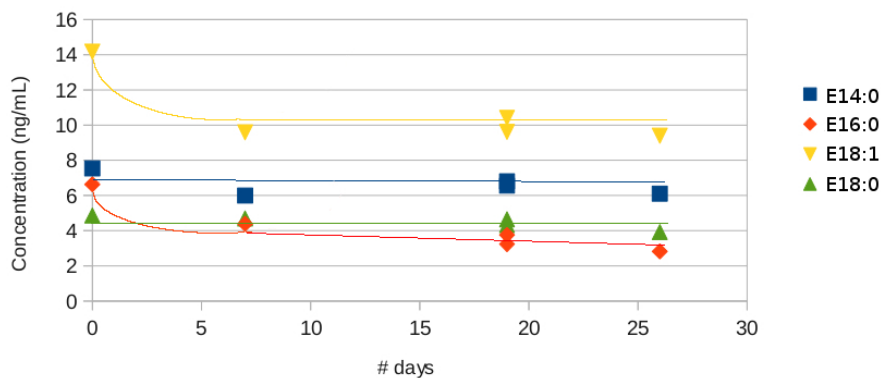
Figure 7.3: Stability of the freshly prepared FAEEs in methanol that were stored at -30℃ for a month.

Stock standard solutions for this method were prepared in methanol. It was decided to investigate the stability of these solutions during various freeze-thaw cycles as well as over a timespan of days. When the samples were stored for 26 days at -30℃, a decrease for ethyl oleate in the first week was most apparent. In addition, the difference between the samples within one day was studied too. This explains the double data points on day 19 and indicated that ethyl oleate and ethyl palmitate decreased in concentration in the first week of storing (see Figure 7.3). Other FAEEs were relatively stable.
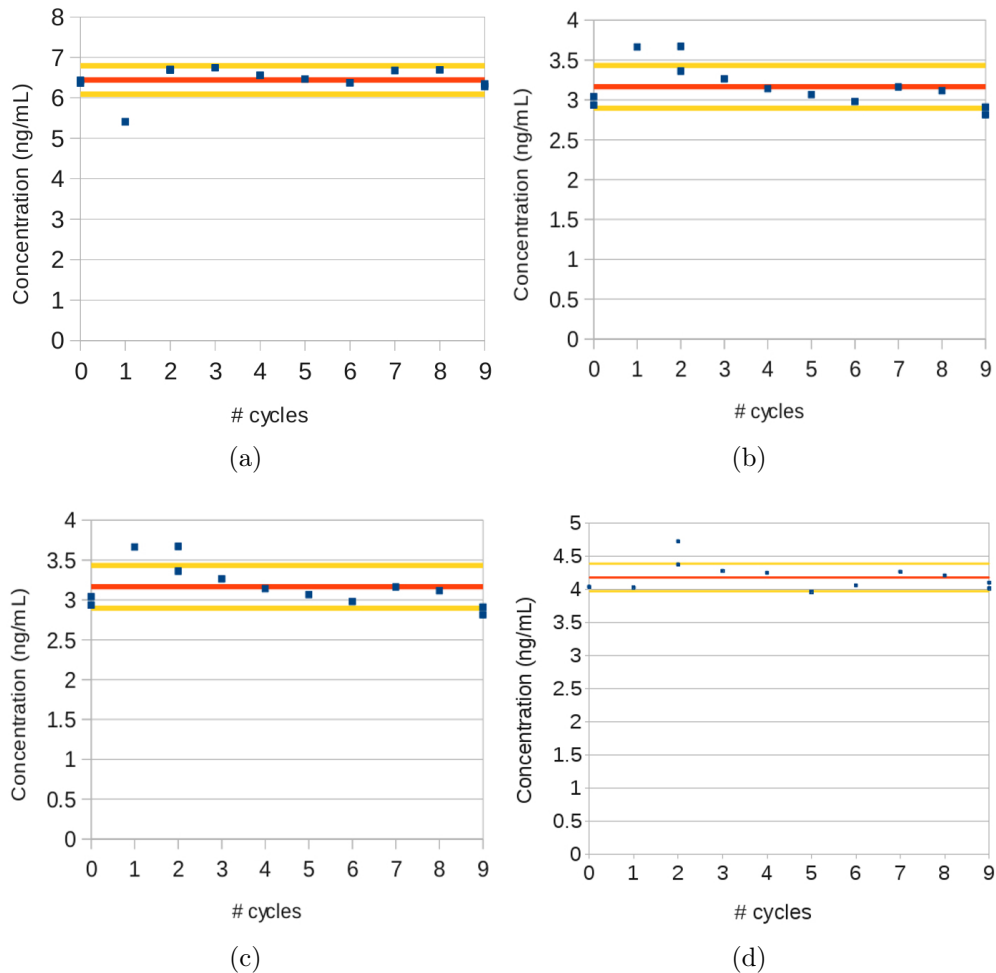
Figure 7.4: FAEEs subjected to freeze–thaw cycles in methanol: a) ethyl myristate, b) ethyl palmitate, c) ethyl oleate and d) ethyl stearate. The average of the measurements is shown as a red line and the standard deviation are indicated by the yellow lines.

The CV was relatively low for FAEEs for the freeze-thaw cycles: the CV was 5.4% for ethyl myristate, 5.0% for ethyl stearate, 8.4% for ethyl palmitate and 6.1% for ethyl oleate (see Figure 7.4). The variation was lower than the inter-day and intra-day variation of the spiked samples.

The measurements of the hair samples from drinkers are shown in Table 7.7. Three negative EtG hair tests were obtained of which two were obtained from hair of heavy drinkers. Five positive EtG hair tests were obtained of which four

belonged to heavy drinkers. The other high EtG concentration was detected for a social drinker (SD) who would be considered a heavy drinker based on the EtG concentration detected in her hair. In another experiment also a remarkably high EtG concentration was observed in the decontamination steps of the hair of the subject. It is possible that the high EtG concentration was caused by the use of a hair product that contained EtG as was described to be possible by Sporkert et al. [78], since the self-report of this subject was low to moderate drinking. The FAEEs concentration in the hair from this subject would indeed indicate only social drinking. Looking at the FAEEs concentrations three positive hair tests were obtained belonging to heavy drinkers and five negative hair tests of which three belonged to a subject who drinks heavily. Note that the false positive and false negatives obtained with the EtG test would have been rebutted with the FAEEs hair test and vice versa.

Table 7.7: EtG and FAEEs concentration from the hair samples of drinkers detected with MeOH-two-aliquot method. HD: heavy drinker, SD: social drinker and ND: not detected.

| Hair sample | A | B | C | D | E | F | G | H |
Drinking behaviour	HD	SD	HD	SD	HD	HD	HD	HD
EtG (pg/mg)	23	16	116	543	29	33	40	30
Sum FAEEs (ng/mg)	1.57	0.27	0.26	0.22	7.75	0.54	ND	1.57
Ethyl myristate (ng/mg)	ND	ND	ND	ND	0.35	ND	ND	0.13
Ethyl palmitate (ng/mg)	1.33	ND	ND	0.22	0.77	0.22	ND	0.70
Ethyl oleate (ng/mg)	0.24	0.27	0.26	ND	6.29	0.32	ND	0.41
Ethyl stearate (ng/mg)	ND	ND	ND	ND	0.42	ND	ND	0.10

7.2.2 The validation of method 2: MeOH-SPE-method

The second method described in Figure 7.1 yielded the following intra-day and inter-day results (see Tables 7.8, 7.9, 7.10, 7.11 and 7.12). The precision was expressed as CV (see Equation 4.4 on page 63) and the accuracy was calculated with Equation 4.5 on page 63. The figures in the tables are in red when the CV was above 20% and the accuracy below 90% or above 110%. The detection limit of this method was calculated at 0.04 ng/mg for ethyl palmitate, 0.06 ng/mg for ethyl myristate, 0.12 ng/mg for ethyl oleate and 4 pg/mg for EtG.

Table 7.8: Summary intra-day and inter-day study of ethyl myristate for MeOH-SPE method.

Actual amount (ng/mL)	Intra-day study (n=3)			Inter-day study (n=9)		
	Average (ng/mL)	CV (%)	Accuracy (%)	Average (ng/mL)	CV (%)	Accuracy (%)
5.0	5.7	3.1	114.9	5.7	3.1	114.9
17.5	16.3	9.8	93.0	16.3	9.8	93.0
37.5	35.4	2.3	94.3	35.4	2.3	94.3

Table 7.9: Summary intra-day and inter-day study of ethyl palmitate for MeOH-SPE method.

Actual amount (ng/mL)	Intra-day study (n=3)			Inter-day study (n=9)		
	Average (ng/mL)	CV (%)	Accuracy (%)	Average (ng/mL)	CV (%)	Accuracy (%)
5.0	3.6	19.4	72.5	3.6	19.5	72.5
17.5	14.7	5.5	83.9	14.7	5.5	83.9
37.5	40.5	5.9	108.1	40.5	5.9	108.1

Table 7.10: Summary intra-day and inter-day study of ethyl oleate for MeOH-SPE method.

Actual amount (ng/mL)	Intra-day study (n=3)			Inter-day study (n=9)		
	Average (ng/mL)	CV (%)	Accuracy (%)	Average (ng/mL)	CV (%)	Accuracy (%)
10.0	10.3	17.35	102.8	10.3	17.4	102.8
35.0	35.1	15.0	100.2	35.1	15.0	100.2
75.0	111.2	17.5	148.3	91.2	23.9	121.6

Table 7.11: Summary intra-day and inter-day study of ethyl stearate for MeOH-SPE method.

	Intra-day study (n=3)			Inter-day study (n=9)		
Actual amount (ng/mL)	Average (ng/mL)	CV (%)	Accuracy (%)	Average (ng/mL)	CV (%)	Accuracy (%)
5.0	4.9	4.1	97.3	4.8	13.1	95.5
17.5	17.0	1.9	96.9	17.0	11.0	97.4
37.5	46.2	4.5	123.1	42.0	18.7	111.9

Table 7.12: Summary intra-day and inter-day study of EtG for MeOH-SPE method.

	Intra-day study (n=3)			Inter-day study (n=9)		
Actual amount (ng/mL)	Average (ng/mL)	CV (%)	Accuracy (%)	Average (ng/mL)	CV (%)	Accuracy (%)
0.75	1.06	30	141	0.76	49	102
1.75	1.67	5	96	1.63	13	94
3.75	3.16	2	84	2.81	27	75

The measurements of the concentrated hair pool showed a CV of 35.9% for the sum of the 4 FAEEs (see Figure 7.5) and no EtG was detected in the concentrated hair pool.
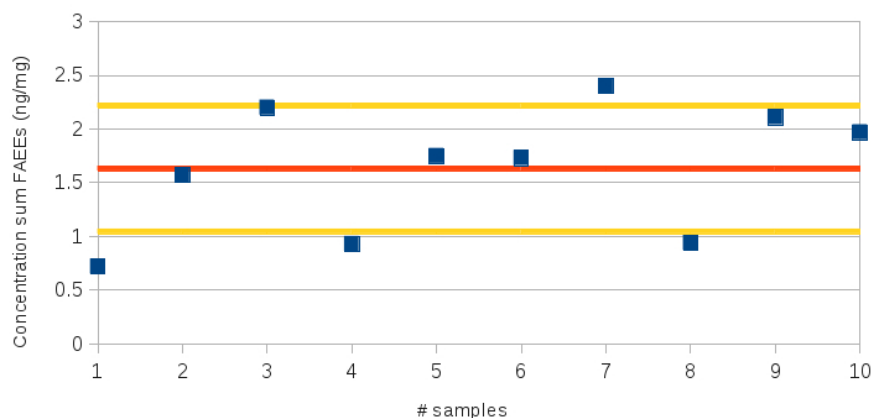
Figure 7.5: The sum of the FAEEs that was detected with MeOH-SPE method in ten different hair samples from the concentrated hair pool.

For this method, the stock solutions were also prepared in methanol and thus the stability of the samples in methanol over various freeze-thaw cycles was examined. As is shown in Figure 7.4 the variation due to freeze-thaw cycles was relatively low for FAEEs in MeOH; the CV was 5.4% for ethyl myristate, 5.0% for ethyl stearate, 8.4% for ethyl palmitate and 6.1% for ethyl oleate.

The measurements of the hair samples of drinkers are shown in Table 7.13. As stated before the EtG concentration found in the hair of subject D was remarkably high and may be explained by the use of a hair product as opposed to chronic drinking. It was also observed that one heavy drinker had a negative EtG hair test and another heavy drinker had a negative FAEEs hair test. The rest of the tests correctly classified the social drinkers and heavy drinkers as such. Also here was observed that the false positive and false negative of an EtG test would have been rebutted with the FAEEs hair test and vice versa.

Table 7.13: EtG and FAEEs concentration from the hair samples of drinkers detected with MeOH-SPE method. HD: heavy drinker, SD: social drinker and ND: not detected.

| Hair sample | A | B | C | D |
Drinking behaviour	*HD*	*SD*	*HD*	*SD*
EtG (pg/mg)	20	13	77	454
Sum FAEEs (ng/mg)	2.62	0.25	0.20	0.23
Ethyl myristate (ng/mg)	ND	ND	ND	ND
Ethyl palmitate (ng/mg)	1.33	ND	ND	0.23
Ethyl oleate (ng/mg)	1.02	0.25	0.20	ND
Ethyl stearate (ng/mg)	0.17	ND	ND	ND

7.2.3 The validation of method 3: DMSO-heptane method

The third method described in Figure 7.1 is similar to the industry standard for the detection of FAEEs in hair. The main difference lies in the extraction. In this work 30 minutes of sonication was used (as reported by Zimmermann and Jackson [215]), whereas Pragst [114] does not use sonication and shakes the sample in this mixture instead for 14 h. The method that is presented here yielded the following intra-day and inter-day results (see Tables 7.14, 7.15, 7.16, 7.17 and 7.18). The precision was expressed as CV (see Equation 4.4 on page 63) and the accuracy was calculated with Equation 4.5 on page 63. The figures in the tables are in red when the CV was above 20% and the accuracy below 90% or above 110%. This method may have been more repeatable for the FAEEs when phosphate buffer was added prior to HS SPME, since this is described as a repeatable method for FAEEs by Pragst [114]. The detection limit of this method was calculated at 0.02 ng/mg for ethyl myristate, 0.03 ng/mg for ethyl palmitate and ethyl stearate, 0.08 ng/mg for ethyl oleate and 7 pg/mg for EtG. Information about the inter-day study lacked for EtG because this method was not robust and the detection of the EtG failed in the QCs, which may be related to the repeatability of the SPE method.

Table 7.14: Summary intra-day and inter-day study of ethyl myristate for DMSO-heptane method.

Actual amount (ng/mL)	Intra-day study (n=3)			Inter-day study (n=9)		
	Average (ng/mL)	CV (%)	Accuracy (%)	Average (ng/mL)	CV (%)	Accuracy (%)
5.0	4.4	7.4	87.4	4.7	24.2	93.2
17.5	16.4	6.4	93.6	17.1	22.7	98.0
37.5	37.7	5.6	100.6	40.7	21.7	108.5

Table 7.15: Summary intra-day and inter-day study of ethyl palmitate for DMSO-heptane method.

	Intra-day study (n=3)				Inter-day study (n=9)		
EtG (pg/mg)	63	27	ND	27	53		
Actual amount (ng/mL)	Average (ng/mL)	CV (%)	Accuracy (%)		Average (ng/mL)	CV (%)	Accuracy (%)
5.0	6.0	101.2	119.8		4.8	12.4	96.9
17.5	15.0	6.2	85.7		16.3	20.6	93.4
37.5	33.3	30.5	88.9		33.8	30.4	90.2

Table 7.16: Summary intra-day and inter-day study of ethyl oleate for DMSO-heptane method.

	Intra-day study (n=3)			Inter-day study (n=9)		
Actual amount (ng/mL)	Average (ng/mL)	CV (%)	Accuracy (%)	Average (ng/mL)	CV (%)	Accuracy (%)
10.0	10.2	19.5	102.4	10.2	16.9	102.1
35.0	35.7	29.3	101.9	31.6	30.7	90.3
75.0	101.4	31.5	135.2	71.68	43.0	95.6

Table 7.17: Summary intra-day and inter-day study of ethyl stearate for DMSO-heptane method.

	Intra-day study (n=3)			Inter-day study (n=9)		
Actual amount (ng/mL)	Average (ng/mL)	CV (%)	Accuracy (%)	Average (ng/mL)	CV (%)	Accuracy (%)
5.0	2.6	37.1	51.9	5.1	16.4	101.4
17.5	14.4	5.1	82.5	14.3	10.3	81.5
37.5	48.6	11.6	129.6	39.3	42.8	104.9

Table 7.18: Summary intra-day study of EtG for DMSO-heptane method.

	Intra-day study (n=3)		
Actual amount (ng/mL)	Average (ng/mL)	CV (%)	Accuracy (%)
0.75	1.40	67.1	187.4
1.75	1.69	69.8	96.6
3.75	2.63	54.5	70.1

The measurements of the concentrated hair pool showed a CV of 28.0% for the sum of the 4 FAEEs (see Figure 7.6) and no EtG was detected in the concentrated hair pool.
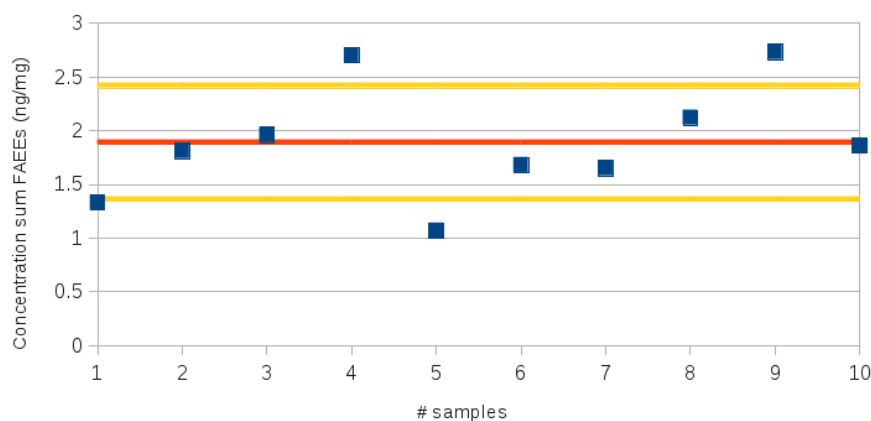
Figure 7.6: The sum of the FAEEs that was detected with DMSO-heptane method in ten different hair samples from the concentrated hair pool.

The stock solutions for the FAEEs in this method were prepared in heptane. Therefore, the stability of the samples in heptane over various days and freeze-thaw cycles was examined. The stability of the FAEEs in heptane when stored for 26 days at -30 °C is shown in Figure 7.7. A decrease of ethyl oleate, ethyl palmitate and ethyl stearate was observed in the first week. The measurement error within one day was relatively small (see the double measurements on day 7 and 19) compared to this decrease.

The CV from the different freeze-thaw cycles was relatively low for the FAEEs. The CV was 7.2% for ethyl myristate, 12.6% for ethyl palmitate, 7.3% for ethyl oleate and 7.3% for ethyl stearate (see Figure 7.8).
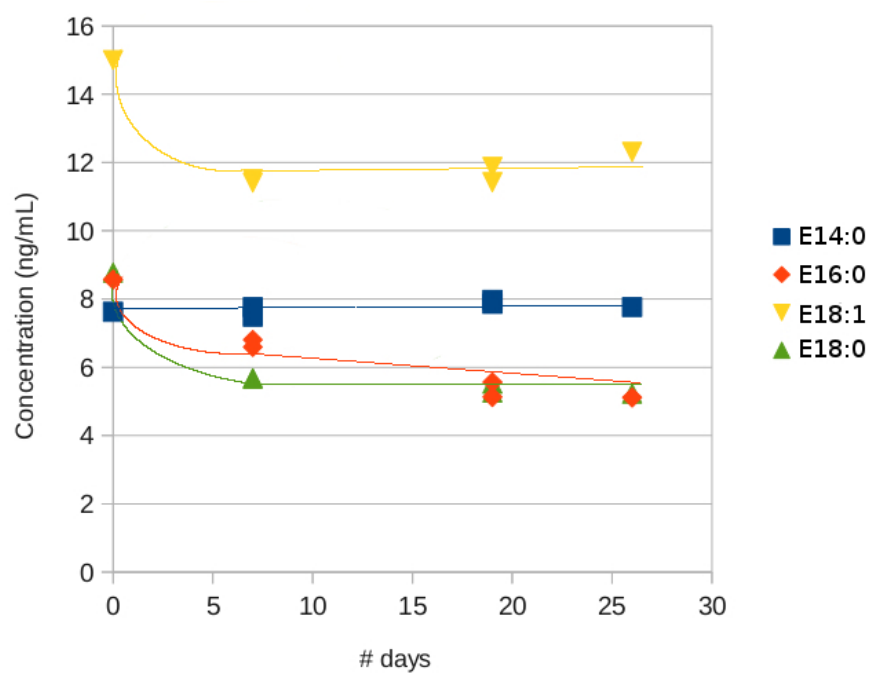
Figure 7.7: Stability of the freshly prepared FAEEs in heptane that were stored at -30 °C for a month.

Table 7.19: EtG and FAEEs concentration in hair samples of drinkers detected with DMSO-heptane method. HD: heavy drinker, SD: social drinker and ND: not detected.

| Hair sample | B | C | E | F |
Drinking behaviour	SD	HD	HD	HD
Sum FAEEs (ng/mg)	0.41	0.33	8.54	1.09
Ethyl myristate (ng/mg)	ND	ND	0.30	0.21
Ethyl palmitate (ng/mg)	0.12	ND	0.83	0.24
Ethyl oleate (ng/mg)	0.29	0.33	7.29	0.64
Ethyl stearate (ng/mg)	ND	ND	0.12	ND
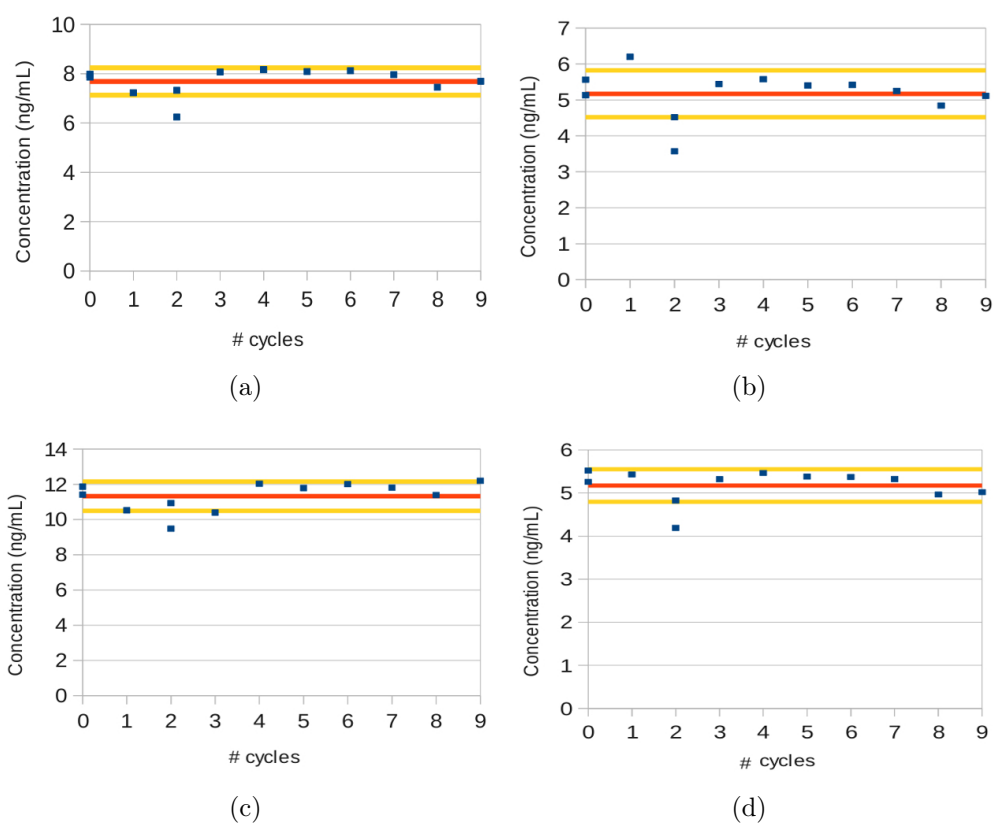
Figure 7.8: FAEEs subjected to freeze-thaw cycles in heptane: a) ethyl myristate, b) ethyl palmitate, c) ethyl oleate and d) ethyl stearate. The average of the measurements is shown as a red line and the standard deviation are indicated by the yellow lines.

7.2.4   The validation of the industry standard for the extraction of EtG from hair

The most common single extraction method for EtG is the extraction with deionized water (see Appendix E). For details see the article of Paul et al. [170] here a different GCMS method was used (see Section 6.3). Only EtG was measured using this method, because no ethyl myristate was observed in the extraction of the hair sample from a chronic heavy drinker (see Section 5.3). The detection limit of this method was calculated at 3 pg/mg for EtG.

Table 7.20: Summary intra-day and inter-day study of EtG for the industry standard for EtG extraction from hair.

Actual amount (ng/mL)	Intra-day study (n=3)			Inter-day study (n=9)		
	Average (ng/mL)	CV (%)	Accuracy (%)	Average (ng/mL)	CV (%)	Accuracy (%)
0.75	0.71	4.7	94.8	0.73	9.4	97.3
1.75	1.58	3.4	90.0	1.64	10.6	93.7
3.75	3.52	1.4	93.9	3.40	19.3	90.7

The results of the measurements of the hair samples of drinkers are shown in Table 7.21. One of the five hair samples gave a false negative, but the others correctly classified the subject's drinking behaviour.

Table 7.21: EtG concentration in hair samples of drinkers detected with the industry standard for EtG extraction. HD: heavy drinker, SD: social drinker and ND: not detected.

Hair sample	A	B	D	E	F
Drinking behaviour	*HD*	*SD*	*SD*	*HD*	*HD*
EtG (pg/mg)	63	27	ND	27	53

7.3 The comparison of the combined methods based on the validation studies with each other and the industry standard

The industry standard method for EtG analysis proved to be superior to other approaches providing the lowest detection limit, the best intra-day and inter-day repeatability and best extraction efficiency was obtained (as Chapter 5 showed and the hair samples of drinkers here indicated). This method is specifically developed for EtG and is not suitable for the FAEEs as was shown in Chapter 5. For FAEEs the lowest detection limit was obtained for method 3 where the heptane layer was analysed for FAEEs. However, method 1, where the analytes were extracted in methanol and divided into two aliquots, was much suitable for the combined detection of EtG and FAEEs.

Method 1 was the most suitable method for the combined extraction and detection of EtG and FAEEs.

- It had a good repeatability for the QCs of the five analytes compared to the other combined methods with a variation in accuracy between 84% and 111% for the FAEEs and 95% to 118% for EtG.

- The stability of the FAEEs in methanol was better than that of the FAEEs in heptane. The CVs of the freeze-thaw cycles were lower and no decrease in ethyl stearate was observed for the FAEEs in methanol. This means that a calibration curve of the methanol samples can be obtained quickly from the samples that are stored in the freezer at -30 °C.

- Method 1 is easy to adopt by the industry. Method 2 would be more difficult to implement due to the more complex nature of the procedure and the possibility of mixing up samples during the SPE procedure.

- The combined method would save 12 to 24 hours per sample compared to the two separate methods and may require less consumables compared to the industry standard of the single tests.

- An extraction with methanol is often used for the extraction of drugs in hair [227] and may thus be used as a screening tool for the detection of various drugs of abuse.

- Method 1 was successfully applied to eight hair samples from subjects that were either social drinkers or heavy drinkers.

- Method 1 showed a good linearity with a correlation coefficient (r^2) above 0.99 of the spiked drug concentrations from 0.3 to 5 ng/mL for EtG, 3 to 50 ng/mL for ethyl myristate, ethyl palmitate, ethyl stearate and 6 to 100 ng/mL for ethyl oleate. The lowest calibrator was less than 40% of the cut-off value of an analyte which is one of the requirements of a quantitative hair test [37].

- The extraction of FAEEs from the hair samples of drinkers and the concentrated hair pool were comparable with the method that was similar to the industry standard (as was previously suggested in Chapter 5). Note that the inhomogeneity of the hair pool may have contributed to the variation of the concentrations that was observed (as also Mönch et al. argued [228]).

Method 1 may be improved by adding phosphate buffer prior to HS SPME. The repeatability for the FAEEs was also relatively low for the FAEEs with the extraction with the DMSO/heptane mixture, which is the industry standard whereas Pragst at al. have shown that this method in combination with phosphate buffer was a robust method [114].

7.4 A summary of the best method that was developed for the combined detection of EtG and FAEEs in hair

Method 1 was compared to the other methods tested the best and fully validated. Standard curves were linear ($r^2 > 0.99$) from 0.3 ng/mL to 5 ng/mL for EtG and from 3 ng/mL to 50 ng/mL for the FAEEs. The limit of detection was between

0.04 and 0.09 ng/mg for the FAEEs and 5 pg/mg for EtG when 30 mg hair was used. No carry over was detected following injection of twice the concentration of the highest calibrator (1.67 ng/mg for the three FAEEs, 3.34 ng/mg for ethyl oleate and 167 pg/mg for EtG for 30 mg of hair). Neither EtG nor FAEEs were found above the LOQ in the blank samples. The intra-day variation (n=3) and inter-day variation (n=9) were 84–111% of the actual value with a coefficient of variation of 3–30% for the FAEEs and the accuracy for the spiked EtG hair control samples were between 95% and 118% with a CV of less than 23%. Moreover, this is a more time efficient and simpler method that uses less chemicals and may therefore reduce the cost compared to the separate analysis of the two alcohol markers EtG and FAEEs.

Chapter 8

Statistical evaluation of EtG and FAEEs hair testing for chronic alcohol consumption

As was discussed in Chapter 1 the quantification of EtG and FAEEs in hair testing can be used to indicate (heavy) drinking. Often laboratories interpret the test results with a cut-off value (see Figure 8.1). The threshold for EtG is suggested to be 30 pg/mg [38] and for the FAEEs the cut-off values for the sum of the 4 FAEEs are set to 0.5 ng/mg for hair shorter than 3 cm and 1 ng/mg for longer hair [38]. Measurements above this value indicate heavy drinking according to the SoHT [38]. The choice of these cut-off values is established on relatively small databases is previously discussed in Chapter 2.

An alternative solution to deal with these uncertainties to the use of a cut-off value may involve the application of the Bayesian approach in which likelihood ratios (LRs) are calculated by experts [229]. This was suggested to be used for forensic evidence in 1977 by Lindley [230]. DNA analysis is one of the front runners; the framework used in DNA analysis since the 1990s is based on quantifying the strength of evidence with a likelihood ratio [231]. The description of the detected concentrations of drugs and drug metabolites in hair is not yet adjusted to this framework. The application of the Bayesian approach can be described
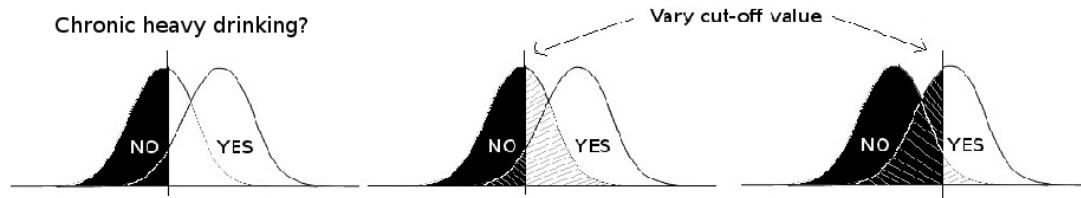
Figure 8.1: A general representation of the distribution of concentrations of two distinct alcohol consuming groups: infrequent drinkers versus chronic heavy drinkers and the classification of these groups based on a cut-off value. An overlap in the concentrations that occur in both these groups is evident and therefore the selection of a cut-off value would result in false negatives, false positives or both.

as follows: the judge or jury can use the LR to update their beliefs about a set of competing propositions based on the evidence presented to them. The question addressed in court, whether someone has been abstinent, a social drinker or chronic heavy drinker cannot be answered directly in a scientific way. The prior odds are required to answer this question and the assessment of the prior odds is subjective since it entails the probability of someone's drinking behaviour prior to the test result.

In this chapter it will be explained why the Bayesian theory is a more transparent approach. In addition, it will be shown that the Bayesian theory can cope with more information when the binary system of a negative and positive test result is replaced by an ordinal scale of the concentration found in hair. Moreover, it is easier to understand the impact of a likelihood ratio than the currently used sensitivity and specificity of a test, for these do not easily translate to individual cases [180]. Also, in this work the Bayesian Theory is applied to calculate the LRs of FAEEs and EtG tests for both abstinence and chronic heavy drinking. Though most LRs from this work originate from a relatively small database and should not be used in casework, however, it does show how the Bayesian approach can be applied when the calculated LRs are used and is useful as proof of concept. A confidence interval (CI) is calculated and indicates the uncertainty in the LRs that are calculated. The value of the evidence for the use of both EtG and FAEEs tests was determined to support the claims of Pragst and Yegles [25]

and Süße et al. [101] that the use of both tests would increase the evidential value. The definitions used in this work for heavy drinker, social drinker and teetotaller are the same as for those in the field (see Section 4.1). However, with the Bayesian theory two additional terms were introduced: the 'drinkers' (everyone excluding the 'teetotallers') and the 'infrequent drinkers' (everyone except the 'heavy drinkers').

8.1 Data sets used for the determination of the relation between the concentration of EtG and FAEEs in hair with one's drinking behaviour

For EtG two sets of data were used for the determination of the LRs. One based on a smaller sample group (referred to as 'initial database') and one based on a larger database (and referred to as 'larger database'). For the FAEEs three databases were used; 'Hastedt's database' and also a 'initial' and 'larger database'. The larger databases only contain information to distinguish the chronic heavy drinkers from the infrequent drinkers, whereas the smaller sized database also contains information to distinguish the teetotallers from the drinkers.

8.1.1 Compiling the 'initial database' linking the concentration of EtG and FAEEs in hair to drinking behaviour

The 'initial database' contained relatively few subjects and various steps were required before the database could be used for the calculation of the LRs.

8.1.1.1 Gathering EtG and FAEEs concentrations linked to one's drinking behaviour from published articles

To form a complete data set, in this work referred to as the 'initial database', data from several scientific articles were combined to obtain information about the concentrations of these markers within the three different consumption groups. For EtG eleven sources were used and for the FAEEs eight (see Table 8.1). These articles provided information about the frequency of the selected concentration ranges found for the persons categorized as teetotallers, social users and heavy drinkers based on mainly self-reports. Their findings are summarized in Tables 8.2 and 8.3 for the EtG and FAEEs concentrations in hair, respectively. More articles are available with EtG and FAEEs concentrations, however, these either lack the exact figures (e.g. Süße et al. [118]) or it is unknown to which of the consumption groups these concentrations belong to (e.g. Bendroth et al. [47], Pragst et al. [3] and Albermann et al. [119]) and can therefore not be used for the calculations.

Table 8.1: The published articles used for the formation of the 'initial database'.

Marker	*Published articles*	*Marker*	*Published articles*
EtG	Álvarez et al. [190], Kharbouche et al. [59], Gareri et al. [79], Jurado [58], Alt et al. [158], Janda [107], Yegles [186], Appenzeller et al. [60], Kerekes et al. [76], Shi et al. [218] and Pirro et al. [81].	**FAEEs**	Auwärter et al. [115, 223], Pragst [114], Wurst [6], Hartwig [80], Yegles [186], Gareri et al. [79] and DeGiovanni et al. [61].

Table 8.2: The published values for the concentration of EtG in the hair of teetotallers, social drinkers and heavy drinkers.

Drinking behaviour	Source	EtG concentrations in pg/mg
Teetotallers	Yegles2004 [186]	<LOQ <LOQ
	Pirro2011 [81]	<LOQ <LOQ <LOQ <LOQ <LOQ <LOQ
	Kerekes2008 [76]	<LOQ <LOQ
	Janda2002 [107]	<LOQ <LOQ <LOQ <LOQ
	Alt2000 [158]	<LOQ <LOQ <LOQ <LOQ
	Shi2010 [218]	<LOQ <LOQ <LOQ <LOQ
Social drinkers	Yegles2004 [186]	<LOQ <LOQ <LOQ <LOQ
	Pirro2011 [81]	22 <LOQ <LOQ 12 30 20 <LOQ 22 <LOQ 30 12
	Kerekes2008 [76]	3 3 3 <LOQ <LOQ 4 3 7 12 12 8 10 17 8 17 15
	Janda2002 [107]	<LOQ <LOQ 55 <LOQ <LOQ
	Alvarez2009 [190]	440 3090 1870 790 1560 2930 830 1960 4590 2770 2320 3720 4200 3580 530
	Alt2000 [158]	<LOQ <LOQ <LOQ <LOQ <LOQ <LOQ
	Shi2010 [218]	<LOQ <LOQ 11 <LOQ
Heavy drinkers	Yegles2004 [186]	30 44 304 415 46 140 305 193 42 76 531 1774 3380 214 327 166 361 255 2043 72 887
	Pirro2011 [81]	37 206 94 35 614 382 229 243 220 218 58 89
	Kerekes2008 [76]	35 180 41 38 29 176
	Jurado2004 [58]	60 80 190 460 750 70 80
	Janda2002 [107]	<LOQ 13157 205 <LOQ 19 <LOQ 34 517 <LOQ 312 118 33 162 410 4110 244 318 565 1070 <LOQ 236 1026 455 2042 3612 322 371
	Appenzeller2007 [60]	116 71 233 137 40 36 24 65 261 140 26 78 126 259 88
	Alt2000 [158]	218 4025 485 344 <LOQ 287 2165 651 250 529 295 287 481 <LOQ 650 840 119 338 388 196
	Shi2010 [218]	10 14 12 22 15 11 48 40 29 55 78
	Kharbouche2009 [59]	80 679 273 712 61 819 538
	Gareri2011 [79]	96 23 4

Table 8.3: The published values for the concentration of FAEEs in the hair of teetotallers, social drinkers and heavy drinkers.

Drinking behaviour	Source	FAEEs concentration in ng/mg
Teetotallers	DeGiovanni2007 [61]	<LOQ <LOQ <LOQ 0.21 <LOQ <LOQ 0.24 <LOQ 0.22
	Auwärter2004 [223]	0.32 0.39 0.02 0.06 0.02 0.18 0.11 0.27 0.17 0.44 0.14 0.82 1.21
	Auwärter2001 [115]	0.09 0.17 0.37 0.10 0.06
	Pragst2001 [114]	<LOQ <LOQ <LOQ <LOQ <LOQ <LOQ <LOQ <LOQ
	Hartwig2003 [80]	0.39
Social drinkers	DeGiovanni2007 [61]	0.43 0.36 0.12 0.85 0.22 0.98 0.25 0.15 0.25 0.35
	Auwärter2004 [223]	0.08 0.19 0.11 0.13 1.20 0.16 0.25 0.08 0.08 0.67 1.96 0.32 0.29 0.87 1.38 0.76
	Auwärter2001 [115]	0.47 0.35 0.20 0.26 0.29 0.43 0.25 0.24 0.61 0.75 0.35 0.85 0.28
	Wurst2004 [6]	1.11 0.84 1.06
	Pragst2001 [114]	0.84 <LOQ 0.07 0.12 0.09 0.08 0.19 0.54 0.13 <LOQ
	Hartwig2003 [80]	0.66 0.35 0.19 0.09 0.32
Heavy drinkers	Yegles2004 [186]	1.23 0.70 0.71 4.16 0.65 3.38 0.85 1.15 20.48 2.83 11.70 30.60 9.80 3.50 10.20 7.30 3.30 13.20 11.10 1.30 1.30
	DeGiovanni2007 [61]	0.76 1.79 1.50 3.36 0.21 1.38 4.61 6.29 0.90 1.01 0.92 0.49 0.22 0.38 0.84 0.57 0.87 3.62 0.06 0.09 5.21 2.46
	Auwärter2004 [223]	1.49 3.90 2.82 3.09 1.31 1.04 4.49 2.35 9.09 5.24 1.64 2.00
	Auwärter2001 [115]	2.20 2.00 1.30 4.30 0.20 4.60 0.92 1.60 1.30 5.10 0.97 3.60 7.50 11.60 1.40 2.50 4.40 6.10 4.40 10.80 7.10 13.50 3.20 4.80 11.10
	Wurst2004 [6]	2.06 3.19 1.71 1.69 1.22 0.73 0.84 0.90 0.39 1.07 1.45 1.91 1.04 1.75 0.53
	Pragst2001 [114]	3.51 3.17 1.22 1.53 0.97 3.85 4.57 26.86 12.20 6.11 0.81 3.34 2.04 4.51 1.18 2.37 14.04 1.76 1.73 2.49 2.61
	Hartwig2003 [80]	1.40 4.40 11.10 2.20 8.20 11.70 16.30 2.80 5.70 2.10 3.50 9.60 12.00 1.70 1.30 1.80 11.90 3.00
	Gareri2011 [79]	4.61 0.60 1.32

8.1.1.2 Excluding information from the 'initial database'

Certain data were excluded. Box plots were made (see Figures 8.2 and 8.3 for EtG and FAEEs concentrations in hair respectively) to show the concentration values of each of the three groups: teetotaller, social drinker and heavy drinker and to investigate the database on outliers. Values that are separated from the box end with more than three box lengths are denoted by an asterisk and are considered to be outliers and were removed unless an explanation could be provided for this observation. The asterisks indicating significantly higher concentrations found within the group of heavy drinkers were not considered as outliers, because heavy drinkers may vary in how much they drink; from 60 grams of alcohol per day up to possibly much more. Also the data from Auwärter et al. [223] were not excluded despite the relatively high concentrations that were found for teetotallers and social drinkers compared to other research data (see Figure 8.3). The difference is small and they have used similar methods compared to those of other researchers. Hence, there is no apparent reason to exclude their data from the database. The asterisks that represent the data from Álvarez et al. [190] in Figure 8.2, on the other hand, showed significantly higher concentrations for social drinkers compared to other articles. Süße et al. [118] did state that self-reports of 'heavy' social drinkers may not be accurate since they tend to under-report, but this does not explain the results of Álvarez et al. since all of the fifteen social drinkers they analysed had a high concentration of EtG in hair, higher than in fact the current cut-off value to determine chronic heavy drinking (30 pg/mg). In addition to the high concentrations for self-reported social drinkers, a remarkably high detection limit and range of calibration curve were used in comparison to other methods that were available. Therefore the data from Álvarez et al. [190] were excluded from the database.

The methods used to obtain the data of EtG and FAEEs concentrations in hair for the three distinct drinking groups vary in quantification limit. When the limit was higher than the value of the lowest concentration category in the ordinal scale the data points below the quantification limit were excluded. In 2000 Skopp et al. [39] and Alt et al. [158] had a detection limit of 2 ng/mg and mentioned as lowest detected concentration 119 ng/mg. In 2002 Janda et al. [107] demonstrated

a detection limit of 51 pg/mg, but provided concentrations as low as 19 pg/mg. Two years later, Jurado [58] indicated to have a detection limit of 25 pg/mg. It is unclear in what category those values 'below the detection limit' fall in the frequency table and therefore these numbers were excluded.
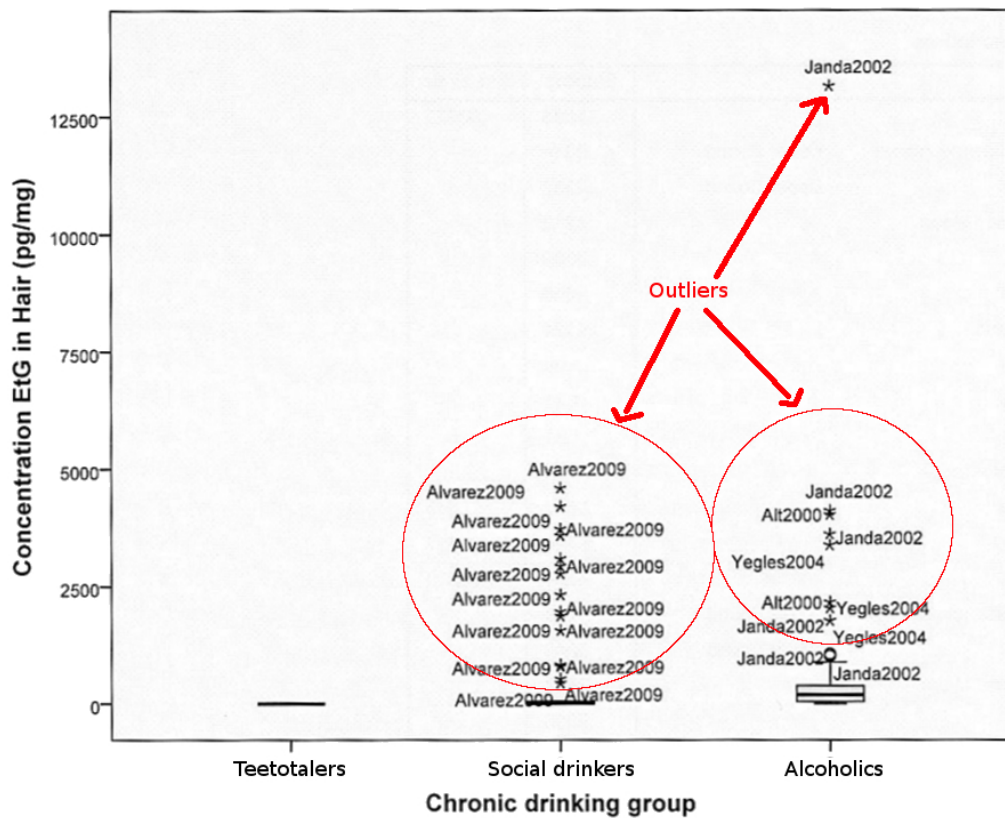
Figure 8.2: A box plot of the published concentrations of EtG in hair in each of the three drinking groups: teetotallers, social drinkers and alcoholics (i.e. chronic heavy drinkers). Outliers are represented as asterisks and clearly marked. The data presented are from [39, 58–60, 76, 79, 81, 107, 158, 186, 190, 218].

Figure 8.3: A box plot of the published concentrations of EtG in hair in each of the three drinking groups: teetotallers, social drinkers and alcoholics (i.e. chronic heavy drinkers). Outliers are represented as asterisks and the data presented are from [6, 61, 79, 80, 114, 115, 186, 223].

8.1.1.3   The frequency databases made from the 'initial database' based on an ordinal scale

A frequency database (Table 8.4) was constructed, from which the LRs were established for the FAEEs tests. The frequency database was built by measuring the occurrence of the event of detecting a value in that particular concentration range within a defined population which was collected from the literature (Table 8.3) and the summation of two different groups as described in Equation 4.1 on page 48. The thresholds for the ranges were selected based on the following information:

- A concentration of FAEEs above 0.20 ng/mg is expected to contradict abstinence [25].

- A value of 0.5 ng/mg and higher is generally accepted as an indication for chronic heavy drinking for hair that is shorter than 3 cm [38].

- A value of 1 ng/mg and higher is generally accepted as an indication for chronic heavy drinking for hair that is longer than 3 cm [38].

- In the database used no teetotallers or social drinkers were observed with levels equal and higher than 2.0 ng/mg (see Table 8.3), therefore this value is use as a threshold too for the evaluation of the classification of chronic heavy drinkers.

Also for the evaluation of the EtG test an ordinal scale was used. Therefore, a frequency database was constructed for EtG (Table 8.5) by measuring the occurrence in Table 8.2 of a value in a particular concentration range. The thresholds for the ranges were selected based on the following information:

- A concentration of EtG below 7 pg/mg is expected to indicate abstinence [37] and is used as the limit for re-granting confiscated driving license in Germany [119].

- A value of 30 pg/mg and higher is generally accepted as an indication for chronic heavy drinking [38].

- Previously, 50 pg/mg was used as a conservative threshold to indicate heavy

Table 8.4: The frequency table of the concentration of FAEEs in hair of different drinking groups. Data were obtained from [6, 61, 79, 80, 114, 115, 186, 223].

Drinking behaviour	FAEEs concentration in ng/mg				
	≤ 0.20	0.21–0.49	0.5–0.99	1.00-1.99	≥ 2.00
Teetotallers (n=42)	29	11	1	1	0
Social drinkers (n=57)	19	21	12	5	0
Heavy drinkers (n=141)	2	7	19	35	78
Drinkers (n=198)	21	28	31	40	78
Infrequent drinkers (n=99)	48	32	13	6	0
Weighted drinkers (n=3032)	966	1072	628	289	78
Weighted infrequent drinkers (n=288)	111	102	53	22	0

drinking [117].

Table 8.5: The frequency table of the concentration of FAEEs in hair of different drinking groups. Data were obtained from [59, 60, 76, 79, 81, 186, 218].

Drinking behaviour	EtG concentration in pg/mg			
	< 7	7–29	30–49	≥ 50
Teetotallers (n=19)	19	0	0	0
Social drinkers (n=37)	19	15	2	1
Heavy drinkers (n=122)	1	12	15	94
Drinkers (n=159)	20	27	17	95
Infrequent drinkers (n=56)	38	15	2	1
Weighted drinkers (n=2623)	1285	1026	150	162
Weighted infrequent drinkers (n=130)	76	45	6	3

8.1.2   'Hastedt's database' linking the concentration of the FAEEs in hair to drinking behaviour

This data set was provided by M. Hastedt from the Charité, Universitätsmedizin Berlin, which is the raw data from the article published by Hastedt et al. [232]. 1057 post-mortem hair samples were analysed for FAEEs and the database included information about the subject's drinking behaviour based on medical history and police reports. From the 1057 in that study 502 cases were known alcohol abusers and in 168 cases it was probable that there was only social consumption. These were respectively used to represent the group of chronic heavy drinkers and social drinkers and thus only used to establish the LR in favour heavy drinking. Since the exact concentrations were present in this raw data set, a frequency database was made in a similar way as was done for the 'initial' database (see Section 8.1.1.3).

8.1.3   Compiling the 'larger databases' linking positive and negative tests to drinking behaviour

The data that were excluded for the initial database could be used for the larger database. Previously the data that could not be categorized for the ordinal scale, due to the lack of exact figures or an insufficient low quantification limit, but can be classified as positive and negative tests for the use of the larger database. Values ≥ 1.00 ng/mg for FAEEs and ≥ 30 pg/mg for EtG were identified as positive test results for chronic heavy drinking. For EtG the database included the same sources as previously described for the 'initial database' as well as the work of Skopp et al. [39], Süße et al. [118], Kronstrand et al. [188] and Appenzeller et al. [60] (see Table 8.12). For the large FAEEs frequency database where only information about the classification and positive/negative test outcome were examined only one source was used and this was the work of Süße et al. [118] (see Table 8.14).

8.2 The interpretation of the frequency databases with the traditional cut-off method

The following can be concluded when Table 8.5 is reviewed with the traditional cut-off method. Thirteen heavy drinkers had a concentration of EtG in their hair that is lower than 30 pg/mg, which is the current cut-off value of which values below are considered to be infrequent drinkers. Three social drinkers had concentrations of EtG in their hair that were higher than the cut-off value. One of them even had a concentration higher than 50 pg/mg. Though all teetotallers had a value below the cut-off that indicates abstinence (7 pg/mg), 20 drinkers (including one heavy drinker) also had an EtG concentrations below this value. A low EtG concentration may be explained by the extraction from hair by harsh hair treatment as was suggested by Morini et al. [233]. Higher values than those expected can be ascribed to under-reporting as was done by Süße et al. [101]. A similar interpretation of the other frequency databases can be expected. From this it is unclear how one can infer someone's drinking behaviour based on only an EtG concentration from hair and when one should take into account an alternative explanation for the EtG concentration detected in hair. It is clear that false positives and false negatives do exist in the database presented and that these may be explained by certain factors other than the subject's (claimed) alcohol consumption. The specificity and sensitivity of the EtG test can be calculated and though this says something about the accuracy of this test, and various tests can be compared using these figures, it is unclear what it says about a specific case as is also argued by McGee [234].

8.3 The interpretation of the frequency databases with the Bayesian theory

The first step for the interpretation of the frequency databases with the Bayesian theory was the calculation of the likelihood ratios. The LRs are the values of the evidence and were calculated from the frequency databases with Equations 4.12

and 4.13 (see pages 68 and 69). The frequency Tables 8.4 and 8.5 (based on the 'initial database') were used to calculate the LRs including the 95% CI on an ordinal scale and based on different hypotheses: in favour of heavy drinking (LR1 in Tables 8.6 and 8.7) and in favour of drinking (LR2 in Tables 8.8 and 8.9). Sample size correction was applied when no subjects with a certain concentration range were found and the LR was infinitive. For instance, no teetotallers were observed with EtG concentrations in hair above 7 pg/mg, this may be because teetotallers only have concentrations below this value or it may be a rare event and therefore not found in this relatively small database. For the sample size correction of the current data set with 19 teetotallers, imagine that the 20th teetotaller that would have been analysed would have a concentration of 7 pg/mg or higher. The chance of the detection in this range given someone is a teetotaller is then: 1/20 instead of 0/19. The LR would subsequently be around 17, which is 'moderate' evidence and not 'very strong' support as was estimated before the sample size correction. A larger database (with n teetotallers) would result in a value closer to zero $(\frac{1}{n+1})$ if it is impossible for a teetotaller to have a value ≥ 7 pg/mg.

To correct for the relatively high amount of heavy drinkers in the drinking group in the database compared to the UK population, the groups were weighted prior to their summation to form the drinking group. A similar correction was performed for the group of the infrequent drinkers. As expected this correction affected the LRs obtained for the different concentration ranges, however the verbal equivalent remained the same (see the weighted LRs in Tables 8.6, 8.7, 8.9 and 8.12) with the exception for FAEEs values equal and above 1 ng/mg for which the strength of the evidence was smaller for the weighted version. However, this value was still well within the 95% CI calculated for the not weighted version. No CI was calculated for the weighted version because in the calculation the number of social drinkers was manipulated and this would affect the CI, which would then only be a theoretical value for a larger sample size with the same distribution and not a practical uncertainty measure. Hence, the weighted LRs were not considered for the interpretation of the frequency databases with the Bayesian theory.

From the calculated LRs based on the initial database, a general trend was ob-

served in which likelihood ratios in favour of drinking and heavy drinking (LR1) increased with a increasing concentration of EtG or FAEEs (see Tables 8.6 and 8.8 for FAEEs and 8.7 and 8.9 for EtG). In court, two questions arise: if someone is a chronic heavy drinker and if someone has been abstinent. As is clear from the LRs in the tables more support can probably be obtained for heavy drinking than for abstinence (cf. Tables 8.6 and 8.7 with Tables 8.8 and 8.9 respectively).

8.3.1 The interpretation of the EtG and FAEEs concentration detected in hair to test for abstinence

It was found that stronger support can probably be obtained for drinking than for abstinence (see the LRs in Tables 8.8 and 8.9). This is in line with the accepted view that abstinence cannot be proven, but merely be excluded [27,72,109]. This may be due to a significant overlap in EtG concentration of alcohol drinkers and teetotallers below the cut-off value of 7 pg/mg or may be related with the relatively high cut-off value for EtG. Lees et al. [128] argued that a lower cut-off value (and thus also lower detection limit) may be more appropriate since a relative high number of drinkers are not classified as drinkers with a cut-off value of 7 pg/mg. Note that a trade-off exists between the number of correctly classified teetotallers and the drinkers being classified as teetotallers and a decrease in cut-off value may decrease the percentage of drinkers that are classified as teetotallers, but may also result in a decrease of correctly classified teetotallers.

The measurement of EtG in hair may be more appropriate to prove abstinence than the measurement of FAEEs in hair. This was also suggested by the consensus of the SoHT [109] and the guidelines of European workplace drug and alcohol testing in hair [111] in which only EtG in hair is mentioned to refute claims of abstinence. In Table 8.9 it is shown that EtG concentrations equal to and above 7 pg/mg were at least 'moderate' support for drinking and for FAEEs the LR was 'moderate' for values ≥ 1.00 ng/mg (see Table 8.8). The latter is relatively high considering that this value equals the cut-off value used to indicate chronic heavy drinking, whereas for EtG the threshold for abstinence is significantly lower than the the cut-off used for heavy drinking (7 pg/mg versus 30 pg/mg). Hence, EtG

tests to prove abstinence are preferred over FAEEs hair tests.

8.3.2   The interpretation of the EtG and FAEEs concentration detected in hair to test for chronic heavy drinking

The LRs calculated for positive and negative EtG tests used to indicate heavy drinking from the 'larger database' are in agreement with the LRs calculated with Equation 4.8 on page 66 from the sensitivity and specificity published by Süße et al. [118] and partially with the work of Politi et al. [69] (cf. Table 8.7 with Table 8.10). The value of a negative EtG test result (LR-) and thus for EtG concentrations in hair below 30 pg/mg) was for the 'larger database' 0.11 (see Table 8.7) which falls within the LRs derived from the work of Politi et al. [69] (0.06 and 0.11) and Süße et al. [118] (0.26) (see Table 8.10). The LR values indicate that a negative EtG test may be 'limited' support for infrequent drinking. For positive test results for an EtG hair test the value is considered to be 'moderate' support for heavy drinking based on both the 'initial database' and the 'larger database' used in this research as well as the data presented by Süße et al. [118]. Politi et al. [69], however, paints another picture with a specificity of 100%. This would mean that infrequent drinkers are correctly classified as infrequent drinkers with the 30 pg/mg cut-off value. This is in contrast with the information in Table 8.7 for which concentrations between 30 and 50 pg/mg were found in hair of self-reported social drinkers. It is likely that the specificity of 100% stated by Politi et al. [69] was overestimated as is supported by the lower specificity described by Süße et al. [118].

The calculated LRs for the FAEEs from the 'initial database' are in agreement with the LRs obtained from the sensitivity and specificity mentioned in various articles (cf. Table 8.6 with 8.11). The calculated LR– for the FAEEs shows that a negative test is only 'limited' evidence in favour of being a infrequent drinker due to relatively high number of false negatives. The LR+ for the FAEEs is approximately 13, which is 'moderate' support for heavy drinking, which is in agreement with the work of Süße et al. [118], but is relatively high compared to

the work of Wurst [6] and Pragst and Balikova [27] for which a positive test is 'limited' support for chronic heavy drinking. When the confidence intervals are considered, this verbal equivalent is also included in the range of LRs from the 'initial database' (see Table 8.6).

The 'initial databases' are relatively small. In the 'larger database' 288 of the 1207 infrequent drinkers have EtG concentrations in hair above or equal to 30 pg/mg (see Table 8.12). This means that based on this database an EtG concentration in hair above or equal to 30 pg/mg is only 'limited' support for heavy drinking. This is not only low compared to the values obtained from the 'initial database', but also low compared to the LRs calculated from the specificity and sensitivity (see Table 8.10). The verbal equivalent of the LR 'limited' evidence to support heavy drinking for concentrations equal and higher than 30 pg/mg falls within the 95% confidence interval for the smaller database. Hence, the value of the evidence may be over-estimated for EtG in hair; Kintz [38] lays out the consensus of the SoHT on hair testing for chronic excessive alcohol consumption 2011 and states that "The cut-off for EtG in hair to strongly suggest chronic excessive alcohol consumption is proposed at 30 pg/mg scalp hair measured in the 0–3 up to 0–6 cm proximal segment." Factors that are mentioned in the literature to explain discrepancies between one's chronic drinking behaviour and EtG concentrations include: harsh hair treatment and contamination from EtG containing hair products. The possibility of elevated concentrations of EtG in hair is not acknowledge in the consensus of the SoHT [38] and may be one of the reasons of the difference in how strong the evidence is perceived by the SoHT and in this work.

The evidential values obtained from the 'initial database' (see Table 8.6) were similar to those of two other larger databases: 'Hastedt's database' (see Table 8.13) and the 'larger database' (see Table 8.14). Also with these larger databases the support in favour of and against heavy drinking is relatively low. The confidence intervals of each of the concentration ranges calculated from these databases fall within those calculated from the relatively small database in Table 8.6 with the exception of concentrations above 2 ng/mg (cf. Table 8.6 with 8.13). This is probably because even with the sample size correction for the initial database the

possibility is underestimated of the detection of concentrations above 2 ng/mg in hair of an infrequent drinker. The value of the evidence to support chronic drinking based on a FAEEs is thus much lower than how the value of the evidence of this test seems to be perceived. The consensus of the SoHT proposes cut-off values for the sum of the four esters in hair at 0.5 ng/mg scalp for hair measured in the 0–3 cm proximal segment and at 1.0 ng/mg scalp hair for the proximal 0–6 cm segment to "strongly suggest chronic excessive alcohol consumption" [38]. This may be related to the prior odds for normal hair testing: a high level of alcohol consumption may be expected when the FAEEs tests are performed in a laboratory to confirm chronic drinking. In that case, the prior odds are higher than for a randomly chosen individual and this makes it more likely that a positive test result is indeed a positive test result for chronic alcohol consumption. For randomly picked individuals like in work placement testing the over-estimation of the diagnostic strength of the FAEEs hair test for chronic heavy drinking may be an issue and caution is required for drawing conclusions in this situation as also Hastedt et al. [235] suggested.

Table 8.6: The LRs and 95% confidence level for different FAEEs concentration ranges under the hypotheses of being a heavy drinker versus an infrequent drinker.

Value of the evidence	FAEEs concentration in ng/mg						
	≤ 0.20	0.21–0.49	0.50–0.99	1.00–1.99	≥ 1.00	≥ 2.00	< 1.00
LR1	0.03	0.15	1.0	4.1	13	∞ (or 55[a])	0.21
95% CI	0.01–0.12	0.07–0.33	0.50–2.0	1.8–9.4	6.1–29	N/A (or 7.8–391[a])	0.15–0.30
$1/LR1^{b,c}$	34	6.5					4.7
95% CI	8.5–137	3.0–14					3.4–6.6
Verbal equivalent LR^d	moderate[b]	limited[b]	inconclusive	limited	moderate	very strong or (moderate[a])	limited[b]
Verbal equivalent CI^d	limited to moderate strong[b]	limited to moderate[b]	limited[b] to limited	limited	limited to moderate	N/A (or limited to moderate strong[a])	limited[b]
LR1 weighted	0.037	0.14	0.74	3.2	10	∞[e]	0.22
Verbal equivalent LR1 weighted	moderate[b]	limited[b]	limited[b]	limited	moderate	very strong[e]	limited[b]

[a] when the sample size correction is applied 1/100 is used instead of 0/99 for the chance that an infrequent drinker has a value within this concentration range
[b] strength of evidence to support the hypothesis that the person tested is not a heavy drinker
[c] for LR1 < 1
[d] for LR > 1
[e] no sample size correction can be applied, because the number of infrequent drinkers is modified

Table 8.7: The LRs with 95% confidence level for different EtG concentration ranges under the hypotheses of being a heavy drinker versus an infrequent drinker.

Value of the evidence	EtG concentration in pg/mg					
	< 7	7–29	30–49	≥ 30	≥ 50	< 30
LR1	0.01	0.37	3.4	17	43	0.11
95% CI	0.00–0.09	0.18–0.73	0.81–15	5.5–50	6.2–302	0.07–0.19
LR1 weighted	0.014	0.28	2.7	12.9	33	0.11
1/LR1[a]	83	2.7				8.9
95% CI	12–588	1.4–5.4				5.3–15
Verbal equivalent for LRs[c,d]	*moderate [b]*	*limited [b]*	*limited*	*moderate*	*moderate*	*limited[b]*
Verbal equivalent for CI	*moderate to strong[b]*	*limited[b]*	*limited[a] to moderate[c]*	*limited to moderate*	*limited to moderate strong*	*limited[b] to moderate[b]*

[a] for $LR1 < 1$
[b] strength of evidence to support the hypothesis that the person tested is not a heavy drinker
[c] where $LR > 1$
[d] for both LR1 and LR1 weighted

Table 8.8: The LRs with 95% confidence level for different FAEEs concentration ranges under the hypotheses of being a drinker versus a teetotaller. For the LR values below 1, the 1/LR is presented to show the value of the evidence in favour of the hypothesis of the tested person being a teetotaller. For drinkers and infrequent drinkers a correction is applied that represents the ratio of the drinking groups in the UK population to obtain LR2 weighted.

Value of the evidence	FAEEs concentration in ng/mg						
	≤ 0.20	$0.21\text{--}0.49$	$0.50\text{--}0.99$	$1.00\text{--}1.99$	≥ 1.00	≥ 2.00	> 0.20
LR2	0.15	0.54	6.6	8.5	25	inf (or 17[a])	2.9
95% CI	0.10–0.24	0.29–0.99	0.92–47	1.2–60	3.6–174	N/A (or 2.4–118[a])	1.8–4.6
1/LR2[b]	6.5	1.9					
95% CI	4.1–10	1.0–3.4					
Verbal equivalent for LR[c]	limited[b]	limited[b]	limited	limited	moderate	very strong (or moderate[a])	limited
Verbal equivalent for CI[c]	limited to moderate[b]	limited[b]	limited[b] to moderate	limited to moderate	limited to moderate strong	very strong (or very strong (or limited to moderate strong[a])	limited
LR2 weighted	0.46	1.4	8.7	4.0	5.1	inf[d]	2.2
Verbal equivalent for LR[c]	limited[b]	limited	limited	limited	limited	limited	limited

[a] when the sample size correction is applied 1/43 is used instead of 0/42 for the chance that a teetotaller has a value within this concentration range
[b] strength of evidence to support the hypothesis that the person tested is not a heavy drinker
[c] for LR > 1
[d] no sample size correction can be applied

Table 8.9: The LRs with 95% confidence interval (CI) for different EtG concentration ranges under the hypotheses being a drinker versus a teetotaller. For the LR values below 1, the 1/LR is presented to show the value of the evidence in favour of the hypothesis of the tested person being a teetotaller.

Value of the evidence	EtG concentration in pg/mg	
	< 7	≥ 7
LR2	0.13	∞ (or 17^a)
95% CI	0.08–0.19	n/a (or 2.6–118^a)
LR2 weighted	0.49	inf (or 10^a)
1/LR2^b	8.0	
95% CI	5.3–12	
Verbal equivalent for LR^c	*limited^b*	*very strong (or moderate^a)*
Verbal equivalent for LR2 weighted^c	limited^b	very strong (or limited^a)
Verbal equivalent for CI^c	*limited^b to moderate^b*	*- (or limited to moderate strong^a)*

^{a} when sample size correction is applied
^{b} strength of evidence to support the hypothesis that the person tested does not drink alcohol
^{c} for $LR > 1$

Table 8.10: The LRs and their verbal equivalents for positive and negative EtG hair tests calculated from the sensitivity and specificity published in different articles.

	Sensitivity	Specificity	LR+	LR-	1/LR-
Politi2007 [69]	0.89 or 0.94^a	1.0	∞ (890 or 940^b)	0.11 or 0.06	9.1 or 17
Verbal equivalent^c			very strong (moderate strong^b)		limited or moderate
Suse2011 [118]	0.75	0.97	25	0.26	3.9
Verbal equivalent^c			moderate		limited

^{a} respectively 3 months and 2 weeks of EDI < 60 grams
^{b} when for the specificity 0.999 is used instead of 1.0
^{c} for $LR > 1$

Table 8.11: The LRs and their verbal equivalents for positive and negative FAEEs hair tests calculated from the sensitivity and specificity published in different articles.

	Sensitivity	Specificity	LR+	LR-	1/LR-
Wurst2004 [6]	1.00^a	0.90	10.00	0.00	∞ (or 900^b)
Wurst2004 [6]	0.944^c	0.90	9.44	0.06	16.07
Wurst2004 [6]	0.889^d	0.90	8.89	0.12	8.11
Wurst2004 [6]	0.556^e	0.90	5.56	0.49	2.03
Verbal equivalent^f	N/A	N/A	limited		limited to very strong
Pragst2006 [25]^g	0.9	0.9	9.0	0.1	9.0
Verbal equivalent^f	N/A	N/A	limited		limited
Suse2011 [118]^h	0.77	0.96	19.25	0.24	4.17
Verbal equivalent^f	N/A	N/A	moderate		limited

^{a} for a cut-off of 0.29 ng/mg
^{b} when for the sensitivity 0.999 is used instead of 1.00
^{c} for a cut-off of between 0.4–0.46 ng/mg
^{d} for a cut-off of 0.7 ng/mg
^{e} for a cut-off of 1.0 ng/mg
^{f} for $LR > 1$
^{g} for a cut-off of 0.5 ng/mg
^{h} for a cut-off of 1.00 ng/mg

Table 8.12: The frequency database and LRs for positive and negative EtG hair tests of the 'larger database'. CI: confidence interval. The data presented are from [39, 58–60, 76, 79, 81, 107, 118, 158, 186, 188, 218].

	EtG concentration in pg/mg	
Drinking behaviour	< 30	≥ 30
Teetotallers (n=389)	345	44
Social drinkers (n=818)	574	244
Heavy drinkers (n=455)	125	330
Infrequent drinkers (n=1207)	919	288
Weighted infrequent drinkers (n=2667)	1944	723
Value of the evidence		
LR1 (95% CI)	0.36 (0.31–0.42)	3.1 (2.7–3.4)
1/LR1 (95% CI)^a	2.77 (2.38–3.23)	
LR1 weighted	0.38	2.7
Verbal equivalent LR^{b,c,d}	limited^e	limited

^{a} for $LR1 < 1$
^{b} for $LR > 1$
^{c} both weighted and not weighted
^{d} this includes the confidence interval for the non-weighted ratio of infrequent drinkers
^{e} strength of evidence to support the hypothesis that the person tested is not a heavy drinker

Table 8.13: The frequency database and LRs with its 95% CI of FAEEs hair tests on an ordinal scale of the 'Hastedt's database'.

Drinking behaviour	FAEEs concentration in ng/mg					
	≤ 0.20	0.21–0.49	0.5–0.99	1.00–1.99	≥ 2.00	≥ 1.00
No. alcoholics (n=493)	67	66	81	205	288	
No. non-alcoholics (n=168)	61	47	23	21	16	37
Value of the evidence						
LR1	0.37	0.47	1.2	1.3	4.3	4.6
1/LR1	2.7	2.1				
95% CI of LR1[a]	1.5–15	1.2–7.2	0.75–1.6	0.88–1.8	3.8–4.8	4.2–4.7
Verbal equivalent LR[a]	limited[b]	limited[b]	limited	limited	limited	limited
Verbal equivalent for 95% CI of LR[a]	limited[b]–moderate[b]	limited[b]	limited[b]–limited	limited[b]–limited	limited	limited

[a] for LRs above 1

[b] in favour of infrequent drinking

Table 8.14: Frequency database of positive and negative FAEEs test results for chronic drinking of the different drinking groups and the derived LRs from the work of Süße et al. [118].

Drinking behaviour	FAEEs test result	
	Negative	Positive
Teetotallers (n=242)	191	51
Social drinkers (n=793)	493	300
Heavy drinkers (n=346)	80	266
Infrequent drinkers (n=1035)[a]	684	351
Weighted infrequent drinkers (n=1659)[b]	1072	587
Value of the evidence		
LR1	0.35	2.3
95% CI	0.29–0.43	2.1–2.5
LR1 weighted	0.36	2.17
1/LR1[c]	2.9	
95% CI	2.4–3.5	
verbal equivalent LR[d,e]	limited[f]	limited
Verbal equivalent 95% CI[d]	limited[f]	limited

[a] the sum of teetotallers and social drinkers
[b] the sum of teetotallers and social drinkers corrected for ratio in UK population
[c] for $LR1 < 1$
[d] for $LR > 1$
[e] for both weighted and non-weighted LR
[f] support for infrequent drinking

8.3.3 The conclusions based on the interpretation of the EtG and FAEEs hair tests with the Bayesian theory

It is difficult to determine the value of the evidence of a test for a specific person of whom his or her chronic alcohol consumption needs to be established when the traditional cut-off method is used. With the Bayesian theory, the value of the evidence can be calculated (the LR or verbal equivalent of the LR) and the statistics can be converted to be used in a specific case. In this way one is able to translate the data set to the specific value of the evidence to support one's drinking behaviour given a certain test result.

The trend found with the initial database is clear and logical: an increase in EtG and FAEEs concentration in hair corresponds with an increasing value of evidence in favour of heavy drinking. This is in line with what you might expect, however, this was largely ignored previously with the use of a traditional cut-off value. In this work a relatively small database was used for the estimation of LRs on an ordinal scale, this may have resulted in the large confidence intervals that are observed. Therefore the exact figures found in this are not as important as the proof of concept provided and that this research is transparent about its accuracy. It is also interesting to note that values found in this work do correspond with the published work of Süße et al. [118], Hastedt et al. [232] and larger databases that were composed based on a binary scale. It only differs from Politi et al. [69] based on their unlikely high calculated specificity of 100% meaning that 100% of the infrequent drinkers would be classified correctly. Multiple times it was found that the EtG and FAEEs hair tests may provide merely 'limited' evidence to support chronic heavy drinking, despite being rather strong diagnostically for heavy drinking compared to other tests (see Appendix C). This indicates that the value of evidence of a positive EtG or FAEEs hair test may not strongly suggest chronic excessive alcohol consumption as was stated by Kintz in the consensus of the SoHT on hair testing for chronic excessive alcohol consumption 2011 [38]. The perceived difference in the value of evidence may be explained by the fact that often an EtG hair test is performed when someone is accused of heavy drinking, which changes the prior odds (the odds that someone is indeed a heavy drinker

before the test results are in) and as a consequence the posterior odds (the odds that someone is a heavy drinker taking into account the test results).

8.4 The effect of simultaneous detection of EtG and FAEEs on the evidential value

It is often claimed that the combined use of EtG and FAEEs tests increases the value of the evidence. Süße et al. [101] and Pragst and Yegles [25] state that the additional use of other markers may increase the reliability of the interpretation due to different sensitivities to sources of error: either false negatives or false positives. It is, however, not proven statistically nor is an estimation provided for the increase of the value of evidence. Therefore, in this section calculations are presented for the first time to indicate that EtG and FAEEs values are independent and that when both tests are used the value of the evidence is higher than when only one test is used.

EtG and FAEEs test results (described as x and y respectively) are independent, when the probability of x given y is the same as the probability of x and the probability of y is the same as y under the condition x for independent tests. The $\text{LR}_{indep.x,y}$ of two positive tests is obtained by simply multiplying the individual likelihood ratios of the EtG positive and FAEEs positive test. In that case, LR_{both+} is: $17 \times 13 = 2.2 \times 10^2$ when the data from Tables 8.6 and 8.7 are used. By having both tests positive instead of only one changes 'moderate' support into 'moderate strong' support for heavy drinking. The same increase in verbal equivalent of the LRs is found when the sensitivity and specificity from Süße et al. [101] were used (see e.g. Table 8.11).

Data from Wurst et al. [7] were used to calculate whether EtG and FAEEs hair tests are independent for the infrequent drinkers. The assumption was made that the women tested by Wurst et al. were infrequent drinkers, because: i) they were in the third semester of their pregnancy and most women drastically reduce their alcohol consumption when they are pregnant [236] ii) hair samples were taken on a voluntary basis, five refused to participate, iii) participants were not selected

based on a suspicion of drinking alcohol during the pregnancy, but merely because they were pregnant and iv) the self-reports indicated that the women drink little to nothing during their pregnancy. Wurst et al. [7] stated how many of the 103 pregnant volunteers tested positive and negative for both EtG and the FAEEs and this was converted to probabilities of a test result by dividing the frequency by the total sample size (see Table 8.15). The cut-off value for EtG is slightly lower than the currently used cut-off value (i.e. 25 pg/mg instead of 30 pg/mg) and the probabilities of EtG- may have been smaller and EtG+ may in fact have been larger. The impact, however, is unknown of changing the cut-off value and the effect of the slightly lower cut-off value was assumed to be negligible.

Table 8.15: The probabilities of positive and negative test results for 103 pregnant women. Cut-off values for EtG: 25 pg/mg and for the FAEEs 1 ng/mg. Data from Wurst et al. [7].

	FAEEs+	FAEEs-
EtG+	0.00 (0/103)	0.04 (4/103)
EtG-	0.03 (3/103)	0.93 (96/103)

The values for specificity dependence were calculated with Equation 4.10 (see page 67) and were low (-0.001, -0.010, 0.010 and -0.001 for the specificity of 97% for EtG and 96% for the FAEEs). Furthermore, the specificity covariance (1.2% of maximal value) was also low. Hence, the addition of the FAEEs test to the EtG testing protocol increases the percentage of non-alcoholics who are correctly identified.

Similar calculations were performed for a group of assumed alcoholics (see Equation 4.11 on page 67). Table 8.16 contains information about FAEEs and EtG hair tests of 116 people who had a value for CDT above 1.3% [118] and were therefore assumed to be alcoholics. Especially because this test is used when heavy alcohol consumption is suspected and no factors that render the test less accurate apply [237].

With a sensitivity for EtG of 75% and sensitivity for FAEEs of 77%, and the values from Table 8.16 as the probabilities, the variances were calculated at 0.040, 0.095, 0.002 and 0.056. In summary, an overall low and positive dependence (6% of the

Table 8.16: The probabilities of positive and negative test results of 116 heavy drinkers: persons with a CDT test equal or higher than 1.3%. Cut-off values for EtG: 30 pg/mg and for the FAEEs 1 ng/mg. Data obtained from [118].

	FAEEs+	FAEEs-
EtG+	0.62	0.08
EtG-	0.19	0.11

maximal value) was found for the sensitivity variance. This means an increase in the percentage of alcoholics who are correctly identified when both tests are used instead of a EtG or FAEEs hair test alone.

Low variances of specificity and sensitivity are desirable when the tests are used in combination, because the EtG and FAEEs can then be assumed to be conditionally independent. The analysis of test dependence among EtG and FAEEs tests indicated a low negative specificity covariance and low positive sensitivity covariance. Biologically this can be expected due to the difference in metabolism, distribution, and incorporation in hair caused by the distinct chemical properties of the two metabolite groups. This is in agreement with Pragst and Yegles [25] who found no proportionality between the two markers.

Hence, the tests are independent when the tests are dichotomized based on the cut-off values that are currently used and specified by the SoHT. It cannot be assumed that this is generally true for all concentrations as Pragst et al. [3] and Süße [238] have previously demonstrated with their study of the correlation between the two tests. The value of the evidence for chronic heavy drinking with a positive test result for either EtG or FAEEs compared to two positive tests is increased from 'moderate' to 'moderate strong' support.

8.5 The practical application of the Bayesian theory on EtG and FAEEs hair tests

In Western society alcohol is a generally accepted drug. Therefore, when alcohol markers are tested for pre-employment as part of an application procedure the question is not whether someone is abstinent, but whether someone is a heavy drinker (an alcoholic to be more specific). The prior odds are small when no other evidence is at hand. In the UK two out of a hundred women are alcoholics and for men this is six out of a hundred [239]. Since no data are available for applicants in particular, the average values for the UK were used to indicate what a positive EtG or FAEEs test result implies.

Example 1: A man tests positive

A male applicant tests positive for EtG. The posterior odds in favour of being an alcoholic are then LR+ times the prior odds (see Equation 4.6 on page 65). The posterior odds are: $25 \times \frac{0.06}{0.94} = 1.6$ when the calculated LR+ is used from the sensitivity and specificity presented by Süße et al. [118] and the prior odds are calculated based on the percentage of alcoholics in the male UK population (6% according to a website about alcoholism [239]). A LR can also be seen as a indication of how many times more (or less) likely a test result of a given level is obtained when someone is an alcoholic or is not an alcoholic [229, 240]. It is thus only 1.6 times more likely that this person is an alcoholic than a non-alcoholic. This can be converted to a probability (with Equation 4.7 from page 65), resulting in a probability of approximately 62% of being an alcoholic.

Example 2: A woman tests positive

For a female applicant who tests positive for FAEEs, the posterior odds is:
$19 \times \frac{0.02}{0.98} = 0.39$ with a probability of 28%, when the calculated LR+ from the data of Süße et al. [118] and the prior odds based on the female UK population of which 2% is an alcoholics are used [239]. This means that even though the test is positive, it is more likely that the woman tested is a non-alcoholic than an alcoholic while the LR provided 'moderate' support for alcoholism. This is remarkably different from the man in example 1, who had a 62% chance of being

an alcoholic based on a LR that also provided 'moderate' support for alcoholism.

A comparison of men and women

The difference for men and women in posterior probabilities for randomly chosen individuals due to the difference in prior probabilities is observed both for a single positive test and when the two tests are used and both subjects test positive for both markers. With a combined LR+ for EtG and FAEEs of: $19 \times 25 = 4.8 \times 10^2$, a posterior odds of 31 is obtained for men and of 9.8 for women. The probability for men being an alcoholic given two positive tests is: 97% and for women 91%. Hence, for two positive tests the difference between men and women is smaller because the evidence is stronger ('moderate strong' instead of 'moderate') and the impact of the prior odds therefore is diminished.

Example 3: A man with certain concentrations of EtG and FAEEs

Instead of the cut-off value, the calculated LRs for a particular range of concentrations may be used (see Tables 8.6 and 8.7). In this example, a random man is tested and thus a prior odds is assumed of $0.06/0.94$. He had an EtG concentration of 40 pg/mg and FAEEs concentration of 1.5 ng/mg in hair. This would be seen as a clear sign for alcoholism when the test is dichotomized. For the combined LR of this example either $3.4 \times 4.1 = 14$ or $17 \times 13 = 2.2 \times 10^2$ is obtained depending on which category is used (resp. 30–50 or ≥ 30 for EtG and 1.00–1.99 or ≥ 1.00 for FAEEs). It is considered to be 14 times as likely to find this evidence given that someone is an alcoholic than a non-alcoholic when the lower LRs are used. This is approximately 220 times as likely for the higher LRs. As a result the combined LR is 'moderate' or 'moderate strong' support for alcoholism and a posterior odds of $14 \times \frac{0.06}{0.94} = 0.90$ or $2.2 \times 10^2 \times \frac{0.06}{0.94} = 14.07$ and posterior probabilities of 47% or 93% are calculated (using Equation 4.7 on page 65). This means that the prior probability of 6% is significantly increased with the evidence provided. The concentration range used, may thus have an impact on the posterior probability. Whereas the higher values represent a binary approach, the smaller values are chosen for a smaller interval and therefore excludes the extremely high tests results that are only observed for heavy drinkers. The latter is in favour of the tested person and results in a lower posterior probability. Note that a multiplication of the LRs of different concentration ranges

other than below and above the cut-off value for heavy drinking may not be correct because it has not been proven that these are independent from each other, this calculation is done purely for demonstrative purposes.

Discussion of examples

Since the prior odds are in general smaller for pre-employment testing for which heavy drinking is not suspected than for legal or medical testing in which alcoholism is suspected, caution is required for the interpretation of these test results. This is in line with what was suggested by Kintz and Mangin [74] in 1995 for hair testing in general when hair testing was relatively new. They stated that "the use of hair analysis for employee and pre-employment drug testing is premature and cannot be supported by current information on hair analysis for drugs of abuse". In addition they stated that hair may be useful when it is supported by other evidence of drug use. Though more than a decade of research has passed, one clearly still needs to be cautious about the conclusions that can be drawn. Other evidence to support a claim of chronic heavy drinking, drinking or abstinence is therefore recommended. Hastedt et al. [235] also stress that the results obtained by hair analysis can only be a part of a proper assessment aiming to answer the question whether the employee is addicted. These types of alcohol testing, however, are currently undertaken as the guideline of Agius and Kintz suggests [111]. In addition, the working examples show that testing for both alcohol markers is highly recommended as is in agreement with the practical work done by Hastedt et al. [235].

From the examples it is clear that several limitations do exist with the Bayesian approach. Firstly, the sample group that is used to calculate the prior odds may not be similar to the group that is tested. The difference between the prior odds for men and women discussed in the second example shows that the prior odds are very important when one test is positive (when the LR used is 'moderate' support for heavy drinking) and less important for stronger evidence such as two positive tests (with a LR indicating 'moderate strong' support for heavy drinking). Secondly, although test results at the extremes of the distribution provide the strongest evidence, the estimates of the likelihood ratios at these values is very imprecise due to the sparse data at the extremes. Therefore in this work it was

chosen not to have a category with extremely high concentrations, whereas it is acknowledged that more information about especially the higher concentration ranges for the alcohol metabolites are interesting for the EtG and FAEEs tests interpretation. However, test results are much more likely to obtain a value in the middle of the distribution and not have an extremely high value; extremely low does exist with these tests and was relatively common for teetotallers.

At times the LR that are calculated will not be correct. When the LRs are calculated in a group where hair treatment is not an issue (i.e. no bleaching prior to an EtG test and no use of alcohol-containing hair products prior to the FAEEs hair test) the LRs need to be corrected for in case hair treatment is an issue for that particular subject. It is logical that the LR for a positive FAEEs test should decrease in case external contamination has occurred from hair products containing alcohol or FAEEs and that the LR of a negative EtG test should be increased in favour of (heavy) drinking. A model can be made that shows the qualitative inference, based on the models that were presented in Section 2.3.6. This was done and shown in Figures 8.4 and 8.5.

The positive FAEEs report will increase the probabilities of both external contamination and chronic elevated FAEEs concentrations in blood (see Figure 8.4(a)). If the signs of the nodes are then set to zero, increased knowledge of external contamination will further increase the probability of elevated FAEEs in hair (sign changes from zero to +). At the same time the probability of elevated FAEEs in blood will be reduced (see Figure 8.4(b)).

The negative EtG report will increase the probability of removal (bleaching in this case) and a chronic low concentration of EtG in blood (see Figure 8.5(a)). If the signs of the nodes are then set to zero, it is clear that the low concentration reported may be explained by bleaching and therefore bleaching will decrease the concentration of EtG in hair. Also, the probability of elevated EtG in blood will be increased, since it would be masked by the removal from hair (see Figure 8.5(b)).

In addition, to the observations from the working examples the calculations indicate how the databases with LR values can be used for casework. There is no
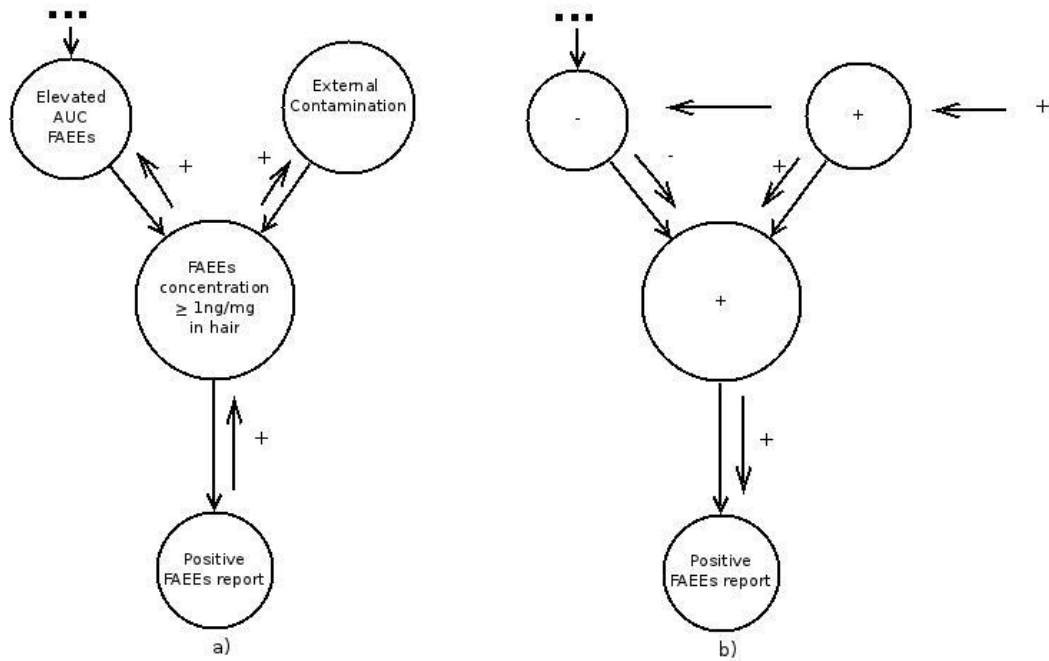
Figure 8.4: Model for an interfered LR result of FAEEs in hair due to external contamination. a) qualitative network for positive FAEEs report and b) qualitative belief propagation for the use of hair products that contain FAEEs or alcohol, where the + or - sign in the nodes indicate an increased or decreased probability and the + or - sign near the arrows show how the factors in the nodes influence each other given their probabilities.

need to calculate these LR values for a specific case, a value can be obtained from a table that is produced in the scientific field much like is done in this section. As stated previously, the LR values do need to represent the same group as the subject that undergoes hair testing. Especially when no background information is obtained concerning the hair products and hair treatment that is used by the subject (see Section 2.3.6).
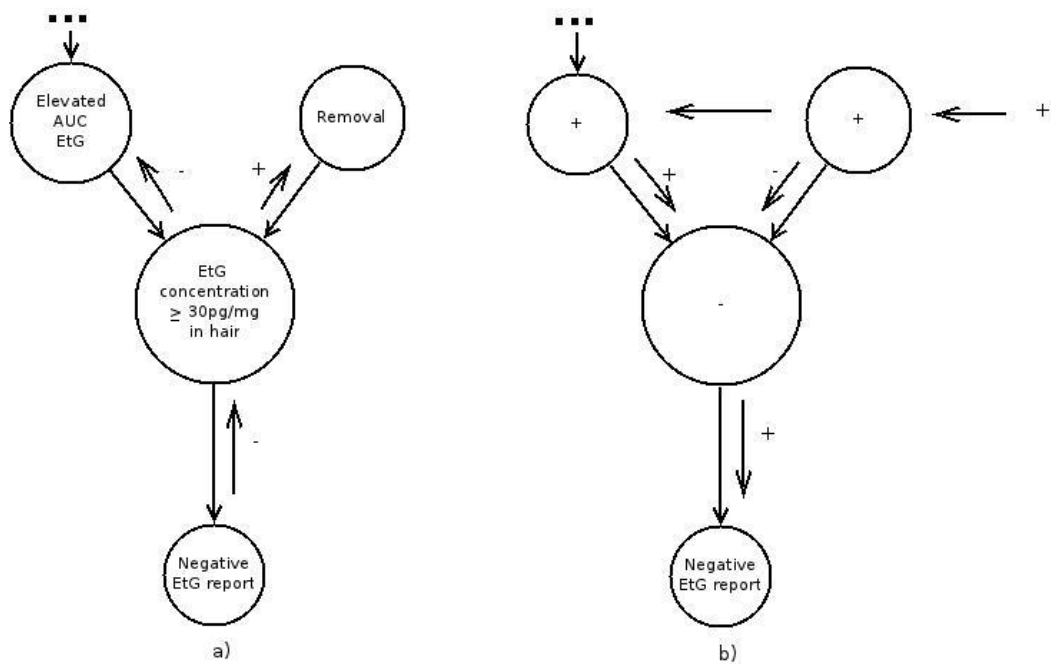
Figure 8.5: A model for an interfered LR result of EtG in hair due to bleaching. a) qualitative network for a negative EtG report and b) qualitative belief propagation when the hair is bleached. The + or - sign in the nodes indicates an increased or decreased probability and the + or - sign near the arrows show how the factors in the nodes influence each other given their probabilities.

8.6 Conclusion

A new application for the LR and Bayesian approach is introduced. For alcohol analysis in hair merely a cut-off value is currently used to distinguish between a positive and negative test result, whereas the application of the Bayesian theory is a better approach. More information is preserved about the exact concentrations and the prior odds are taken into account in a transparent procedure. EtG and FAEEs tests in hair are not yet described in this manner, whereas other forensic evidence is reviewed with the Bayesian approach. The reason that these methods should be considered in this field is that the evidence contains a degree of uncertainty (i.e. it cannot be concluded with a 100% degree of certainty that someone is a heavy drinker or not based on the concentration of EtG or FAEEs found in hair) and the Bayesian theory can account for these uncertainties. It is believed that when the evidence under the current cut-off system is verbally explained it may not be sufficiently clear because this translation step may lead to a less transparent and less scientifically based statement. The judiciary can better evaluate evidence and it is clear to them what assumptions are made to determine the evidential value when evidence is interpreted scientifically.

The database of approximately a hundred subjects for EtG and FAEEs hair test results indicated a higher evidential value to merely exclude abstinence and not to conclude abstinence. Stronger support was obtained for heavy drinking than for abstinence and EtG in hair seemed to be more suitable as test for abstinence than the FAEEs. This is in line with what is found in the literature (see [27, 72, 109]).

The likelihood ratios and their 95% confidence intervals calculated in this work indicate that evidence obtained with an EtG or FAEEs test in hair may only provide 'limited' support for drinking or heavy drinking. The 'initial database' had a CI from 'limited' to higher values for the strength of the evidence, and the larger databases including 'Hastedt's database' showed with a 95% confidence interval that the hair tests only provide 'limited' support for heavy drinking. The specificity and sensitivity of the FAEEs and EtG hair test may be well above other tests for heavy drinking and therefore better diagnostically, but for a low prior odds the posterior probability, the probability that someone is a heavy

drinker, is still relatively low when only one test is used as was shown in the working examples. This indicates that the value of evidence of a positive FAEEs or EtG hair test does not strongly suggest chronic excessive alcohol consumption in contrary to what was stated in the consensus of the SoHT on hair testing for chronic excessive alcohol consumption 2011 [38]. The perceived difference in the value of evidence may be explained by the fact that often an EtG hair test is performed when someone is accused of heavy drinking, which changes the prior odds (the odds that someone is indeed a heavy drinker before the test results are in) and as a consequence the posterior odds (the odds that someone is a heavy drinker taking into account the test results). The same accounts for testing for abstinence for which values ≥ 7 pg/mg for EtG and > 200 pg/mg for the FAEEs in hair is seen to "strongly suggest repeated alcohol consumption" [109]. However, in this consensus it is clearly stated that it is "not advisable to use the results of hair testing for alcohol markers in isolation", which refers to the prior odds that is discussed in this work. Also, as this work has proven the two tests can be combined to increase the evidential value of a positive and negative outcome.

The Bayesian theory is more than a calculation, it is a way of thinking. In court the Bayesian calculations that determine the conclusions should not be disputed, but the assumptions required for the analysis should and will be disputed as is pointed out in detail by Fenton and Neil [241]. The scientist should not have to reason in court about the Bayesian calculation themselves, much like lay persons do not need to understand how the calculator works in order to accept the results of calculations as being correct to a sufficient level of accuracy. The scientist should explain their findings in a clear and concise manner. In this work examples are provided for the likelihood ratios that provide intuitive information. It is logical that higher concentrations of alcohol metabolites would constitute a higher value of evidence in favour of (chronic heavy) drinking. It is also clear that evidence that offers only limited support for the hypothesis that someone is a chronic heavy drinker is not convincing enough (beyond reasonable doubt) when the test was performed without suspecting chronic heavy drinking and without any other evidence.

It is important to realize that the Bayesian theory brings transparency. In this

work the confidence interval was calculated for the value of the evidence that was mostly ignored in the work to establish a cut-off value. Kharbouche et al. [242] did calculate the CI for the receiver operating characteristic (ROC), which says something about how good the test is for the population not an individual as is the case for the calculations in this chapter. Also, the Bayesian theory is transparent in the sense that is shows why a scientist cannot directly address the question in court whether someone is a chronic heavy drinker or not, based on the hair test. Remember, the prior odds can majorly affect the posterior probability which in turn is the statement about one's drinking behaviour after hearing the evidence. In addition, in contrast with the traditional cut-off method more information can be obtained because the data are not dichotomized based on a cut-off value.

Chapter 9

Overall discussion and conclusion

9.1   The development of a combined method

A method that detects both EtG and FAEEs was developed. This combined method may be commercially more desirable than the use of the two separate methods currently in use (see Appendices E and F), since the analysis of both EtG and the FAEEs can result in stronger evidence to support someone's drinking behaviour than a test for a single marker. In our method development various steps in the sample preparation and analysis were examined to ensure the maximum sensitivity. This was important because the sensitivity was likely to be an issue because the two alcohol markers are completely different in terms of their chemical and physical properties (see Table 1.2 on page 14).

This work showed BSTFA to be a more suitable derivatisation reagent than PFPA since the latter caused accelerated degradation of the GC column and SPME fibre. It also showed that longer heating times and the addition of ethyl acetate during the derivatisation with BSTFA increased the derivatised product EtG-BSTFA. The EI full scan spectra revealed that possibly only four out of the five hydroxyl groups of EtG were substituted with TMS during the derivatisation process.

The optimised sample preparation and analysis steps were used to develop three combined extraction and detection methods (see Table 9.1). For this method the

hair was first segmented and approximately 30 mg was decontaminated with 1 mL DCM and then with 1 mL of methanol. Subsequently, the hair was dried and cut in mm pieces. The hair was accurately weighed and the analytes were extracted with 30 min sonication in one millilitre of methanol. 450 µL of the methanol extract was analysed for EtG and the other for the FAEEs. For EtG the methanol was partially evaporated, the standard SPE method (see Table 4.2 on page 55) was used with an Oasis MAX cartridge for EtG, and the clean extract was evaporated to dryness, derivatised with 20 µL BSTFA in the presence of 20 µL EA at 80 ℃ for at least 2 hours, left to cool and then analysed. For the FAEEs the aliquot was evaporated to dryness in a 10 mL HS vial and subjected to HS SPME with the 65 µm PDMS-DVB fibre (for the specifics of the HS SPME procedure refer to Table 4.4 on page 57). For the GCMS settings, oven temperature programme and ions detected in CI SIM refer to Tables 4.5, 4.6 and 4.7 in Chapter 4. The carrier gas was helium and the flow was set at 1 mL/min. For CI methane gas was used and this provided the most sensitive and specific ionisation method for the FAEEs because less fragmentation occurred and the protonated molecular ions were more abundant than the molecular ions in EI.

The combined analysis of EtG and FAEEs with methanol extraction was fully validated. Standard curves were linear (r^2 >0.99) from 0.3 ng/ml to 5 ng/ml for EtG and from 3 ng/ml to 50 ng/ml for the FAEEs. The limit of detection was between 0.04 and 0.09 ng/mg for the FAEEs and 5 pg/mg for EtG when 30 mg hair was used. No carry over was detected following injection of twice the concentration of the highest calibrator. Neither EtG nor FAEEs were found above the LOQ in the blank samples. The intra-day variation (n=3) and inter-day variation (n=9) were 84–111% of the actual value with a coefficient of variation of 3–30% for the FAEEs and the accuracy for the spiked EtG hair control samples were between 95% and 118% with a coefficient of variation (CV) of less than 23%. Moreover, this method saves at least 12-24 hrs and uses less chemicals and may therefore reduce the cost compared to the separate analysis of the two alcohol markers EtG and FAEEs.

Table 9.1: The optimal conditions used in this work.

When	this is preffered over	this/ these
For storage	silanized glassware	not silanized glassware
For sample preparation	not silanized glassware	silanized glassware
For storage and sample preparation	glassware	plastic containers
For the storage of the FAEEs	MeOH	heptane
For decontamination of hair	DCM and MeOH	water+DCM, n-heptane, water+n-heptane, 2×DCM, diethyl ether+acetone, water+acetone+methanol, methanol, methanol+acetone, water+acetone and water
For the combined extraction	MeOH	water, heptane+DMSO, acetone, methanol+water and methanol+DCM
For the analysis of the FAEEs	CI	EI
For the sensitivity of the method	SIM	MSMS
For the FAEEs	short evaporation	longer and more frequent evaporation steps
After derivatisation with PFPA	longer evaporation	shorter evaporation
For the derivatisation of EtG	BSTFA	PFPA
For HS SPME	65 µm PDMS-DVB fibre	85 µm PA, 65 µm CW-DVB, 75 µm CAR-PDMS, 55/30 µm DVB-CAR-PDMS, 7, 30 and 100 µm PDMS

9.2 Interpretation of alcohol hair tests and the results of these tests

A higher ethyl oleate concentration in real hair samples may be the result of more efficient incorporation as indicated by our studies on reference hair by soaking

a blank hair sample in a solution of FAEEs in equal concentrations, followed by washing. Here final analysis showed a relatively high concentration of ethyl oleate compared to the other FAEEs.

The analysis of both EtG and the FAEEs can result in stronger evidence to support someone's drinking behaviour than a test for a single marker. This was previously suggested, but in this work it was for the first time indicated statistically. EtG and FAEEs tests were shown to possibly be statistically independent. This is probably caused by the difference in chemical properties which affects analyte formation processes, analyte distribution over the body, analyte incorporation mechanism in hair and different methods to possibly decrease the amounts of analytes in hair (e.g. harsh hair treatment). In addition, other products (containing either FAEEs/alcohol or EtG) may result in the incorporation of the metabolites in hair, possibly resulting in a false positive test result.

A new application for the Bayesian (including LR) approach is suggested for the interpretation of alcohol metabolites in hair. At the moment, for the interpretation of alcohol analysis in hair merely a cut-off value is used to distinguish between a positive and negative test result. A Bayesian approach is better because it allows for a more gradual scale of how likely it is to find this concentration in hair with two opposing hypotheses (e.g. heavy drinking versus not heavy drinking). Hence, more information is preserved about the exact concentrations and the prior odds are taken into account. The scientist needs to articulate the assumptions that are made which makes the Bayesian approach a transparent procedure. More importantly, with this approach, the value of the evidence is expressed in a statistically sound manner which can relatively easily be translated to the specifics of the case. It is important to note that the prior odds can majorly affect the posterior probability which in turn is the statement about one's drinking behaviour after hearing the evidence. Another reason why the Bayesian approach should be considered in this field is that the evidence contains a degree of uncertainty and the Bayesian theory can account for these uncertainties. Briefly, EtG and FAEEs tests in hair are not yet described in this manner, whereas other forensic evidence is reviewed with the Bayesian approach and the interpretation of the evidence in this manner should be considered in this field.

The use of the Bayesian approach was demonstrated on a relatively small database (approximately a hundred subjects for EtG and FAEEs hair tests) as well as larger databases that did not contain certain details. The small database resulted in relatively large 95% confidence intervals, but the observations that were made were generally in line with the literature. The results indicated a higher evidential value to merely exclude abstinence and not to conclude abstinence. This is in line with what is described in the consensus for abstinence by the SoHT [109]. EtG in hair is a more suitable test for abstinence than the FAEEs. This is also reflected in the consensus of the SoHT [109]. Stronger support was obtained for heavy drinking than for abstinence. The likelihood ratios did paint another picture than what the literature suggests. The values for the support of chronic drinking are relatively low. The likelihood ratios and their 95% confidence intervals calculated in this work indicate that evidence obtained with an EtG or FAEEs test in hair may only provide 'limited' support for drinking or heavy drinking. The small database used has a CI from 'limited' to higher values for the strength of the evidence, but a larger database showed (with a 95% confidence interval) only 'limited' support for heavy drinking. This means that for a low prior odds the posterior probability, the probability that someone is a heavy drinker is still relatively low when only one test is used, as was shown in this research.

9.3 Future research

Based on the investigations that are presented in this thesis, more work can be done on the topic of EtG and FAEEs analysis. A couple of ideas are presented in the next sections.

9.3.1 More research into a combined method of EtG and the FAEEs

A combined injection with a sufficiently high sensitivity was not achieved in this research. This may be achieved with LC. No derivatisation step is required

if LCMS is used, which makes the sample preparation easier for the combined detection. Furthermore, the interference that was increased due to the derivatisation step will not be observed now. In addition, a combined method can be developed for other matrices (see Section 9.3.2) that have a higher concentration of the alcohol markers. Therefore this combined method would not need the type of sensitivity that caused the combined injection to be discarded.

9.3.2 The use of other matrices to investigate someone's chronic drinking behaviour

Hair is not always available and other keratin matrices store the alcohol metabolites too. Fingernails and bonetissue may be an interesting alternative when hair is not available [243]. More so, fingernails contain EtG and the FAEEs in a higher concentration than in hair (29.1 ±55.6 pg/mg versus 9.48 ±22.3 pg/mg) [244]. The feasibility and cut-off values for these other matrices need to be established.

Hair from other sites than scalp hair may be worth looking into. The difference between social drinkers and heavy drinkers appears to be bigger in pubic hair than scalp hair if one looks at the work of Pirro et al. [81]. For this reason a negative pubic hair sample is considered to strongly confirm abstinence according to the consensus of the SoHT [109]. Note that pubic hair has at least one disadvantage over that of scalp hair; it is considered to be an intimate sample by the scenes of crime handbook [245]. This means that the test can be perceived as more invasive and that in casework more regulation applies. An intimate sample can only be taken by a registered medical practitioner or registered nurse. Also at least two non-intimate samples suitable for the same means of analysis should be taken during the investigation which are proved to be insufficient for the analysis (in quality or quantity) [245]. Other non-head hair samples that may be looked at are: beard, chest, arm, leg and axillary hair. These different hair types have been investigated for the alcohol metabolites by Pirro et al. [81], Hartwig [80], Süße et al. [101], Pianta et al. [246] and Kerekes et al. [76], but with a small sample group.

9.3.3 More accurate determination of the LRs for alcohol analysis in hair

When the view is accepted that the Bayesian theory is important in hair testing, more research can be done to generate larger databases and discover the true value of the LRs with probably smaller confidence intervals than those obtained in Chapter 8. The minimal size of the database depends on the ratio of the three different groups and the estimates in this work (see Tables 9.2 and 9.3 calculated with the method described by Simel et al. [182]) can be used as a guideline. The LRs of the larger database can then be used in casework and the preliminary findings in this work can be evaluated properly. This larger database can be evaluated with a similar approach as described in this work. It would be an advantage if the data set would be from one source to avoid possible aggregation problems that exist with the use of several sources. Also, when both EtG and FAEEs concentrations in hair are obtained for the subjects, the dependency can be calculated for EtG and FAEEs for different concentrations. Other information can also be collected to aid in the investigation such as hair products that are used. To establish the drinking behaviour of a subject, different methods can be used such as an interview, an anonymous questionnaire and the opinion of friends and family. Comasco et al. discovered in their study that adolescents in Sweden under-reported in the questionnaire compared to interviews [130]. Hence, to obtain more robust knowledge about someone's drinking behaviour more than one method can be used and data can be excluded when contradictory information is obtained.

9.3.4 Larger databases to calculate the effect on the LR

It is known that not only alcohol consumption affects the concentration of EtG and FAEEs in hair. It has been suggested that diabetics have a different alcohol metabolism [247], also ethnic biases exist [248] and hair growth agents may affect the concentration of EtG and FAEEs in hair. Süße et al. [101] concluded that physical fitness or obesity do not affect the FAEEs hair test. As is often the case

Table 9.2: Total sample size required for LR+$_{min}$=10 for EtG with different ratios of heavy drinkers to not heavy drinkers.

$r = n_1/n_2$	n_1 (no. heavy drinkers)	n_2 (no. not heavy drinkers)	Total sample size
0.05	9	180	189
0.1	17	170	187
0.5	76	152	228
1.0	150	150	300
2.0	298	149	447
3.0	446	149	595
5.0	742	149	891
10.0	1481	149	1630

Table 9.3: Total sample size required for LR+$_{min}$=10 for the FAEEs test with different ratios of heavy drinkers to not heavy drinkers.

$r = n_1/n_2$	n_1 (No. heavy drinkers)	n_2 (No. not heavy drinkers)	Total sample size
0.05	14	269	285
0.1	24	242	266
0.5	111	221	332
1.0	218	218	436
2.0	434	217	651
3.0	648	216	864
5.0	1080	216	1296
10.0	2160	216	2376

with these relatively small databases, no consensus is reached on these points for different research points in different directions. Working together and developing a large database in which all possible factors are considered will shed some light on the current controversies surrounding hair testing. In addition, a principle component analysis may be performed with this database, which may be more accurate than assuming that one factor has a direct influence on the EtG or FAEEs hair test.

9.4   Final synopsis

This study has shown that the combined information of EtG and FAEEs concentration in hair increases the evidential value of positive hair tests as was previously only hypothesized in the literature. Therefore a combined analysis of EtG and FAEEs in hair is better than using a single hair test of either EtG or FAEEs. This research aimed to have a combined analysis of EtG and FAEEs in hair so that in a single test the information on both markers could be obtained, which may be less expensive for the industry than running two single tests one for EtG and one for FAEEs. This research has shown that a combined extraction and detection is possible for two very different groups of analytes: EtG and FAEEs. The fully validated combined method where these analytes are extracted together saves time and possibly money. The feasibility of implementing this combined work in the industry is promising when a combined injection without the significant loss of the sensitivity of the analytical method is achieved.

In this work the Bayesian theory is recommended to be used for the interpretation of hair tests for the first time to the knowledge of the author. This way the controversies raised in the beginning of hair research (see Chapter 2), which definitely are not yet solved, may be cleared up. It is also clear from the examples given in Chapter 8 how the Bayesian theory can be applied in casework and represented in court. This would have a major impact on the interpretation of hair tests. For both the scientific world as the legal world more data is required for final conclusions based on the interpretation with the Bayesian theory. In this work, a clear and transparent setting is used as proof of concept. This indicated that when the concentration of a alcohol marker also the support for (heavy) drinking increases and that binary systems with a cut-off value unnecessary reduce the available information. It has also shown that the evidence of a hair test can be combined with other evidence. This proof of concept also indicated that abstinence is more difficult to proof with these tests than heavy drinking, which was suggested before in the literature but not yet shown with a statistical assessment as was done here. Moreover, this theory provides insight in what an expert can and cannot say which is important in order not to mislead

the judiciary. With this approach the data interpretation is made explicit for the likelihood ratios can only be determined with certain information that is either known or assumed. This aids in open discussion amongst the experts. The new application of the Bayesian approach may thus be very useful in a legal setting to indicate the strength of the evidence obtained with the hair test and show what is considered for the interpretation of the evidence as well as for scientific research to address the controversies that are raised concerning hair analysis. When likelihood ratios cannot be calculated, because not enough background information is available, it is suggested to use a more qualitative approach and recommended to use both alcohol markers. Briefly, the Bayesian way of thinking is suggested in this research and would open the door to a good conversation about the hair test results amongst scientific researchers and with lay persons, whereby experts will be reminded of the boundaries of their knowledge.

Appendices

Appendix A

Questionnaire

Title of Project	Monitoring Alcohol Consumption through Hair Analysis
Name of Researcher	L. Bossers - PhD student
Research Facility	The University of Glamorgan
Reference ID number	....
Date	....

You are kindly requested to complete this form. However, it is not a prerequisite to participate in this study. If you prefer not to fill in the questionnaire or only answer some of the questions, you can still participate in this research study.

If you do fill out the form, you can be assured that every effort will be made to ensure confidentiality of any identifying information obtained in connection with this study. Obtained data will only be used for research purposes. No information will be disclosed to third parties.

If you have any questions or concerns regarding the research, please do not hesitate to contact Lydia Bossers at lbossers@glam.ac.uk or 07 831 989 474.

QUESTIONS

Please try to answer the questions as honest and accurately as you can. All information will be treated in strict confidence.

Please tick the box with the most applicable statement.

Drinking habits

1. In the last year, how often did you have an alcoholic drink?
   [ ]   Never
   [ ]   Monthly or less
   [ ]   2–4 times a month
   [ ]   2–3 times a week
   [ ]   4 or more times a week

2. How many drinks containing alcohol did you ave on a typical day when you were drinking?
   [ ]   1 to 2
   [ ]   3 to 4
   [ ]   5 to 6
   [ ]   7 60 9
   [ ]   10 or more

3. Has your drinking behaviour changed over the last year? If yes, please indicate in which way. For instance, periods of abstinence (duration and when), increase or decrease of average alcohol intake.
   . . . .
   . . . .

If you need more writing space, you can use the back of this sheet.

Hair history

1. Have you gone swimming in a swimming pool in the last three months?
   [ ]   No
   [ ]   Yes, approximately . . . times

2. When is the last time you washed your hair with shampoo?
   [ ]   Less than 24 hours ago
   [ ]   Less than 7 days ago
   [ ]   Less than 18 days ago
   [ ]   More than a month ago

3. Please indicate how many times you have used the following hair care products in the last three months.

   - Shampoo: . . . .

   - Hair conditioner: . . . .

   - Hair colouring products: . . . .

   - Hair bleaching products: . . . .

   - Permanent wave products: . . . .

   - Hair spray: . . . .

   - Hair wax: . . . .

   - Hair gel: . . . .

   - Other, namely . . . . . . : . . . .

4. If you can remember or look it up, please indicate the brand and/or percentage of alcohol in each of the hair care products you have used in the last three months.

   - Product: Brand, Percentage

   - Shampoo: . . . . , ... %

   - Hair conditioner: . . . . , ... %

   - Hair colouring products: . . . . , ... %

   - Hair bleaching products: . . . . , ... %

   - Permanent wave products: . . . . , ... %

   - Hair spray: . . . . , ... %

- Hair wax: ...., ...%
- Hair gel: ...., ...%
- Other, namely ....: ......%

Appendix B

Consent form

CONSENT FORM RESEARCH STUDY

Title of Project	Monitoring Alcohol Consumption through Hair Analysis
Name of Researcher	L. Bossers – PhD student
Research Facility	Faculty of Health, Sport and Science at the University of Glamorgan
Date	. . . .

Please tick the box on left side to confirm a statement

[ ]   I confirm that I have read and understand the information sheet date ... for the above study.

[ ]   I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without any medical care/treatment or legal rights being affected.

[ ]   I understand that data collected during the study, may be looked at by responsible individuals/researchers from the University of Glamorgan.

[ ]   I understand that any information provided is confidential, ant that no information that could lead to the identification of any individual will be disclosed in any reports on the project, or to any other party. No identifiable personal data will be published or shared.

[ ]   I give permission to use any data obtained during this study for research purposes concerning alcohol analysis only.

[ ]   I agree to take part in the above research study

[ ]   I understand that this is for initial alcohol research and, therefore, no information about the alcohol concentration can be obtained by the participant or linked to the participant.

[ ]   I have had the opportunity to ask any questions about this study.

[ ]   I have been given a copy of this consent form.

_____

Signature of research participant

After completion of the consent form:

- One copy should be provided to the participant

- One copy should be provided to the participant's physician or therapist at the rehabilitation centre

- The original form should be kept in the research file of the responsible researcher at the University of Glamorgan

_____   _____   _____
Name of Participant   Date   Signature

_____   _____   _____
Name of Witness   Date   Signature

_____   _____   _____
Name of Researcher   Date   Signature

[ ]   The researcher has to tick the box on the left to confirm all the boxes on the consent form have been ticked and subsequently has been signed by the participant and a witness before using hair samples for research.

Appendix C

The performance of different markers for alcoholism

Different markers exists for alcoholism. In Table C.1 different markers are mentioned with their sensitivity and specificity.

Table C.1: Different markers for alcoholism can be compared based on their sensitivity and specificity.

Marker (abbreviation)	Half life	Sensitivity	Specificity	Source
Mean corpuscular volume of erythrocytes (MCV)	2–4 months	34–40%	95%	[249]
		<50%	34–89%	[41]
		<50%	>90%	[250]
		40–50%	80–90%	[251]
Gamma-glutamyltransferase (GGT)	2–3 weeks	47–52%	99%	[249]
		30–50%		[41]
		30–50%	40–90%	[250]
		54–65%	91–97%	[252]
		45–50%	55–100%	[251]
Carbohydrate-deficient transferrin (CDT)	2–3 weeks	34–39%	98%	[249]
		60% in men, 29% in women	92%	[253]
		73% in men, 41% in women	90%	[252]
		12–45%	80–95%	[251]
		82%	97%	[254]
GGT-CDT combination (γ-CDT or GGT-CDT)	2–3 weeks	90%	98%	[249]
		75–90%	91–87%	[252]
Aminotransferases (AST, ALT)	2–3 weeks	39–50%	87% and 95%	[249]
AST		25–60%	-	[251]
ALT		15–40% for ALT	-	[251]
AST		>90%	>90%	[250][a]
ALT		>80%	>80%	[250][a]
Hair Ethyl glucuronide (EtG)	N/A	100%	95%	[36]
			89%	[188]
		100%	98%[b] and 100%[c]	[108]
Hair Fatty acid ethyl esters (FAEEs)	N/A	70%[d] or 90%[e]	nearly 100%[d] or 90%	[27]
		100%	94.4%	[6]

[a] for monitoring drug abuse
[b] with HPLC
[c] with immunonephelometry
[d] for 1.0 ng/mg
[e] for 0.5 ng/mg

Appendix D

Review determination cut-off values

In Table D.1 a summary of the review of the cut-off determination of EtG in hair is presented and in Table D.2 for the FAEEs in hair.

Table D.1: The grounds on which cut-off values for EtG in scalp hair are determined in different articles. EDI=ethanol daily intake, ROC=receiver operator curve, n=total sample size of the data set used.

Source	Data set	Conclusion	Analysis
Skopp et al. 2000 [39]	n=14. Medical reports of heavy drinking were obtained from 4 of them, 7 were known to consume alcohol on a socially accepted level and 3 children were teetotallers. None of the subjects underwent hair treatment.	No EtG was detected for social drinkers and teetotallers. The detection of EtG in hair may help to identify some subjects who have consumed alcohol.	Comparing concentrations for different groups
Alt et al. 2000 [158]	n=31. 5 children, 6 self-reported social drinkers consuming up to 20 grams of alcohol per day and 20 alcoholics. The alcoholics were diagnosed as such medially of which 16 hair analysis were performed post-mortem	Alcoholism strongly assumed when EtG is detected, but heavy alcohol consumption is not excluded when EtG is not detected.	Comparing concentrations for different groups
Janda et al. 2002 [107]	n=97. 5 children, 5 self-reported social drinkers, 27 post-mortem cases that have a history of alcohol abuse and 60 subjects were sampled when they were hospitalized for alcohol withdrawal.	No correlation was found between self-reported alcohol consumption and EtG concentration in hair. No proof for abstinence can be provided with this test, but test can be used as proof of drinking.	Comparing concentrations for different groups and correlation graph
Politi et al. 2006 [255]	n=50. 22 known alcoholics, 21 subjects with a self-reported EDI between 2 and 60 g and 7 teetotallers.	Concentration EtG in hair appears to correlate with EDI.	Evaluation positive and negative results for different cut-off values
Appenzeller et al. 2007 [60]	n=15. All subjects were enrolled in a treatment programme for alcoholics.	A correlation was found for EtG concentrations in hair and the self-reported drinking amounts.	Statistical analysis of the correlation
Morini et al. 2009 [116]	n=98: 23 had a 3 month EDI of below 60 g per day and 75 equal or higher levels according to self-reports.	A cut-off value of 27 pg mg^{-1} of EtG in hair to distinguish heavy drinkers from others is appropriate and confirms that EtG in hair is a sensitive and specific marker due to the sensitivity of 92% and specificity of 96%.	ROC analysis and Mann Whitney test
Concheiro et al. 2009 [256]	n=17, all withdrawal patients	EtG in urine and in hair proved to be a powerful tool for monitoring abstinence in these patients	Comparing EtG and ethanol in urine with EtG hair test
Pragst et al. 2010 [3]	n=174, alcohol consumption unknown.	30 pg mg^{-1} EtG in the proximal hair segment 0–3 cm seems to be an optimal compromise for the discrimination between social drinking and excessive alcohol consumption.	Evaluation positive and negative results based on various cut-off values
Süße et al. 2011 [118]	n=261 of which 111 are abstinent or low moderate drinkers and 150 drink excessively according to self-reports. Total data set: n=1872.	A cut-off value for EtG in 3 cm hair segments is selected of 30 pg mg^{-1} based on the sensitivity of 75% and specificity of 97%.	ROC analysis of a small selection of the data set
Albermann et al. 2011 [122]	n=835 of unknown alcohol usage, tests were performed to re-grant driving license.	The variation of the cut-off value for EtG in hair had a minor impact on the number of positive and negative tests for abstinence.	Evaluation amount positive and negative tests for different cut-off values
Kharbouche et al. 2011 [242]	n=125: 43 teetotallers, 44 low-risk drinkers and 38 at-risk drinkers	Values of EtG in hair above 9 pg mg^{-1} suggest an alcohol consumption of 20 to 30 g per day and values above 25 pg mg^{-1} suggest a consumption of more than 60 g per day.	ROC analysis and Kruskal-Wallis test
Kronstrand et al. 2011 [188]	n=44 of which 21 drinkers who participated with this study consumed 32 or 16 g per day for 3 months and 23 were abstinent.	All were below cut-off value for chronic heavy drinking of 30 pg mg^{-1}.	Evaluation concentrations detected
Lees et al. 2012 [128]	n=100 volunteers. Categorized into 4 groups based on a questionnaire: 81 teetotallers, 8 lower risk drinkers, 30 increased risk drinkers and 19 high risk drinkers.	29 samples were above the cut-off value for EtG in hair, which entails 57.9% of high risk, 45.5% increased risk and 9.8% of lower risk. EtG in hair is thus a marker with low sensitivity.	Statistics based on a previously determined cut-off values

Table D.2: The grounds on which cut-off values of FAEEs in hair are determined in different articles.

Source	Data set	Conclusion	Analysis
Auwärter et al. 2001 [115]	n=47. 19 alcoholic patients in withdrawal treatment, 10 fatalities with a history of excessive alcohol consumption, 13 self-reported social drinkers and 5 teetotallers.	Not ambiguously possible to confirm or refute the claim of abstinence because teetotallers can have low FAEEs concentrations in hair. Social drinkers had values up to 0.85 ng mg^{-1} and concentrations for heavy drinkers were found between 0.35 and 13.5 ng mg^{-1}.	comparing concentrations for different groups
Pragst et al. 2001 [114]	n=41. 21 fatalities known to have abused alcohol, 10 social drinkers ranging from 30 to 60 grams per week and 10 teetotallers.	Alcoholics are clearly distinguishable from those of social drinkers and teetotallers based on FAEEs concentration in hair.	Comparing concentrations for different groups
Hartwig et al. 2003 [80]	n=28. 1 teetotaller, 5 moderate social drinkers and 22 fatalities of known (based on police reports and medical files) heavy drinkers.	Cases of chronic excessive alcohol consumption were characterized as such in almost all samples with a previously established cut-off value of 1.0 ng mg^{-1} for FAEEs in hair.	Comparing concentrations for different groups
Wurst et al. 2004 [6]	n=48. 18 alcohol-dependant patients in detox, 10 social drinkers and 10 teetotallers. The classification was done based on self-reports and the detection of other markers for alcoholism.	A cut-off value of 0.4 ng mg^{-1} for the FAEEs in hair was chosen based on sensitivity of 94.4% and specificity of 90%. No correlation between concentration of FAEEs in hair and total alcohol consumption last month.	ROC and correlation analysis (Kruskal Wallis test)
Auwärter et al. 2004 [223]	n=50. 13 teetotallers (children and adults who definitely did not drink), 16 self-reported and to the researchers familiar social drinkers, 12 fatalities with a history of excessive alcohol abuse (legal or medical files), 9 unclear alcohol anamnesis.	When the concentration FAEEs divided by the concentration of Squalene is below 0.6 ng μg^{-1} abstinence is indicated and above 1.5 ng μg^{-1} alcohol abuse. However, due to high values for some that cannot be explained by drinking this new measure cannot replace the FAEEs concentration fully.	Comparing concentrations for different groups
Yegles et al. 2004 [186]	n=25. 10 alcoholics from withdrawal treatment of which 5 self-reported to be abstinence, 11 fatalities with documented excessive alcohol consumption, 4 self-reported moderate social drinkers and 3 children (strict teetotallers).	FAEEs concentration only observed above 1 ng mg^{-1} for excessive alcohol consumption. Previously found cut-off value is thus confirmed.	Comparing concentrations for different groups
De Giovanni et al. 2007 [61]	n=32. 12 alcoholics, 10 social drinkers and 10 teetotallers.	Despite the slight overlap in concentration between the group, the results show the usefulness of these biochemical markers in the diagnosis of alcoholism.	Comparing concentrations for different groups
Pragst et al. 2008 [25]	n=124. 38 self-reported moderate social drinkers, 65 known alcoholics, 21 death cases with a history of heavy drinking.	For a cut-off value of 0.5 ng mg^{-1} for FAEEs in hair the test could differentiate between heavy drinkers and social drinkers with a sensitivity of 90% and a specificity of 90%.	ROC analysis
Pragst et al. 2010 [3]	n=174, alcohol consumption unknown.	0.50 ng mg^{-1} FAEEs in the proximal hair segment 0-3 cm seems to be an optimal compromise for the discrimination between social drinking and excessive alcohol consumption.	Evaluation positive and negative results based on various cut-off values
Süße et al. 2011 [118]	n=229 for FAEEs in hair of which 92 are abstinent or low moderate drinkers and 137 drink excessively according to self-reports. Total data set: n=1872.	A cut-off value for FAEEs in 6 cm hair segments of 1.0 ng mg^{-1} is suitable with a sensitivity of 77% and a specificity of 97%.	ROC analysis of a small selection of the data set

Appendix E

Summary EtG methods

Table E.1: Summary of the different methods that are used for the detection of EtG in hair.

Source	Sample preparations	Extraction	Work-up	Analytical method	Detection limit
[257]	Dry hair segments (1–3 cm) were washed with DCM and MeOH, then dried under N₂, 10–100 mg weighed.	The hair was cut in small pieces (1–5 mm) and water and DIS in a methanolic solution was added. The sample was centrifuged, incubated overnight at room temperature then 2 h of ultrasonication.	Sample was centrifugate.	LC was used. The m/z ions monitored for EtG: 221/75, 221/85, 221/113 and for EtG-D5: 226/75, 226/85	LOD: 1 pg mg⁻¹ LOQ: 4 pg mg⁻¹
[44]	10–50 mg hair was washed with 2 ml water and 2 ml acetone and dried.	2 ml water and 2.5 ng internal standard: 25 µl of 10 ng/ml EtG-D5 in MeOH was added to pulverized hair. Then 2 h ultrasonication at 40 °C.	Centrifugation. Clean-up with SPE Cleanscreen: Conditioning with 1 ml of 1% formic acid, addition sample, washing with 1 ml water, EtG is eluted with 2 ml 1% formic acid in MeOH mixture. Derivatisation: 40 µl HFBA for 15 min at 80 °C. Remaining reagent evaporated prior and after derivatisation.	HS SPME in combination with GCMS. Extraction and injection was performed with 75 µm CAR-PMDS SPME fibre for 10 min at 90 °C. GC setting used, were: a fused silica capillary column, l=20m x 0.15mm ID x 0.30 µm film thickness, a Helium flow of 1 ml/min, in splitless mode, injection port temperature of 250 °C, transfer line temperature of 250 °C and an oven temperature of 75°C for 1 min then increased by 20 °C/min to 300 °C. MS settings: ionized with methane at a pressure of 3 Torr and temperature of 250 °C, collision cell filled with Argon at 1 mtorr and 10 eV, third Q set on 1200 V. Transitions 596/427 and 601/423 were used as quantifiers and 596/288 and 601/288 as qualifiers.	LOQ: 2.8 pg/mg LOD: 0.6 pg/mg
[134]	100 mg hair washed with 1 ml dichloromethane and then with 1 ml MeOH.	Incubated overnight with deionized water spiked with 10 ng EtG-D5 used as internal standard. The following day the sample was sonicated for 2 h.	The sample was centrifuged at 13,000 rpm and then objected to SPE to reduce ion suppression. Dimethyl butylamine cartridge was used. The cartridge was activated with 1 ml MeOH, 1 ml ACN before loading the extracted organic solution. After washing a step with 1 ml hexane, EtG was eluted with 1 ml deionized water.	LCMSMS: 8 µl was injected, on a C18 column with a flow rate of 200 µl/min. m/z values used: 221/75, 221/85 and 221/221 for EtG and 226/75, 226/85 and 226/226 for EtG-D5.	LOD: 2 pg/mg LOQ: 3 pg/mg
[3] (similar to [19, 47, 107])	Between 30–50 mg hair, cut in small pieces and washed in a 1.5 ml Eppendorf-vial with 1 ml DCM for 5 min and 1 mL MeOH for less than 1 min.	water and 27.5 ng EtG-D5 in 50 µL acetonitrile/water were added to the washed hair and sonicated for 30 min. The mixture was incubated for 2 days.	Manual SPE was performed on Oasis-MAX cartridges (3 ml, 60 mg). Conditioning with 3 ml MeOH and 3 ml water, then the addition of an aqueous sample, and washing in 1 ml 5% NH₃ in water and 1 ml 1% NH₃ in MeOH. Drying in air stream, eluted with 1.8 ml 1% formic acid in MeOH. Evaporate eluate at 60 °C in nitrogen stream and the residue is then dissolved in the mobile phase.	For LC the Thermo Hypercarb Column (100mm x 2.1 mm x 5 µm, ThermoFisher Scientific) was used under isocratic conditions with acetonitrile/ 0.1% formic acid (8:92, v/v) as the mobile phase, a flow rate of 0.3 ml/min and a run time of 5 min. Injection volume was 50 µl. ESI in negative mode, spray voltage 4 kV, Temperature: 330 °C, nitrogen flow 10 l/min, nebulizer pressure 40 psi, cone voltage: 100 V, collision energy: 12 eV and dwell time 300 ms. Following transitions were measured: EtG 221.2/75.1 and 221.2/85.1 of which the latter was used for quantification.	LOD: 2 pg/mg LOQ: 6 pg/mg

Table E.2: Summary of the different methods that are used for the detection of EtG in hair – *continued from previous page.*

Source	Sample preparations	Extraction	Work-up	Analytical method	Detection limit
[189]	Hair was washed with 5 ml water and 5 ml acetone and dried.	The hair was cut in pieces of 1–2 mm, and 25 mg of hair sample was used for the analysis. Hair spiked with 25 ml internal standard solution (EtG-D5, 500 ng/ml in methanol) and 25 ml methanol. Then, 1 ml of water and 1 ml of acetonitrile were added and the tubes were vortexed and incubated for 16 h (overnight) at RT and in the dark. Samples were sonicated for 90 min.	Centrifugation prior to SPE. Varian-aminopropyl cartridges (conditioned with methanol, water and acetonitrile), which after washing with 1 ml hexane and drying under vacuum were eluted wit 1 ml of Milli-Q water. Evaporated to dryness using a nitrogen stream, at approx. 80 °C.	Dried extracts were reconstituted with 50 ml water and 150 ml acetonitrile and 3 µl was injected on the LCMSMS. Luna HILIC column (150 mm, 3 mm, 5 mm) (Phenomenex, Torrance, CA, USA) was used with gradient conditions: at 0.4 ml/min with a mobile phase composed by (A) ammonium acetate 2 mM in water and (B) acetonitrile. The initial mobile phase composition was 10% A, these conditions were maintained for 0.5 min, then the aqueous phase was increased up to 70% A in 2.5 min with a linear gradient. After keeping this composition for 2 min, the original mobile phase (10% A) was restored in 0.3 min and this was preserved until 14 min for column equilibration. Nitrogen served both as collision-induced dissociation gas, drying gas (set at 12 l/min) and nebulizer gas (set at 35 psi). The source temperature was maintained at 350 °C, the capillary voltage was set at 4000 V and the fragmentor voltage at 100 V. m/z ions used: 221.0/85.0 was set as quantifier and 221.0/74.9 as qualifier, and for EtG-D5 the corresponding transitions 226.0/85.0 (quantifier) and 226.0/74.9 (qualifier).	LOQ: 20 pg/mg
[59]	30 mg hair was washed with deionised water and acetone for 5 min and then dried.	20 ng of DIS and 1 ml of distilled water were added to the pulverized hair that was cut with scissors and then powdered in ball mill for 2 min. Followed by ultrasonic incubation for 2 h.	Centrifugate for 840 × g for 10 min prior to SPE. Supernatant was applied to an OasisMAX cartridge (3 ml, 30 mg) conditioned with 2 ml MeOH and 2 ml deionised water. The cartridge was then washed with 1 ml 5% Ammoniumhydroxide and 1 ml MeOH. EtG was eluted from the column by using 2 ml MeOH/formic acid (98:2, v/v) and dried under nitrogen. The derivatisation was done with 100 µl PFPA for 30 min at 80 °C, then dried under nitrogen and reconstituted in 50 µl hexane.	GC: direct injection of 2 µ in split-less mode on a fused silica capillary column with a 5% phenyl-95% methyl-polysiloxane stationary phase. (15 m x 0.25 mm ID x 0.25 µm film thickness). The Helium flow was 1 ml/min, temperature injection port at 250 °C, the temperature of the transfer line at 275 °C, temperature of the oven was initially 70 °C (for 1 min), 35 °C/min increased to 120 °C, then 5 °C/min to 150 °C and finally 70 °C/min to 300 °C. MS settings: methane as reagent gas at 8–8.5 Torr, ion source temperature at 150 °C, electron multiplier at 1200 V and m/z values measured: 347/163 (for quantification EtG), 347/119 (for identification EtG) and 352/163 (EtG-D5).	LOD: 3.0 pg/mg LOQ: 8.4 pg/mg

Table E.3: Summary of the different methods that are used for the detection of EtG in hair – *continued from previous page.*

Source	Sample preparations	Extraction	Work-up	Analytical method	Detection limit
[76]	Hair was washed with water and acetone and dried.	20–30 mg sample was cut and pulverized and then added to 2 ml water. After 2 h of sonication and 5 ng of EtG-D5 was added.	First the sample was centrifuged (5000 rpm for 10 min). SPE was performed by applying supernatant to OasisMAX (waters) cartridges, conditioned with 2 ml MeOH and 2 ml water. EtG was eluted by washing with 1 ml of water/ ammonia (5%) solution and 2 ml of MeOH. Columns were dried under vacuum for 5 min. Then 2 ml of 2% formic acid in MeOH was used to elute EtG. The mixture was evaporated under nitrogen using a heated metal block at 37 °C. Derivatisation of EtG was done with PFPA for 30 min at 60 °C. Then, the sample was dried under N_2. EtG was reconstituted in 50 μl ethyl acetate.	Direct injection in the GC system with 30 m x 0.25 mm x 0.25 μm column, a helium flow of 1 ml/min and an oven temperature of 100 °C for the first 2 min, heated to 170 °C at 10 °C/min and then to 300 °C at 40 °C/min. EtG was detected with NCI in SIM. CI gas was methane and m/z values were for EtG: 496, 349 and for EtG-D5: 501.	LOD: 0.7 pg/mg LOQ: 2.3 pg/mg
[117]	The hair was washed with 2 dichloromethane bathes of 5 ml for 2 min.	2 ml water was added to 50 mg of cut hair and 10 ng DIS. Then submitted to ultrasonication for 2 h.	SPE with an OasisMAX column.	LC was done with a ACQUITY BEH HILIC column with a gradient of nitroglycerin and formate buffer. Then, MSMS with the m/z values, for EtG: 221/85 and 75, for EtG-D5: 226/75	LOD: 2 pg/mg LOQ: 10 pg/mg
[170]	10 mg hair sectioned and then washed with MeOH.	1 ml de-ionised water and 200 μl of EtG-D5 daily sub-dilution were added. Sonication overnight.	Anion exchange SPE was performed with Water OasisMAX, conditioned with 1 ml MeOH, 1 mL de-ionised water. 500 μl of sample was then loaded onto the column. Elution by sequential washes with 1 ml 5% ammonia solution in water, 1 ml water, 1 ml MeOH. Then column dried under full vacuum for 5 min. 1 ml of 5% acetic acid in MeOH was used to elute the analyte. The sample was evaporated to dryness and derivatised with 10 μl ethyl acetate and 10 μl BSTFA at 80 °C for 20 min.	GC: 2 μl injected after sample was cooled down. Column: 15 m x 0.25 mm DF=0.25 μm. Temperature of the injector and detector: 280 °C Oven temperature starts at 50 °C for 1 min, was increased to 120 °C at 20 °C/min, further increased to 300 °C at 75 °C/min, and a final gradient of 50 °C/min was applied till 320 °C. MSMS in EI was used for the analysis. m/z values for EtG: 261/143 and 266/143 for EtG-D5. Collision cell was set at 10.0 V.	LOD: 0.01 ng/ml LOQ: 0.02 ng/ml
[47]	100 mg of 5 cm or less of the proximal segment was washed with dichloromethane and methanol (see [19]).	The hair sample was cut into small fragments. Incubated overnight with deionised water and internal standard followed by 2 h ultrasonication (see [19]).	-	UPLC with a 1.8 μm 2.1 mm x 100 mm high strength silica (HSS) trifunctional C18 column preceded by 0.2 μm column filters. Binary gradient: A) 0.1% formic acid in water, pH 2.85 and B) 100% nitroglycerin with a flow rate of 400 μl/min, 0 min A 99%, B 1%; 2 min, A 88%, B 12%; 2.2 min, A 0% and B 100%; 2.21 min, A 98%, B 2%. Detected with ESI-MSMS in negative ion mode. Temperature nebulizer and desolvation gas (nitrogen): 400 °C, the capillary voltage of 3.5 kV, multiplier at 650 V, extractor at -3 V, REF lens at 1 V, cone gas flow at 50 l/h, desolvation gas flow at 1000 l/h, ion energy I at 0.5 V and II at 2.0 V, entrance and exit potential of 1 V, collision gas (argon) flow at 0.35 ml/min and m/z values used of 221/84.9 for EtG and 226/85 for EtG-D5.	LOQ: 2.5 pg/mg

Table E.4: Summary of the different methods that are used for the detection of EtG in hair – *continued from previous page*.

Source	Sample preparations	Extraction	Work-up	Analytical method	Detection limit
[19] (also used in [134])	100 mg hair was washed with 1 ml DCM, vortexed for 30 s and sonicated for 10 min, solvent was removed and 1 ml MeOH was added and vortexed for 20 s, dried at RT with N₂-flow.	Hair was cut in 1–2 mm pieces, 700 μl water, 20 μl of DIS solution in MeOH of 500 μg/l were added and incubated overnight at 25 °C and then sonicated for 2 h.	Centrifuged at 13000 rpm for 10 min.	8 μl was injected on the LC and analysed in the negative mode with ESI-MSMS with m/z values, for EtG: 221/75, 221/85 and for EtG-D5: 226/75, 226/85.	LOQ: 3 pg/mg LOD: 2 pg/mg
[58]	100 mg hair washed with water and acetone (15 min each) and dried.	The hair was finely cut and DIS (50 μl of 1 ng/ml) as well as 2 ml water were added. The sample was sonicated for 2 h and left at RT overnight.	The sample was centrifuged and the upper layer was evaporated to dryness under N₂ oxygen flow at 40 °C. Derivatisation was performed with 100 μl of PFPA at RT for 30 min then dissolved with 50 μl of n-hexane.	1 μl was injected in the GC system wit ha capillary column DB-1 (25 m x 0.2 mm ID x 0.33 μm film thickness). The temperature of the injector and interface were: 220 °C and 280 °C. The helium flow was set at 1 ml/min. The oven temperature started at 70 °C for 2 min, increased till 280 °C at 20 °C/min and held at 280 °C for 4 min. Analysed with EI-MS, with an electronic impact mode of 70 eV, ion source temperature of 230 °C and m/z values for EtG: 333, 234, 495 and for EtG-D5: 338, 239 and 500.	LOD: 25 pg/mg LOQ: 50 pg/mg
[186] (also used in [60])		The postmortem samples were washed with deionised water. All samples were then washed twice with n-heptane. Hair was pulverised in a ball mill. 2 ml water and 5 ng EtG-D5 in 10 μl water were added to about 30 mg of pulverised sample and sonicated for 2 h.	For SPE aminopropyl NH2 columns were used, conditioned with 3 ml methanol, 3 ml water and 3 ml acetonitrile. Then the sample was applied to cartridge. Columns were washed with n-hexane and dried during 15 min by a strong vacuum. Elution was performed with 2 ml of H₂O/NH₃ 2%. For derivatisation 100 μl / 70 μl PFPA/PFPOH was added at 90 °C for 30 min. The mixture was dried under nitrogen at 40 °C and reconstituted in 50 μl of ethyl acetate.	2 μl was injected on the GC column: HP-Ultra 2 capillary column (12 m x 0.2 mm x 0.33 μm film thickness). The injector temperature: 260 °C. Helium carrier gas flow: 1 ml/min. The oven temperature started at 70 °C for 2 min, subsequently was increased to 280 °C with 20 °C/min. NCI, with methane and m/z values for EtG of: 496, 347 and for EtG-D5: 501, 352	LOQ: 2-4 pg/mg
[107]	100 mg (or less) hair washed with methanol and acetone.	Hair was cut in small pieces then 200 ng EtG-D5 and 1.5 ml water was added. Ultrasonication 3 h at 50 °C.	To the sample 3.5 ml nitroglycerin was added and centrifuged at 3000 rpm for 15 min. The clear supernatant was applied to aminopropyl (NH₂) cartridge, conditioned with 3 ml methanol, 3 ml de-mineralised water and 3 ml acetonitrile. Then washed with 3 ml n-hexane and a strong vacuum for 15 min was applied. EtG was eluted using 1.8 ml of water:25% ammonia (98:2 v/v). Flow rate: 0.5 ml/min. Elute was evaporated to dryness under a stream of N₂ using a heated metal block at 30 °C. Residue was redissolved with 100 μl 0.1% formic acid.	10 μl was injected on the LC equipped with a polar-end-capped phenyl-hexyl-reversed phase column (Synergy Polar-RP 150 mm x 2 mm x 4 μm) and a guard column (4 mm x 2 mm) packed with the same reverse material. The mobile phase was water with 1% nitroglycerin and 0.1% formic acid (v/v) and the flow rate was 0.2 ml/min. Post column: MeOH and acetonitrile at flow rate of 200 μl/min. Negative ESI was used to analyse the sample, with a temperature of the turbo-spray-source of 3500 °C, -4 kV ionisation voltage, -25 V orifice and -160 V ring voltage, collision energy of 14 eV with N₂ CAD gas and m/z values used: 221/71 (for EtG) and 226/75 (for EtG-D5).	LOD: 51 pg/mg LOQ: 102 pg/mg

Table E.5: Summary of the different methods that are used for the detection of EtG in hair – *continued from previous page*.

Source	Sample preparations	Extraction	Work-up	Analytical method	Detection limit
[158]	2–8 cm hair segments washed with methanol and acetone (10 ml for 5 min).	To 100 mg finely cut hair 100 ng EtG-D5 and 2 ml demi water was added. Then 2 h sonicated at 30 °C.	Centrifuged at 3000 rpm for 5 min. Then evaporated under N₂ at 30 °C. Derivatisation was performed with 50 μl pyridine and 100 μl BSTFA, the sample was vortexed and placed in an incubator at 90 °C for 30 min. Then the residue was dissolved in 50 μl ethyl acetate.	GC system: 30 m x 0.25 mm x 0.25 μm film thickness HP-5ms in splitless mode with valve closed for 0.5 min, temperature of the injector: 250 °C, temperature detector: 280 °C, temperature of the oven starts at 100 °C for 2 min, increased at 15°C/min to 250°C and then a 20°C/min gradient to 280°C. m/z values used for EtG: 160, 261, 405 and for EtG-D5: 165, 266, 410.	LOD and LOQ not provided. LOQ < 119 pg/mg
[39]	Hair samples were washed with 5 ml ether then 5 ml acetone for 10 min.	Segments were pulverized. To 50 mg hair 50 ng methyl glucuronide, 0.25 ml distilled water and 1 ml MeOH were added. After incubation at RT for 5 h, ultrasonication took place for 3 h at 30 °C.	The mixture was centrifuged at 14000 g for 10 min and 0.8 ml of the supernatant was filtrated and evaporated to dryness under N₂. The residue was derivatized with 30 μl MSTFA for 60 min at 70 °C, then evaporated to dryness and redissolved in 70 μl n-hexane.	1 μl was injected in the GCMS system. Column used: CP-sil 5, 12 m x 0.25 mm, id: 0.4 μm film thickness. The oven temperature started at 60 °C for 1 min and was increased to 320 °C at 30 °C/min and maintained at 320 °C for 1 min. The m/z values for EtG: 261, 292, 375, 405 and for methyl glucuronide: 391.	LOD: 2.2 ng/mg LOQ: 5 ng/mg

Appendix F

Summary FAEEs methods

Table F.1: Summary of the different methods that are used for the detection of FAEEs in hair.

Source	Sample preparation	Analytical method	Detection limit
[172]	About 100 mg hair was accurately double washed with methanol (2 ml for about 2 min each). Extraction of the FAEEs from hair with overnight incubation in n-hexane/DMSO mixture (4 and 0.5 ml respectively) with alpha-colestane as internal standard. Followed by a clean-up step on aminopropyl-NH_2 SPE cartridges that were conditioned with DCM and hexane. The analytes were extracted by n-hexane and DCM, evaporated under nitrogen and dissolved in n-hexane with direct injection on the GCMS.	GCMS (SIM): 88, 213, 256 for E14:0, 101, 241, 284 for E16:0, 101, 265, 310 for E:18:1 and 157, 269, 312 for E18:0.	0.01 ng/mg
[3]	As Pragst et al. [114], but instead of chloroform n-heptane is used. After the extraction the sample was cooled down, to enable the separation of the frozen DMSO layer by decanting. After which the n-heptane solution was evaporated with nitrogen. HS SPME was performed on the residue with 0.5 g NaCl and 1 ml 0.1 M phosphate buffer at a pH of 7.6.	GC MS (SIM) [a]	0.01 (for E18:0) to 0.04 ng/mg (for E18:1)
[101]	About 50 mg hair was washed twice with each 8 ml n-heptane for 15 min by gently shaking. Both wash solutions were collected, united in a 20 ml head space vial and the solvent was evaporated. 50 mg were exactly weighed and placed into a 4 ml glass vessel with screw cap and Teflon septum. 5 ml of the working solution of the four FAEEs-D5 in chloroform [b], 0.5 ml DMSO and 2 ml n-heptane were added. The vessels were tightly closed and vigorously shaken with the ThermoTwister comfort mixer at orbital speed 9 for 15 h at 25 °C. Then, the mixture was cooled below 0 °C and the n-heptane phase was decanted into a 20 ml head space vial. The solvent was completely evaporated at RT by a nitrogen stream. To the residue 1 ml phosphate buffer (0.1 M, pH = 7.6) and 0.5 g NaCl were added. For HS-SPME the sample was preheated for 5 min at 90 °C and 250 rpm agitation, head space adsorption for 30 min at 90°C and 250 rpm agitation and desorption for 5 min at 250 °C.	GC oven temperature was 3 min at 90 °C, then at 20 °C/min increased to 300 °C, then 5 min kept constant at 300 °C. Temperatures of the injector, the interface, the ion source and the quadrupole were: 250 °C, 280 °C, 230 °C and 150 °C respectively. m/z values used in MS for ethyl myristate-D5 (ethyl myristate): 106, 162, 261 (101, 157, 256); ethyl palmitate-D5 (ethyl palmitate): 106, 162, 289 (101, 157, 284); ethyl oleate-D5 (ethyl oleate): 93, 106, 315 (88, 101, 310) and ethyl stearate-D5 (ethyl stearate): 106, 162, 317 (101, 157, 312).	LOD in ng/mg: 0.008, 0.022, 0.026 and 0.010 for E16:0, E18:1 and E18:0 respectively. LOQ in ng/mg: 0.074; 0.027; 0.074; 0.087; 0.032 (same series)
[186]	External degreasing was performed with n-heptane. For the extraction, the hair was cut in mm pieces and to 30 mg of this hair sample 0.5 ml DMSO, 2 ml n-heptane and 20 ng of each of the internal standards in 10 μl chloroform was added. Then shaken during 20 h at 25 °C. HS SPME was automatically carried out with a 65 μm PDMS/DVB fibre. [c]	GCMS (SIM) [d]	0.01–0.04 ng/mg

[a]for details see Pragst et al. [114]

[b]the concentration of. ethyl myristate-D5, ethyl palmitate-D5, ethyl stearate-D5 and ethyl oleate-D5: 4.4, 9.6, 7.5, 11.3 mg/ml

[c]For more details is referred to Pragst et al. [114]

[d]for details see Pragst et al. [114]

Table F.2: Summary of the different methods that are used for the detection of FAEEs in hair – *continued from previous page.*

Source	Sample preparation	Analytical method	Detection limit
[115]	20–50 mg was cut into 1 mm pieces. Washed twice with n-heptane for 1 min at 25 ^{o}C. After centrifugation both heptane washings were separated and combined in a 10 ml head space vial 40 ng of each DIS in 20 μl of chloroform was added. For the washing layers: the solvent was evaporated with a N_2 stream at 40 ^{o}C. For the internal hair concentration: to washed hair 0.5 ml DMSO, 2 ml of n-heptane and 20 μl of the DIS (40 ng in chloroform) were added and the mixture was shaken for 15 h at 25 ^{o}C. With centrifugation the n-heptane phase was separated and transferred into head space vial. Then HS SPME as described by Pragst et al. [114].	GCMS (SIM) [a].	-
[114]	Hair was washed with dodecyl sulphate. The extraction was performed with a DMSO/n-heptane mixture. 50 mg of washed and dried hair was put in a 4 ml glass vessel and 25 μl of DIS of 2 μg/ml each in Chloroform was added. The mixture was vigorously shaken for 14 h. After centrifugation hexane phase was separated into a 10 ml head space vial. Solvent was evaporated at 40 ^{o}C under a nitrogen stream. To the residue 1 ml phosphate buffer (0.1 M, pH of 7.6) and 0.5 g NaCl were added. The mixture was preheated for 5 min at 90 ^{o}C and 250 rpm, head space adsorption with PDMS/DVB SPME fibre was done for 30 min at 90 ^{o}C and 150 rpm and the desorption took place in the GC inlet for 15 min at 260 ^{o}C.	The GC system was equipped with HP5-MS capillary column (28 m x 0.25 mm x 0.25 μm), the helium flow was set at 1.0 ml/min, splitless injection for 3 min was performed, the temperature of the injector, interface, ion source and Quadrupole were set at: 260 ^{o}C, 280 ^{o}C, 230 ^{o}C and 106 ^{o}C respectively. The oven temperature started at 100 ^{o}C for 2 minutes then increased at 20 ^{o}C/min up to 300 ^{o}C, and kept at 300 ^{o}C for 5 min. The m/z values looked at with MS are: 93, 106, 162, 218, 261 (for E14:0-D5); 88, 101, 157, 213, 256 (for E14:0); 93, 106, 246, 289 (for E16:0-D5); 88, 101, 157, 241, 284 (for E16:0); 93, 106, 315 (for E18:1-D5); 88, 101, 310 (for E18:1); 93, 106, 162, 274, 317 (for E18:0-D5); 88, 101, 157, 269, 312 (for E18:0).	0.01 (for E18:0) to 0.04 (for E18:1) ng/mg

[a]for details see Pragst et al. [114]

Appendix G

MS method development EtG-PFPA

The FS of EtG-PFPA in PCI mode shows a high abundancy for m/z 333 (see Figure G.1(b)). This m/z value was also used by Jurado et al. [58]. The other two m/z values including the molecular ion that were observed by Jurado et al. were not observed in the mass spectrum. Instead a high abundancy was obtained for m/z 451 and m/z 289. These ions were suggested for the SIM method of the PFPA in the positive mode. However, a more sensitive (higher peak area) and probably more specific method was obtained in the negative mode.

Negative ions can be measured with the mass spectrometer in the CI mode, which is referred to as NCI (negative chemical ionization). The fragmentation of EtG-PFPA results in negative ions and a good response in negative mode CI can be obtained [44] (and cf Figure G.1(a) with G.2(a)). The selection of negative ions instead of positive ions may result in a more specific method since fewer negative ions are formed than positive ions [164] and thus less matrix interference can be expected.

For EtG-PFPA the most significant ions in negative CI were: 496, 347, 349 and 238 (see Figure G.2(b)). This is in agreement with the methods that were published by Yegles et al. [186] who used the ions 347 and 496 and Kerekes et al. [76] who used 349 and 496 as m/z values for their SIM method. In this research 496 was selected for a SIM method and 347 because this m/z value is more abundant than m/z 349. In Chapter 4 it was described that 3 ions were used for the SIM methods. Since NCI is more specific than most acquisition methods two ions for identification and quantification purposes should be sufficient. Hence, for NCI SIM of EtG-PFPA 496 and 347 were selected and the formation is shown

in Figure G.3(a).

For EtG-PFPA a product ion scan in NCI with m/z 347 as precursor showed two m/z peaks: one at 119 and one at 163 (see Figure G.4). These two transitions were also used by Kharbouche et al. [59]. Hence for a tandem MS method in CI with EtG-PFPA the transitions $347 \rightarrow 119$ and $347 \rightarrow 163$ were chosen. These transitions are shown in Figure G.5.
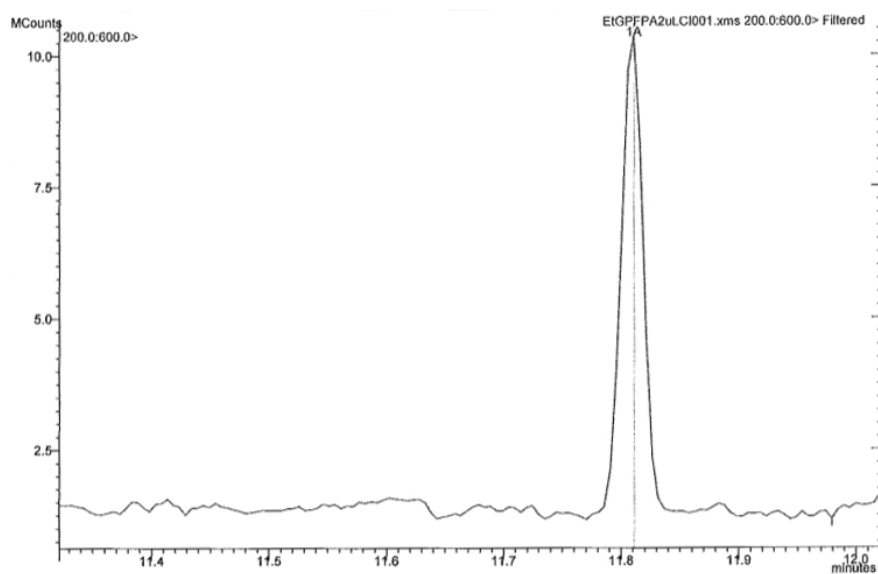
(a)
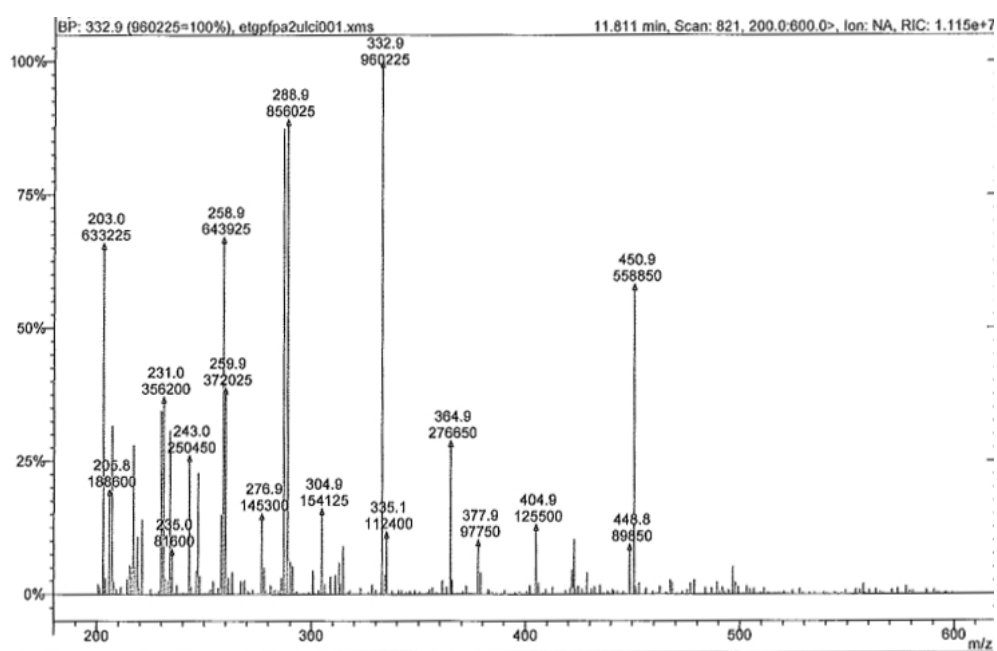
(b)

Figure G.1: Chromatogram and corresponding full scan mass spectrum of EtG-PFPA acquired using PCI.
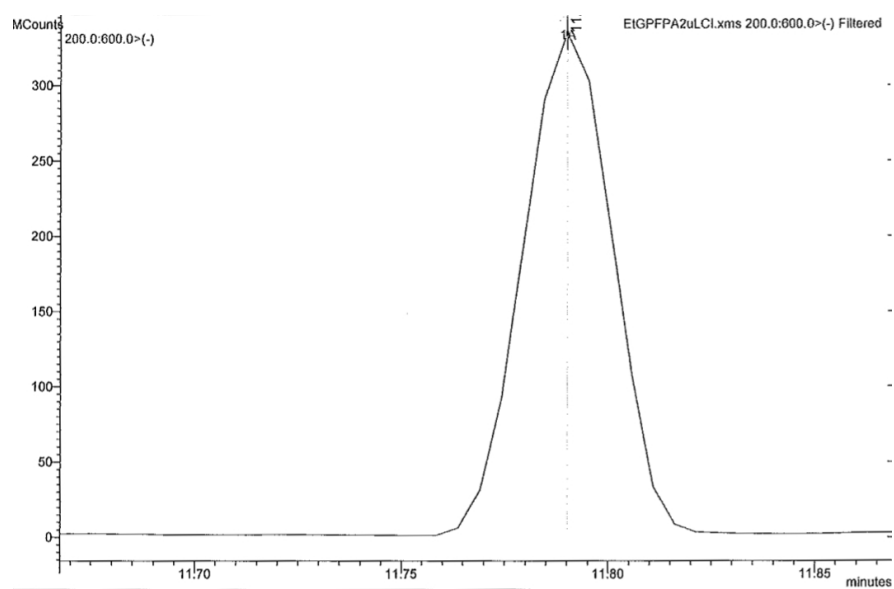
(a)
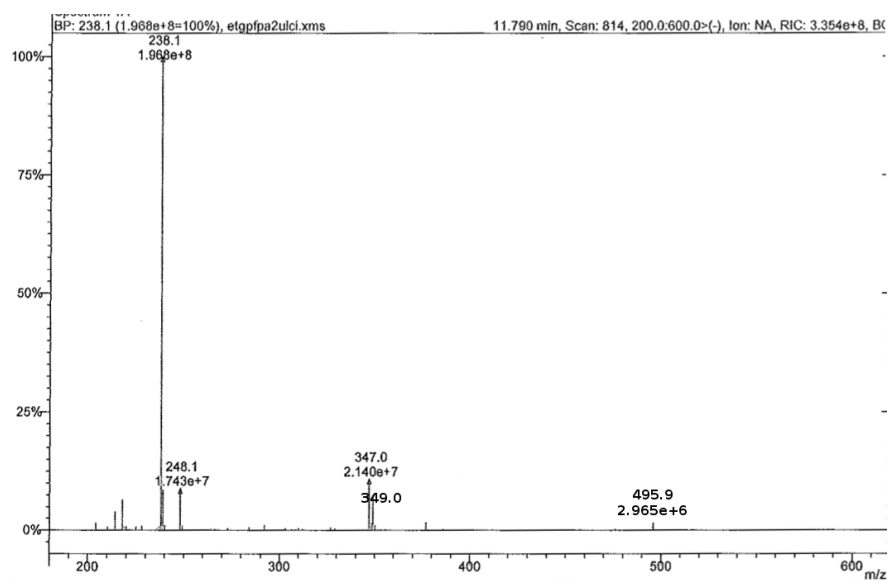
(b)

Figure G.2: Chromatogram and corresponding full scan mass spectrum of EtG-PFPA acquired using NCI.
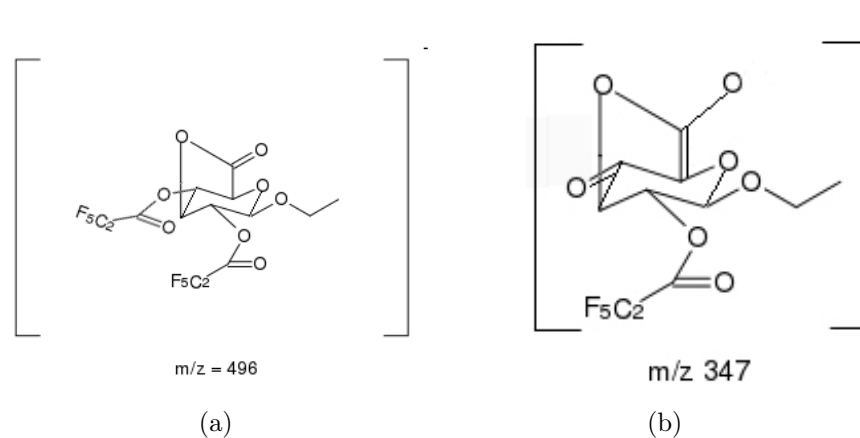
Figure G.3: Chemical structure of the EtG-PFPA ions observed in NCI: a) the molecular ion and b) the fragment with m/z 347. Adjusted from Kharbouche et al. [59].
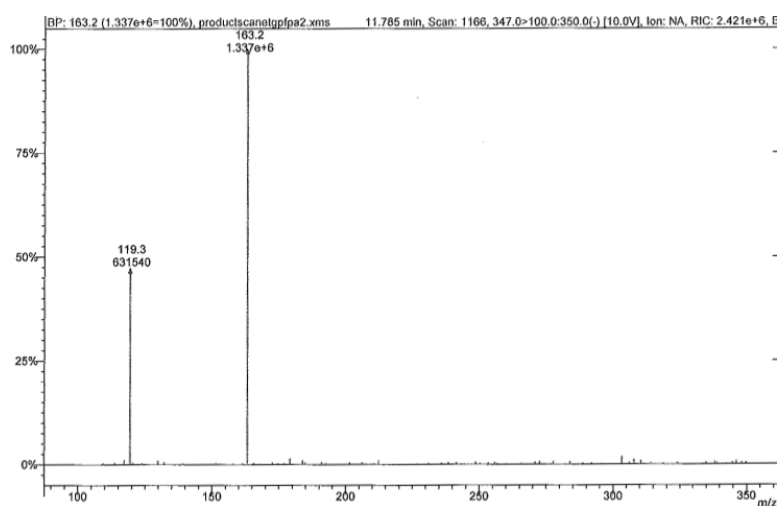
Figure G.4: Product ion spectrum of a fragment of EtG-PFPA acquired using NCI at m/z 347.

(a)

(b)
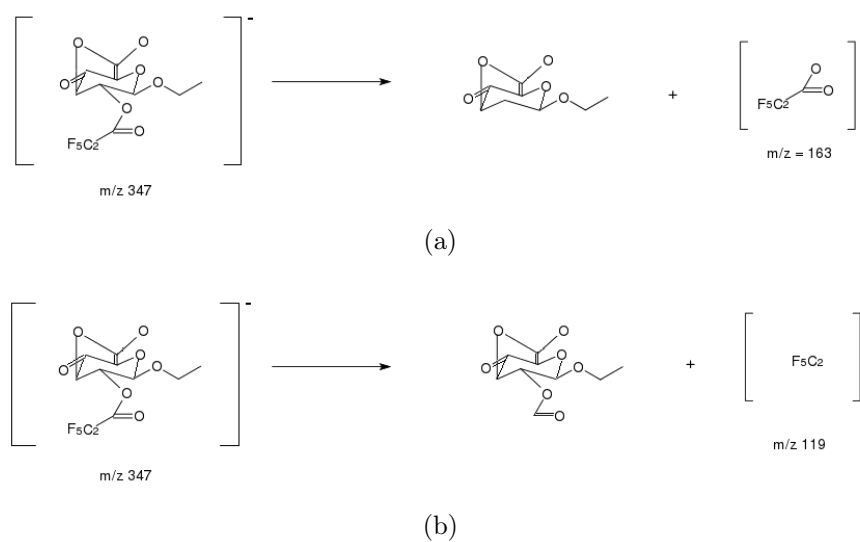
Figure G.5: The fragmentation reaction of an EtG-PFPA fragment with 347 Da to a) 163 Da and b) 119 Da. Adjusted from Kharbouche et al. [59].

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