UNIVERSIDADE DA BEIRA INTERIOR

Ciências

Hair Analysis for Alcohol Biomarkers:

Assessing Excessive Consumption in a Student Population

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Tese para obtenção do Grau de Doutor em

Bioquímica

(3ºCiclo de estudos)

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Covilhã, Junho de 2016

This project was financed by the Programa de investigação de saúde de 2012 (reference 125895) from the Fundação Calouste Gulbenkian.

FUNDAÇÃO
CALOUSTE
GULBENKIAN

Thesis overview

This thesis is structured into five main chapters:

Chapter I consists of a general introduction on the problematic of alcohol consumption, consumption monitoring approaches, and proposed objectives. In this part a review article is presented (manuscript 1), including a literature review of alcohol biomarkers used for the assessment of alcohol consumption.

Chapter II presents the experimental results obtained during the PhD, and includes information presented in the format of research articles:

> Manuscript 2 - Determination of ethyl glucuronide (EtG) and fatty acid ethyl esters (FAEEs) in hair samples.
>
> Manuscript 3 - Determination of ethyl glucuronide in hair to assess excessive alcohol consumption in a student population.
>
> Manuscript 4 - Alcohol consumption assessment in a student population through combined hair analysis of ethyl glucuronide and fatty acid ethyl esters.

Chapter III consists of a general discussion of the presented results, also complementing the information presented in the manuscripts.

In chapter IV the main conclusions of the doctoral work are summarized.

Finally, in chapter V future perspectives are presented.

Acknowledgements

One of the most crucial parts in this project was the collection of hair samples from the numerous students. This was a specially challenging task, which required a lot of patience, effort, explanations and persistence to finally reach the required number of samples. This would have not been possible without the help of my laboratory colleagues. Too all of them, a cheerful thank you!

I'd like to thank to my supervisor Eugenia Gallardo, for the opportunity and share of knowledge about several aspects during the entire PhD, as well as the valuable discussions, corrections and help on the various obstacles. To my co-supervisor Mário Barroso, for his prompt availability at every encountered problem, correction of manuscripts, share of opinions and for the entire knowledge and opinions provided on hair testing. To my co-supervisor Luís Passarinha, for his availability and opportunities for collaboration in other research projects.

To my friends and colleagues, David and Fabiana thank you for all your help, not only during sample collection, but also in the laboratory. Particularly to David for his efficient abilities in persuading students to participate in the study, and not giving up even after days of collection, even after reaching eminent frustration. And Fabiana for her amazing speed at cutting hair, and constant will to help. To both of you, thank you also for the great times spent in the laboratory, and motivation by the good mood that became routine during the long days of work. Without you, all this could have not been the same.

To my parents, Manne and Hedy, and my brother Jolle, I'd like to thank for their continuous support and believe on my academic work, even if it meant that due to the workload we couldn't meet often for several weeks. Your support and believe meant a lot and gave me much help and motivation during this entire time. To you Hedy, thank you also for all the help in Coimbra.

To Catarina, I own you more than any gratitude I could write in these lines. You've been my best friend and accompanied me day and night at every step of this project. You were present at every challenge, victory and defeat during this entire time, and were there for every decision that had to be taken and every problem to be solved. Despite to say that every opinion you gave and discussion about all kinds of obstacles were of great value to me, and I can't recall how many times that helped me to keep on track with the work that had to be done. I should

also not forget the patience you had during all this time, every part of this project over the past years was made in a constant hurry, running against time. And even with the stress this brought to our lives, the fact that you remained patient and supportive, especially all those times when everything seemed to be going down the drain, is to be praised and shows the fabulous person you are. Thank you for everything, and most importantly, thank you for being yourself!

3 Years ago I would have never guessed that I would be writing the following lines. In fact, I would have never imagined that during this project something as surprising and wonderful, as you Lisa, would happen. Long before you were born you already took part in this project, you were present when hair was collected from students, during the entire time we spend working in the laboratory, and, from birth until now, during the entire writing process. You can't imagine in how many ways you helped me during my work, you were the motivation that kept your mother and me working hard and spending excessive hours in the laboratory. And then after birth, every step of growth was an explosion of happiness to your parents' eyes. You wouldn't believe how much inspiring and motivating every babble and every smile you make is to us, and how often it made my day and made me realize how lucky I am. Thank you for being a part of my life!

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"The world is still a weird place, despite my efforts to make clear and perfect sense of it."

Hunter S. Thompson

Abstract

Alcohol consumption within the student population has become an increasing health concern in several countries. The use of alcohol has deleterious consequences on intellectual performance of students, and can be associated with a variety of risky situations, as injuries, unplanned and unsafe sex, sexual and non-sexual violence, property damage and reckless driving. Monitoring alcohol consumption in the student population is therefore important to identify the degree of the problem and to assess consumption trends, allowing also for the implementation of adequate policies, as well as verifying their effectiveness.

Alcohol consumption can be monitored using statistics, such as survey studies performed on the population by means of questionnaires. These approaches have relative efficiency and are widely used; however, considerable limitations are associated to their use, making them unreliable for clinical and/or forensic purposes. Alcohol biomarkers are measurable substances in a biological sample, whose presence indicates some form of exposure to alcohol, and their use to assess alcohol exposure in clinical and forensic scenarios has been applied in a variety of biological specimens in the past years. One of the most notable of these specimens is hair, which presents several advantages, including non-invasive collection, ease of availability, long windows of detection and low chance of adulteration. Hair testing for alcohol exposure relies mainly on the analysis of two biomarkers, ethyl glucuronide (EtG) and fatty acid ethyl esters (FAEEs). These markers have been widely applied in several studies, and were shown to present good sensitivity and specificity.

Therefore, the objective of the present work was to evaluate the alcohol consumption on a university student population using two main approaches, one based on questionnaire analysis, and the second on the analytical determination of alcohol biomarkers in hair. A total of 1192 hair samples, and respective self-completion questionnaire were collected, 975 samples were analysed thereof.

Through analysis of the questionnaires it was found that alcohol consumption started mostly at the age of 15, and that most of the students considered their consumption as moderate, while almost one-third consumed alcohol excessively at least once in the last month. Beer was reported to be the most consumed beverage, and drinking was widely preferred accompanied. Places for alcohol consumption are mostly public places, such as coffee bars. Higher ingested quantities of alcohol per drinking occasion were associated with the male genre, but both genres presented mean alcohol volumes indicating risky and excessive drinking. The use of other substances was also assessed, including tobacco, which was a habit in one-third of the students. The incidence of the use of illicit substances was similar, and cannabis was the most consumed substance, but mainly on rare occasions. The use of illicit substances was however associated with drinking events in more than 50% of the students.

For alcohol biomarker analysis in hair, a method was developed for the analysis of EtG. The compound was extracted from the hair matrix by ultra-sonication with water, clean-up using mixed anion-exchange solid-phase extraction (SPE) and analysis by liquid chromatography coupled to *tandem* mass spectrometry (LC-MS/MS). A second method was developed for the analysis of four FAEEs (ethyl myristate, palmitate, oleate and stearate). The compounds were extracted from the hair matrix by incubation with heptane, extracts were cleaned-up with aminopropyl SPE and analysed by gas chromatography coupled to *tandem* mass spectrometry (GC-MS/MS). Both methods were validated according to guidelines from the Food and Drug Administration (FDA) and International Conference on Harmonization (ICH). Assessed parameters included sensitivity, limits, linearity, precision and accuracy, recovery, stability and matrix effect. The methods were found to be selective, with a limit of quantification of 3 pg/mg for EtG and 30 pg/mg for each of the four FAEEs. Linearity was obtained in the ranges of 3 – 500 pg/mg for EtG and 30 – 5000 pg/mg for FAEEs, and precision and accuracy were found acceptable according to the guidelines. Overall recovery ranged from 74.79% and 97.90%, and processed EtG samples were stable for at least 96h, and FAEEs samples for at least 24h. Matrix effects were evaluated for EtG analysis and were not significant.

Higher concentrations of both biomarkers were obtained for the male genre, and at the universities of Beira Interior and Coimbra. Based on hair sample results, individuals were categorized as abstinent, moderate or excessive drinkers, according to cut-off concentrations proposed by the Society of Hair Testing (SoHT) for both biomarkers. These values are 7 pg/mg of EtG, and 0.2 ng/mg (for 0-3 cm segments) or 0.4 ng/mg (for 0-6 cm segments) of FAEEs, to distinguish abstinence from moderate drinking. To distinguish moderate from excessive drinking, a value of 30 pg/mg of EtG, and 0.5 ng/mg (for 0-3 cm segments) or 1.0 ng/mg (for 0-6 cm segments) of FAEEs was used. At the proposed cut-off values, EtG presented good sensitivity (60-81.6%) and specificity (56.3–90.3%), while for FAEEs good specificity was obtained (87.5–100%), but sensitivity varied between 30.5% and 100%.

In order to verify if the currently proposed cut-off values are adequate for the studied population, receiver operating characteristic (ROC) analysis was performed, to determine the optimal cut-offs based on the results. Optimal EtG cut-off concentrations were determined to be 7.30 and 29.85 pg/mg, respectively for abstinence and excessive drinking, which are similar to those proposed by the SoHT. For FAEEs, optimal concentrations were 0.185 and 0.378 ng/mg for abstinence, at 0–3 cm and 0–6 cm hair segments, respectively, both similar to the proposed values. At the cut-off for excessive drinking, similar cut-off values were obtained for both lengths (0.817 and 0.889 ng/mg), but these are not similar to the proposed values. However, they confirm that harmonization of the excessive drinking cut-off value for FAEEs can be possible, at a concentration close to 0.8 ng/mg, regardless the length of the hair segment.

The use of hair washing products, such as hair conditioner and mask, was found to be associated with lower concentrations of EtG in hair. Cosmetic treatments as bleaching and/or dyeing were associated with the same effect. No effect was associated with the use of hairspray, gel or wax,

and for the FAEEs there was no observable effect associated with any of the mentioned products. This demonstrates the importance of documenting the use of hair washing products and cosmetic treatments during sample collection, and the high care that must be taken during result interpretation, considering this information when positivity or negativity for a hair sample is to be given.

Both biomarkers correlated with the self-reported consumption habit, while only EtG correlated with the ingested quantities of alcohol per occasion. Inconclusive cases were obtained during combined interpretation of EtG and FAEEs. These cases could mostly be explained by the use of hair products and cosmetic treatments; however, in a number of cases where no simple explanation could be presented, the results of EtG analysis were in agreement with the questionnaire data. This indicates that EtG should be regarded as the first choice in alcohol consumption assessment, while FAEEs should be used to confirm the results from EtG analysis.

Only 56.3% of the self-reported abstinent cases could be confirmed by combined analysis of EtG and FAEEs; for moderate drinkers this percentage was 71.6% and for excessive drinkers 60%. Alcohol consumption underestimation or overestimation are assumed as major reasons for this percentages. Combined alcohol biomarker analysis showed that the student population is majorly composed of moderate drinkers (69.39%), followed by abstinent (18.82%) and excessive drinkers (11.69%).

Overall, hair analysis for alcohol biomarkers proved to be a powerful tool for the assessment of alcohol consumption in a student population, with considerable advantages. Hair is a sample easy to collect through non-evasive manners, easily stored and the chances of adulteration are low. Therefore, it has good potential to be applied in population studies, especially because it can complete and confirm a great part of the information obtained by questionnaire analysis with added reliability, promoting as such sound and defensible results.

Key-Words:

Alcohol consumption, student population, alcohol biomarkers, ethyl glucuronide, fatty acid ethyl esters

Resumo Alargado

O álcool é a substância psicoativa mais consumida a nível mundial, e o seu consumo representa um problema de saúde crescente em diversos países. O consumo de álcool na população jovem é bastante comum, com particular destaque e incidência nos estudantes universitários. Deste modo, a população estudantil é considerada como um grupo de risco, no qual o consumo de álcool é associado a baixas performances a nível intelectual. O consumo excessivo de álcool pode ainda provocar diversos comportamentos de risco, nomeadamente sexo não planeado e não seguro, violência sexual e de outras naturezas, danos materiais e condução rodoviária irresponsável. Desta forma, a monitorização do consumo de álcool na população estudantil é fundamental para avaliar o panorama dos hábitos de consumo, bem como identificar tendências emergentes, permitindo a implementação de políticas de combate e avaliação da respetiva eficácia.

O consumo de álcool é frequentemente avaliado por estudos de inquérito, que recorrem à análise estatística de resultados obtidos através de questionários. Este método, apesar de possibilitar a recolha de uma vasta informação, apresenta algumas limitações, nomeadamente em relação à veracidade da informação recolhida, dado que o participante é passível de exagerar ou inclusivamente mentir nas respostas fornecidas. Assim, e de modo a obter resultados verdadeiros, é fundamental o recurso a outras ferramentas. O uso de métodos analíticos para determinação de biomarcadores de álcool em amostras biológicas assume grande interesse no contexto clínico e forense, podendo ainda ser usados como confirmação de dados obtidos através de questionários. Um biomarcador de álcool é uma substância passível de ser mensurável, e a sua presença na matriz biológica é normalmente indicativa de exposição. Nos últimos anos, o cabelo tem sido uma matriz amplamente estudada, com inúmeras vantagens, nomeadamente a sua fácil recolha por métodos não-invasivos, apresenta uma larga janela de deteção e é difícil de adulterar. A monitorização da exposição ao álcool através da análise de cabelo incide fundamentalmente em dois biomarcadores, o etil glucuronido (EtG) e os ésteres etílicos dos ácidos gordos (FAEEs). Estes dois marcadores têm sido usados em diversos estudos nos últimos anos, e apresentam boa sensibilidade e especificidade.

O presente trabalho teve como objetivo principal avaliar o consumo de álcool na população estudantil universitária portuguesa com recurso a dois métodos: questionários de autopreenchimento e determinação analítica de biomarcadores de consumo de álcool (EtG e FAEEs) em amostras de cabelo. A população foi equitativamente distribuída por 9 universidades portuguesas, 1192 amostras de cabelo foram recolhidas bem como os respetivos questionários. Em 975 amostras foi possível proceder à análise e determinação dos biomarcadores de álcool pelos métodos analíticos desenvolvidos.

Os resultados dos questionários indicam que a idade de início de consumo de álcool se situa entre os 12 e os 18 anos, sendo que a maioria admite ter iniciado o consumo aos 15 anos. Em relação aos hábitos de consumo, os estudantes consideram o seu consumo como moderado, enquanto cerca de um terço da população assume ter consumido álcool em excesso no último mês. A bebida mais consumida é a cerveja, e a grande maioria prefere beber acompanhado e em locais públicos, como café-bar. Ao género masculino está associada uma ingestão de maiores quantidades de álcool por ocasião, contudo ambos os géneros apresentaram volumes de álcool indicativos de consumo de risco e excessivo. Nos questionários foi ainda recolhida informação sobre o consumo de outras substâncias, nomeadamente tabaco e substâncias ilícitas, e tendo aproximadamente um terço da população admitido o uso destas substâncias, com maior incidência no género masculino. A canábis é a substância ilícita mais consumida, sendo que maioria da população admite consumi-la raramente, contudo 50% dos participantes admitem utilizar substâncias ilícitas em conjunto com o álcool.

Tendo em conta as características físico-químicas dos biomarcadores de álcool, EtG e FAEES, foram desenvolvidos e otimizados dois métodos analíticos. Para a análise do EtG, a amostra de cabelo foi sujeita a ultra-sonicação em água, para extração do composto da matriz, seguindo-se extração em fase sólida (SPE) e análise por cromatografia líquida acoplada a espetrometria de massa em *tandem* (LC-MS/MS). Relativamente aos FAEEs, foram analisados quatro compostos (miristato, palmitato, oleato e estearato de etilo), tendo os compostos sido extraídos do cabelo por incubação em heptano. Os extratos foram sujeitos a SPE e analisados por cromatografia gasosa acoplada a espetrometria de massa em *tandem* (GC-MS/MS). Ambos os métodos analíticos foram validados de acordo com normas internacionalmente aceites para a validação de métodos bioanalíticos, nomeadamente da *Food and Drug Administration* (FDA) e *International Conference on Harmonization* (ICH). Os parâmetros avaliados foram sensibilidade, limites, linearidade, precisão e exatidão, recuperação, estabilidade e efeito matriz. Ambos os métodos mostraram ser seletivos, com limites de quantificação de 3 pg/mg, para o EtG, e de 30 pg/mg, para cada um dos FAEEs analisados. Os intervalos de linearidade situaram-se entre os 3–500 pg/mg e 30–5000 pg/mg, para o EtG e FAEEs, respetivamente. A precisão e exatidão revelaram ser aceitáveis de acordo com as normas utilizadas. A recuperação variou entre os 74,79% e os 97,90%, e as amostras processadas de EtG revelaram ser estáveis durante pelo menos 96h, enquanto que para os FAEEs foram estáveis pelo menos 24h. O efeito matriz foi apenas estudado para o EtG, não tendo sido considerado significativo.

A análise das amostras de cabelo mostra que o género masculino apresenta maiores concentrações de ambos os biomarcadores, sendo que as universidades da Beira Interior e de Coimbra foi onde foram registadas as concentrações mais elevadas. Os participantes foram divididos de acordo com o tipo de consumo: abstinente, moderado e excessivo, consoante as concentrações de *cut-off* propostas pela *Society of Hair Testing* (SoHT) para cada biomarcador. Os valores propostos para distinguir consumo abstinente de moderado são de 7 pg/mg para o EtG, e de 0,2 ng/mg (para segmentos de 0-3 cm) ou 0,4 ng/mg (para segmentos de 0-6 cm)

para os FAEEs. Para distinguir consumo moderado de excessivo, foi usado o valor de 30 pg/mg para o EtG, e 0,5 ng/mg (para segmentos de 0-3 cm) ou 1,0 ng/mg (para segmentos de 0-6 cm) de FAEEs. O EtG apresentou boa sensibilidade (60–81,6%) e especificidade (56,3–90,3%) para cada *cut-off* proposto; para os FAEEs foi obtida boa especificidade (87,5–100%), mas a sensibilidade foi muito variável, tendo oscilado entre 30,5% e 100%.

Com o propósito de verificar se os *cut-off* atualmente propostos pela SoHT se adequam à população em estudo, foi elaborada análise através das curvas ROC (*receiver operating characteristic*), e com base nos resultados obtidos foram determinados valores de *cut-off* ótimos. Para o EtG, os valores determinados para o consumo abstinente e excessivo, foram de 7,30 e de 29,85 pg/mg, respetivamente. Para os FAEEs, a SoHT recomenda que seja tido em conta o comprimento do segmento do cabelo. Assim, os valores determinados para abstinência foram de 0,185 e de 0,378 ng/mg para os segmentos de 0-3 cm e 0-6 cm, respetivamente. No que respeita o consumo excessivo, foram obtidos valores semelhantes para os diferentes segmentos, de 0,817 e de 0,889 ng/mg, respetivamente. Os valores de consumo excessivo determinados para o EtG são similares aos propostos pela SoHT, contudo o mesmo não acontece para os FAEEs. Ainda assim, a proximidade dos valores obtidos para o consumo excessivo para estes compostos apoia a possibilidade de usar o mesmo valor de *cut-off* independentemente do comprimento do segmento analisado, considerando-se um valor de cerca de 0,8 ng/mg como adequado.

Foi ainda possível avaliar se o uso de tratamentos e produtos cosméticos interferiam na determinação dos biomarcadores de álcool. Os resultados obtidos para o EtG mostraram que o uso de produtos de lavagem, tais como acondicionador e máscara, estão associados a concentrações mais baixas, assim como tratamentos cosméticos como descoloração e/ou pintura. Por outro lado, o uso de produtos como laca, gel ou cera não mostraram ter qualquer efeito associado. Não foi observado qualquer efeito associado a tratamentos ou produtos específicos no caso dos FAEEs. Ainda assim, estes resultados reforçam a necessidade e importância de documentar os hábitos de higiene (por exemplo, número de lavagens) bem como o uso de produtos e tratamentos cosméticos, aquando da recolha da amostra. Estas informações devem ainda ser tidas em consideração na interpretação dos resultados e os casos tratados com particular atenção.

Tendo em conta os resultados para o EtG e FAEEs em combinação, foi possível verificar a existência de correlação entre a resposta nos questionários, em relação aos hábitos de consumo, e a concentração determinada para ambos os biomarcadores. Para o EtG, foi ainda possível encontrar correlação em relação às quantidades de álcool ingeridas por ocasião. Contudo, foram obtidos alguns casos inconclusivos durante a interpretação combinada de EtG e FAEEs. Em parte, isto pode ser justificado devido ao uso de produtos e tratamentos cosméticos, uma vez que estes podem influenciar a determinação dos biomarcadores em cabelo; contudo, não existe uma justificação simples para o elevado número de casos inconclusivos. Ainda assim, os resultados do EtG estavam em concordância com a informação

recolhida nos questionários, o que reforça a ideia de que o EtG é um biomarcador mais fiável, devendo ser a primeira escolha para determinar o consumo álcool em amostras de cabelo, enquanto os FAEEs devem ser usados para confirmar os resultados da análise de EtG.

A análise combinada de EtG e FAEEs mostra que a população estudantil é maioritariamente composta por consumidores moderados (69,39%), seguido de abstinentes (18,82%) e excessivos (11,69%). Contudo, a análise combinada dos biomarcadores apenas permitiu confirmar 56,3% dos casos de consumo abstinente reportado nos questionários, e 71,6% e 60 % para o consumo moderado e excessivo. Tendo em conta que os questionários são de autopreenchimento, os participantes são suscetíveis de subestimar ou sobrestimar os seus hábitos do consumo aquando do preenchimento, o que permite justificar os resultados obtidos.

No geral, a análise de biomarcadores de álcool em cabelo mostrou ser uma poderosa ferramenta para a determinação de consumo de álcool na população estudantil. O cabelo é uma matriz que, para além de ser de fácil recolha, por métodos não-invasivos, é fácil de armazenar e difícil de adulterar. Por estes motivos, apresenta um grande potencial para ser aplicado a estudos populacionais, especialmente porque é capaz de completar e confirmar grande parte da informação obtida por questionários, com elevada veracidade.

Palavras-chave:

Consumo de álcool, população estudantil, biomarcadores do consumo de álcool, etil glucuronido, ésteres de ácidos gordos.

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List of Abbreviations

5-HIAA	5-hydroxyindoleacetic acid
5-HT	5-hydroxytryptamine
5-HTOL	5-hydroxytryptophol
5-MMIA	5-Methoxy-2-methyl-3-indoleacetic acid
AcH	Acetaldehyde
ADH	Alcohol dehydrogenase
ALDH	Aldehyde dehydrogenase
ALT	Alanine aminotransferase
ANOVA	Analysis of variance
APA	Acetaldehyde protein adducts
APCI	Atmospheric pressure chemical ionization
AST	Aspartate aminotransferase
AUC	Area under the curve
BAC	Blood alcohol concentration
BSTFA	N,O-Bis(trimethylsilyl)trifluoroacetamide
C18	Octadecylsilane
C8	Octylsilane
CAS	Chemical Abstract Service
CDG	Congenital disorders of glycosylation
CDT	Carbohydrate-deficient transferrin
CE	Capillary electrophoresis
CI	Chemical ionization
CIEF	capillary isoelectric focusing
CSF	Cerebrospinal fluid
CITP	Capillary isotachophoresis

CoA	Coenzyme A
CV	Coefficient of variation
d2	Double-deuterated
d4	Tetra-deuterated
d5	Penta-deuterated
DBS	Dried blood spots
DLLME	Dispersive liquid-liquid microextraction
DMSO	Dimethylsulphoxide
DUS	Dried urine spots
ED	Electrochemical detection
EI	Electron impact
EIA	Enzyme immunoassays
ELISA	Enzyme-linked immunosorbent assay
ELSD	Evaporative light-scattering detection
EMCDDA	European Monitoring Centre for Drugs and Drug Addiction
ESI	Electrospray ionization
ESPAD	European School Survey Project on Alcohol and Other Drugs
EtG	Ethyl glucuronide
EtS	Ethyl sulphate
EU	European Union
FAEEs	Fatty acid ethyl esters
FDA	Food and Drug Administration
FID	Flame ionization detection
FN	False-negative
FP	False-positive
GC	Gas chromatography
GGT	Gamma-glutamyl transferase

GTOL	5-hydroxytryptophol glucuronide
HAA	Haemoglobin associated acetaldehyde
Hb	Haemoglobin
HDL	High density lipoproteins
HFBA	Heptafluorobutyric anhydride
HILIC	Hydrophilic interaction liquid chromatography
HPLC	High pressure liquid chromatography
HS	Headspace
ICH	International Conference on Harmonization
ICPMS	inductively coupled plasma mass spectrometry
IEF	Isoelectric focusing
IS	Internal standard
$K_{o/w}$	Octanol-water partition coefficient
LC	Liquid chromatography
LLE	Liquid-liquid extraction
LLOQ	Lower limit of quantification
LOD	Limit of detection
MAE	Microwave-assisted extraction
MALDI	Matrix-assisted laser desorption/ionization
MAX	Mixed anion-exchange
MCV	Mean corpuscular volume
ME	Matrix effect
MRM	Multiple reaction monitoring
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MW	Molecular weight
NACE	Non-aqueous capillary electrophoresis

NAD^+	Oxidized nicotinamide adenine dinucleotide
NADH	Reduced nicotinamide adenine dinucleotide
PAGE	Polyacrylamide gel electrophoresis
PBut	Phosphatidylbutanol
PED	Pulsed electrochemical detection
PEth	Phosphatidylethanol
PFPA	Pentafluoropropionic anhydride
PLD	Phospholipase D
PMF	Peptide mass fingerprint
PProp	Phosphatidylpropanol
R^2	Coefficient of determination
ROC	Receiver operating characteristic
RP	Reverse-phase
RT	Retention time
SD	Standard deviation
SLE	Supported liquid extraction
SoHT	Society of Hair Testing
SPDE	Solid-phase dynamic extraction
SPE	Solid-phase extraction
SPME	Solid-phase microextraction
SSRI	Selective serotonin reuptake inhibitor
Tf	Transferrin
TN	True-negative
TOF	time-of-flight
TP	True-positive
UA	Universidade de Aveiro
UALG	Universidade do Algarve

UBI	Universidade da Beira Interior
UC	Universidade de Coimbra
UDP	Uridine diphosphate
UE	Universidade de Évora
UL	Universidade de Lisboa
UM	Universidade do Minho
UP	Universidade do Porto
UTAD	Universidade de Trás-os-Montes e Alto Douro
UV	Ultraviolet
WADA	World Anti-doping Agency
WBAA	Whole blood associated acetaldehyde assay

List of Publications

Articles:

David Oppolzer, Mário Barroso, Eugenia Gallardo, "Bioanalytical procedures and developments in determination of alcohol biomarkers in biological specimens.", *Bioanalysis*, Vol. 8, No. 3, 229-251, 2016.

David Oppolzer, Mário Barroso, Luís Passarinha, Eugenia Gallardo, "Determination of ethylglucuronide (EtG) and fatty acid esters (FAEEs) in hair samples.", *Biomedical Chromatography*, Accepted, 2016.

David Oppolzer, Mário Barroso, Eugenia Gallardo, "Determination of ethyl glucuronide in hair to assess excessive alcohol consumption in a student population.", *Analytical and Bioanalytical Chemistry*, Vol. 408, No. 8, 2027-2034, 2016.

David Oppolzer, Eugenia Gallardo, Luis Passarinha, Mário Barroso, "Alcohol consumption assessment in a student population trough combined hair analysis of ethyl glucuronide and fatty acid ethyl esters.", *Addiction*, Submitted, 2016

Oral presentations during the doctoral work:

David Oppolzer, Mário Barroso, Eugenia Gallardo, "Diagnóstico de consume excessive de álcool em estudantes universitários através da análise de etilglucoronido em cabelo.", II Conferência Nacional de Medicina Legal e Ciências Forenses, 29th and 30th October (2015), Coimbra, Portugal.

David Oppolzer, Mário Barroso, Eugenia Gallardo, "Hair analysis to assess excessive alcohol consumption in a student population.", 20th Meeting of the Society of Hair Testing (SoHT), 3-6th May (2015), São Paulo, Brazil.

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Posters presentations during the doctoral work:

Mário Barroso, David Oppolzer, Eugenia Gallardo, "Evaluation of ethanol consumption in university students: Hair analysis for specific biomarkers in 975 cases.", XXI Congresso Español y V Iberoamericano de Toxicología, 17-19th June (2015), León, Spain.

David Oppolzer, Mário Barroso, Eugenia Gallardo, "Development and validation of an analytical method for the determination of ethylglucuronide in hair by LC-MS-MS.", 20th Meeting of the Society of Hair Testing (SoHT), 3-6th May (2015), São Paulo, Brazil.

David Oppolzer, Mário Barroso, Luís Passarinha, João Queiroz, Eugenia Gallardo, "Determinação de etilglucoronido em amostras de cabelo por LC-MS/MS: um importante biomarcador do consumo de etanol", I Conferência Nacional de Medicina Legal e Ciências Forenses, 30th and 31st October (2014), Coimbra, Portugal.

Chapter I - General Introduction

1.1 Introduction

Ethanol is one of the most abundantly consumed teratogens amongst humans, and it is present in all alcoholic beverages. Alcohol is, directly or indirectly, the cause of several diseases, direct and indirect injuries, including violence, homicides and suicides [1–3], and 5.9% of all deaths worldwide are attributed to alcohol consumption [3]. Alcohol consumption prevails worldwide, and represents an important social-economic issue in several countries. The European Union (EU), presents the highest alcohol consumption in the world [1]. The average hospital expenditures for alcohol treatments per capita in 15 European countries was found to be of 8.5 euros and 1.76% of hospital days were attributable to alcohol treatment [4], and public expenditures for alcohol treatment exceed even those for the treatment of illegal drug addiction [4].

Excessive and irresponsible consumption reflects at either social, economic or health levels, but also at third parties, as occurs in the case of alcohol consumption during pregnancy, a trigger for domestic violence, traffic accidents or even those professions where drinking can cause or impair reactions capable of jeopardizing the life of others [1,2,5,6].

1.2 Alcohol Consumption in Students

Alcohol consumption amongst students is an increasing health concern in many countries [2]. According to the 2014 report of the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA), results from a survey indicate that almost two-thirds of the students report drinking alcohol at least once in the last month, of which 20% were intoxicated [7]. While alcohol consumption begins at or even before the age of 13 [8], university students are more likely to binge drinking (characterized by a high number of drinks per occasion) and heavy episodic drinking (characterized by periods of high consumption) [2,9]. Alcohol consumption by students can have deleterious consequences on both school and academic performances, and has been associated to injuries, unplanned and unsafe sex, sexual and non-sexual violence, property damage and reckless driving [2,8,9].

It is therefore evident that monitoring alcohol consumption in the student population is of high importance. Firstly, for the evaluation of the impact of alcohol consumption on health, socio-economic and other harms. Secondly, to assess the degree of the problem, identify developing consumption trends and to develop and adopt adequate policies regarding alcohol consumption

[2]. Additionally, monitoring and evaluating alcohol consumption regarding existing alcohol policies is crucial to evaluate their effectiveness and to strengthen their impact [1].

1.3 Alcohol Monitoring

Statistics provided by government agencies are relatively well standardized and usually reliable. Besides, they have the advantages that, due to the number of international agencies participating in those statistics, clear and standardized results can be collected for harms of alcohol consumption and abuse on public health, economy and other issues. However, by being a human product, they are susceptible to being culture-specific and may be biased. For instance, one must consider that underestimation is very common in sales statistics due to other, licit or illicit, sources of supply; and morbidity or health treatment statistics are heavily influenced by the degree of development of alcohol treatment programmes, since higher specialized treatments are more likely to identify a disorder as alcohol-related [1].

Another monitoring approach is by surveys analysis. These are typically performed on the effectiveness of governmental policies, as well as on the population, to assess alcohol consumption by means of questionnaires. However, great limitations must be noted on this approach: information might be omitted or over-emphasized in governmental policies surveys in order to hide policy failures or stress the success of a given policy, respectively. In addition, high limitations are also associated to population surveys; besides the obvious implications of the number of participants that abstain from responding in such studies on the gathered information, consumption is often underestimated. Moreover, when dealing with adolescent and student populations, both underestimation and overestimation are likely to occur [1].

These above-mentioned alcohol monitoring approaches present several limitations, mostly associated to the fact that different facts may skew the results, individuals may misclassify their consumption and/or omit or over-emphasize certain information in the surveys, and obviously interpretation differences exist between individuals.

1.4 Alcohol Biomarker Analysis

1.4.1 Alcohol Biomarker

It is evident, especially when dealing with clinical and forensic scenarios, that such approaches as mentioned above are inefficient for the monitoring of alcohol consumption. Also, it is often important in these fields to know the current effect of alcohol on an individual and obtain a drinking history. Additionally, alcohol consumption is usually assessed in terms of quantities to which an individual was exposed [10].

Alternative approaches that cannot be influenced by the participants' opinion and evaluate directly the degree of alcohol consumption are therefore preferred. In this context, analytical techniques can provide an answer to many of the mentioned limitations. This requires two important variables: a substance or molecule that is present or altered only in cases where alcohol was consumed, termed biomarker; and a biological specimen, where the biomarker can be found and successfully analysed. An alcohol biomarker can be defined as a measurable substance in a biological sample, whose presence indicates some form of exposure to alcohol. The biomarker plays a very important role, since it will have to: be able to discriminate between alcohol consumption (true exposure - sensitivity) and endogenous alcohol production (lack of exposure - specificity), remain in the biological specimen for a sufficient amount of time until analysis, and its detection should be relatively easy to perform. On the other hand, the biological specimen also plays a crucial role: its collection should ideally be an easy, non-invasive process to the individual, the collected sample should be in sufficient amounts, and the biomarkers should be stable in the sample. Overall, the process to analyse one or more biomarkers in a specific biological sample, should be a reliable, fast, preferably easy and inexpensive procedure [6].

1.4.2 Manuscript 1

BIOANALYTICAL PROCEDURES AND DEVELOPMENTS IN THE DETERMINATION OF ALCOHOL BIOMARKERS IN BIOLOGICAL SPECIMENS

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Bioanalysis, Vol. 8, No. 3, 229-251, 2016

Review

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Bioanalysis

Bioanalytical procedures and developments in the determination of alcohol biomarkers in biological specimens

Excessive alcohol consumption is a global problem, and consequently its evaluation is of great clinical and forensic interest. Alcohol biomarkers have been the focus of several research works in the past decades, with new compounds being studied in more recent years. The main objective of this review is to discuss topics for an analyst to consider when evaluating alcohol consumption through the analysis of alcohol biomarkers in biological specimens. For this, existing alcohol biomarkers will be reviewed, including carbohydrate-deficient transferrin, 5-hydroxytryptophol, ethanol, hemoglobin-associated acetaldehyde, fatty acid ethyl esters, ethyl glucuronide, ethyl sulfate and phosphatidylethanol. Additionally, their potential will be discussed, as well as analytical considerations, main challenges, limitations, data interpretation and existing methodologies for their determination in biological specimens.

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First draft submitted: 9 September 2015; Accepted for publication: 11 November 2015; Published online: 22 January 2016

Keywords: cut-off value • effect-based biomarkers • exposure-based biomarkers • sensitivity • specificity

Ethanol is a teratogen present in all alcoholic beverages, and its consumption accounts for one of the most common drug habits, reported addiction and teratogenic effects on individuals worldwide. Irresponsible and/or excessive consumption is usually involved in situations that penalize either the consumer or other individuals, often in crime situations [1]. Evaluation of alcohol consumption is, therefore, of great importance, and this has been traditionally done by using self-report questionnaires or by the clinical history of the subject; however, these methods are indirect approaches, presenting poor sensitivity [2–5]. Alcohol consumption assessment through analysis of biomarkers provides an answer to these limitations. Ethanol has toxic effects in the organism, affecting the mean corpuscular volume (MCV), enzymes such as GGT, ALT, ASP, CDT and the serotonin metabolites 5-hydroxytryptophol (5-HTOL) and 5-hydroxyindoleacetic acid (5-HIAA) (Figure 1) [5]. Almost all the ethanol that enters the organism is rapidly metabolized to acetaldehyde and acetate by two reaction pathways: via the cytoplasmic enzyme alcohol dehydrogenase, and involving hydrogen peroxide and the enzyme catalase [6]. Part of the remaining ingested ethanol undergoes nonoxidative metabolism through four main paths, originating ethylglucuronide (EtG), ethylsulfate (EtS), fatty acid ethyl esters (FAEEs) and phosphatidylethanol (PEth) (Figure 1) [3,5]. The molecules affected by ethanol and its direct metabolites are suitable for use as biomarkers of alcohol consumption. Several research works were developed in the past decades using these compounds as biomarkers; however, with the variety of published results, it becomes difficult to interpret data and establish standard laboratory procedures for analysts. Therefore, in this paper, an extensive review of existing biomarkers is

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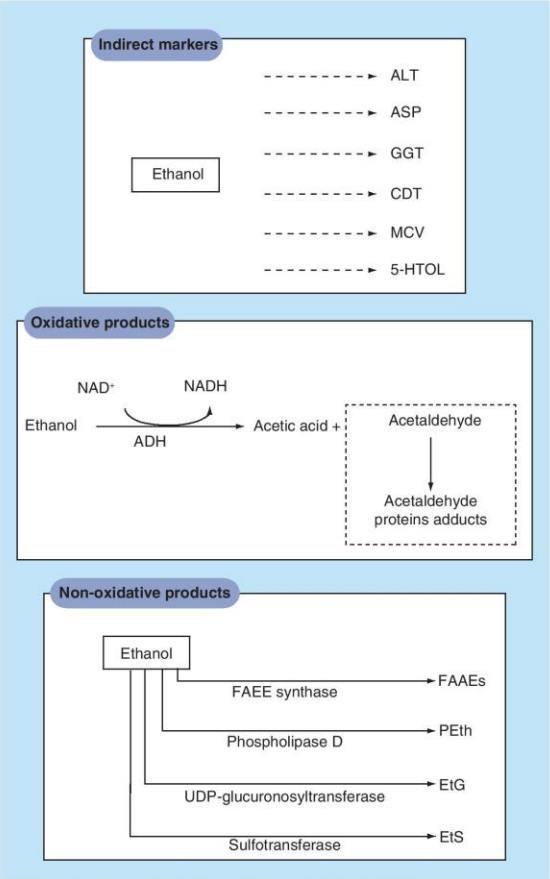

Figure 1. Schematic representation of the tissue or organ, oxidative and nonoxidative pathways of the alcohol metabolism and formation of the alcohol biomarkers reviewed in this paper.
5-HTOL: 5-hydroxytryptophol; EtG: Ethyl glucuronide; EtS: Ethyl sulfate; FAAE: Fatty acid ethyl ester; MCV: Mean corpuscular volume; PEth: Phosphatidylethanol.

presented, including reviewing advances on analytical methodologies and biological specimens' analysis since 2001, as well as discussing data interpretation for the use of the mentioned compounds as alcohol biomarkers.

Effect-based biomarkers

Established biochemical markers

Traditional biomarkers for determining alcohol exposure are based on the alteration of biochemical molecules. Established markers are GGT, ALT, AST and MCV. These biomarkers can be determined using routine laboratory methods, and have been widely used for monitoring chronic drinking, having become part of routine blood chemistry profiles in medical examinations [5]. However, several limitations are associated when used as alcohol biomarkers, especially concerning the limited specificity due to different diseases, genetic variance, smoking and consumption of certain drugs; in addition, proper reference intervals need to be adjusted according to genre, age and body weight [3,5].

Carbohydrate-deficient transferrin

Transferrin (Tf) is an iron-transporting glycoprotein containing terminal sialic residues in its structure, which in number can vary in humans from 1 to 8 [5,7]. The major isoform present in humans contains four residues (tetrasialo-Tf), but following extensive alcohol consumption the amounts of isoforms containing less sialic residues increase (disialo-Tf and sialo-Tf), while the remaining isoforms remain unaffected [5,7]. Tf molecules containing two or less sialic residues are collectively termed as CDT. Therefore, CDT is an effective biomarker of heavy alcohol consumption, as increased levels of these isoforms are observed in individuals that consume moderate-to-high quantities of ethanol [7]. Due to this, CDT has the limitation of not being suitable for monitoring abstinence or low-occasional drinking. CDT shows good specificity (60–96%) but poor sensitivity (31–91%), since individuals can show low levels despite having consumed excessive alcohol; also, false positives may occur due to genetic variance, liver diseases, congenital disorders of glycosylation and medications [3,8]. Another limitation is associated to the fact that high quantities of ethanol need to be ingested for CDT isoforms to increase significantly. Therefore, CDT is used in clinical and forensic scenarios only to assess chronic moderate-to-heavy alcohol consumption [7,9]. During abstinence periods CDT values take approximately 2–3 weeks to fall back into the reference levels [9].

Analytical considerations

Analytically, CDT is assumed as the sum of disialo-Tf and sialo-Tf relative to the total amount of Tf (% CDT), to compensate for variations in total Tf concentration [7,9,10]. Different Tf isoforms were originally detected using isoelectric focusing; however, this technique is difficult to perform and too complex to be used in routine laboratories. Recently, a direct immunoassay procedure was introduced in the market. In Table 1, an overview of newly developed methods for CDT analysis in biological samples

is presented. High pressure LC (HPLC) methods with visible detection at 460–470 nm were developed using anion exchange [9] and reverse-phase columns [11]. CE methods were developed using UV detectors at 200 nm [12]. Commercial HPLC and CE kits are available, and both techniques allow the determination of different Tf isoforms; however, CE relies on the peptide bond absorbance measure at 200 nm, where various other biomolecules could interfere, while the HPLC method relies on the specific absorbance of the iron–transferrin complex (460–470 nm) [9]. The lack of standardization is the great limitation of CDT use as biomarker; MS would be best technique for a reference CDT method, and, as seen on Table 1, studies have been performed using ESI-TOF-MS, ESI-TOF, MALDI-TOF, MALDI-TOF-MS, CE-ESI-TOF/MS and LC-ESI-MS, with the ESI sources operated in positive ionization mode. Of all these papers, only one reported the online coupling of immunoaffinity LC to the MS detector [13], while in the remaining, a previous separation of the Tf variants on a separate step was performed, either through ion-exchange chromatography and collection of the CDT fractions [14–16] or native polyacrylamide gel electrophoresis [11]. This is the main reason why no MS-based method for clinical CDT measurement has been developed [11]. To date, the HPLC method is regarded as the best candidate CDT reference method [5,9,14]. Another problem related to CDT analysis in serum and plasma is due to the fact that most available techniques (immunoassays, CE and HPLC) are commercially available and different cut-off values are proposed by the manufacturers, and this feature shows even more the lack of standardization and the need for a reference method for CDT analysis with a uniform cut-off. Additionally, when using CDT, the limitations of lack of sensitivity and the short detection window of 2 weeks have to be considered [8].

5-hydroxytryptophol

Ethanol interacts in the metabolism of serotonin (5-hydroxytryptamine, 5-HT) affecting some of its metabolites, namely 5-HTOL and 5-HIAA. After alcohol consumption, 5-HTOL levels increase, altering the 5-HTOL/5-HIAA ratio [7,19]. Instead of 5-HTOL alone, the ratio of 5-HTOL to 5-HIAA is used as a biomarker of alcohol consumption to compensate for interferences from variations of sample dilution and serotonin turnover [20]. The 5-HTOL/5-HIAA ratio is a marker for recent alcohol exposure [7,19,20], and does not appear to be affected by consumption of 5-HT-rich foods, selective serotonin reuptake inhibitors or genetic variance [19].

Analytical considerations

5-HTOL and 5-HIAA are electroattractive compounds and, therefore, suitable for HPLC analysis with electrochemical detection using potentials ranging from 620–700 mV versus an Ag/AgCl reference electrode [20,21]. However, the limits of quantification obtained with this equipment are insufficient to determine 5-HTOL in biological specimens, since it normally occurs at low concentrations [19,20,22]. An alternative procedure was based on the analysis of 5-HTOL using GC coupled to MS (GC–MS) after derivatization with pentafluoropropionic acid anhydride, but this approach implies analysis of the ratio with two different systems with obvious implications on the precision of the results [19,22]. LC–MS/MS techniques allow for the simultaneous detection of both metabolites during the same chromatographic run and for the determination of the low concentrations of 5-HTOL or glucuronide (GTOL) [19,22,23]. These techniques are used in the most recent developed methods for 5-HTOL analysis [19,22–24]. Internal standards include 5-methoxy-2-methyl-3-indoleacetic acid, a compound not present in the organism [19] and deuterium-labeled versions of 5-HTOL glucuronide (GTOL-d4) [23,24], 5-HTOL (5-HTOL-d4) [25] and 5-HIAA (5-HIAA-d2) [24,25]. Concerning extraction procedures, liquid–liquid extraction (LLE) is reported [19,25], as well as SPE using C18 sorbents [23].

Toxicological considerations

5-HTOL, GTOL and 5-HIAA are detected in urine. 5-HTOL can be found in urine mostly in the conjugated form with GTOL, but also with sulfate, although at a lesser extent; 5-HIAA is found in urine in its free

Table 1. Analytical methods for the determination of carbohydrate-deficient transferrin in biological specimens.

Biological sample (amount)	Analytical technique	Sample treatment	Ref.
Serum (100 µl)	HPLC-visible	Precipitation	[9]
Serum	CIEF-UV HPLC-UV PMF	Native-PAGE	[11]
Serum (200 µl)	CE-UV	Dilution	[12]
Serum (25 µl)	LC–MS	Dilution	[13]
Plasma	ESI-TOF-MS	Anion exchange chromatography	[14]
Serum and plasma (30 µl) CSF (4 ml)	LC–MS	Anion exchange chromatography	[15]
Serum (100 µl)	MALDI-TOF-MS	Anion exchange chromatography	[16]
Plasma	CE-TOF/MS	–	[17]
Serum (500 µl)	MALDI-TOF ESI-Q-TOF	HPLC-UV/ICPMS	[18]

CIEF: Capillary isoelectric focusing; CSF: Cerebrospinal fluid; ICPMS: Inductively coupled plasma MS; PMF: Peptide mass fingerprint.

form [7,20]. Since 5-HTOL is present in urine mostly as the GTOL derivative, the analysis of its free form requires sample hydrolysis [19,21,25]. The increasing levels of 5-HTOL can be found in urine during 11–19 h after alcohol consumption, supporting the reason why this is a biomarker for recent alcohol consumption [19,22]. An established cut-off value for distinguishing alcohol consumption from abstinence 14 h prior to sampling is assumed at 15 pmol/nmol for the ratio 5-HTOL/5-HIAA [19–22]. However, it has to be taken into account that 5-HTOL should be regarded to date as a potential alcohol biomarker only, since its routine application is still scarce.

Exposure-based biomarkers

Ethanol

The most commonly used approach to determine recent alcohol exposure, is by analyzing ethanol itself [6,26]. Direct measurement of ethanol presents several advantages when compared with other biomarkers: ethanol is the agent of intoxication, the pathological effects are related to its concentration in tissues, its measurements are highly sensitive and specific, and ethanol is rapidly distributed into the water present in the body allowing the molecule to be sampled in a variety of body fluids [6,21,27]. However, direct measurement of ethanol has the limitation of its short half-life, being rapidly metabolized and concentrations vary in different body fluids or tissues. So, estimating ethanol concentrations among the different body compartments and establishing relationships between compartment concentrations can be a complex task, since these variations are also affected by interindividual variations such as sex or body mass [6].

Analytical considerations

Ethanol presents high volatility, therefore, the most common employed analytical method is GC with headspace injection coupled to a flame ionization detector (HS-GC-FID) [21,26,28–31]. Few methods report the use of GC–MS [25,32]. In some cases, a sample preparation procedure is applied using headspace solid-phase microextraction (HS-SPME) [28,33]. Internal standards in GC are usually organic solvents as *n*-propanol [25,30,32], *t*-butanol [28,29,31,33] or acetonitrile [26]. Another commonly used approach to determine ethanol is through the enzymatic reaction using the enzyme alcohol dehydrogenase, as happens during metabolism. Ethanol is converted by ADH into acetaldehyde reducing the coenzyme NAD^+ into NADH during the process [6,34]. The increase of NADH results in an increase of absorbance at 340 nm [34]. Other enzymatic approaches are based on the formation of different color intensities [6,29].

Toxicological considerations

Since alcohol distributes into body water, it could be measured theoretically in any body fluid. Measurements in whole blood or expired air (breath) have, however, become the standard approaches [6]. Most analytical advances since the year 2001 were performed using blood samples [21,25,28,29,31–34]. When ethanol is determined in other body fluids, the measured concentrations are usually standardized by converting to the equivalent concentration in blood [6]. The blood alcohol concentration is of important clinical and forensic value, since it correlates directly with the impairment degree of an individual, as blood is the main route for alcohol distribution in the body. Ethanol has the property of partitioning from an aqueous solution into a vapor, during a deep inhalation of air into the lungs, diffusing out of the pulmonary capillary blood into the air and reaching equilibrium concentration [3,6]. Based on this, ethanol is analyzed in breath, which shows good correlation with the blood alcohol concentration; however, considerable variability exists in the measured breath alcohol concentration [3,6]. Despite this variability, a uniform breath to blood partitioning coefficient has been defined, allowing for the use of different commercial available devices (breath analyzers) used routinely for breath alcohol analysis both for clinical and forensic applications [6].

Methods have also been developed for urine samples [26,28,30,31,34]. Urine, oral fluid and sweat are specimens that contain similar concentrations of ethanol as plasma at the time of production; however, due to differences in the time of fluid production and ethanol measurement, different ethanol concentrations between these fluids and plasma are found [6]. These samples are useful for determining if alcohol consumption has occurred up to hours before analysis [3,6]. Ethanol has also been determined in vitreous humor specimens in cases of post-mortem sampling [28,31].

Hemoglobin-associated acetaldehyde

Acetaldehyde (AcH) is the main metabolite of the oxidative metabolism pathway of ethanol and can be detected in blood, but its measurement is inaccurate and this metabolite is eliminated very fast from the organism, making AcH itself a poor biomarker [6,35]. However, AcH is extremely reactive, binding covalently to many proteins (AcH protein adducts) including hemoglobin (Hb), whose adducts formed with acetaldehyde (HAA) can be detected and used clinically to identify cases of heavy alcohol consumption [4,6]. The levels of AcH protein adducts remain elevated in blood during 1 month after alcohol consumption [4]. A limitation of this biomarker is the fact that variations in enzyme aldehyde dehydrogenase (as is the case of the

slow variant ALDH2*2) result in increasing levels of AcH [6,36], medications interfering with ALDH might result in the same effect [6], and false positive results might originate due to the formation of AcH in blood after sample collection [4].

Analytical considerations

Hemoglobin-associated acetaldehyde (HAA) can be assayed by immunoassays [4,6]; however, these lack specificity, since it becomes difficult to select specific adducts for the development of antibodies, and the development of reproducible and specific antibodies is challenging [4,35]. Cation-exchange LC allows for the separation and identification of different modified hemoglobin fractions [35], and has been implemented for HAA detection with fluorescence detectors, an assay known as the 'whole blood-associated acetaldehyde assay' [6,36]. However, most analytical methods found in literature to determine HAA in biological samples date before the year 2001, and to date no routine method for determining HAA has been applied. Only recently two new methods were developed for the determination of HAA in blood samples using CE-MS with positive ESI [35] and LC-TOF-MS using cation-exchange columns [37].

Fatty acid ethyl esters

FAEEs are minor ethanol metabolites formed by the reaction of ethanol with endogenous free fatty acids and acyl-CoA/fatty acids in blood and tissues, catalyzed by the enzyme fatty acid ethyl ester synthase and acyl-CoA/ethanol *O*-acetyltransferase [4,38]. FAEEs have been known since 1960s to be biotransformation products of ethanol [39], but only in 2001 were proposed as biomarkers for ethanol exposure [40]. The FAEEs comprise over 20 different species, and ethyl laurate (E12), ethyl myristate (E14), ethyl palmitate (E16), ethyl palmitoleate (E16:1), ethyl stearate (E18), ethyl oleate (E18:1), ethyl linoleate (E18:2), ethyl linoleate (E18:3) and ethyl arachidonate (E20:4) are the main compounds analyzed in biological specimens (Table 2). FAEEs are mainly found in blood and have the ability to accumulate in several organs, adipose tissue, meconium and hair [41].

Analytical considerations

Table 2 resumes the analytical methods used for FAEEs analysis in biological specimens. The FAEEs are nonpolar compounds of lipophilic nature, and therefore, analysis is widely performed using GC–MS. Regarding the ionization source, chemical ionization (CI) [42–46] and electron impact (EI) [47–50] are used; however CI sources are preferred by some authors since with EI sources identical fragments are obtained [44,51]. GC-FID [52,53] and GC–MS/MS with CI sources [46] have also been employed. LC–MS/MS methods were developed, using reverse-phase columns with C8 stationary phases, with ESI sources operated in the positive mode [51,54–56]. Deuterated internal standards of E14, E16, E18 and E18:1 are commercially available and are widely used [1,38,45,47–49,56,8,57,58]; however, ethyl heptadecanoate was used in several other papers, especially when other FAEEs species are included [42–44,46,51–55,59–61]. As seen in Table 2, sample clean-up procedures are mostly based on HS-SPME and SPE, while more recently microwave-assisted extraction and supported liquid extraction have also been applied. Regarding SPE, due to the nature of FAEEs, normal-phase aminopropyl cartridges are widely preferred, but anion exchange cartridges were also used [44]. In solid samples, as hair and meconium, an extraction step is performed previously to the clean-up procedure, this is mostly achieved using LLE with acetone-hexane or incubation with DMSO and hexane or heptane.

Toxicological considerations

In blood, the FAEEs present a two-phase kinetic elimination profile persisting at least 24 h after alcohol intake, being then hydrolyzed by the action of FAEE hydrolases or distributed into tissues [4,59,62]. Detection of FAEEs in blood is, therefore, limited to recent consumption [4]. When analyzing serum, it has to be taken into account that *in vitro* formation of FAEEs occurs when samples are stored at room temperature for at least 1 day, however, storage at 4°C, or -80°C up to 2 days, does not alter the concentration [3]. Inter-genre variation also exists in blood and plasma, the peak concentration of males is found to be approximately twofold higher [3]. Hair and meconium analysis require sensitivity, since the compounds are found at much lower concentrations; in addition, sample amount, especially in the case of hair, can be very low, and a high chromatographic background from complex samples is expected [4]. Another consideration in hair and meconium is the variation of the concentrations of the different FAEEs species, therefore, the results in these specimens are considered as a sum of the analyzed FAEEs concentration. FAEEs are mainly incorporated in the hair from the sebum [3,40]. This incorporation is not affected by pigmentation [40]; however, regular use of hair products containing at least 10% ethanol can elevate the concentrations [3,49]. Several species were studied in hair but only four were found to correlate with the use of alcohol, ethyl myristate, ethyl palmitate, ethyl oleate and ethyl stereate [38,40]. The use of FAAEs as alcohol biomarkers in hair was originally proposed by Pragst and coworkers using HS-SPME and GC–MS [38].

Table 2. Analytical methods for the determination of fatty acid ethyl esters in biological samples.

Compounds	Biological sample (amount)	Analytical technique	Extraction	Clean up	LOD	LLOQ	Ref.
Myristate, palmitate, oleate, stearate	Hair (20 mg)	GC–MS	Incubation (DMSO-heptane)	HS-SPME	0.004–0.014 ng/mg	0.011–0.031 ng/mg	[1]
Myristate, palmitate, oleate, stearate	Hair (30 mg)	GC–MS	LLE (DMSO-heptane)	SPME	0.008–0.026 ng/mg	0.027–0.087 ng/mg	[8]
Myristate, palmitate, oleate, stearate	Hair (50 mg)	GC–MS	LLE (DMSO-heptane)	HS-SPME	0.001–0.04 ng/mg	0.04–0.12 ng/mg	[38]
Laureate, myristate, palmitoleic, palmitate, oleate, stearate	Neonatal hair of babies (20 mg)	GC–MS	Incubation (DMSO-heptane)	SPE (aminopropyl)	0.008–0.02 pmol/mg	0.008–0.22 pmol/mg	[42]
Palmitoleic, palmitic, linoleic, oleic, stearic, arachidonic	Meconium (0.5–1 g)	GC–MS	LLE (acetone-hexane)	SPE (normal phase)	0.05 ng/mg	–	[43]
Laurate, myristate, palmitate, stearate, oleate, linoleate, linolenate, arachidonate, docosahexaenoate	Meconium (0.5 g)	GC–MS	LLE (hexane)	SPE (anion-exchange)	0.05–1 ng/mg	–	[44]
Laurate, myristate, palmitate, palmitoleate, stearate, oleate, linoleate, arachidonate	Meconium (0.1 g)	GC–MS	–	SPME	0.065–0.1 ng/mg	0.010–0.150 ng/mg	[45]
Laurate, myristate, palmitate, oleate, stearate	Hair (30 mg)	GC–MS/MS	Comparison of three solvents	HS-SPME	0.002–0.03 ng/mg	–	[46]
Myristate, palmitate, stearate	Hair (50 mg)	GC–MS	Incubation (DMSO-heptane)	HS-SPME	5–800 ng/mg	10–1800 ng/mg	[47]
Myristate, palmitate, stearate	Meconium (0.5 g)	GC–MS	MAE with hexane/water	–	0.05–0.1 ng/mg	0.100–0.500 ng/mg	[48]
Myristate, palmitate, oleate, stearate	Hair (30 mg)	GC–MS	Incubation (DMSO-heptane)	HS-SPME	0.02–0.1 ng/mg	0.05–0.2 ng/mg	[49]
Myristate, palmitate, oleate, stearate	Sebum	GC–MS	MAE with acetone/hexane	SPME	3–10 pg per sample	10–30 pg per sample	[50]
Laurate, linolenate, myristate, arachidonate, palmitoleate, linoleate, palmitate, oleate, stearate	Meconium (1 g)	LC–MS/MS	LLE (acetone-hexane)	SPE (aminopropyl)	11.3–15.9 ng/g	29.3–51.2 ng/g	[51]
Laurate, myristate, palmitate, stearate, linoleate, linolenate	Meconium (0.5 g)	GC-FID	LLE (acetone-hexane)	SPE (aminopropyl)	50 ng/g	100 ng/g	[52]

FID: Flame ionization detector; HS-SPME: Headspace solid-phase microextraction; LLE: Liquid–liquid extraction; MAE: Microwave-assisted extraction; SLE: Supported liquid extraction.

Table 2. Analytical methods for the determination of fatty acid ethyl esters in biological samples (cont.).

Compounds	Biological sample (amount)	Analytical technique	Extraction	Clean up	LOD	LLOQ	Ref.
Myristate, palmitate, palmitoleate, oleate, linoleate, linolenate, arachidonate	Meconium (1 g)	GC-FID	LLE (acetone-hexane)	Silica column chromatography	–	–	[53]
Laurate, myristate, palmitate, palmitoleate, oleate, linoleate, linolenate, stearate, arachidonate	Meconium (0.5 g)	LC–MS/MS	LLE (acetone-hexane)	SPE (aminopropyl)	0.01–0.08 nmol/g	0.02–0.27 nmol/g	[54]
Laurate, myristate, palmitate, palmitoleate, stearate, oleate, linoleate, linolenate, arachidonate	Meconium (1 g)	LC–MS/MS	LLE (acetone-hexane)	SPE (aminopropyl)	–	0.12–0.20 nmol/g	[55]
Laurate, myristate, linolenate, palmitoleate, arachidonate, linoleate, palmitate, oleate, stearate	Meconium (1 g)	LC–MS/MS	LLE (methanol)	SLE	15–50 ng/g	5–50 ng/g	[56]
Myristate, palmitate, oleate, stearate	Meconium (0.05 g)	GC–MS	LLE (acetone-hexane)	HS-SPME	6.3–11.9 ng/g	18.8–35.8 ng/g	[57]
Myristate, palmitate, oleate, stearate	Skin surface lipids	GC–MS	–	HS-SPME	0.3–1 ng per sample	1–3 ng per sample	[58]
Palmitate, stearate	Serum	GC–MS	LLE (acetone-hexane)	SPE (aminopropyl)	–	–	[59]
Palmitate, stearate, oleate, linoleate, arachidonate, eicosapentaenoate, docosahexaenoate	Liver and adipose tissue (1 g)	GC–MS	LLE (acetone-hexane)	SPE (aminopropyl)	–	–	[60]
Palmitate, oleate, stearate	Plasma (0.5 ml)	GC–MS	LLE (hexane)	SPE (aminopropyl)	–	5–10 nM	[61]
Myristate, palmitate, oleate, stearate	Hair (30–100 mg)	GC–MS	Incubation (DMSO-heptane)	SPE (aminopropyl)	–	0.01 ng/mg	[65]

FID: Flame ionization detector; HS-SPME: Headspace solid-phase microextraction; LLE: Liquid–liquid extraction; MAE: Microwave-assisted extraction; SLE: Supported liquid extraction.

Since then, several methods appeared for hair analysis, including adaptations of the mentioned method. One important achievement of the performed studies is the establishment of cut-off values for identifying abstinence, social moderate drinking and chronic excessive drinking. Establishing proper cut-off values is a challenging task, and much controversial results are reported in the literature. FAEEs concentrations tend to increase with the hair length [40], different cut-off values are, therefore, proposed for the hair segment length. In the latest Consensus of the Society of Hair Testing (SoHT) of 2014, for segments with 0–3 cm and 0–6 cm, a value of 200 and 400 pg/mg, respectively, is suggested for social drinking, and 500 and 1000 pg/mg, respectively, for chronic excessive drinking [63]. Compared with well-established biochemical markers as CDT, hair FAEEs have the advantage of longer detection windows and enhanced specificity and sensitivity [8]. Different FAEEs species can also be detected in meconium, to assess alcohol consumption during pregnancy. However, oppositely to hair, there is no agreement on the species to be monitored in meconium, with different authors reporting the analysis of different species (Table 2). Observing the mentioned table, ethyl palmitate and oleate are the most analyzed species, while ethyl laurate and myristate are analyzed at a lesser extent. In fact, several authors exclude these last two species as biomarkers in meconium, since large quantities are found in cases where mothers have not consumed alcohol [43,51,52,55]. Cut-off values for discriminating exposure to alcohol during pregnancy and no exposure have been proposed at 50 ng/g [43], 2 nmol/g (~600 ng/g) [45,48,51,52,55,57] and 500 ng/g [64]. 2 nmol/g has been mostly used and become internationally accepted; however, further research is needed in establishing proper cut-off values in meconium. Studies in meconium have reported high sensitivities and specificities (close to 100%) when ethyl laurate and myristate are not considered [45,52,57]. FAEEs were also analyzed in tissues such as skin surface lipids, sebum, liver and adipose tissue (Table 2).

Ethyl glucuronide

Ethyl glucuronide (EtG, ethyl-β-D–6 glucuronic acid) is a phase II metabolite of ethanol, formed by the conjugation of glucuronic acid with ethanol via the enzyme uridine diphosphate glucuronyltransferase [4,5,7]. It represents around 0.6–1.5% of the ingested ethanol dose [3,66], and is a stable, nonvolatile and water miscible metabolite [7,39,67]. EtG presents very high specificity and sensitivity, even exceeding those of other alcohol biomarkers. This is of outmost importance, since it is only detectable in those cases where alcohol consumption has occurred [68–70].

Analytical considerations

EtG is highly polar, of acidic nature, with high affinity to aqueous solvents. EtG is mostly analyzed using GC and LC techniques; however, immunoassays and CE methods have also been described. As shown in Table 3, few papers developed methods using ELISA. A commercial enzyme immunoassay is also available, but although being cost-effective techniques when compared with others and presenting relative high specificity, the commercial enzyme immunoassay is still regarded as a screening tool, and several authors highlight the importance of confirming the results by GC or LC methods [3,71]. CE is used only in some papers, usually with UV detection at 214 [72,73] and 254 nm [74] and recently with MS detection [75]. GC and LC techniques are mostly coupled to MS or MS/MS detection; however, methods were also developed coupling HPLC to pulsed electrochemical detection. GC–MS and GC–MS/MS are operated both using EI [70,76–80] and negative CI ionization modes [62,66,81–83]. In the case of LC–MC detectors, ESI sources operated in the negative ionization mode are most widely used [30,31,39,56,8,64,67–69,84–117]; however, two works reported the use of APCI sources in order to overcome the matrix effect that typically occurs on ESI sources [118,119]. Due to the nonvolatile nature of EtG, derivatization is required for GC techniques, and the use of agents as heptafluorobutyric anhydride [81,82], pentafluoropropionic anhydride [62,66,80,83], *N,O*-Bis(trimethylsilyl)trifluoroacetamide [70,76–79,120] or acetic anhydride [71] has been described. LC techniques provide, therefore, an advantage since no derivatization is required; however, separation of EtG on a reverse-phase analytical column requires mobile phases with higher contents of aqueous solvents, which may be problematic when ESI sources are used [69,101]. Solutions to this problem have passed by enhancing ionization through postcolumn addition of acetonitrile [39,90,91,96,98,104,106,109,113,118] or 2-propanol [31,89,110,111], using hydrophilic interaction LC columns allowing for the use of mobile phases with higher organic solvent content [30,68,69,84,86,92,94,108], using porous graphitic carbon columns [64,83,90,93,97,116,118] or the use of a mixed-mode reverse-phase/weak anion exchange stationary phase [101]. Regarding the internal standard, deuterated EtG (EtG-d5) is commercially available and widely used for MS-based techniques; however, when MS detectors are not used methylglucuronide [121], propylglucuronide [122] and 2,4-dimethylglutaric acid [72] may be used. The clean-up procedure of choice is SPE, mostly employing anion exchange or aminopropyl sorbents. Other used clean-up procedures include HS-SPME and microwave-assisted extraction. Extraction of EtG from solid samples requires solvents with high aqueous content or pure water, several

approaches are reported using incubation and/or ultrasonication during different times with these solvents, as shown in Table 3.

Toxicological considerations

After elimination of ethanol, EtG can be found in blood for up to 14 h [4,102] and for up to 4 days in urine [116]. In post-mortem blood, EtG allows to differentiate between alcohol consumption prior to death and post-mortem formation of ethanol [31], with high reported sensitivities and specificities (close to 100%) [31,116]. However, decomposition and formation of EtG is possible in this sample [31], and the same can occur in urine; although EtG is stable for up to 5 weeks when stored at 4°C; bacterial contamination can alter the concentrations of EtG, which may lead to false results [3,111]. Therefore, the combined detection of EtG and EtS has been recommended to improve clinical specificity [2]. In urine, Hernández Redondo and coworkers have suggested an approach to improve stability by inhibiting bacterial growth using dried urine spots [111]. In blood, EtG has also been determined in DBS, which presents an advantage in forensic scenarios when no other sample is available [103]. There is no general agreement on the cut-off for urine and blood; currently a concentration of 0.1 mg/l in urine is used for proving abstinence according to the German driving license guidelines [91,120]; however, other authors propose higher concentrations as 0.3 mg/l [118], 1 mg/l [30] or 1.1 mg/l [117]. One method was also developed for the determination of EtG in oral fluid [102]. EtG incorporates into hair at very low amounts, in the range of picograms, thus requiring sensitive analytical methods. The incorporation is not affected by natural hair color, but cosmetic treatment as bleaching, perming, dying of hair and abundant hair washing may lower the concentrations of EtG; however, the use of hair products including alcohol-based does not appear to influence EtG concentrations; hair analysis for EtG alone has the disadvantage of not being suitable for proving complete abstinence, only low social drinking [63]. In the 2014 Consensus, the following recommendations were drawn: during sampling, the type of cosmetic hair treatment should be noted and considered during data analysis; EtG should be the first choice in abstinence assessment; and in the same thought, a positive EtG result cannot be overruled by a negative FAEEs result [63]. Powdering of hair results in increased extraction efficiencies when compared with cut-powdered hair with scissors [90,114], therefore, powdering is preferred unless similar recoveries are shown when different procedures are used [63]. Currently, the following cut-offs are suggested by the SoHT for EtG in hair: 7 pg/mg for social moderate drinking and 30 pg/mg for chronic excessive drinking [63]. Different population studies using EtG in hair to monitor alcohol consumption have reported sensitivities and specificities close to 100% (overall sensitivity of nine selected studies is 96% and of specificity is 99%) [123]. EtG can also be sampled using hair from different body sites, except for axillary hair, and the same cut-off values are assumed; however, the time period represented by these samples should be considered [63]. EtG has also been detected in fingernails and dental tissue, using similar sample preparation procedures as used for hair as shown in Table 3. Several methods have also been developed using meconium samples, and since 2010, different studies have been conducted in order to establish proper cut-off values for distinguishing alcohol consumption and abstinence during pregnancy. Morini and coworkers proposed the use of 1.5 nmol/g [124], while Bakdash and coworkers determined a value of 274 ng/g [64]. In some papers, a value of 2 nmol/g is also suggested [125–127]; however, Morini and coworkers suggested a revision of this value based on their results [126]. Recently, Pichini and coworkers suggested the use of 0.9 nmol/g when using the ELISA immunoassay with high sensitivity (100%) and specificity (78%) [127]; however, in the same work the authors considered lowering the cut-off value. This latter data clearly indicates the need of further and extensive research in determining proper cut-off values in meconium. Considering alcohol consumption during pregnancy, EtG was also determined in other relevant samples, as placenta and placental perfusate. Moreover, EtG was also determined in post-mortem samples, such as vitreous humor, bone marrow, liver, muscle and fat tissue.

Ethyl sulfate

EtS (ethyl hydrogen phosphate or sulfovinic acid) is also a phase II metabolite of ethanol, obtained by its conjugation with sulfate via the superfamily of cytosolic sulfotransferases [3,5,7,129,130]. Only a small amount (0.1%) of the ingested alcohol undergoes this process [131,132]. EtS is a stable compound and a biomarker of recent alcohol exposure [132].

Analytical considerations

Similarly to EtG, EtS is a highly polar, nonvolatile compound with affinity for aqueous solvents [132], therefore, analysis can be performed using the same instrumentation, analytical columns and ESI source conditions; due to this aspect LC–MS/MS using negative ESI sources is most commonly used (Table 4). In fact, the first chromatographic method for EtS was proposed by Helander and Beck by extending the analysis time of an established LC–MS method for EtG [97]

Table 3. Analytical methods for the determination of ethyl glucuronide in biological samples.

Biological sample (amount)	Analytical technique	Extraction	Clean up	LOD	LLOQ	Ref.
Urine (0.1 ml)	LC–MS/MS	Precipitation (acetonitrile)	–	0.13 mg/l[†]	0.001 mg/l[†]	[30]
Vitreous humor, blood and urine (100 µl)	LC–MS/MS	Vitreous humor and urine: precipitation (methanol), Blood: precipitation (acetonitrile)		0.05 mg/l	0.05 mg/l	[31]
Urine (0.05 ml)	LC–MS/MS	Dilution	–	0.025 mg/l	0.05 mg/l	[39]
Meconium (0.1 g)	LC–MS/MS	Dilution	SPE (anion exchange)	5 ng/g	5 ng/g	[56]
Hair (30 mg)	GC–MS	Ultrasonication (water)	SPE (aminopropyl)	2 pg/mg	4 pg/mg	[62]
Meconium (0.01–0.02 g)	LC–MS/MS	Dilution	Filtration	0.010 ng/g	0.030 ng/g	[64]
Hair (30 mg)	GC–MS/MS	Ultrasonication (water)	SPE (anion exchange)	3 pg/mg	8.4 pg/mg	[66]
Hair (100 mg)	LC–MS/MS	Ultrasonication (water)	SPE (anion exchange)	10 pg/mg	20 pg/mg	[67]
Hair (25 mg), meconium (0.2 g)	LS-MS/MS	Hair: incubation and ultrasonication (water-acetonitrile), Meconium: ultrasonication (water-acetonitrile)	SPE (aminopropyl)	–	Hair: 20 pg/mg, meconium: 50 ng/g	[68]
Hair (30 mg)	LC–MS/MS	Ultrasonication (water)	SPE (CleanScreen EtG)	4 pg/mg	10 pg/mg	[69]
Hair (100 mg)	GC–MS	Incubation (hexane-water)	MAE	100 pg/mg	300 pg/mg	[70]
Urine and serum (0.2 ml)	ELISA GC–MS	Precipitation (methanol)	–	0.25 mg/l	–	[71]
Serum (0.1 ml)	CE-UV	Precipitation (acetonitrile)	SPE (CleanScreen EtG)	0.25 mg/l	0.50 mg/l	[72]
Serum	CE-UV	–	–	0.079 mg/l	–	[73]
Serum	CE-UV CITP-condutivity	Dilution	–	9.8 nM	–	[74]
Serum (0.5 ml), urine (1 ml)	CE-MS	Urine: dilution	SPE (CleanScreen EtG)	Dilution: 2 mg/l, extracts: 0.2 mg/l	–	[75]
Hair (20 mg)	GC–MS/MS	Ultrasonication (water)	SPE (anion exchange)	5 pg/mg	10 pg/mg	[76]
Hair (10 mg)	GC–MS/MS	Ultrasonication (water)	SPE (anion exchange)	0.01 ng/ml	0.02 ng/ml	[77]
Urine (0.475 ml)	GC–MS	–	MAE	0.005 mg/l	0.1 mg/l	[78]
Urine and serum (1.5 ml)	GC–MS	Precipitation (acetonitrile)	SPE (aminopropyl)	Serum: 0.037 mg/l, urine: 0.168 mg/l	Serum: 0.173 mg/l, urine: 0.560 mg/l	[79]
Hair (100 mg)	GC–MS	Ultrasonication and incubation (water)	–	0.025 ng/mg	0.05 ng/mg	[80]

[†]Data taken from the cited source references.
CITP: Capillary isotachophoresis; DUS: Dried urine spots; EtG: Ethyl glucuronide; HS-SPME: Headspace solid-phase microextraction; MAE: Microwave-assisted extraction; PED: Pulsed electrochemical detection.

Table 3. Analytical methods for the determination of ethyl glucuronide in biological samples (cont.).

Biological sample (amount)	Analytical technique	Extraction	Clean up	LOD	LLOQ	Ref.
Placenta and perfusate (950 µl)	GC–MS	–	HS-SPME	Placenta: 13.7 ng/g Perfusate: 1.6 ng/ml	Placenta: 41.6 ng/g Perfusate: 4.8 ng/ml	[81]
Hair (10–50 mg)	GC–MS/MS	Ultrasonication (water)	SPE (CleanScreen EtG) HS-SPME	0.6 pg/mg	2.8 pg/mg	[82]
Hair (50 mg)	GC–MS	Incubation (water)		0.68 pg/mg	2.4 pg/mg	[83]
Fingernails (50 mg)	LC–MS/MS	Ultrasonication (water-acetonitrile)	–	0.00048 mg/l	0.00161 mg/l	[84]
Hair and fingernails (10–50 mg)	LC–MS/MS	Ultrasonication and incubation (water)	SPE (anion exchange)	0.008 pg/mg	2 pg/mg	[85]
Hair (200 mg)	LC–MS/MS	Ultrasonication (water-acetonitrile)	Filtration	0.05 pg/mg	0.18 pg/mg	[86]
Hair	LC–MS/MS	Ultrasonication (water)	–	–	5 pg/mg	[87]
Hair (50 mg)	LC–MS/MS	Ultrasonication and incubation (water-methanol)	–	0.5 pg/mg	1 pg/mg	[88]
Fingernails (30 mg)	LC–MS/MS	Ultrasonication (water)	–	3 pg/mg	10 pg/mg	[89]
Hair (75 mg)	LC–MS/MS	Incubation and ultrasonication (water)	–	1.7 pg/mg	2.3 pg/mg	[90]
Urine (0.1 ml)	LC–MS/MS	Precipitation (methanol)	–	0.005 mg/l	0.019 mg/l	[91]
Hair (30 mg)	LC–MS/MS	Ultrasonication (water)	SPE (CleanScreen EtG)	1 pg/mg	3 pg/mg	[92]
Hair (100 mg), urine (0.1 ml)	LC–MS/MS	Urine: precipitation (methanol), Hair: ultrasonication and incubation (water)	–	Hair: 25 pg/mg, urine: 0.1 mg/l	Hair: 50 pg/mg, urine: 0.25 mg/l	[93]
Hair and pubic hair (50 mg)	LC–MS/MS	Ultrasonication (water)	SPE (anion exchange)	2 pg/mg	10 pg/mg	[94]
Hair (30 mg)	LC–MS/MS	Ultrasonication (water)	SPE (CleanScreen EtG)	–	2 pg/mg	[95]
Hair (100 mg)	LC–MS/MS	Ultrasonication and incubation (water)	–	2 pg/mg	3 pg/mg	[96]
Urine (0.01 ml)	LC–MS	–		0.05 mg/l	0.1 mg/l	[97]
Urine (0.1 ml)	LC–MS/MS	Precipitation (methanol)	–	0.052 mg/l	0.152 mg/l	[98]
Urine (0.1 ml)	LC–MS/MS	Dilution	–	0.03 mg/l	0.10 mg/l	[99]
Bone marrow, liver, muscle and fat tissue (1 g)	LC–MS/MS	Precipitation (methanol)	SPE (aminopropyl)	–	–	[100]
Post-mortem urine (0.05 ml)	LC–MS/MS	Dilution	–	–	0.1 mgl	[101]

[1]Data taken from the cited source references.
CITP: Capillary isotachophoresis; DUS: Dried urine spots; EtG: Ethyl glucuronide; HS-SPME: Headspace solid-phase microextraction; MAE: Microwave-assisted extraction; PED: Pulsed electrochemical detection.

Table 3. Analytical methods for the determination of ethyl glucuronide in biological samples (cont.).

Biological sample (amount)	Analytical technique	Extraction	Clean up	LOD	LLOQ	Ref.
Oral fluid (200 µl)	LC–MS/MS	–	SPE (anion exchange)	–	4.4 ng/ml	[102]
DBS (200 mg)	LC–MS/MS	Dilution	Filtration	0.1 mg/l	0.3 mg/l	[103]
Placenta and fetal tissue (0.5 g)	LC–MS/MS	Precipitation (acetonitrile)		13 pmol/g	22 pmol/g	[104]
Urine (0.5 ml)	LC–MS/MS	–	SPE (anion exchange)	0.04 mg/l	0.13 mg/l	[105]
Serum (0.5 ml)	LC–MS/MS	Precipitation (acetonitrile)	–	0.04 µmol/l	0.2 µmol/l	[106]
Urine and serum (0.1 ml)	LC–MS/MS	Urine: centrifugation (acetonitrile), Serum: precipitation (acetonitrile)	–	Urine: 0.05 mg/l, Serum: 0.03 mg/l	Urine: 0.15 mg/l, serum: 0.8 mg/l	[107]
Dental tissue	LC–MS/MS	Ultrasonication (water-acetonitrile)	–	0.48 pg/mg	1.61 pg/mg	[108]
Hair (100 mg)	LC–MS/MS	Water (ultrasonication)	SPE (aminopropyl)	51 pg/mg	102 pg/mg	[109]
Blood (0.05 ml), DBS (10 µl)	LC–MS/MS	Blood: dilution, DBS: extraction with methanol	–	–	0.1 mg/l	[110]
DUS and urine	LC–MS/MS	Extraction with methanol	–	0.05 mg/l	0.175 mg/l	[111]
DBS (200 mg)	LC–MS/MS	Ultrasonication (water)	–	0.1 mg/l	0.3 mg/l	[112]
Meconium (0.2 g)	LC–MS/MS	Ultrasonication (acetonitrile)	SPE (aminopropyl)	1.5 ng/g	5 ng/g	[113]
Hair (50 mg)	LC–MS/MS	Incubation (water)		0.46 pg/mg	2.29 pg/mg	[114]
Hair (50 mg)	LC–MS/MS	Incubation and ultrasonication (water-methanol)		3 pg/mg	10 pg/mg	[115]
Post-mortem blood (0.2 ml)	LC–MS	Precipitation (methanol)		0.02 mg/l	0.06 mg/l	[116]
Blood (0.2 ml), urine (0.1 ml)	LC–MS/MS	Blood: precipitation (methanol) Urine: dilution	–	Blood: 0.03 mg/l, urine: 0.17 mg/l	Blood: 0.06 mg/l, urine: 0.37 mg/l	[117]
Urine (0.125 ml)	LC–MS	Precipitation (acetonitrile)	SPE (aminopropyl)	0.05 mg/l	0.1 mg/l	[118]
Urine (0.02 ml)	LC–MS/MS	Dilution	–	–	0.1 mg/l	[119]
Urine (0.6 ml)	GC–MS	–	SPE (CleanScreen EtG)	0.05 mg/l	0.08 mg/l	[120]
Urine (1 ml)	HPLC-PED	Precipitation (acetonitrile)	SPE (aminopropyl)	0.03 mg/l	0.1 mg/l	[121]
Urine (0.2 ml)	HPLC-PED	–	SPE (aminopropyl)	0.01 mg/l	0.02 mg/l	[122]
Meconium (0.25 g)	ELISA	–		–	–	[127]
Hair (50 mg)	LC–MS/MS	Ultrasonication (water-acetonitrile-HCl)	SPE (aminopropyl)	1 pg/mg	2.6 pg/mg	[128]

[a]Data taken from the cited source references.
CITP: Capillary isotachophoresis; DUS: Dried urine spots; EtG: Ethyl glucuronide; HS-SPME: Headspace solid-phase microextraction; MAE: Microwave-assisted extraction; PED: Pulsed electrochemical detection.

Table 4. Analytical methods for the determination of ethyl sulfate in biological samples.

Biological sample (amount)	Analytical technique	Extraction	Clean up	LOD	LLOQ	Ref.
Urine (0.1 ml)	LC–MS/MS	Precipitation (acetonitrile)	–	0.2 mg/l†	0.001 mg/l†	[30]
Vitreous humor, blood and urine (0.1 ml)	LC–MS/MS	Vitreous humor and urine: precipitation (methanol), Blood: precipitation (acetonitrile)	–	0.05 mg/l†	0.04 mg/l†	[31]
Urine (0.05 ml)	LC–MS/MS	Dilution	–	0.025 mg/l	0.05 mg/l	[39]
Meconium (0.1 g)	LC–MS/MS	LLE (methanol)	SPE (anion exchange)	2.5 ng/g	2.5 ng/g	[56]
Serum (0.5 ml), urine (1 ml)	CE-MS	Dilution	SPE (anion exchange)	Dilution : 2 mg/l, extracts: 0.2 mg/l	–	[75]
Urine (0.1 ml)	LC–MS/MS	Precipitation (methanol)	–	0.005 mg/l	0.015 mg/l	[91]
Urine (0.1 ml)	LC–MS/MS	Dilution	–	0.020 mg/l	0.08 mg/l	[99]
Post-mortem urine (0.05 ml)	LC–MS/MS	Dilution	.	–	0.1 mg/l	[101]
Placental and fetal tissue (0.5 g)	LC–MS/MS	Precipitation (acetonitrile)	–	23 pmol/g	40 pmol/g	[104]
Serum (0.5 ml)	LC–MS/MS	Precipitation (acetonitrile)	–	0.08 µmol/l	0.40 µmol/l	[106]
Urine and serum (0.1 ml)	LC–MS/MS	Urine: centrifugation, serum: precipitation (acetonitrile)	–	Urine: 0.03 mg/l, Serum: 0.01 mg/l	Urine: 0.1 mg/l, serum: 0.03 mg/l	[107]
Blood (0.05 ml), DBS (10 µl)	LC–MS/MS	Blood: precipitation (acetonitrile), DBS: extraction with methanol	–	–	0.1 mg/l	[110]
DUS and urine	LC–MS/MS	Extraction with methanol	–	0.1 mg/l	0.340 mg/l	[114]
Meconium (0.2 g)	LC–MS/MS	LLE (acetonitrile)	SPE (aminopropyl)	0.3 ng/g	1 ng/g	[113]
Blood (0.2 ml), urine (0.1 ml)	LC–MS/MS	Blood: precipitation (methanol), Urine: dilution	–	Blood: 0.007 mg/l, Urine: 0.06 mg/l	Blood: 0.02 mg/l, urine: 0.16 mg/l	[117]
Urine (0.125 ml)	LC–MS	Precipitation (acetonitrile)	SPE (aminopropyl)	0.05 mg/l	0.1 mg/l	[118]
Urine (0.02 ml)	LC–MS/MS	Dilution	–	–	100 ng/ml	[119]
Urine	LC–MS	Dilution	–	0.5 µmol/l	–	[129]
Urine (0.1 ml)	LC–MS/MS	Dilution	–	0.05 mg/l	0.11 mg/l	[130]
Urine	CE-MS	–	SPE (anion exchange)	–	–	[131]
Urine and serum (0.5 ml)	CE-UV	Urine: dilution, serum: precipitation with silver acetate	SPE (anion exchange)	–	0.6–2 mg/l	[132]
Urine (0.1 ml)	CE-UV	Dilution	–	–	–	[133]

†Data taken from the cited source references.
DUS: Dried urine spots; LLE: Liquid–liquid extraction.

Table 5. Analytical methods for the determination of phosphatidylethanol species in biological samples.

PEth species	Biological sample	Analytical technique	Extraction	Clean up	LOD	LLOQ	Ref.
16:0/16:0, 16:0/18:1, 16:0/18:2	Blood (100 µl)	LC–MS, LC–MS/MS	Lipid LLE (2-propanol:hexane)	–	<0.02 µmol/l	<0.1 µmol/l	[134]
48 homologs	Blood (300 µl)	LC–MS/MS	Lipid LLE (2-propanol:hexane)	–	0.04 µmol/l	–	[135]
16:0/18:1, 16:0/18:2	Blood (100 µl)	LC–MS/MS	Lipid LLE (2-propanol:hexane)	–	0.009 µmol/l	16:0/18:1, 16:0/18:2: 0.03 µmol/l total PEth: 0.10 µmol/l	[136]
16:0/18:1	Blood (300 µl)	NACE-UV	Lipid LLE (2-propanol:hexane)	–	0.4 µmol/l	–	[137]
16:0/16:0, 18:1/18:1	Blood (300 µl)	LC–MS/MS	Lipid LLE (2-propanol:hexane)	–	16:0/16:0: 14 ng/ml, 18:1/18:2: 17 ng/ml	16:0/16:0: 45 ng/ml, 18:1/18:2: 54 ng/ml	[138]
16:0/16:0, 16:0/18:1, 18:1/18:1	Blood (200 µl)	LC–MS/MS	Lipid LLE (2-propanol:hexane)	–	16:0/16:0,18:1/18:1: 0.4 nmol/l 16:0/18:1: 0.9 nmol/l	16:0/16:0: 1.5 nmol/l, 16:0/18:1: 3.1 nmol/l, 18:1/18:1: 1.2 nmol/l	[139]
18:1/18:1, 16:0/18:1	DBS and blood (100 µl)	LC–MS/MS	Lipid LLE (2-propanol:hexane)	–	18:1/18:1: 8.86 ng/ml 16:0/18:1: 24.4 ng/ml	18:1/18:1: 22.7 ng/ml, 16:0/18:1: 87.3 ng/ml	[140]
16:0/18:1	Blood	LC–MS/MS	Precipitation	DLLME	10 ng/ml	30 ng/ml	[141]
17 different species	Blood	LC–MS, LC–MS/MS	Lipid LLE (2-propanol:hexane)	–	0.0005 µM	0.001 µM	[142]
18:1/18:1	HDL	LC-TOF-MS	Lipid LLE (2-propanol:hexane)	–	1 ng/ml	–	[143]
16:0/18:1	DBS	LC–MS/MS	Extraction with methanol	–	2 ng/ml	8 ng/ml	[144]
16:0/18:1, 16:0/18:2	Blood (1 ml)	LC–MS/MS	Lipid LLE (2-propanol:hexane)	–	–	20 ng/ml	[145]
16:0/18:1	Post-mortem blood (300 µl) and organs	LC–MS/MS	Lipid LLE (2-propanol:hexane)	–	–	0.7 µmol/l (0.2 nmol)	[146]
Total PEth	Blood	HPLC-ELSD	Lipid LLE (2-propanol:hexane)	–		0.2 nmol	[147]
16:0/16:0, 16:0/18:1 18:1/18:1	Blood (300 µl)	NACE-MS	Precipitation (methanol) LLE (chloroform)	–	16:0/16:0: 0.1 µM	16:0/16:0: 0.4 µM	[148]
16:0/16:0, 16:0/18:1	Breath	LC–MS/MS	–	–	2 pg/filter	5 pg/filter	[149]

DLLME: Dispersive liquid–liquid microextraction; ELSD: Evaporative light-scattering detection; HDL: High-density lipoproteins; LLE: Liquid–liquid extraction; NACE: Nonaqueous capillary electrophoresis; PEth: Phosphatidylethanol.

Table 5. Analytical methods for the determination of phosphatidylethanol species in biological samples (cont.).

PEth species	Biological sample	Analytical technique	Extraction	Clean up	LOD	LLOQ	Ref.
Total PEth	Blood (300 µl)	HPLC-ELSD	Lipid LLE (2-propanol:hexane)	–	0.1 ng/ml	–	[150]
Total PEth	Blood	Immunoassay	Lipid LLE (2-propanol:hexane)	–	–	–	[152]

DLLME: Dispersive liquid–liquid microextraction; ELSD: Evaporative light-scattering detection; HDL: High-density lipoproteins; LLE: Liquid–liquid extraction; NACE: Nonaqueous capillary electrophoresis; PEth: Phosphatidylethanol.

and monitoring the pseudomolar ions of EtS [129]. CE methods were also developed using UV detectors at 220 nm [133] and 214–215 nm [132] and MS detectors with negative ESI sources [75,131]. For confirmation studies using CE, MS detectors are required rather than UV detectors [7]. For the first LC–MS method for EtS, deuterium-labeled EtG was used as internal standard; however, a deuterium-labeled version of EtS has since become commercially available and used in MS methods [30,31,39,56,91,99,101,104,106,107,110,113,117–119,130,131]; in the case of CE-UV, vinylsulfonic acid has been used [132,133].

Toxicological considerations

EtS can be detected in urine, blood and meconium (Table 4). In urine, EtS stability is comparable to that of EtG and is detectable up to 36 h after moderate alcohol consumption [3]. Oppositely to EtG, EtS is found to be degradable in urine only under high bacterial density [3]. EtS has also been detected in blood, DBS and serum (Table 4). No cut-off has been established to date for urine and blood samples, however, a value of 0.1 mg/l has been used for urine [30]. In meconium, EtS has been analyzed together with EtG [56,113,124,126], however, only one work proposed a cut-off value of 0.012 nmol/g [124]. Attempts to analyze EtS in hair samples were performed my Morini and coworkers; however, no correlation with drinking behavior was found [96]. Finally, EtS was also detected in vitreous humor, placental and fetal tissues. Further research is needed for the use of EtS as an alcohol biomarker, especially for the establishment of cut-off values in biological samples as urine, blood and meconium.

Phosphatidylethanol

The term PEth is used to refer a group of metabolites comprising glycerophospholipid homologs with phosphoethanol as head group [2,134]. PEth is actually an abnormal formation of a phospholipid in cell membranes by the transphosphatidylation of a phospholipid by phospholipase D (PLD) in the presence of ethanol [2,134,135]. Under normal conditions and in the presence of water, PLD would hydrolyze phosphatidylcholine to phosphatidic acid and choline; however, in the presence of ethanol due to a much higher PLD specificity constant to ethanol, phosphatidylethanol is formed instead [2,135]. The PEth homologs are named based on: the number of carbon in the carboxylic acid substituent at the first (a) and second (c) position of the glycerol backbone, the respective number of double bonds (b and d) and are represented as 'PEth a:b/c:d' [2,134]. To date, 48 homolog species have been identified [135]; however, PEth 16:0/18:1 and PEth 16:0/18:2 are among the most predomi-

nant species [2,134,136], and therefore, most commonly analyzed (Table 5). PEth presents high specificity and sensitivity, since in theory, it is only formed when ethanol is present, and a clear correlation exists with the reported alcohol consumption [2]. PEth is not determined in blood after single alcohol consumption and accumulation in cells occurs after prolonged exposure to alcohol. Therefore, PEth represents a marker for long-term alcohol abuse [7].

Analytical considerations

Because PEth species do not have sufficient UV absorption groups in their structure, HPLC methods are mostly coupled to evaporative light-scattering detection, MS and MS/MS detection, however, LC-TOF-MS is also described (Table 5). Due to the high molecular weight of the PEth species, GC analysis is not ideal, and this is probably the main reason for which no methods were developed using GC for determining PEth in biological specimens. An immunoassay was also developed using monoclonal antibodies and nonaqueous capillary electrophoresis coupled to UV detection at 200 nm [137] and MS detection. Considering the mentioned techniques, HPLC-evaporative light-scattering detection and nonaqueous capillary electrophoresis-UV measure the sum of the PEth species [2,7,135], only MS detectors have the capability of measuring individual species [2,7,134,135]. All analytical methods coupled to MS detection use negative ESI [134–136,138–146]. Phosphatidylbutanol 18:1/18:1 [138,141,142,147] and phosphatidylpropanol 18:1/18:1 [134,135,139,140,143–145,148] are mostly used as internal standards. However, in one work, deuterium-labeled versions of the species 16:0/18:1 and 16:0/18:2 were synthesized by the authors [136], and recently deuterium-labeled PEth species have become commercially available [149]. The sample preparation procedure for PEth analysis usually employs an LLE procedure for lipids (Table 5) using a mixture in different proportions of hexane and propanol. Variations in the time of addition of solvents are described, the direct application of a hexane:2-propanol (3:2 v/v) mixture is reported [137,143,146,150], as well as step addition of isopropanol is followed by hexane [134,135,138–140,142,147] or heptane [136]. Higher recoveries were reported when using the step addition approach, probably due to the cell membrane disruption and breaking of the lipid-protein linkages by 2-propanol [147]. Extraction of DBS with methanol is also reported, as well as precipitation of blood followed by dispersive liquid–liquid microextraction (Table 5).

Toxicological considerations

As seen in Table 5, PEth is mainly detected in blood, the half-life is of about 4 days, and reports indicate that it can be detected for up to 4 weeks after alcohol has been eliminated from the body [2,7,139]. PEth is stable in blood for 3 days at 4°C or -80°C; however, at room temperature, or even at -20°C PEth is still formed in the presence of ethanol [146]. Activation of PLD by freezing is suggested as the reason for formation of PEth at -20°C, so storage under this temperature should be avoided [146]. For the establishment of cut-off values it has to be taken into account the number, and which PEth homologs are analyzed. Zheng and coworkers suggested the use of 0.70 µmol/l for total PEth, 0.20 µmol/l for PEth 16:0/18:1 and 0.18 µmol/l for PEth 16:0/18:2 [136]. In another work, Stewart and coworkers used 20 ng/ml individually for the species 16:0/18:1 and 16:0/18:2 [145], and 30 ng/ml was suggested for 16:0/18:1 by Cabarcos and coworkers [141]. PEth analysis in blood for detecting heavy drinking demonstrates good clinical efficiency, with high sensitivity and specificity [151]. However, one major limitation of blood PEth analysis is the fact that it is suitable as a marker for heavy drinking only [7,151]. PEth was also detected in DBS, high-density lipoproteins and post-mortem abdominal fat and tissues (Table 5). Recently, PEth analysis of the species 16:0/16:0 and 16:0/18:1 was conducted in a noninvasive manner using breath analysis. Only 16:0/18:1 was detected in samples of alcohol consumers; however, no difference of PEth concentration was found between abstinence and recent consumption, therefore, the authors suggested that the PEth breath test would represent chronic and not acute drinking [149]. These were, however, very preliminary results and further research is needed for the use of PEth in breath analysis.

Conclusion

Although traditional biochemical markers as MCV and the enzymes ALT, AST, GGT, are widely used in clinical scenarios, they present several limitations and disadvantages for the use as alcohol biomarkers, mainly due to the lack of specificity and to the fact that they are influenced by several metabolic conditions, diseases and genetic variations. CDT is affected by some diseases and genetic variations, but has become an established alcohol biomarker with advantages over the above mentioned markers; however, the lack of standardization and cut-off value establishment remains as its greatest limitation. The 5-HTOL/5-HIAA ratio and ethanol are effective biomarkers, but evaluate only recent alcohol consumption, requiring sampling and analysis close to the time period of alcohol consumption. HAA presents several possibilities, however, the methodological obstacles impaired the development of novel methods and its use as an alcohol biomarker in recent years. In this manner, nonoxidative metabolites

of ethanol as FAEEs, EtG, EtS and PEth, overcome some of the limitations presented by other markers. However, due to their relative recent use as biomarkers, further research is needed in order to standardize their use and establish cut-off values for a proper result interpretation. Based on the review here provided, EtG surely looks like the biomarker with the most potential, especially because, depending on the biological specimen, it allows differentiating recent from chronic alcohol consumption, with high specificity and selectivity, and thus covers a clinical and forensic important time window between short-term markers, such as ethanol itself, and long-term markers, such as CDT.

Future perspective

Alcohol consumption analysis is a very attractive field in clinical and forensic applications, and accurate diagnostic is one of the major obstacles to be achieved in future. The lack of standardization and specificity of commonly used biomarkers urges the continuous research of novel and effective biomarkers, as was discussed here. The continuous development of techniques and tools in the field of proteomics and 'omics' areas can be of great advantage in the search of novel proteins and molecules targeted exclusively by alcohol consumption and, consequently used as biomarkers. On the other hand, further developments and research using sensitive techniques as MS can overcome several obstacles related to the use of alcohol biomarkers. In the future, one of the main challenges on alcohol biomarker research will not only be the establishment of proper cut-off values for both new and well-established biomarkers, but also the search of stable, noninvasive and readily available biological specimens. In this manner, the recent report of the use of breath analysis for PEth detection is very promising since it raises possibilities of analyzing alcohol exposure through noninvasive manners.

Financial & competing interests disclosure

The authors acknowledge Fundação Calouste Gulbenkian for financing of this project (Investigação e Saúde – reference: 125895), the program COMPETE and the Fundação para a Ciência e Tecnologia (PEst-OE/SAU/UI0709/2014). D Oppolzer acknowledges the Programa Operacional Regional do Centro 2007–2013 QREN (Programa 'Mais Centro') in the form of a fellowship (CENTRO-07-ST24-FEDER-002014, reference TPCR-2-004). The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

Executive summary

Effect-based biomarkers

- Traditional biochemical markers GGT, ALT, ASP and mean corpuscular volume present several disadvantages when used as alcohol biomarkers, mainly due to limited specificity.
- CDT is an effective alcohol biomarker to assess moderate-to-heavy consumption, and it is determined mainly in serum.
- The 5-hydroxytryptophol/5-hydroxyindoleacetic acid ratio is used as a marker for recent alcohol exposure. Both compounds are determined in urine; however analysis of 5-hydroxytryptophol requires analytical techniques with higher sensitivities.

Exposure-based biomarkers

- Ethanol is a highly sensitive and specific marker sampled in a variety of body fluids, but represents only very recent exposure due to being rapidly metabolized.
- Hemoglobin-associated acetaldehyde presents potential for the use as alcohol biomarker, however, very few methodological advances were studied in recent years.
- Formed through nonoxidative metabolism of ethanol, fatty acid ethyl esters, ethyl glucuronide, ethylsulfate and phosphatidylethanol are direct alcohol markers, maintaining part of the ethanol molecule.
- Nonoxidative alcohol markers have been the focus of several works in the past years, presenting great efficiency for the assessment of alcohol exposure. Biological specimens as hair, meconium, blood or urine have shown good results for those markers.

Future perspective

- Developments in the 'omics' area can be of great assistance in the search of new molecules to be used as alcohol biomarkers.
- One of the great challenges to be achieved is the standardization and establishment of adequate cut-off values for the existing biomarkers in different biological specimens.

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1.5 Hair Analysis as a Tool to Monitor Alcohol Consumption

1.5.1 Hair Physiology

Hair is a non-homogenous epidermal outgrowth, formed 3 – 5 mm below the skin surface in the hair follicle, which obtains the necessary metabolic material for the growing hair from a rich capillary system that surrounds the follicle [11]. The hair shaft is formed by three concentric structures, cuticle, cortex and medulla (Figure 1a and 1b). These structures play different roles on the characteristics of hair [12], and are formed of keratinized cells that are glued by the cell membrane complex (Figure 1b) [11]. Hair is composed of 65–95% proteins (mainly keratin), 15–35 % water, 1–9% lipids and less than 1% minerals [13,14]. The cell membranes consist mostly of protein and protein-lipid complexes, which are more vulnerable to chemical and mechanical actions. This part of hair is also a diffusion point for incorporation and elimination of drugs, especially those of lipophilic nature. Melanocytes are located in the basement membrane of the cortex and are responsible for pigmentation, they play also an important role in the incorporation of basic drugs in hair [11].

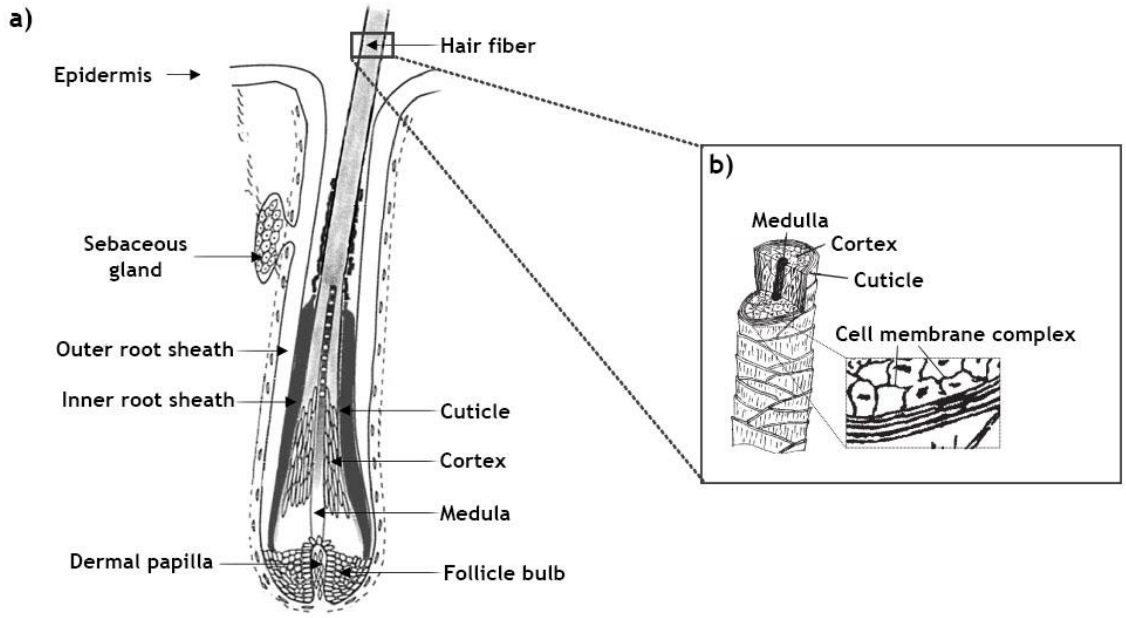


Figure 1. Schematic representation of a hair follicle (a), and structure of the human hair shaft (b). Adapted from Kronstrand et al., 2006 [13] and Pragst et al., 2006 [11]

Each hair is associated to a sebaceous gland and is in proximity of sweat glands (Figure 2). The sebaceous gland surrounds the hair duct to the upper part of the root to ensure that new hair is bathed in sebum for 2–3 days before reaching surface. The sweat glands hydrate the hair

shaft and contribute to the incorporation of hydrophilic drugs. The growth rate of scalp hair is approximately 0.35 mm per day [13], ranging from 0.6 to 1.4 cm per month [11,12,14].

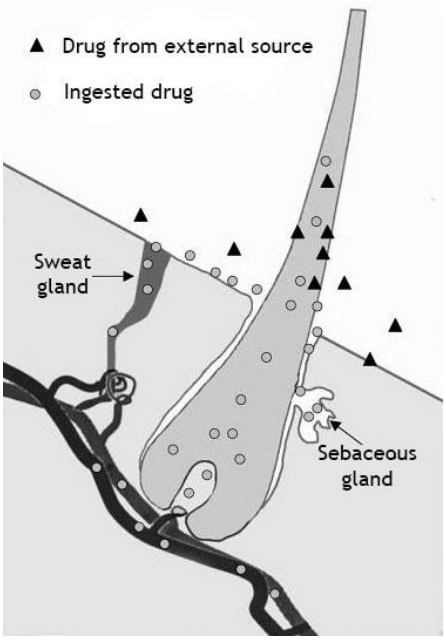


Figure 2. Representation of the sweat and sebaceous glands surrounding the hair follicle, and mechanisms for substance incorporation into hair. Source: Kronstrand *et al.*, 2006 [13]

1.5.2 Incorporation of Substances in Hair

There are three different mechanisms for the incorporation of different substances into hair, as can be seen in Figure 2. The blood supply provided by the capillary system surrounding the follicle is one of these routes. To penetrate the growing hair matrix the substances need to diffuse across its cell membrane, depending on the compounds' lipid solubility and the pH of both the cell and plasma. This transport is also selective to unbound substances only [12,13].

Another route is incorporation from sweat and sebum produced by nearby glands, after the hair emerges from skin [12,13]. As stated above, these secretions are in direct contact with the hair shaft, facilitating incorporation. Additionally several substances, including their metabolites, are excreted in sweat [14].

The third route of incorporation is through external contamination from smoke, vapours, dirty hands or different products containing the drugs [12–14]. When substances come in contact with hair through by any of those means, dissolution into sweat will occur. Consequently, the drugs will incorporate into hair, and at this point it will be almost impossible to distinguish

between a consumed substance and external contamination [12,14]. A clear example of this is seen in scientific literature, where positive cases for ethyl glucuronide in hair are due to its presence in some hair-care products (e.g. herbal tonics) [10,15,16].

1.5.3 Advantages of Hair Testing

Hair testing for substances presents several advantages when compared to other more traditional specimens as blood and urine, from which the most evident is the non-invasive sample collection procedure, which protects individual privacy and enhances the examinee's collaboration. Another powerful advantage is the ability to provide retrospective time windows for substance use that range from weeks to months or even years, depending on the hair length and analysed substance. It is even possible to perform segmental analysis to assess a substance exposure history for an individual, taking into account the growth rate and variations between individuals. Moreover, hair has also the advantage of a low risk of adulteration, and in case of suspicions and/or breaches in the chain of custody it is possible to get an identical sample from the same individual, or at least a sample representing a similar timeline [11,12].

1.5.4 Analytical Considerations in Hair Testing

Hair is a complex matrix and for a reliable analysis it is essential that results interpretation is performed carefully and possibilities of external contamination considered. The analysis procedure of a hair sample becomes thus a complex task. Different steps are required for hair analysis, including sample decontamination, extraction of analytes, sample clean-up and analytical detection, which will be briefly discussed below.

1.5.4.1 Decontamination

An important consideration to be taken into account during hair analysis is the possibility of a substance being determined due to environmental contamination and not following active consumption, which might originate false-positive results [12]. For this reason hair samples

should be cleaned from external contamination. This decontamination procedure is also very important to eliminate residues of hair products, sebum, sweat, dust and other contaminants from hair, since these can lead to increased analytical background noise. The solvents used during the decontamination procedure should remove external interferences as much as possible, but should not extract the incorporated compounds. There is no agreement on the washing procedures reported in literature, however the thorough choice of adequate solvents can be crucial for a correct analytical outcome of hair analysis [11].

1.5.4.2 Isolation of Analytes from the Hair Matrix

For the analysis of incorporated compounds, these have to be extracted from the hair matrix usually through solubilisation or digestion. For the choice of the correct procedure, the structure of the compounds and their properties have to be taken into account. Different procedures include the use of several solvents (as methanol, hexane or water), supercritical fluid extraction, and acidic, alkaline or enzymatic digestion. Before this process and to facilitate extraction, hair samples are usually cut into fragments of 1–3 mm, or pulverized using a grinder. Grinding procedures have resulted in increased extraction efficiencies for some compounds, however some sample material may be lost during the process [10,11].

1.5.4.3 Clean-up of Hair Extracts

Impurities and hair constituents present in hair extracts hinder the direct analysis of these solutions, and therefore a further clean-up step is usually deemed necessary. The applied techniques are similar to those used for other specimens as blood and urine, and include liquid-liquid extraction (LLE), solid-phase extraction (SPE), headspace solid-phase microextraction (HS-SPME) and headspace solid-phase dynamic extraction (HS-SPDE). SPE is commonly used due to its laboratory availability and ease of automation, however HS-SPME coupled to GC-MS shows great possibilities due to its automation by online coupling, and due to the fact that it is a miniaturized technique [11,17].

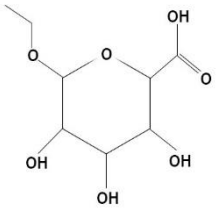
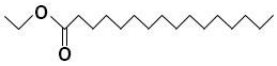
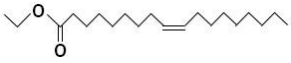
1.5.4.4 Detection Techniques

Hair analysis is challenging in what concerns the analytical instruments necessary for the detection of compounds. In the high complexity of hair samples, the analytical method will have to allow unambiguous detection and quantification of the compound(s) of interest, presenting high selectivity. Additionally, the concentrations of the drugs are commonly very low and samples are usually available in small amounts. Depending on the nature of the compound to be studied, liquid (LC) or gas chromatography (GC) may be used for compound separation. In order to be able to detect low concentrations, the coupling of gas or liquid chromatographers to mass spectrometry (MS) or tandem mass spectrometry (MS/MS) is necessary for the high sensitivities provided and also the capability for unambiguous identification of the involved compounds [11].

1.5.5 Hair Testing for Alcohol Exposure

Ethanol determination in hair is not possible due to its volatile nature, fast metabolism and to the fact that it is not durably incorporated into hair [11,18]. Therefore, hair analysis for evaluation of alcohol consumption is performed via the determination of the minor ethanol non-oxidative metabolites, ethyl glucuronide (EtG) and fatty acid ethyl esters [FAEEs, ethyl myristate, palmitate, oleate and stearate]. The structures and physical and chemical properties of these alcohol biomarkers are presented in Table 1.

Table 1. Structure and chemical properties of the alcohol biomarkers EtG and FAEEs. Information was gathered from the database of PubChem [19].

Compound	Structure	CAS[a]	Molecular Formula	MW[b] (g/mol)	$K_{o/w}$[c]
Ethyl Glucuronide		17685-04-0	$C_8H_{14}O_7$	222.2	-1.4
Fatty Acid Ethyl Esters					
Ethyl myristate		124-06-1	$C_{16}H_{32}O_2$	256.4	6.7
Ethyl palmitate		628-97-7	$C_{18}H_{36}O_2$	284.5	7.8
Ethyl oleate		111-62-6	$C_{20}H_{38}O_2$	310.5	8
Ethyl stearate		111-61-5	$C_{20}H_{40}O_2$	312.5	8.9

[a] CAS: Chemical Abstract Service

[b] MW: Molecular weight

[c] $K_{o/w}$: octanol-water partition coefficients

1.5.5.1 Properties of Ethyl Glucuronide and Incorporation in Hair

EtG is formed during the phase II metabolism of ethanol by conjugation with glucuronic acid mediated by UDP – glucuronyltransferase. Considering the octanol/water partition coefficients ($K_{o/w}$), the hydrophilic nature of EtG becomes evident. The mechanism of incorporation of EtG into hair is not clear yet, but since EtG has been determined in sweat [20] deposition from sweat is believed to be the main route [21]. However, other authors have shown that EtG is determined in hair of rats, which lack sweat glands in their fur [18]. Based on this, the bloodstream was found likely to also display a major role in the incorporation of EtG into hair. EtG only incorporates weakly due to its acidic properties (pKa = 3.21), which results in very low concentrations in hair, usually in the range of a few picograms per milligram of hair (pg/mg) [18,22]. This highlights the need of sensitive and selective techniques for the analysis of this compound in hair, and the fact that mass spectrometric detection is mandatory.

Hair care and cosmetic treatment products and habits may affect the concentration of EtG in hair. As already mentioned above, some hair products, like herbal hair tonics, may contain EtG in their formulation, and consequently increase the concentration of this compound in hair [10,15,16]. Conversely, cosmetic treatments, as bleaching or dyeing, may result in a washout effect of EtG [23–27], lowering concentrations; however, this may also occur due to intensive hair washing [28,29].

Despite being present in a few hair-care products, external contamination of hair by EtG is improbable, therefore the decontamination procedure is more focused on removing impurities that may interfere with analysis. There is no general accepted procedure, however non-swelling agents as acetone and dichloromethane are preferred to avoid EtG extraction [10]. Often, a sequential washing procedure with solvents of different polarities (water, methanol, acetone, dichloromethane, ether, and heptane) is used [24].

Grinded powdered hair results in increased extraction of EtG, and is usually preferred when compared to cut hair [10,30,31]; however, this procedure may be used if similar extraction efficiencies are obtained [32].

The SoHT currently proposes the use of a concentration of 7 pg/mg for distinguishing between abstinence and social moderate drinking, while a concentration equal or superior to 30 pg/mg is indicative of chronic excessive drinking. Both cut-off values are valid at 0-3 cm and 0-6 cm segments [32].

1.5.5.2 Properties of Fatty Acid Ethyl Esters and Incorporation in Hair

FAEEs are formed from free fatty acids, triglycerides, lipoproteins or phospholipids in the presence of ethanol, by action of cytosolic and microsomal FAEE synthases as well as by unspecific enzymes. As can be seen from Table 1, these compounds have linear structures, and the intact ethyl group of ethanol is maintained. FAEEs are non-polar in nature, and this may be observed by the $k_{o/w}$, thus facilitating lipophilic incorporation into hair, which occurs mainly from sebum [10,11].

The use of hair treatments as bleaching, perming or dyeing may affect the concentration of FAEEs in hair. Additionally, the use of ethanol-containing hair products may originate false-positive results. Due to the effects of the use of hair products on hair results for both EtG and FAEEs, the use of those products should therefore be documented during sample collection and carefully considered during results interpretation [10,29,32]. In addition, a combined interpretation of both markers' concentrations is recommended [5,10,23,29,33].

During hair sample treatment for FAEEs analysis it is important that the use of plastic material is avoided. The decontamination procedure to be applied will have to remove the FAEEs present in the external lipids, but avoid their extraction from hair. The solvent used is usually *n*-heptane for the high solubility of the external lipids in this non-polar solvent. Interestingly, contrary to EtG analysis, grinding procedures are unsuitable for FAEEs analysis, since these results in analyte losses due to the evaporation of FAEEs [10].

Due to the fact that FAEEs tend to increase with the hair length [34], the SoHT currently proposes the use of different cut-off values at 0-3 cm and 0-6 cm segments. A concentration of 0.2 ng/mg (at 0-3 cm segments) or 0.4 ng/mg (at 0-6 cm segments) is proposed to distinguish between abstinence and social moderate drinking. Concentrations equal or superior to 0.5 ng/mg (at 0-3 cm segments) or 1.0 ng/mg (at 0-6 cm segments) are indicative of chronic excessive drinking [32].

1.6 Objectives

The objective of the present work was to evaluate the alcohol consumption on a university student population by two main approaches, one based on questionnaire analysis, and another based on the analytical determination of alcohol biomarkers in hair. The following specific objectives were defined:

1. Collection of hair samples at nine Portuguese public universities (Aveiro, Algarve, Beira Interior, Coimbra, Évora, Lisboa, Minho, Porto and Trás-os-Montes e Alto Douro), and gathering a self-completion questionnaire from each participant.
2. Interpretation of the questionnaires to assess alcohol consumption habits, patterns and trends, and comparison with published data concerning the year 2011.
3. Development and optimization of analytical methods for the determination of alcohol biomarkers in hair:
    a. Extraction of EtG from hair, sample clean-up by solid-phase extraction and analysis by liquid chromatography coupled to tandem mass spectrometry;
    b. Extraction of FAEEs from hair, sample clean-up by solid-phase extraction and analysis by gas chromatography coupled to tandem mass spectrometry;
    c. Validation of both methods according to internationally accepted guidelines for bioanalytical method validation.
4. Analysis of students' hair samples for EtG and FAEEs, considering the cut-off values proposed by the SoHT, and:
    a. General demographic and gender assessment of biomarker analysis results;
    b. Verification of the adequacy of the currently proposed values for the studied population through ROC analysis;
    c. Evaluation of the effects of hair products and cosmetic treatments on EtG and FAEEs concentrations in hair;
    d. Evaluation of the congruence between self-reported consumption habits and alcohol biomarker analysis.

Chapter II - Experimental

2.1 Study Outline and Description

The present study is characterized as an epidemiological, descriptive and cross-sectional study. The entire sampling and analysis procedures were anonymous, and were performed in a complete random manner. Sampling was performed between March 2013 and September 2014, at random times during the day(s) of collection. Each participant was informed about the objectives of the study and of the gathered information before sampling, and a written consent was provided, consisting in a brief description of the project and applied methodologies (Attachment 1). The project was submitted and approved by the ethical committee of the Faculdade de Ciências da Saúde (FCS) from the Universidade da Beira Interior (UBI).

The study population in this work consisted of students from nine Portuguese universities: Aveiro (UA), Algarve (UALG), Beira Interior (UBI), Coimbra (UC), Évora (UE), Lisboa (UL), Minho (UM), Porto (UP) and Trás-os-Montes e Alto Douro (UTAD). In order to obtain an adequate selection of samples and significant number of samples to be analysed in each targeted institution, the representative number of necessary samples was calculated. This was performed using the Epi Info ™ (version 7.1.4) software (Centers for Disease Control and Prevention, USA). A 90% frequency of alcohol consumption was assumed [35], and based on the number of students enrolled in the academic year of 2012/2013 or 2013/2014, the number of necessary participants for each university was calculated assuming a 95% confidence interval. These data are provided in Table 2.

Table 2. Number of students enrolled in Portuguese universities in the academic year of 2013/2014, or 2012/2013.

University	Enrolled students*	Sample size
Universidade de Aveiro (UA)	13779**	137
Universidade do Algarve (UALG)	9708	137
Universidade da Beira Interior (UBI)	6931**	136
Universidade de Coimbra (UC)	23958	138
Universidade de Évora (UE)	8970	137
Universidade de Lisboa (UL)	67005	138
Universidade do Minho (UM)	18490	138
Universidade do Porto (UP)	31385	138
Universidade de Trás-os-Montes e Alto Douro (UTAD)	7808	136

*Data obtained from the Direção-Geral de Estatísticas da Educação e Ciência in the academic years of 2013/2014. **academic year of 2012/2013. [36]

Criteria for participation in the study included a minimum age of 18 and attendance of an academic degree; additionally, participants were required to fill a self-completion questionnaire (Attachment 2) and to accept the collection of at least 100 mg of hair. This sample was cut as close as possible to the scalp in the posterior part of the head, in order to avoid being aesthetically noticeable, to standardize the collection site and because the growth rate in this area is relatively uniform. In addition, more hairs are actively growing in this area of the scalp. The end of the hair segment closer to the cut was identified with a string, since only the proximal 6 cm to the hair root are analysed for alcohol biomarkers [32]. Until processing, samples were stored in paper envelopes at room temperature. A total of 1192 samples were collected.

2.2 Questionnaire Results Interpretation

2.2.1 Distribution by Gender

Of the total participants that answered the question concerning their gender (N = 1184), 821 (68.88%) were females and 363 (30.45%) were males (Figure 3a). As can be observed in Figure 3b, this pattern was very intense in 5 universities (UALG, UC, UM, UP, UTAD), and with lower differences (but same trend) for the remaining universities; the exception was UE, for which more males participated in the study. This higher number of female participants is likely due to a higher will in providing hair samples than males, since for the latter collection may be aesthetically noticeable due to typically shorter hair. However, one must consider as well that the number of females enrolled in academic courses is higher than that of males [36].

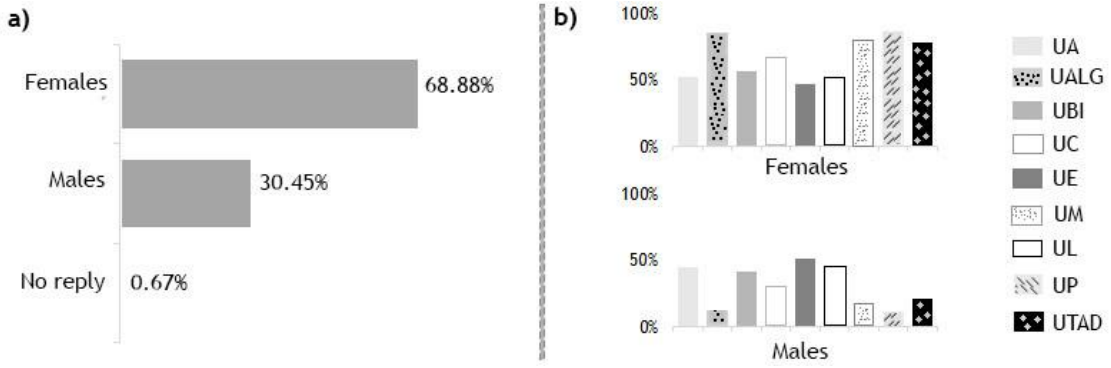


Figure 3. Distribution of the total enquired students according to gender (a), and distribution of gender according to university (b).

2.2.2 Distribution by Age and Start of Consumption

The mean age of the studied population was 21.55 ± 3.18 years (median of 21 years) (Table 3). Observing the nine universities individually, little variation was observed, with the mean age ranging from 19.44 ± 2.31 to 22.89 ± 4.43 years, and the median from 19–23 years.

Table 3. Mean and median age, and age when alcohol consumption started, of the enquired students per university.

University	Age of the participants		Age when consumption started	
	Mean ± SD	Median	Mean ± SD	Median
UA	19.44 ± 2.31	19	15.05 ± 2.11	15
UALG	20.03 ± 2.17	20	14.53 ± 2.11	15
UBI	22.88 ± 2.80	23	14.97 ± 1.82	15
UC	22.89 ± 4.43	21	15.35 ± 2.92	15
UE	21.47 ± 2.75	21	14.57 ± 1.78	15
UL	21.51 ± 2.69	22	14.67 ± 1.98	15
UM	21.51 ± 2.51	21	15.12 ± 2.36	15
UP	21.91 ± 3.37	21	15.21 ± 2.49	15
UTAD	22.25 ± 3.06	22	15.17 ± 3.03	16
TOTAL	**21.55 ± 3.18**	**21**	**14.98 ± 2.35**	**15**

The age at which alcohol consumption started was also asked to the participants. This information is represented as a chart in Figure 4. The age varied widely, however most of the population admitted to have started to consume alcohol around the age 12 to 18, with the majority reporting 15 years (Mean = 14.98 ± 2.35 years, Median = 15 years). Unfortunately, a high number of the enquired individuals did not remember when consumption started. It is also noticeable in Figure 4 that the number of teetotallers, i.e. complete abstainers who had never consumed alcohol, is very low.

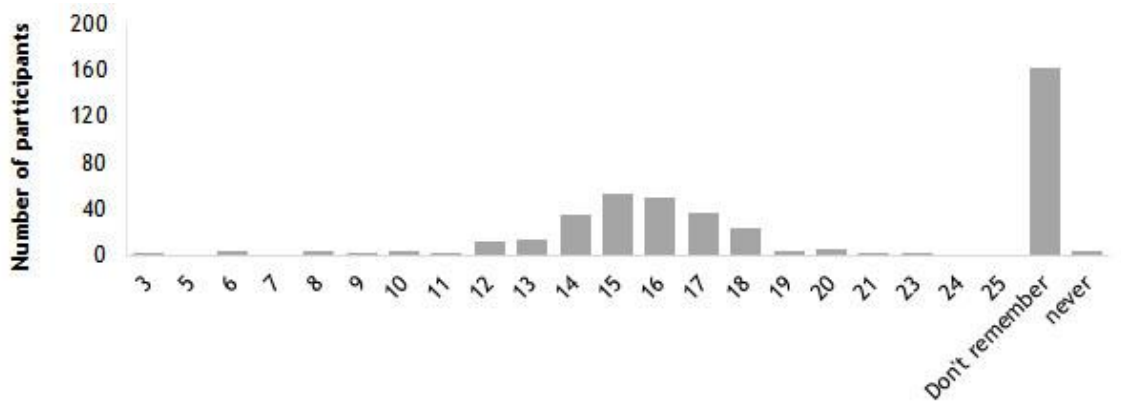


Figure 4. Distribution according to the age at which alcohol consumption started.

Comparing to European data [35], where it was reported that 6 out of 10 students had consumed alcohol at least once at the age of 13, our results show that the mean age is higher, at 15 years. However, considering the variation of the reported ages (Figure 4), we can conclude that the population presents a similar behaviour as the European average. In different study, performed in the Portuguese adolescent population aged 13, half of the inquiries reported experience of alcohol consumption [8], while in the ECTAD (Estudo sobre o Consumo de Álcool, Tabaco e Drogas) study of 2011 (students age 13-18) circa 36.5% of students aged 13 had consumed alcohol, and over 50% had consumed alcohol at the age of 14 [37]. Interestingly, this tendency could not be verified by our study, since only 9.48% (N = 113) individuals reported drinking at or before the age of 13. A comparison is however difficult, since our study was performed on academic students, and cannot represent the same population as the referred study, which was performed in high schools.

2.2.3 Consumption Frequency

Regarding consumption frequency, 7.38% of the participants classified their consumption as abstinent (N = 88), 67.28% as moderate (N = 802), 24.16% as frequent (N = 288) and 0.5% as excessive (N = 6) (Figure 5). The low number of self-reported excessive drinkers can be explained by the general tendency for participants to underestimate their alcohol consumption, however overestimation may also be possible which can possibly explain the low number of abstinent individuals [1]. Nevertheless, variations between gender were found in the excessive drinking group, with 83.33% of the participants (N = 5) belonging to the male gender.

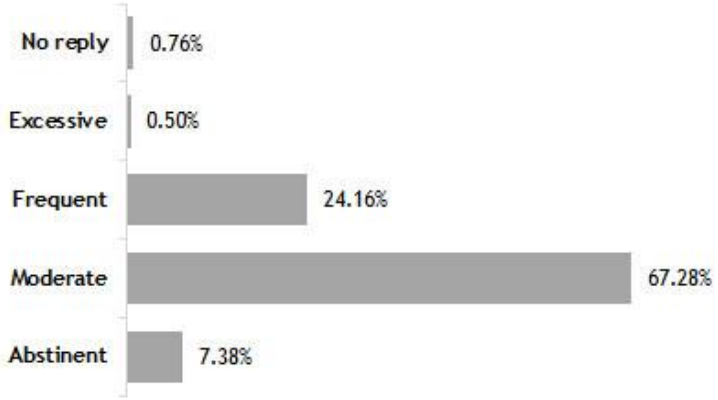


Figure 5. Alcohol consumption frequency of the studied population.

Most students reported to have consumed alcohol in excess at least once in the past month (29.36%), while about one-quarter of the students (25.92%) reported to have never consumed alcohol in excess. Excessive consumption within 1 to 6 months (13.93%), and over 6 months (14.43%) before sampling was less common, with both accounting for less than 30% of the inquiries (Figure 6a). The percentage of heavy drinking during the past 30 days is much higher than that reported in 2011 in the ESPAD [35] and ECTAD [37], but similar to the ones reported in the INME (Inquérito Nacional em Meio Escolar) study of 2011 [38,39], and was also found to be more incident in males (40.50%) than in females (24.36%).This last observation is however in agreement with the information of the European Survey Project on Alcohol and other Drugs (ESPAD), where in the majority of studied countries, the tendency for higher alcohol consumption during the previous 30 days is generally more incident on males [35]. Moreover, the percentages of heavy drinking in the past 30 days, both for males and females, are very similar to those obtained outside the EU, in American college students (32% of females and 43% of males) [37]. This tendency was the same for all universities (Figure 6b).

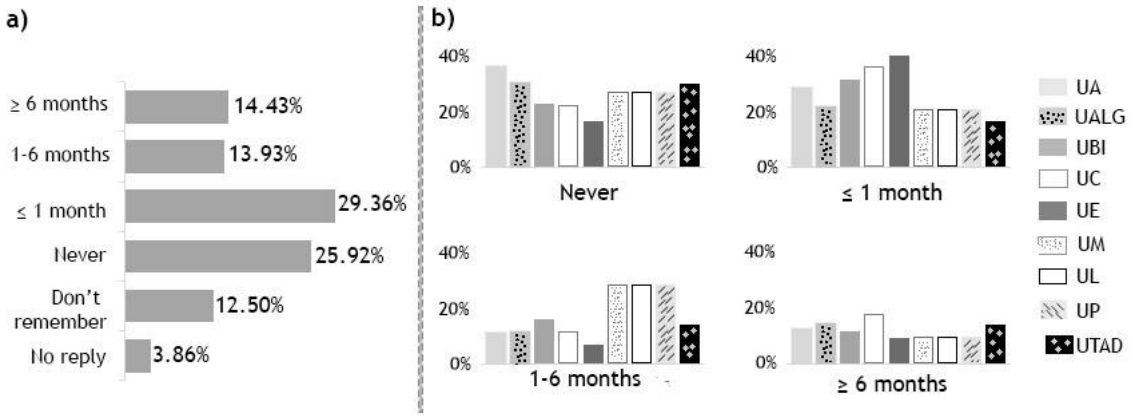


Figure 6. Excessive consumption before the date of sampling of the total population (a), and distribution according to university (b).

2.2.4 Consumption Habits

By observing Figure 7a, it is noticeable that the majority of the participants prefer drinking in group, rather than alone. This tendency was the same for all universities (Figure 7b). The preference of participants to drink alcohol in group can be associated with the information provided in Figure 8. Almost 45% of the students reported that they consumed alcohol in coffee bars, while drinking at home, which would be more associated with drinking alone, was only reported by 7.82%.

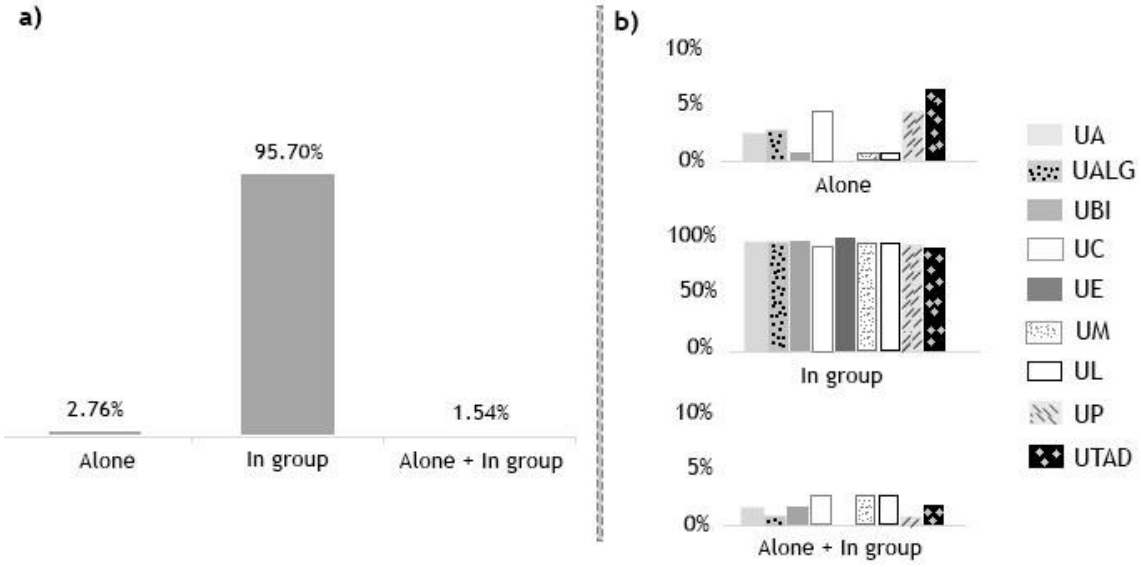


Figure 7. Preference for alcohol drinking in group for the total population (a), and distributed by university (b).

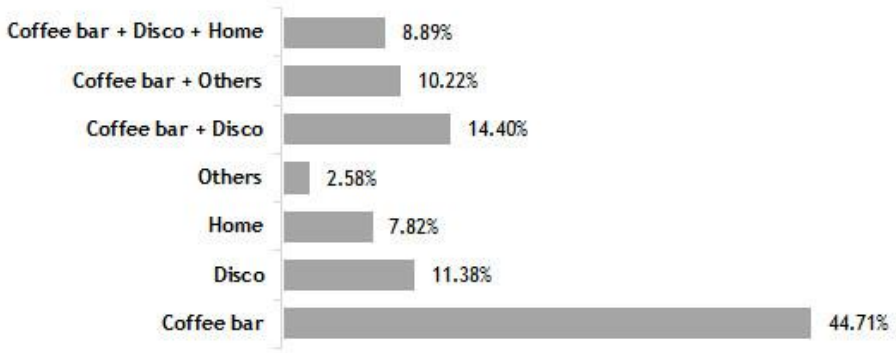


Figure 8. Places where alcohol is more often consumed, for the total population.

The preferred and most consumed drink by the individuals was beer (26.51%), spirits (21.48%) or both (22.40%) (Figure 9a). This tendency was the same for both genders (figure 9b), however consumption of spirits alone was not as common in males as in females. These results are in agreement with the survey of ESPAD, where beer was found as the predominant beverage, and spirits were predominant in part of the female population. Both beverages accounted for 70% of the total alcohol consumption [35]. The same tendency for the most consumed drink is also similarly reported in the ECTAD 2011 [37], INME 2011 [38,39] and Health Behaviour in School-Aged Children (HBSC) 2014 [40] studies.

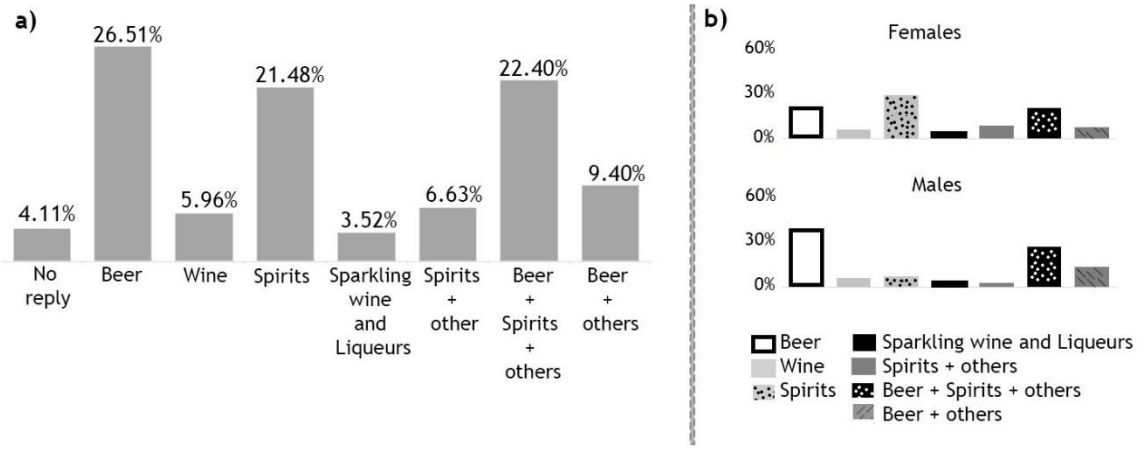


Figure 9. Most consumed drink among the studied population (a), and according to gender (b).

Based on the mean alcohol content of alcoholic beverages, the ingested quantities of pure ethanol were calculated for each university and both genders (Table 4). Mean alcohol content (v/v) in drinks was assumed as follows: 5% in beer, 13% in wine, 25% in sparkling wine and liqueur and 40% in spirits. Due to the great range of self-reported ingested quantities (from 9 to 2000 mL), Table 4 shows both the mean and median in mL of pure alcohol. Comparing this information with the survey of ESPAD in 2011, where males were found to drink one-third more than females (58 *vs* 43 mL), our results confirm higher drinking for males, however the difference between both genders is not so high (96 *vs* 82 mL). One great discrepancy with the results of ESPAD is the ingested quantities (males: 58 mL, females: 43 mL, mean for Portugal: 50 mL), which according to our study are much higher for both genders. Observing the median for males (82.50 mL) and females (60 mL), an increase in ingested alcohol per drinking occasion can be observed relatively to the results of 2011. However, one must also consider the possibility that university students consume higher quantities per occasion, compared to the students aged 15–16 years inquired for the ESPAD study. The higher quantities of ingested alcohol by individuals of the male genre is also reported in other studies, namely the ECTAD 2011 [37], INME 2011 [38,39] and HBSC 2014 [40].

Table 4. Mean and median ingested quantities of pure ethanol (in mL) per drinking occasion, distributed by university and gender.

University	Females		Males	
	Mean ± SD	Median	Mean ± SD	Median
UA	61.56 ± 55.26	52.00	83.28 ± 61.30	79.00
UALG	96.23 ± 112.84	66.00	155.06 ± 315.98	66.00
UBI	60.17 ± 33.44	52.13	99.23 ± 56.03	91.25
UC	69.73 ± 98.47	60.00	97.51 ± 103.24	82.50
UE	199.04 ± 383.66	99.00	119.75 ± 79.65	109.75
UL	80.24 ± 65.65	66.00	88.86 ± 59.33	66.00
UM	105.47 ± 187.67	54.75	81.75 ± 44.93	80.00
UP	64.22 ± 87.32	57.38	81.10 ± 31.42	77.75
UTAD	72.05 ± 68.60	54.75	78.21 ± 53.39	74.25
TOTAL	**81.57 ± 127.49**	**60.00**	**96.11 ± 95.40**	**82.50**

2.2.5 Smoking

Smoking was reported by 36.41% (N = 434) of the participants (Figure 10a), and this percentage was higher in males (42.70%) than in females (33.74%) (Figure 10b). The average number of cigarettes per day was 7.95 and was similar between both genders (7.78 in males, 8 in females). Comparing to the information provided in the ESPAD survey, where 54% of the students reported cigarette smoking and 29% reported smoking in the last 30 days [35], the percentage of students with smoking habits in our study is lower. Also, comparing to the ECTAD 2011, where smoking varied between 17.1% (age 16) and 60.6 % (age 18) [37] and to the INME 2011, where smoking varied between 58%-68% [38,39], the percentages obtained in our study are lower. However, compared to the HBSC 2014 where only 7.5% of the enquiries reported smoking habits [40], the percentages of the present study are higher, however, age differences must be considered.

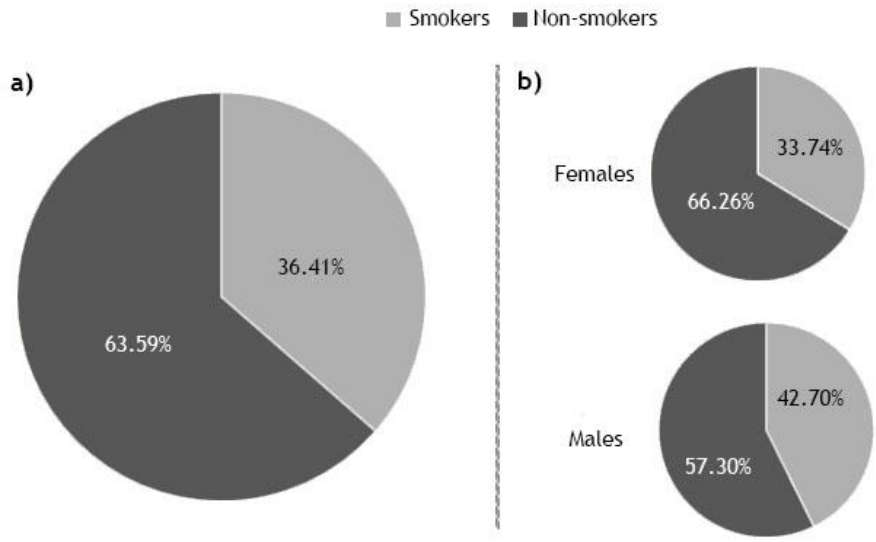


Figure 10. Percentage of smokers in the total population (a), and according to gender (b).

2.2.6 Use of Illicit Substances

27.35% of the participants (N = 326) reported the use of some sort of illicit substance (Figure 11a). This incidence was almost doubled in the male population (40.5%) compared to the female population (21.07%) (Figure 11b). Comparing to data from 2011 [35, 37], our study shows a higher incidence of drug use (27% *vs* 18%), however, as stated above, one must consider the difference in age of the studied populations in both surveys.

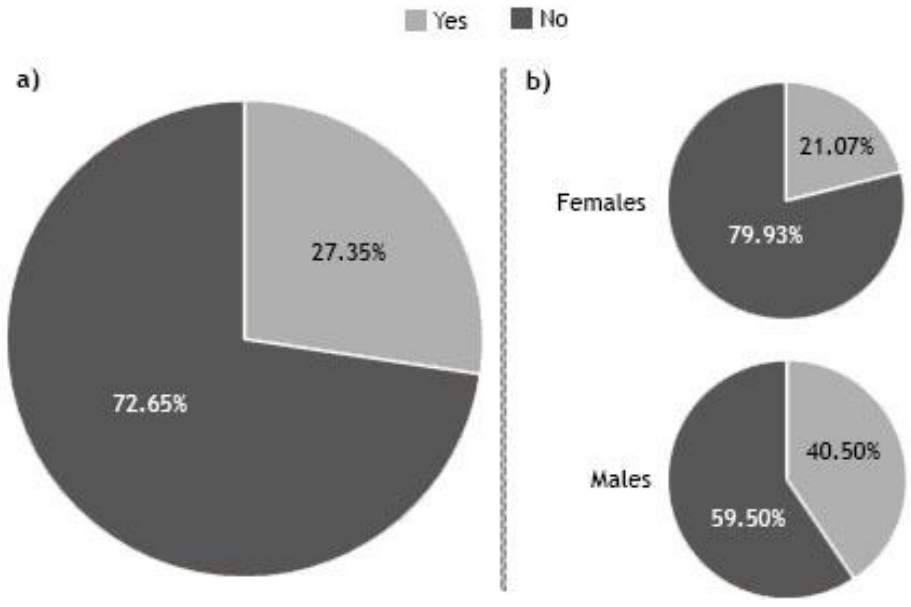


Figure 11. Use of illicit substances in the total student population (a) and according to gender (b).

Drug consumption incidence on the nine universities varied between 11.03% and 36.99%, except for UE where a significantly high percentage of 73.97% was found (Figure 12). Substances were mostly consumed on rare occasions (63.93%), with only 11.07 % and 10.0% of the drug consumers reporting weekly or daily consumption, respectively (Figure 13). The most consumed illicit substance was cannabis, alone (87.14%) or in combination with cocaine and others (Figure 14). Participants were also asked if they consumed these substances together with alcohol (Figure 15a), thereof the majority (53.37%) reported to consume illicit substances together with alcohol. This percentage was identical for each university, except at UBI and UE were an impressive percentage of 86.67% and 85.12% was found (Figure 15b).

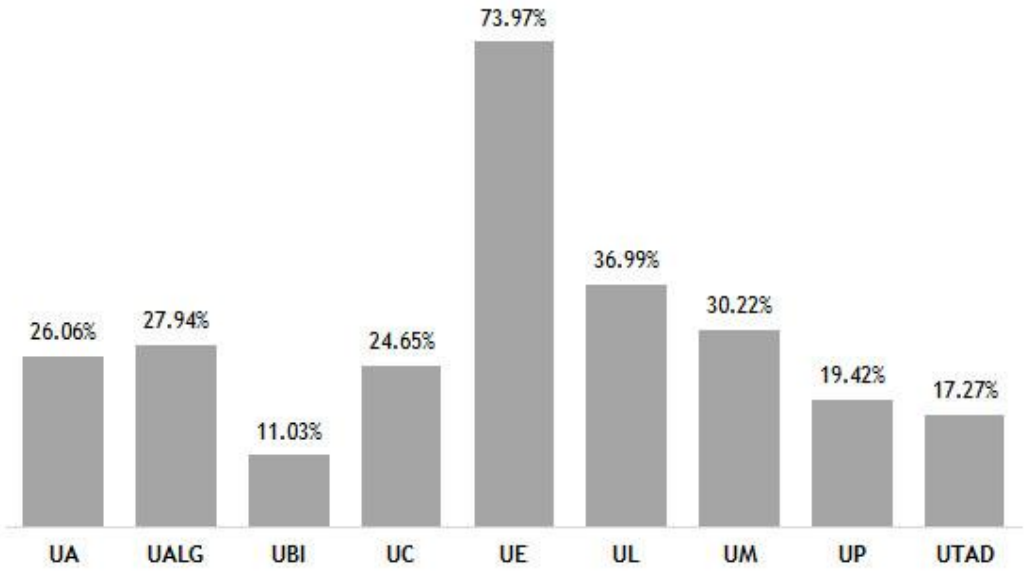


Figure 12. Use of illicit substances according to university

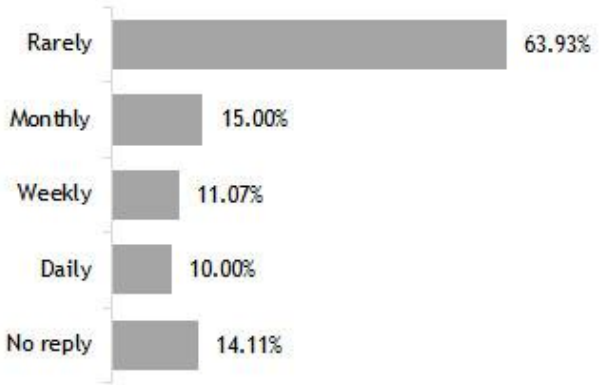


Figure 13. Illicit substance consumption frequency.

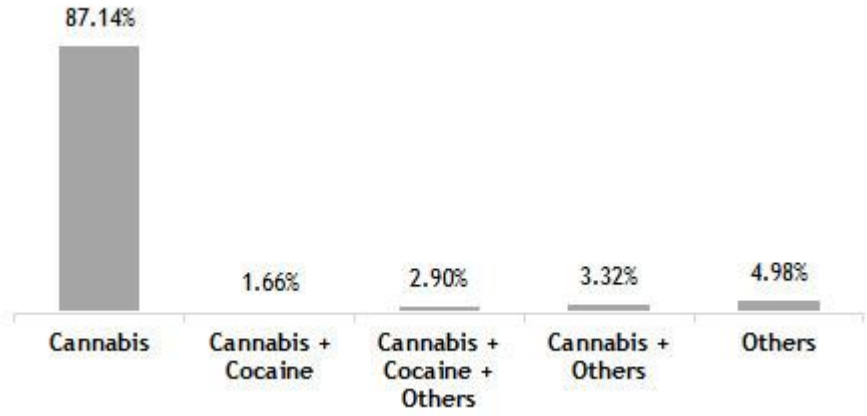


Figure 14. Type of illicit substances consumed by students.

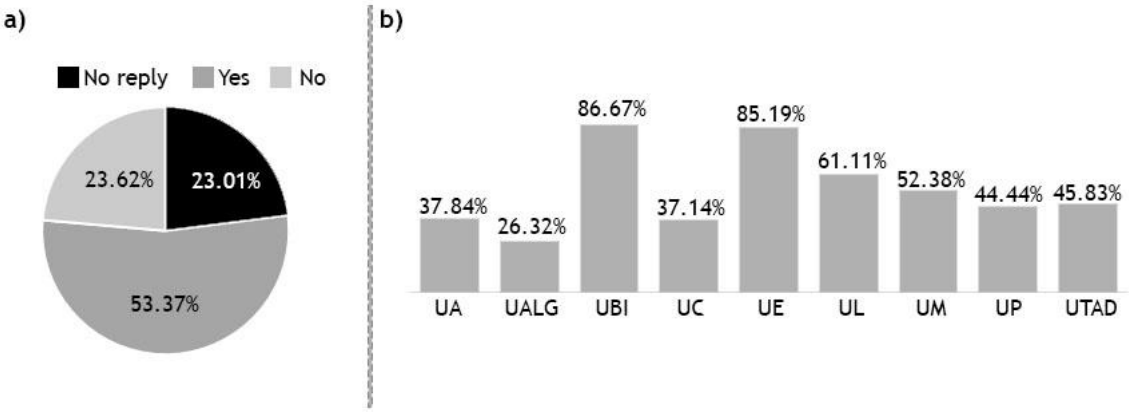


Figure 15. Consumption of illicit substances together with alcohol in the total population (a), and distributed according to university (b).

2.3 Manuscript 2

DETERMINATION OF ETHYL GLUCURONIDE AND FATTY ACID ETHYL ESTERS IN HAIR SAMPLES

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Submitted to the journal Biomedical Chromatography, 2016

(BMC-16-0070, Accepted – June 2016)

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Abstract

Hair testing for alcohol biomarkers is an important and reliable tool for monitoring alcohol consumption. In this work, we propose two methods for assessing alcohol exposure through combined analysis of the biomarkers ethyl glucuronide (EtG) and four fatty acid ethyl esters (FAEEs) species (ethyl myristate, palmitate, oleate and stearate) in hair. 30 mg of hair were processed using solid-phase extraction; EtG was analysed by liquid chromatography-tandem mass spectrometry, while FAEEs were analysed for the first time by gas chromatography-tandem mass spectrometry using electron impact (EI) ionization. Both methods were validated according to internationally accepted guidelines. Linearity was proven between the intervals of 3-500 pg/mg for EtG and 30-5000 pg/mg for FAEEs, and the limits of quantification were 3 pg/mg for EtG and 30 pg/mg for each of the four FAEEs. Precision and accuracy were considered adequate according to the adopted guidelines, processed EtG samples were found to be stable up to 96h left in the injector and processed FAEEs samples up to 24h. Matrix effects for EtG were not significant. Both methods were successfully applied to the analysis of 15 authentic samples, using the cut-off values proposed by the Society of Hair Testing for interpretation. The results were in agreement with the self-reported alcohol consumption in most cases, and demonstrated the suitability of the methods to be applied in routine analysis of alcohol biomarkers, allowing monitoring alcohol consumption using low sample amounts.

Keywords

Alcohol Biomarkers, Ethyl glucuronide, Fatty acid ethyl esters, Hair

1. Introduction

Alcohol consumption is a global problem associated to several social and health-related issues. Directly or indirectly, alcohol is responsible for millions of deaths every year, representing 5.9 % of all deaths worldwide (World Health Organisation, 2014). Its consumption is also an important social-economic issue in several countries; in the European Union alone, public expenditures for alcohol treatment even exceed those for the treatment of illegal drug addiction (Lievens *et al.*, 2014). The development of methodologies that allow for an efficient

evaluation of alcohol exposure is therefore of high importance. Ethyl glucuronide (EtG) and fatty acid ethyl esters (FAEEs) are direct biomarkers of alcohol exposure that contain the carbon atoms of ethanol in their structure, and can only be formed in the presence of alcohol (Auwärter *et al.*, 2001; Pragst *et al.*, 2010). Indeed, these compounds have proven to effectively allow for the estimation of alcohol exposure surpassing some limitations, as the lack of specificity presented by indirect biomarkers, as enzyme analysis and mean corpuscular volume (MCV) (Pragst *et al.*, 2010, Morini *et al.*, 2011; Hastedt *et al.*, 2013; Oppolzer *et al.*, 2016). However, it has to be considered that, even with the exclusive formation of these compounds only when alcohol is present, it does not necessarily mean that all the markers detected in a biological sample are due to consumption, since they can be formed from ethanol naturally present in the organism (Cabarcos *et al.*, 2009; Pragst *et al.*, 2010). Therefore, it is very important to establish proper cut-off values that allow to distinguishing between abstinence (own body ethanol production) and true consumption cases. These cut-off values can also be applied to different categories of drinking amounts, so that EtG and FAEEs can be used to distinguish between abstinence, moderate and excessive alcohol consumption (Morini *et al.*, 2009; Pragst *et al.*, 2010; Hastedt *et al.*, 2012, 2013, Kintz, 2015; Pragst, 2015). Hair has been widely used for the determination of several drugs of abuse, therein including alcohol biomarkers, since mostly these compounds present higher stability in this sample when compared with others as is the case of blood (Auwärter *et al.*, 2001; Barroso *et al.*, 2011). Hair also presents other advantages, for example the drinking behaviour for months before sample collection can be evaluated through segmental hair analysis of 0-3 cm or 0-6 cm proximal segments (Hastedt *et al.*, 2012, 2013; Kintz, 2015; Pragst, 2015; Suesse *et al.*, 2015). Additionally, the sample collection procedure is non-invasive, and the chances of adulteration are very low (Pragst and Balikova, 2006; Barroso *et al.*, 2011). EtG is a highly polar substance, extracted from hair preferably with polar solvents and usually analysed by liquid chromatography methods coupled to mass spectrometry (LC-MS) or tandem mass spectrometry (LC-MS/MS) (Morini *et al.*, 2009; Pragst *et al.*, 2010; Hastedt *et al.*, 2012; Suesse *et al.*, 2012; Cabarcos *et al.*, 2013; Crunelle *et al.*, 2014; Pragst, 2015; Oppolzer *et al.*, 2016). Gas chromatography coupled to mass spectrometry (GC-MS) or tandem mass spectrometry (GC-MS/MS) methods are also used, but require prior derivatization (Pragst *et al.*, 2010; Crunelle *et al.*, 2014; Oppolzer *et al.*, 2016).

FAEEs are hydrophobic compounds, and their extraction from hair is usually performed using non-polar solvents; sample clean-up is mostly performed using headspace solid-phase micro extraction (HS-SPME) and analysis by GC-MS (Auwärter *et al.*, 2001; Pragst *et al.*, 2001; Cabarcos *et al.*, 2009; Pragst *et al.*, 2010; Hastedt *et al.*, 2012, 2013; Suesse *et al.*, 2012; Oppolzer *et al.*, 2016), while a few papers describe SPE procedures (Caprara *et al.*, 2005; Politi *et al.*, 2011; Bertol *et al.*, 2014). Combined use of both biomarkers mentioned above has shown to increase the accuracy of alcohol consumption interpretation (Pragst *et al.*, 2010; Hastedt *et al.*, 2012; Suesse *et al.*, 2012). In this work we propose a method for the determination of EtG by LC-MS/MS and of FAEEs by GC-MS/MS in hair. MS/MS methods were preferably chosen for the enhanced sensitivity and selectivity these detectors present to reach proper limits of quantification and detection, as recommended by the Society of Hair Testing (SoHT) (Kintz, 2015). In both methods SPE was chosen for the fact that this technique is more readily available to laboratories when compared for example with HS-SPME. In this work is also reported for the first time the analysis of FAEEs using tandem mass-spectrometry with an IE ionization source.

2. Experimental

2.1. Reagents

Methanol, acetonitrile, water and isopropanol (MS-grade) from Fisher were acquired from Enzymatic (Santo Antão do Tojal, Portugal). Methanol and heptane (HPLC-grade) were acquired from VWR Internacional (Carnaxide, Portugal). Formic acid (MS-grade) was acquired from Sigma-Aldrich (Sintra, Portugal). Acetone, dichloromethane and ammonium hydroxide from Fisher were purchased from Enzymatic (Santo Antão do Tojal, Portugal). Oasis MAX 3cc SPE cartridges were acquired from Waters (Lisboa, Portugal), Phenomenex Strata NH_2 (100 mg/1mL) and Strata-X-A (30 mg/1mL) SPE cartridges were purchased from Tecnocroma (Caldas da Rainha, Portugal).

2.2. Standards

Ethyl glucuronide and its deuterated analogue (internal standard – IS, EtG-d5) at 100 µg/mL in methanol were purchased from LCG Promochem (Barcelona, Spain). Working solutions were prepared by diluting EtG to 600, 60 and 6 ng/mL, the IS was prepared at 300 ng/mL. The FAEEs ethyl myristate (E 14:0, Myr-Et)), ethyl palmitate (E 16:0, Palm-Et), ethyl oleate (E 18:1, Ole-Et) and ethyl stearate (E 18:0, Stea-Et)) and their respective penta-deuterated analogues (FAEEs-d5, IS) were purchased from LCG Promochem (Barcelona, Spain). Stock solutions of each compound were prepared at 5 mg/mL in heptane, the working solutions containing a mixture of the four FAEEs were prepared by successive dilutions at 6000, 600 and 60 ng/mL. A mixture containing the four ISs was prepared at 3000 ng/mL in heptane. All stock and working solutions were stored in amber glass vials, below -20 ºC.

2.3. Biological samples

Human hair free from all analytes (EtG and the 4 FAEEs) was obtained from children aged 3-6 years, and was used during method development and validation experiments after preparing a homogenized pool of hair. Authentic hair samples were obtained from voluntary university students. Samples were cut as close as possible to the scalp and stored in paper envelopes until analysis, a brief description of the study and applied methodologies was provided to the participants at the moment of sampling. Additionally, at the moment of sampling, participants were asked to self-report their consumption habits as abstinent (complete abstinence to alcoholic beverages for the time-lapse corresponding to the segment length, by assuming a growing rate of 1 cm/month), moderate (rare or occasional consumption during few days per week, less than 60 g of ethanol per day) or excessive (common consumption of high quantities of ethanol or occasional consumption of high quantities of alcohol per occasion, more than 120 g of ethanol per day).

2.4. Hair processing and extraction procedure

The proximal ends of each hair sample were aligned, and the actual length was measured. Whenever available, up to 6 cm proximal segments were analysed, and in cases where the

length exceeded 6 cm, the excess was cut and discarded. For hair samples of less than 6 cm, 3 cm segments were considered. Using these measures, samples were grouped as 0-3 cm or 0-6 cm proximal segments. Each sample was washed sequentially with water, methanol, acetone and dichloromethane, each step lasting 5 min. Samples were allowed to dry at room temperature and then cut into millimetre-sized snippets, as long as necessary until the visual aspect was close to powder. We have compared the results obtained by using our procedure with those obtained after milling of the hair (as stated in the consensus of the SoHT; the samples were grinded in a different laboratory). No significant differences were observed (cut samples had values within ± 15% of the grinded samples, data not shown). Thirty milligrams of hair were weighted for each procedure of analysis.

For the extraction of EtG, 2 mL of deionised water was added to the hair and it was placed into an ultra-sonication bath without heating, for 2h. 20 µL of IS were added and the samples were centrifuged at 800 x g during 10 min. The supernatant was then applied to Oasis MAX SPE cartridges, previously conditioned with 2 mL of methanol and 2 mL of water. Washing was performed with 1 mL of 5% ammonia in water, 1 mL water, 1 mL methanol and 2 mL of hexane. After drying of the sorbent, EtG was eluted with 2 mL of 2% formic acid in methanol. The eluate was evaporated to dryness under a gentle nitrogen stream at 45 ºC and re-suspended in 50 µL of mobile phase (98% of phase A and 2% of B), 10 µL were injected into the LC.

The FAEEs were extracted by incubating the weighted hair with 500 µL heptane during 15h at 45 ºC. The mixture was allowed to cool at room temperature, and 20 µL of IS was added. The solution was then applied to NH_2 SPE cartridges previously conditioned with 1 mL heptane, and eluted with 1 mL heptane and 1 mL dichloromethane. No washing was performed before elution. Eluates were evaporated to dryness under a gentle nitrogen stream at room temperature and re-suspended in 65 µL heptane, 2 µL were injected into the GC. During the entire procedure for FAEEs analysis, the use of plastic (as recipients, vials or gloves) was avoided due to the potential interference of such materials in the analysis (Pragst, 2015).

2.5. Chromatographic conditions

EtG was analysed using a LC-MS/MS system composed of a Jasko X-LC system (Norleq, Porto) with a binary pump, column oven and auto sampler (maintained at 4 ºC) and a 4000 Q TRAP mass detector with a Turbo V Ion source from SCIEX (Madrid, Spain). EtG was separated using a Phenomenex synergi 4u fusion RP 80A (4 µm, 50 x 2.00 mm i.d.) column (maintained at 40 ºC) through a gradient elution at 0.1 mL/min of water with 0.1% formic acid (Phase A) and acetonitrile with 0.1% formic acid (Phase B), using the following program: from 0-2.5 min 98% of phase A and 2% of phase B was passed, changing to 10% of phase A and 90% of phase B at 3 min, and 98% of phase A and 2% of phase B from 3.5-6 min. The retention time of EtG and internal standard was 1.86 min with a total run time of 6 min. The final optimized mass spectrometry conditions for EtG analysis are presented in Table 1. The electrospray ionization (ESI) source was operated in the negative ionization mode at -4500V, temperature was set to 500 ºC, the nebulizer, turbo and curtain gases were set to 50, 53 and 25 psi, respectively. Data analysis was performed using the MultiQuant™ (version 2.0.2) software from SCIEX.

FAEEs were analysed using a HP 7890A GC system equipped with a model 7000B triple quadrupole mass spectrometer (Agilent Technologies, Waldbronn, Germany), purchased from Soquimica (Lisboa, Portugal), a MPS2 auto sampler and PTV-injector purchased from Gerstel (Soquímica, Portugal). The compounds were separated on a capillary column with 5% phenylmethylsiloxane (HP-5 MS, 30 m×0.25 mm I.D., 0.25µm film thickness) from J & W Scientific (Soquímica, Portugal), using helium as carrier gas at a rate of 1 mL/min. The oven program started at 90 ºC held for 0.5 min, followed by increments of 25 ºC/min to achieve 200 ºC and increased 8 ºC/min to the final temperature of 300 ºC, held for 2 min. The total chromatographic run was 20.4 min. The final optimized mass spectrometry conditions for the analysis of the four FAEEs, and respective retention times are given in Table 2. Injection was performed in the *splitless* mode at 250 ºC. The ion source was set to 280 ºC in the positive ionization mode, with a filament current of 35 µA, electron energy of 70 eV, and nitrogen was used as collision gas at a rate of 2.5 mL/min. Data was acquired using the MassHunter WorkStation Acquisition Software rev. B.02.01 (Agilent Technologies).

3. Results and discussion

3.1. Method Optimization

The mass spectrometry conditions for EtG were optimized by direct infusion of EtG and EtG-d5 (at 10 ng/mL) into the mass spectrometer and using the MRM compound optimization tool of the Analyst® software from SCIEX (version 1.5.1). Two transitions were chosen for EtG and one for EtG-d5. The final MRM conditions for the compounds are presented in Table 1. The ion source conditions were optimized by monitoring the detector response during direct infusion of EtG and varying the source conditions, liquid chromatography conditions were optimized using the final mass spectrometry conditions. Regarding sample preparation, 3 different SPE cartridges were previously compared (n=3) (Oasis MAX, Phenomenex Strata-X-A and Phenomenex Strata NH_2), the cartridges and respective protocols were chosen based on papers published by other authors (Kharbouche *et al.*, 2009; Pragst *et al.*, 2010; Suesse *et al.*, 2012; Cabarcos *et al.*, 2013; Crunelle *et al.*, 2014). For comparison, the response was recorded as the ratio between EtG and the IS, added only after extraction. Oasis MAX showed the best behaviour, originating cleaner chromatograms with higher response (0.18±0.01 for Strata NH_2, 4.44±0.31 for Strata-X-A and 5.06±0.03 for Oasis MAX), the clean-up protocol using these cartridges was subsequently further optimized.

Table 1. Mass spectrometry settings of final MRM conditions and retention times for ethyl glucuronide and IS.

	Transition *Quantitative transitions are underlined	**DP (V)**	**EP (V)**	**CE (eV)**	**CXP (V)**	**Dwell time (ms)**	**RT (min)**
EtG	220.932 -> 84.9	-60	-10	-24	-5	100	
	<u>220.932 -> 74.9</u>	-55	-10	-24	-5	100	1.86
EtG-d5	<u>226 -> 84.8</u>	-5	-10	-24	-5	100	

DP – declustering potential, **EP** – entrance potential, **CE** – collision energy, **CXP** – cell exit potential.

The conditions for FAEEs were optimized by performing individual injections of standard solutions at 20 µg/mL in full scan acquisition mode; based on this scan, candidate ions were studied in the product ion mode to build the final ion transitions used in the MRM. Other MRM conditions as the collision energy (CE) and dwell time were optimized by individual injections varying CE voltage and dwell time, the final conditions are summarized in Table 2. Based on

papers published by other authors (Pragst *et* al., 2001; Pragst *et al.*, 2010; Hastedt *et al.*, 2012; Suesse *et al.*, 2012; Bertol *et al.*, 2014), different extraction procedures employing incubations with heptane and combinations of heptane with dimethyl sulfoxyde (DMSO) were tested. Factors as different temperature and times of incubation and the use of agitation were also evaluated. The IS was added only after extraction in all experiments. The highest responses were obtained using 500 µL of heptane. Regarding temperature and time, the response did not seem to increase significantly for temperatures above 45ºC and extraction times longer than 15h, so these values were set as final conditions. Regarding SPE, the choice of the NH_2 sorbent and the procedure was based on literature (Caprara *et al.*, 2005; Politi *et al.*, 2011; Bertol *et al.*, 2014), since this is the most commonly used sorbent for FAEEs extraction using SPE (Oppolzer *et al.*, 2016). The adopted procedures were subsequently further optimized to yield higher recoveries for all the four FAEEs.

Table 2. Mass spectrometry settings and final MRM conditions and retention times for the four FAEEs and respective IS.

Segment	Compound	Transition *Quantitative transitions are underlined	CE (eV)	Dwell time (ms)	RT (min)
1	**Myr-Et**	101 -> 101 101 -> 73	1	21 33.6	6.99
	Myr-Et-d5	106 -> 106		20	6.95
2	**Palm-Et**	101 -> 101 101 -> 73		20.5 226.2	8.66
	Palm-Et-d5	106 -> 106		20	8.62
3	**Ole-Et**	101 -> 101 101 -> 73		34.2 17.2	10.30
	Ole-Et-d5	106 -> 106		20	10.26
	Stea-Et	101 -> 101 101 -> 73		34.2 17.2	10.54
	Stea-Et-d5	106 -> 106		20	10.50

3.2. Method validation

Both methods were validated according to international guidelines from the Food and Drug Administration (FDA) (Food and Drug Administration, 2001), International Conference on Harmonization (ICH) (International Conference on Harmonization, 2005) and the Scientific Working Group of Forensic Toxicology (SWGTOX) (Scientific Working Group of Forensic Toxicology, 2013). The evaluated parameters included selectivity, linearity, precision and accuracy, recovery, stability and, in the case of EtG, matrix effects.

3.2.1. Selectivity

Selectivity was evaluated for EtG and FAEEs by analysing 10 pools of blank hair samples obtained from different teetotallers and children aged 3-6 years. Compound identification was performed following the identification criteria suggested by the World Anti-doping Agency (WADA) (World Anti-Doping Agency, 2010). Briefly, an absolute retention (RT) time within 2% (±0.1 min) of the RT of the compounds in control and standard samples and the presence of two transitions for each compound were assumed. Also, between the two transitions, the maximum relative ion intensities allowed comparing to control samples were as follows: for values higher than 50% (10% tolerance), between 25 and 50% (±20% tolerance), between 5 and 25% (±5% tolerance) and lower than 5% (50% tolerance) (World Anti-Doping Agency, 2010). No interferences were found at the retention time and transitions of the compounds (Figures 1 and 2).

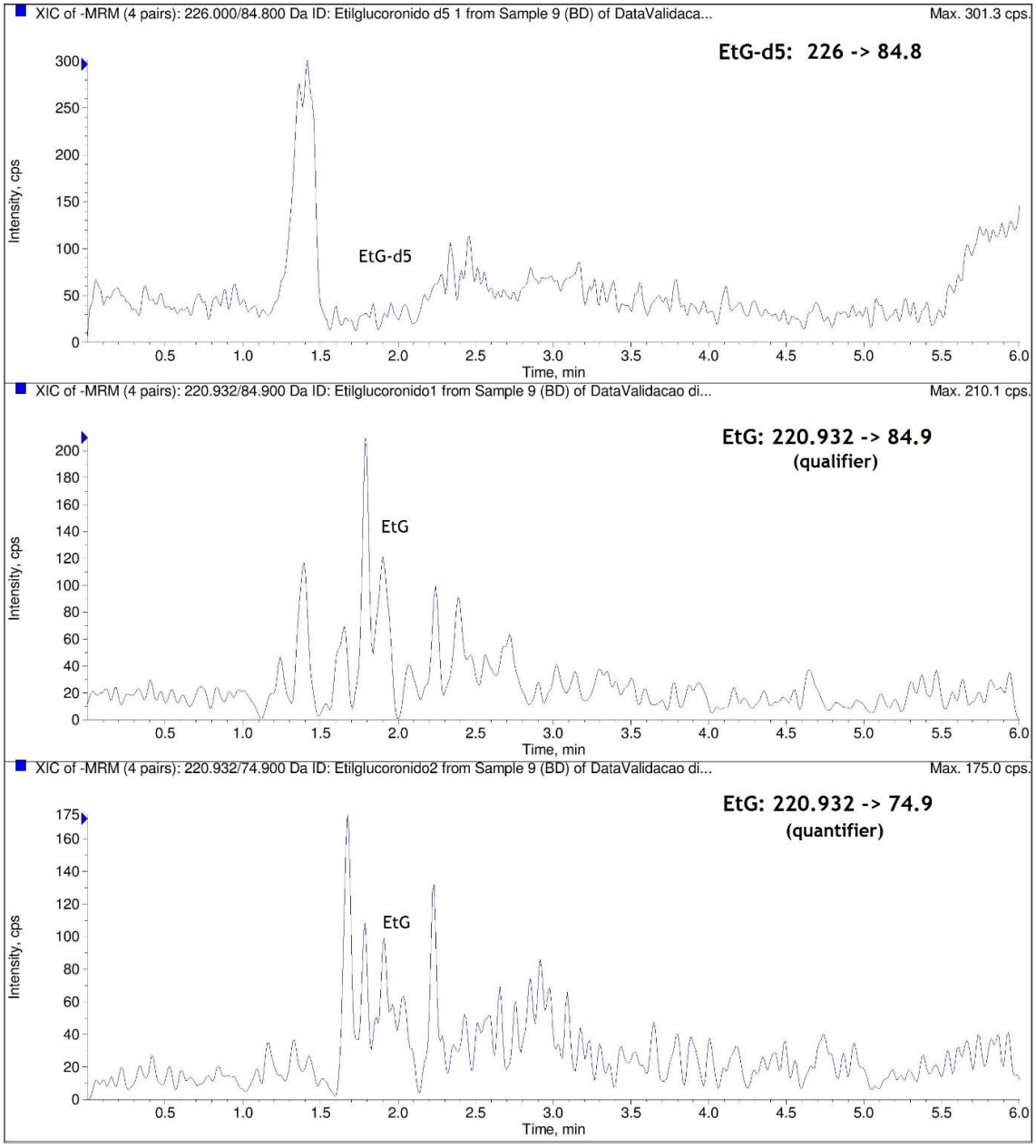

Figure 1. Chromatogram obtained after EtG analysis of a blank sample.

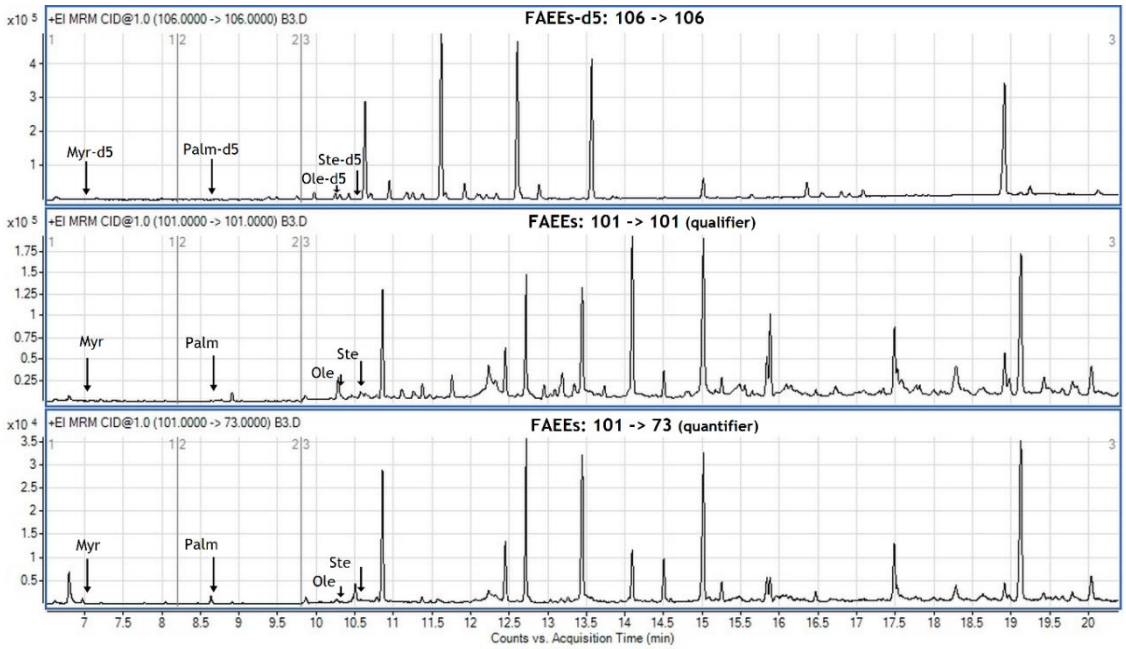

Figure 2. Chromatogram obtained after FAEEs analysis of a blank sample.

3.2.2. Linearity

For defining the linearity of the calibration curves we intended to achieve the limits of quantification proposed in the consensus on alcohol markers from the SoHT (3 pg/mg for EtG and 30 pg/mg for the four FAEEs) (Kintz, 2015). Linearity studies were carried out following a 5-day protocol, spiking 30 mg of blank hair samples with appropriate volumes of working standard solutions in the range of 5-500 pg/mg (EtG) and 30-5000 pg/mg (FAEEs), 20 µL of IS were added in both cases. 6 evenly distributed calibrators were used for EtG (3, 7, 50, 150, 300 and 500 pg/mg) and 7 calibrators for the four FAEEs (30, 100, 400, 600, 1500, 3000 and 5000 pg/mg). Additionally, quality control samples were prepared using 3 authentic hair samples positive for the compounds (EtG and FAEEs), covering the calibration range. For EtG another control sample was added (spiked at 30 pg/mg), covering the cut-off for chronic excessive drinking suggested by the SoHT (Kintz, 2015), and for the FAEEs another two control samples (spiked at 50 and 200 ng/mg). These control samples were prepared every day in triplicate during the five days. One factor to consider when working with hair is the incorporation and fixation of compounds into the hair matrix (Kharbouche *et al.*, 2009), therefore spiked samples cannot accurately represent an authentic hair sample. For this reason, we opted to use

authentic hair samples as controls. The overall calibration curves showed good linearity with coefficients of determination (R^2) higher than 0.99 (Table 3).

Table 3. Linearity data for EtG and FAEEs.

	Weighting factor	Calibration range (pg/mg)	Regression equation		R^2	LLOQ (pg/mg)
			m	b		
EtG		3 - 500	0.008 ± 2E-04	0.025 ± 0.008	0.997 ± 0.004	3
Myr-Et		30 - 5000	7E-04 ± 3E-04	0.032 ± 0.038	0.996 ± 7E-04	30
Palm-Et	$1/x^2$	30 - 5000	0.001 ± 5E-04	0.084 ± 0.119	0.994 ± 0.004	30
Ole-Et		30 - 5000	0.002 ± 8E-04	0.118 ± 0.074	0.998 ± 0.001	30
Stea-Et		30 - 5000	0.001 ± 9E-04	0.076 ± 0.099	0.996 ± 0.001	30

The limits of quantification were considered as the lowest quantifiable concentration with a signal-to-noise ratio of at least 5:1, relative error below ± 20% and a coefficient of variation below 20% (Food and Drug Administration, 2001). In the case of EtG the LLOQ was determined as 3 pg/mg, and was close to values reported by other authors (Kharbouche *et al.*, 2009; Morini *et al.*, 2009; Suesse *et al.*, 2012; Crunelle *et al.*, 2014). The LLOQ for the FAEEs was determined as 30 pg/mg for each of the compounds and, comparing to other authors, few report lower values (Cabarcos *et al.*, 2009; Hastedt *et al.*, 2012; Bertol *et al.*, 2014); however according to the consensus of the SoHT (Kintz, 2015) for assessment of abstinence and chronic excessive alcohol consumption, our limits are quite satisfactory. Only one work is reported in literature for the analysis of FAEEs in hair samples using GC-MS/MS, however with chemical ionization (CI) (Zimmerman *et al.*, 2010). Our results based on a method using a EI source and the same sample amount, shows comparable limits of detection, unfortunately the LLOQ was not evaluated in the cited work. Additionally, our method uses a more readily applicable clean-up procedure (SPE) when compared to HS-SPME. In terms of sample amount our methods shows comparable limits when compared to methods using 30 mg or more (Pragst *et al.*, 2001; De Giovanni *et al.*, 2007; Cabarcos *et al.*, 2009; Süße *et al.*, 2010; Zimmerman *et al.*, 2010; Politi *et al.*, 2011) and when using 20 mg (Caprara *et al.*, 2005; Hastedt *et al.*, 2012). Figures 3 and 4 show representative chromatograms of hair samples spiked with EtG and FAEEs at the LLOQ.

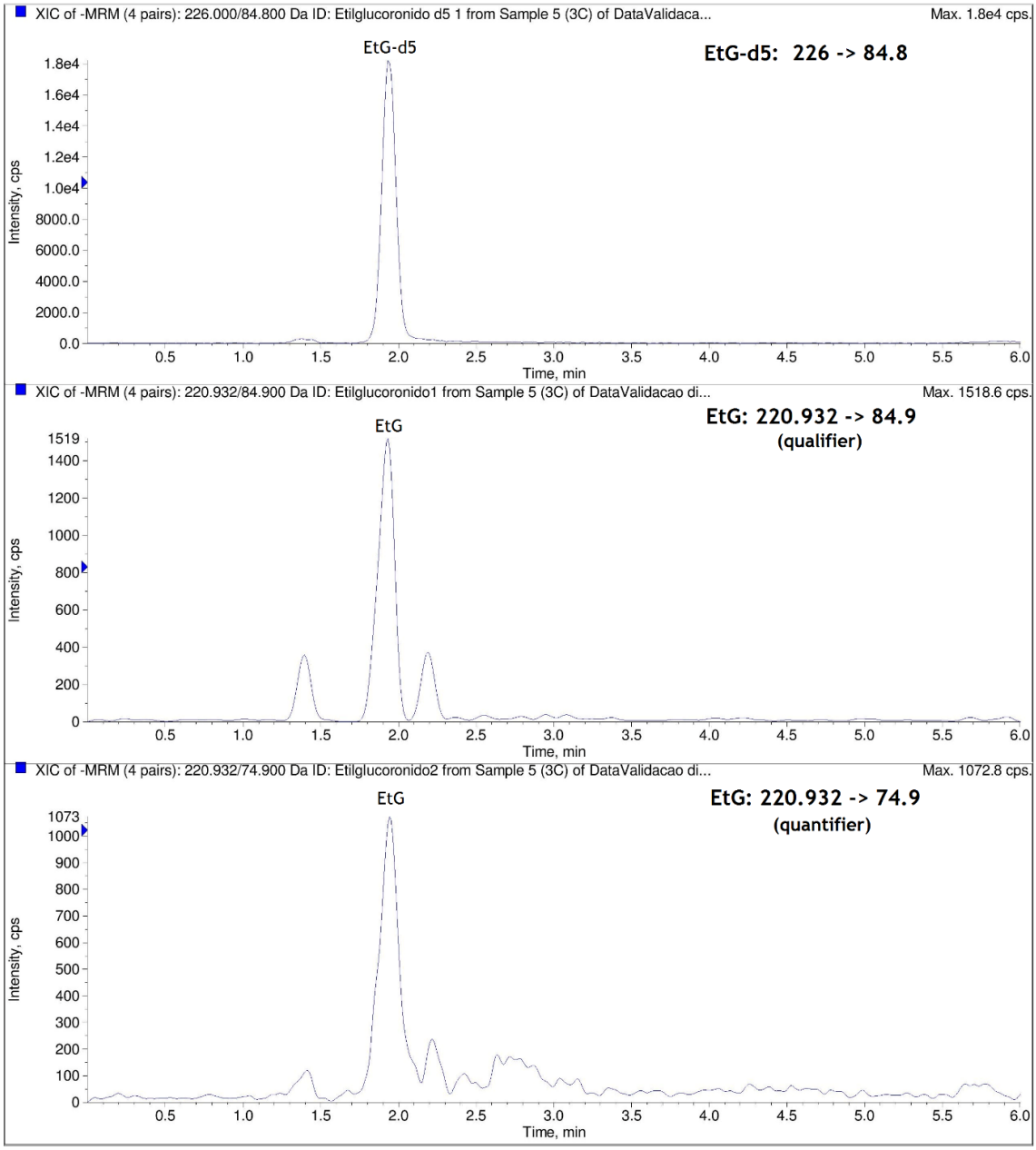

Figure 3. Chromatogram of a sample spiked with EtG at the LLOQ (3 pg/mg).

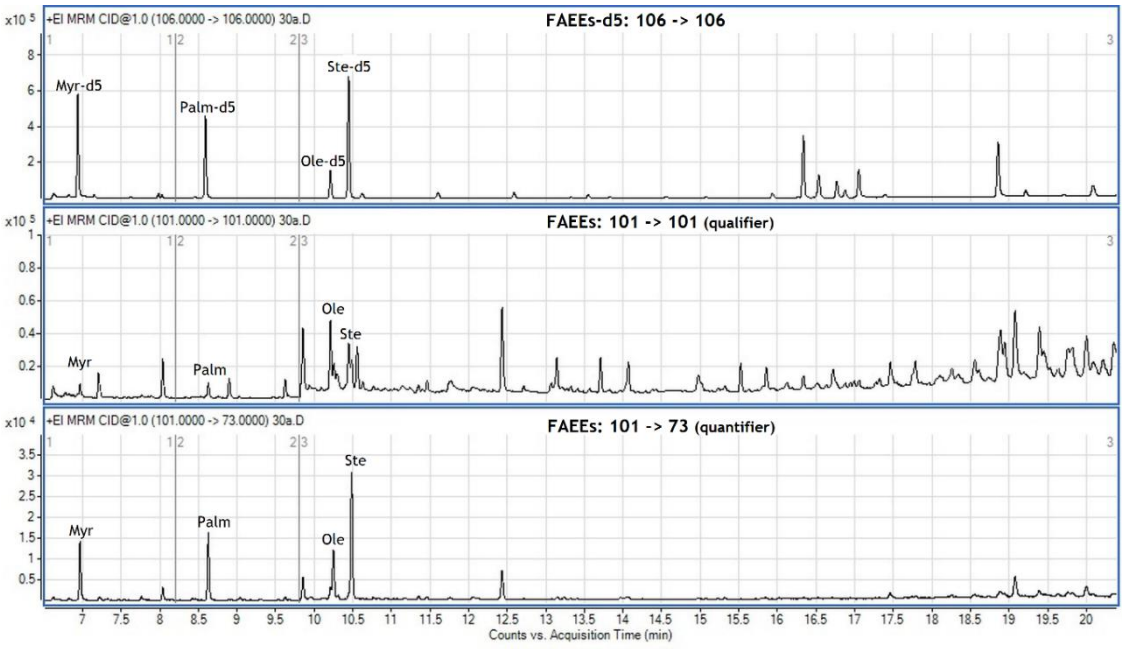

Figure 4. Chromatogram of a sample spiked with the four FAEEs at the LLOQ (30 pg/mg for each compound). Myr – Myristate, Palm – Palmitate, Ole – Oleate, Ste – Stereate.

3.2.3. Precision and accuracy

Between-run precision and accuracy were evaluated using the calibrators prepared and analysed during the 5 days of validation (n=5). Within-run precision and accuracy were evaluated by analysing different concentrations in 5 replicates during the same day [for EtG 4 levels of concentration were used (3, 50, 150, 500 pg/mg), and for FAEEs 3 levels of concentration (30, 600, 5000 pg/mg)]; these results are shown in Tables 4 and 5. Both between- and within-run precision and accuracy were found acceptable according to the guidelines (Food and Drug Administration, 2001; International Conference on Harmonisation, 2005; Scientific Working Group of Forensic Toxicology, 2013). For EtG, the coefficient of variation (CV) was overall lower than 12.22% and the relative error (Bias) was lower than ±7%, for the FAEEs, the CVs were below 11.25% and Bias lower than ±11.36%.

Table 4. Between-run precision and accuracy (n=5).

	Calibrator (pg/mg)	Measured concentration (pg/mg)	CV (%)	Bias (%)
EtG	3	2.97 ± 0.11	3.75	-2.24
	7	7.13 ± 0.6	8.45	5.46
	50	51.62 ± 1.85	3.59	0.80
	150	147.38 ± 3.72	2.52	-0.68
	300	298.99 ± 9.05	3.03	-1.77
	500	489.43 ± 10.49	2.14	-1.37
Myr-Et	30	29.66 ± 0.82	2.75	-1.12
	100	105.00 ± 9.37	8.92	5.00
	400	391.83 ± 22.20	5.67	-2.04
	600	570.25 ± 22.76	3.99	-4.96
	1500	1490.92 ± 60.98	4.09	-0.61
	3000	3066.08 ± 107.24	3.50	2.20
	5000	5076.01 ± 228.45	4.50	1.52
Palm-Et	30	30.04 ± 0.92	3.05	0.13
	100	100.30 ± 9.11	9.09	0.30
	400	388.45 ± 34.24	8.81	-2.89
	600	596.64 ± 47.22	7.91	-0.56
	1500	1498.72 ± 118.56	7.91	-0.09
	3000	3031.29 ± 207.97	6.86	1.04
	5000	5102.70 ± 364.17	7.14	2.05
Ole-Et	30	29.89 ± 0.22	0.74	-0.36
	100	102.19 ± 3.58	3.50	2.19
	400	388.82 ± 24.48	6.30	-2.80
	600	588.21 ± 27.39	4.66	-1.97
	1500	1494.87 ± 75.77	5.07	-0.34
	3000	3012.25 ± 88.83	2.95	0.41
	5000	5143.34 ± 123.05	2.39	2.87
Stea-Et	30	30.18 ± 0.57	1.89	0.60
	100	97.86 ± 5.42	5.53	-2.14
	400	408.23 ± 28.37	6.95	2.06
	600	588.00 ± 26.86	4.57	-2.00
	1500	1464.64 ± 75.79	5.17	-2.36
	3000	3063.04 ± 140.92	4.60	2.10
	5000	5086.74 ± 350.17	6.88	1.73

Table 5. Within-run precision and accuracy (n=6).

	Concentration (pg/mg)	Measured concentration (pg/mg)	CV (%)	Bias (%)
EtG	3	2.79 ± 0.34	12.22	-7.00
	50	52.10 ± 1.87	3.58	4.20
	150	150.87 ± 1.25	0.83	0.58
	500	499.44 ± 3.98	0.80	-0.11
Myr-Et	30	31.63 ± 1.50	4.74	5.42
	600	630.51 ± 60.85	9.65	5.09
	5000	4682.13 ± 282.26	6.03	-6.36
Palm-Et	30	31.81 ± 2.35	7.38	6.03
	600	662.00 ± 39.18	5.92	10.33
	5000	4795.90 ± 539.60	11.25	-4.08
Ole-Et	30	32.49 ± 2.18	6.72	8.31
	600	592.94 ± 53.26	8.98	-1.18
	5000	5004.86 ± 273.11	5.46	0.10
Stea-Et	30	32.34 ± 1.53	4.75	7.81
	600	614.46 ± 49.18	8.00	2.41
	5000	4432.04 ± 320.65	7.23	-11.36

The precision and accuracy at the quality control samples is shown in Table 6, the CVs were below 15% and Bias below ±5.01% in all controls samples. Regarding the authentic samples used as controls, the coefficient of variation indicates excellent precision of the method.

Table 6. Quality control samples data (n=15).

	Control	Measured concentration (pg/mg)	CV (%)	Bias (%)
EtG	Authentic sample #1	33.13 ± 4.34	13.10	-
	Authentic sample #2	61.67 ± 3.92	6.36	-
	Authentic sample #3	130.66 ± 10.28	7.87	-
	Spiked (30pg/mg)	29.99 ± 1.61	5.37	-0.03
Myr-Et	Authentic sample #1	42.01 ± 4.83	11.50	-
	Authentic sample #2	92.17 ± 12.65	13.73	-
	Authentic sample #3	186.85 ± 27.92	14.94	-
	Spiked (50 pg/mg)	49.40 ± 5.00	10.13	-1.19
	Spiked (200pg/mg)	197.39 ± 20.18	10.22	-1.30
Palm-Et	Authentic sample #1	86.37 ± 12.48	14.45	-
	Authentic sample #2	177.27 ± 26.24	14.80	-
	Authentic sample #3	401.80 ± 59.41	14.79	-
	Spiked (50 pg/mg)	49.32 ± 5.24	10.62	-1.36
	Spiked (200pg/mg)	193.92 ± 20.44	10.54	-3.04
Ole-Et	Authentic sample #1	214.65 ± 25.09	11.69	-
	Authentic sample #2	466.49 ± 68.68	14.72	-
	Authentic sample #3	915.16 ± 110.86	12.11	-
	Spiked (50 pg/mg)	50.18 ± 4.61	9.19	0.35
	Spiked (200pg/mg)	189.97 ± 18.50	9.74	-5.01
Stea-Et	Authentic sample #1	64.54 ± 9.43	14.61	-
	Authentic sample #2	128.46 ± 19.18	14.93	-
	Authentic sample #3	253.78 ± 32.86	12.95	-
	Spiked (50 pg/mg)	49.94 ± 4.67	9.36	-0.13
	Spiked (200pg/mg)	200.89 ± 19.99	9.95	0.45

3.2.4. Recovery

The recovery of the SPE procedures was evaluated at three levels of concentration by comparing spiked blank samples with blank samples spiked only after extraction, in both groups the IS was added only after extraction. The assays were performed in triplicate, for EtG the recovery ranged from 74.8% to 82.5%, and for FAEEs from 79.9% to 97.9% (Table 7). These values are quite satisfactory and are close to values reported by other authors (Cabarcos *et al.*, 2009, 2013; Pragst *et al.*, 2010; Hastedt *et al.*, 2013), especially considering the obtained LLOQs.

Table 7. Recovery of all compounds at three levels of concentration (n=3).

	Recovery (%)		
	30 pg/mg	**600 pg/mg**	**5000 pg/mg**
Myr-Et	92.2 ± 5.9	93.3 ± 12.3	77.4 ± 8.4
Palm-Et	92.3 ± 2.6	89.6 ± 16.8	78.3 ± 9.6
Ole-Et	96.0 ± 7.3	92.2 ± 11.2	97.9 ± 7.5
Stea-Et	93.2 ± 9.8	79.9 ± 1.8	96.1 ± 6.6
	3 pg/mg	**50 pg/mg**	**500 pg/mg**
EtG	82.5 ± 1.8	81.0 ± 3.0	74.8 ± 2.4

3.2.5. Stability

Stability of processed samples was evaluated by leaving the quality control samples in the auto-samplers, during at least 24h. For EtG, samples were left for 24, 48, 72, and 96h, for FAEEs samples were only left for 24h due to the volatility of heptane. These samples were then compared to freshly prepared control samples, and quantified using the respective calibration curve. The accuracy relative to the fresh controls and the overall precision during the studied times were calculated, samples were considered stable if Bias was lower than ±15% and CV% lower than 15%. All processed samples were found to be stable during the evaluated time. It has to be noted that the successful stability of EtG extracts is of great advantage to laboratories performing analysis and injections over longer periods of time, such as weekends.

3.2.6. Matrix Effects

Matrix effects and ion suppression phenomena were studied for EtG using the method proposed by Matuszewski and co-workers (Matuszewski *et al.*, 2003). 2 samples sets were prepared, each one in triplicate: set A was prepared by spiking blank hair only after extraction, and set B was prepared by evaporating the same concentration as in A and re-suspending in mobile phase. These data allow to determine the matrix effect (ME) using the following formula ME (%) = set A/set B x 100 (Matuszewski *et al.*, 2003), the matrix effect was found not to be significant (ME > 98%).

3.3. Analysis of authentic hair samples

The described methods are currently being used in laboratory routine analysis, for the evaluation of alcohol consumption amongst university students. The results of 15 samples analysed for EtG and FAEEs are shown in Table 8, together with information gathered from questionnaires of the subjects regarding their self-reported consumption (abstinent, moderate or excessive).

Table 8. Alcohol biomarker analysis results of 15 samples, obtained from voluntary university students.

Sample	Segment lenght (cm)	Alcohol biomarker concentration (pg/mg)						Self-reported consumption
		Myr-Et	Palm-Et	Ole-Et	Stea-Et	$\sum cFAEEs$	EtG	
#1	3.0	n.q.	47.6	38.8	30.1	**116.5**	**n.q.**	**Abstinent**
#2	3.0	34.5	88.4	95.0	34.9	**252.8**	**22.2**	**Moderate**
#3	6.0	138.1	230.6	113.7	87.8	**570.2**	**26.4**	**Moderate**
#4	4.3	37.8	154.2	147.0	85.8	**424.8**	**9.9**	**Moderate**
#5	6.0	187.5	286.8	805.2	182.6	**1362.1**	**136.1**	**Excessive**
#6	6.0	34.7	80.4	53.3	31.0	**199.4**	**6.6**	**Abstinent**
#7	6.0	48.3	148.7	135.5	258.9	**591.4**	**15.1**	**Moderate**
#8	1.2	60.1	94.5	91.5	59.1	**305.2**	**17.0**	**Moderate**
#9	6.0	72.0	163.1	150.0	49.5	**434.6**	**22.3**	**Moderate**
#10	6.0	226.0	244.6	416	312.3	**1198.9**	**147.4**	**Excessive**
#11	6.0	34.7	80.4	53.3	31.0	**199.4**	**6.5**	**Abstinent**
#12	6.0	35.2	73.4	92.8	27.7	**229.1**	**6.7**	**Moderate**
#13	3.0	73.6	72.3	n.q.	n.q.	**145.9**	**5.6**	**Abstinent**
#14	3.1	n.q.	32.3	61.3	n.q.	**93.6**	**5.5**	**Moderate**
#15	6.0	92.1	150.8	162.1	100.7	**505.7**	**7.3**	**Abstinent**

n.q. - not quantifiable (below the LLOQ)

FAEEs were interpreted as a sum of concentrations (∑cFAEEs), since the analysis of individual species is not recommended due to presenting a high variability of concentrations. Similarly, to what occurred in other studies, we found that the most occurring FAEEs species were ethyl palmitate and ethyl oleate (Pragst *et al.*, 2001; Hartwig *et al.*, 2003; Süße *et al.*, 2010). The analysis results were compared to the answers provided by the subjects to verify the efficiency of the developed methodologies, therefore the cut-off values proposed in the current consensus of the SoHT were adopted (Kintz, 2015). Briefly, for 0-3cm segments, an EtG concentration of 7 pg/mg and ∑cFAEEs of 200 pg/mg was used to distinguish an abstinent from a moderate drinker, while an EtG concentration of 30 pg/mg and ∑cFAEEs of 500 pg/mg was used to distinguish a moderate from an excessive drinker. For 0-6cm segments, an EtG concentration of 7 pg/mg and ∑cFAEEs of 400 pg/mg was used to distinguish an abstinent from a moderate

drinker, an EtG concentration of 30 pg/mg and ∑cFAEEs of 1000 pg/mg was used to distinguish a moderate from an excessive drinker. It is important to note at this point that, while we used these values to distinguish an abstinent from a moderate drinker, the SoHT states that a biomarker concentration below the cut-off of abstinence does not contradict self-reported abstinence, but does not necessarily prove strict abstinence (Kintz, 2015). By using these values, in most of the cases the self-reported consumption was confirmed by the results, indicating that the developed methods are suitable for the routine analysis of these compounds. Some discrepancies were however observed, namely in samples 12, 14 and 15. In cases 12 and 14, the participants have considered their consumption to be moderate, but the concentrations of both markers were below the cut-off for moderate drinking. Reasonable explanation for these cases may be the presence of a low moderate drinking behaviour or that the participants were abstinent only during the time period corresponding to the collected segment length, nevertheless, these results do not prove abstinence (Kintz, 2015). The opposite occurs in the case of sample 15, where the participant considered the consumption as abstinent but both markers appear above the cut-off for moderate drinking, indicating repeated alcohol consumption (Kintz, 2015). Similar reasons as mentioned above might explain this case, especially concerning the fact that both markers surpass only slightly the concentrations of the cut-off indicating a low moderate drinking behaviour. One important fact to be noted is that the analysed samples were obtained from subjects that did not report the use of any aggressive cosmetic treatments that could affect alcohol biomarker concentration (bleaching, dyeing, use of alcohol containing products) (Albermann *et al.*, 2011; Suesse *et al.*, 2012; Crunelle *et al.*, 2015; Kintz, 2015; Pragst, 2015). A chromatogram of one of the analysed samples positive for EtG (147.4 pg/mg) is shown in Figure 5 and a sample positive for FAEEs (187.5, 286.8, 805.2 and 182.6 pg/mg of ethyl myristate, palmitate, oleate and stereate, respectively), is shown in Figure 6.

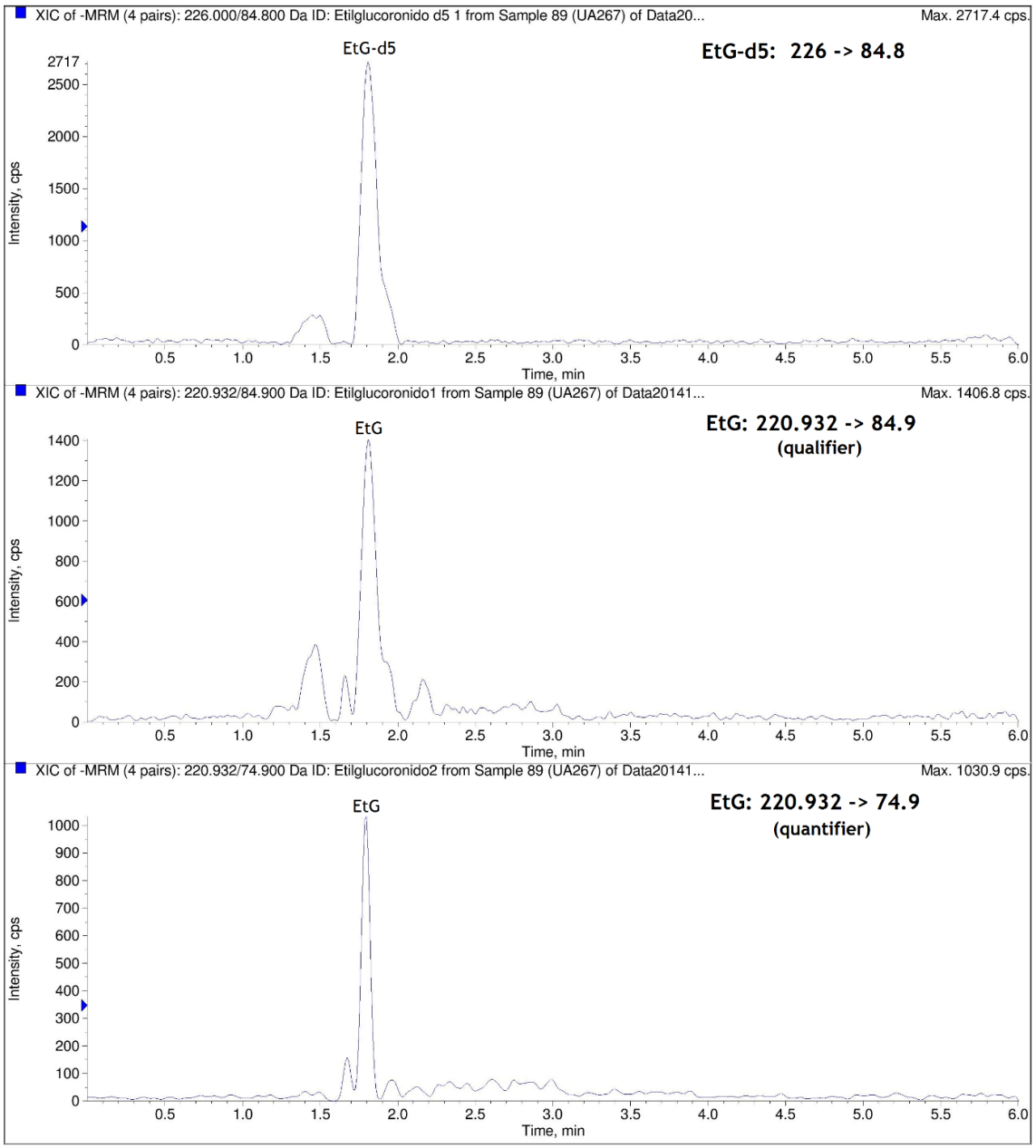

Figure 5. Chromatogram obtained after analysis of an authentic hair sample (sample #10) positive for EtG (147.4 pg/mg).

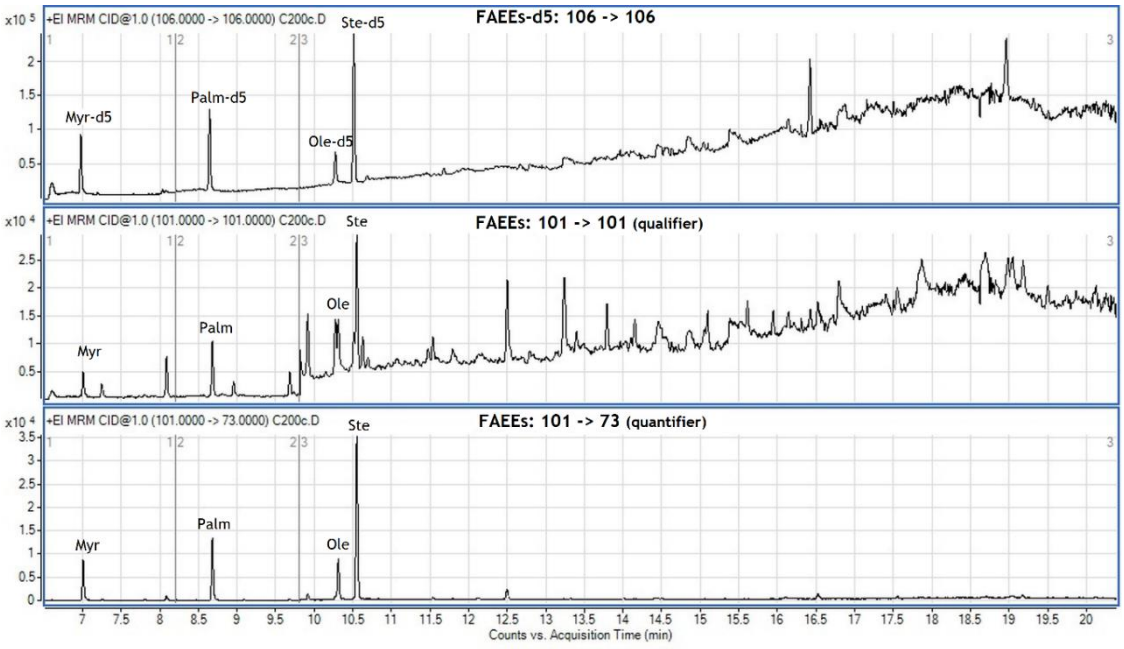

Figure 6. Chromatogram obtained after analysis of an authentic hair sample (sample #5) positive for FAEEs. Myr – Myristate (187.5 pg/mg), Palm – Palmitate (286.8 pg/mg), Ole – Oleate (805.2 pg/mg), Ste – Stereate (182.6 pg/mg).

4. Conclusions

Two methods were developed and validated for the determination of EtG and FAEEs in hair samples. The methods were validated according to internationally accepted guidelines evaluating selectivity, linearity, precision and accuracy, recovery, stability and matrix effects. The methods were found to be linear in the studied range, covering the limit of quantification for determining alcohol exposure suggested by the SoHT. Precision and accuracy were found acceptable according to international guidelines, and the processed samples showed to be stable up to 96h in the injector for EtG and up to 24h for FAEEs. Matrix effects were found inexistent. This paper describes for the first time the analysis of FAEEs using GC-MS/MS with and EI source, presenting and interesting and comparable approach to methods using CI sources, or GC-MS. The methodologies were successfully applied to authentic hair samples proving the applicability of the proposed methods, therefore useful and suitable for the routine analysis of alcohol biomarkers in hair. Both analysis could be performed on each provided sample. The small sample amount used for each analysis (30 mg) presented a great advantage, since it's very common that limited sample amounts are collected in hair analysis, and due to different

reasons further analysis could be required from the same sample, for example for the determination of drugs of abuse.

Acknowledgements

The authors acknowledge the Fundação Calouste Gulbenkian for the financing of this project (Investigação e Saúde - reference: 125895), the POCI - COMPETE 2020 - Operational Programme Competitiveness and Internationalisation in Axis I - Strengthening research, technological development and innovation (Project POCI-01-0145-FEDER-007491) and National Funds by FCT - Foundation for Science and Technology (Project UID/Multi /00709/2013). D. Oppolzer acknowledges the Programa Operacional Regional do Centro 2007-2013 QREN (Programa "Mais Centro") in the form of a fellowship (CENTRO-07-ST24-FEDER-002014, reference TPCR-2-004).

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2.4 Manuscript 3

DETERMINATION OF ETHYL GLUCURONIDE IN HAIR TO ASSESS EXCESSIVE ALCOHOL CONSUMPTION IN A STUDENT POPULATION

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Analytical and Bioanalytical Chemistry, Vol. 408, No. 8, 2027-2034, 2016

Anal Bioanal Chem (2016) 408:2027–2034
DOI 10.1007/s00216-015-9155-7

RESEARCH PAPER

Determination of ethyl glucuronide in hair to assess excessive alcohol consumption in a student population

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Received: 2 September 2015 / Revised: 16 October 2015 / Accepted: 27 October 2015 / Published online: 4 November 2015

Abstract Hair analysis for ethyl glucuronide (EtG) was used to evaluate the pattern of alcohol consumption amongst the Portuguese university student population. A total of 975 samples were analysed. For data interpretation, the 2014 guidelines from the Society of Hair Testing (SoHT) for the use of alcohol markers in hair for the assessment of both abstinence and chronic excessive alcohol consumption were considered. EtG concentrations were significantly higher in the male population. The effect of hair products and cosmetics was evaluated by analysis of variance (ANOVA), and significant lower concentrations were obtained when conditioner or hair mask was used or when hair was dyed. Based on the analytical data and information obtained in the questionnaires from the participants, receiver operating characteristic (ROC) curves were constructed in order to determine the ideal cut-offs for our study population. Optimal cut-off values were estimated at 7.3 pg/mg for abstinence or rare occasional drinking control and 29.8 pg/mg for excessive consumption. These values are very close to the values suggested by the SoHT, proving their adequacy to the studied population. Overall, the obtained EtG concentrations demonstrate that participants are usually well aware of their consumption pattern, correlating with the self-reported consumed alcohol quantity, consumption habits and excessive consumption close to the time of hair sampling.

Keywords Ethyl glucuronide · Hair · Student population · Receiver operating characteristic · Cut-off

Parts of the work have been presented at the 20th Annual Meeting of the Society of Hair Testing.

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Introduction

Alcohol consumption accounts for a severe problem amongst the student population. According to the 2014 report of the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA), results from a survey indicated that almost two thirds of students report drinking alcohol at least once in the last month, of which 20 % were intoxicated [1]. The juvenile student population is at key risk since alcohol has deleterious consequences as poor school performance, relational and sexual problems, delinquency and accidents [2]. Hair testing for alcohol biomarkers is an effective approach to assess alcohol consumption for toxicological and forensic purposes and has been the interest of many studies published over the past years [3–13]. Ethyl glucuronide (EtG), a direct metabolite of ethanol, is an effective alcohol biomarker that correlates well with alcohol intake, and its analysis in hair samples has proven to be highly specific [8, 9, 11, 14, 15]. A consensus for uniform sample analysis and data interpretation was recently reviewed by the Society of Hair Testing [16, 17], providing analysts with proper cut-off values, at 7 pg/mg for abstinence or rare occasional drinking control and 30 pg/mg for excessive drinking. EtG analysis in hair may be affected by hair products and cosmetic treatments which should be considered during sampling and analysis [5–7, 11, 15–20]. Much of the contributions to the international knowledge on hair testing for alcohol biomarkers come from population studies; however, to our knowledge, no study has been conducted to date on a student

population. Therefore, our aim was to assess alcohol consumption in a large student population, by analysing hair samples collected at nine Portuguese universities using a liquid chromatography–tandem mass spectrometry (LC-MS/MS) method previously developed and validated in our laboratory.

Experimental

Reagents and standards

Methanol, acetonitrile, water and isopropanol (MS grade) from Fisher were acquired from Enzymatic (Santo Antão do Tojal, Portugal). Methanol (HPLC grade) was acquired from VWR Internacional (Carnaxide, Portugal). Formic acid (MS grade) was obtained from Sigma-Aldrich (Sintra, Portugal). Acetone, dichloromethane and ammonium hydroxide from Fisher were purchased from Enzymatic (Santo Antão do Tojal, Portugal). Oasis MAX 3-cc SPE cartridges were acquired from Waters (Lisboa, Portugal). EtG and its deuterated analogue (EtG-d5; internal standard, IS) at 100 μg/mL in methanol were purchased from LCG Promochem (Barcelona, Spain). All stock and working standard solutions were prepared in methanol and were stored in amber glass vials below −20 °C.

Biological specimens

Human hair free of EtG was obtained from children aged 3–6 years, and was used for the calibration curves after preparing a homogenized pool of hair. Samples from the student population were collected at nine Portuguese universities: Universidade do Algarve (UAlg), Universidade de Aveiro (UA), Universidade da Beira Interior (UBI), Universidade de Coimbra (UC), Universidade de Évora (UE), Universidade de Lisboa (UL), Universidade do Minho (UM), Universidade do Porto (UP) and Universidade de Trás-os-Montes e Alto Douro (UTAD). Samples were cut as close as possible to the scalp and stored in paper envelopes until analysis. Together with each hair sample, a self-completion questionnaire was provided to the students, and data on age, sex, alcohol consumption habits and use of tobacco, illicit and licit drugs, hair washing products and cosmetics, amongst others, was collected.

Analysis of EtG

Hair samples were analysed for EtG by a method previously developed and validated in our laboratory. Briefly, hair samples were sequentially washed with water, methanol, acetone and dichloromethane, left to dry and cut with scissors to fragments of less than 1 mm. Each analysis was performed using 30 mg of hair, to which 20 μL of a 300-ng/mL IS solution was added (200 pg/mg). EtG was extracted from the hair matrix by 2 h sonication with 2 mL deionized water and centrifuged for 10 min (800×*g*). Supernatants were then applied to OASIS MAX solid-phase extraction (SPE) cartridges, previously conditioned with 2 mL methanol and 2 mL water. A washing step followed using 1 mL of 5 % ammonium in water, 1 mL water, 1 mL methanol and 2 mL hexane, after which the sorbents were dried under vacuum. Samples were eluted with 2 mL of 2 % formic acid in methanol, and the eluate was evaporated to dryness under a gentle nitrogen stream (at 45 °C). The dried residues were re-suspended in 50 μL of mobile phase, and 10 μL was injected into the LC-MS/MS system. The LC-MS/MS system was composed of a Jasko X-LC system (Norleq, Porto, Portugal) with a binary pump, column oven and auto sampler (maintained at 4 °C) and a 4000 Q TRAP mass detector with a Turbo V Ion source from SCIEX (Madrid, Spain). Separation was performed on a Phenomenex synergi 4u fusion RP 80A (4 μm, 50×2.00 mm i.d.) column (maintained at 40 °C) through a gradient elution at 0.1 mL/min of water with 0.1 % formic acid (phase A) and acetonitrile with 0.1 % formic acid (phase B), using the following programme: from 0 to 2.5 min, 98 % of phase A and 2 % of phase B; at 3 min, 10 % of phase A and 90 % of phase B; and from 3.5 to 6 min, 98 % of phase A and 2 % of phase B. The ion source was operated in the negative ionization mode at −4500 V, temperature was set to 500 °C and the nebulizer, turbo and curtain gases were set to 50, 53 and 25 psi, respectively. EtG was monitored using two transitions, 220.9→84.9 and 220.9→74.9, and EtG-d5 was monitored at 226→84.8. The retention time of both compounds was 1.86 min and the total run time was 6 min. The method was validated according to guidelines from the Food and Drug Administration (FDA) and International Conference on Harmonisation (ICH) [21, 22]. The method was selective and presented linearity from 3 to 500 pg/mg with a lower limit of quantification (LLOQ) of 3 pg/mg (Table 1), covering both the LLOQ and cut-off values proposed in the consensus of the Society of Hair Testing (SoHT) [16, 17]. Each calibrator was analysed in five replicates, during the different days of the validation protocol. The between-run precision and accuracy were evaluated at the concentrations of the calibrators (n=5) (Table 2), while within-run precision and accuracy were evaluated at four levels of concentration, including the lower and upper limits of quantification (n=6) (Table 3). Precision and accuracy were found adequate, with all coefficients of variation (CV) lower than 13 % and mean relative errors within ±7 % from the nominal value. The absolute recovery ranged from 75 to 82 %. During analysis of authentic hair samples, a calibration curve was freshly prepared every day and analysed together in the same run.

Table 1 Linearity data for EtG

Weighting factor	Calibration range (pg/mg)	Regression equation		R^2	LLOQ (pg/mg)
		m	*b*		
$1/x^2$	3–500	0.008±2E−04	0.025±0.008	0.997±0.004	3

Statistical analysis

Statistical analysis was performed using the IBM® SPSS® Statistics software (v22) and Microsoft® Excel 2013 (v15). Group mean outcomes were compared for statistical differences by analysis of variance (ANOVA); statistical significance was attributed to p values less than or equal to 0.05. Optimal cut-off value estimation was performed by receiver operating characteristic (ROC) analysis. The specificity and sensitivity plots were built using SPSS. Normality tests and Spearman and Pearson correlation studies were also performed on SPSS.

Results

Sample distribution

Relevant answers to the study provided by the participants are summarized in Table 4. For analytical purposes and to allow comparison of results, participants were asked to classify their consumption of ethanol as abstinent, moderate (occasional consumption of low quantities), frequent (consumption during a few days per week) or excessive (daily consumption of high quantities). This classification was based on the European School Survey Project on Alcohol and other Drugs (ESPAD) of 2011 [23]. A total of 1192 hair samples were collected, 975 of which were analysed. The remaining samples (18.2 %) were excluded due to insufficient sample amount. Up to 6-cm hair segments were analysed; in the case of longer samples, the proximal 6 cm was cut and used for analysis, and shorter samples were completely processed. In all cases, the segment length was recorded and this information can be found in Table 4. The mean EtG concentration obtained for the analysed samples distributed along the nine universities was calculated and is shown in Fig. 1. Of the total samples, the concentrations ranged from 3.1 to 153.4 pg/mg. In females, the concentration range was 3.2 to 153.4 pg/mg with a median of 11.9 pg/mg (average of 15.3 pg/mg), compared to a median of 16.9 pg/mg (average of 21.9 pg/mg) in males (range 3.1–147.4 pg/mg). ANOVA between females' and males' hair EtG showed a statistically significant difference (p value <0.001) for EtG concentrations between sexes.

Based on the determined EtG concentration, samples were grouped into either abstinence, social drinking or chronic excessive drinking. For that effect, the following cut-offs suggested by the SoHT were used: an EtG concentration of 7 pg/mg was used to distinguish abstinence or rare occasional drinking from moderate drinking, and 30 pg/mg was used to distinguish moderate from excessive drinking [16, 17]. Fifty-four samples had EtG concentrations below the LLOQ (3 pg/mg) and were considered not quantifiable; the total samples for each of the groups are presented in Table 4.

Table 2 Between-run precision and accuracy (n=5)

Concentration (pg/mg)	Measured concentration (pg/mg)	CV (%)	Bias (%)
3	3.0±0.1	3.7	−2.2
7	7.1±0.6	8.4	5.5
50	51.6±1.8	3.6	0.8
150	147.4±3.7	2.5	−0.7
300	299.0±9.0	3.0	−1.8
500	489.4±10.5	2.1	−1.4

Effect of washing and cosmetic treatment products

Questionnaire data included the number of hair washes per week, the use of washing products apart from shampoo (hair conditioner or hair mask) and also the use of any sort of cosmetic treatment during the preceding 6 months of sample collection (in order to account even for the longest analysed hair segments of 6 cm). Results were grouped according to the different number of hair washes per week and analysed by ANOVA statistics; however, no statistical difference was observed. For evaluating the effect of washing and cosmetic treatments, the individuals that reported the use of the above-mentioned products were selected and compared with the samples where no use of any of these treatments was reported. The differences between the groups were analysed by ANOVA statistics and are presented in Table 5. Statistically

Table 3 Within-run precision and accuracy (n=6)

Concentration (pg/mg)	Measured concentration (pg/mg)	CV (%)	Bias (%)
3	2.8±0.3	12.2	−7.0
50	52.1±1.9	3.6	4.2
150	150.9±1.2	0.8	0.6
500	499.4±4.0	0.8	−0.1

significant lower EtG concentrations were observed when washing products were used: conditioner, hair mask and both combined, and for the cosmetic treatments: dyeing and dyeing and/or bleaching.

Cut-off value estimation

For comparison, the specificity and sensitivity for the proposed cut-off values were calculated as follows: specificity $= \frac{TP}{TP+FN}$ and sensitivity $= \frac{TN}{TN+FP}$. For this, the total of true-positive (TP), true-negative (TN), false-positive (FP) and false-negative (FN) samples were counted at the cut-off values of 7 and 30 pg/mg. These results are shown in Table 6. The sensitivity and specificity were, respectively, 81.8 and 56.3 % for 7 pg/mg and 60 and 90.3 % for 30 pg/mg. In order to optimize both sensitivity and specificity, optimal cut-off values for this study were estimated. This was done using the EtG concentration and the self-report of the participants' alcohol consumption habit. Optimal cut-off values, specificity and sensitivity were calculated by means of ROC curve analysis, using individually the concentration of EtG versus the data obtained in the questionnaires, regarding how the participants considered their respective consumption habits: abstinent, moderate or excessive drinking. For this, samples were grouped as abstinent or consumers for the determination of the cut-off value for abstinence or rare occasional drinking, and excessive consumers or non-excessive consumers for the cut-off value for excessive drinking. The plots obtained from the ROC curves for abstinence and excessive drinking are shown in Figs. 2 and 3, respectively. Optimal cut-off values were estimated using the best relation between specificity and sensitivity, based on Youden's index (J = specificity + sensitivity − 1) [24]. The concentrations with the highest values for this index were considered as candidate cut-off values. The highest J value was obtained at a cut-off of 7.3 pg/mg (J=0.394) for distinguishing abstinence from moderate drinking and 29.8 pg/mg (J=0.701) for distinguishing moderate from excessive drinking. Table 6 shows the specificity and sensitivity obtained using the suggested cut-offs and using the optimal determined cut-offs for this study.

The sensitivity and specificity of the answers provided in the questionnaire regarding consumption habits were also determined based on the analytical results. For this, the cut-off values proposed by the SoHT were assumed as the "gold standard" against which the answers given in the questionnaires were plotted (particularly the classification of the consumption pattern). Sensitivity and specificity of the responses were calculated at 7 pg/mg, yielding a sensitivity of 0.959 and a specificity of 0.195, and at 30 pg/mg for which a sensitivity of 0.031 and a specificity of 0.997 were obtained.

Correlation studies

Information provided in the questionnaires (Table 4) was correlated with the determined concentration of EtG. Firstly, the

Table 4 Sample distribution according to age, sex, reported alcohol consumption, consumption of other substances, analysed segment length and determined EtG concentration

Age													
17 (n=10)	18 (n=108)	19 (n=124)	20 (n=173)	21 (n=151)	22 (n=125)	23 (n=98)	24 (n=57)	25 (n=45)	26 (n=15)	27 (n=15)	28 (n=12)	29 (n=11)	≥30 (n=29)
Sex													
Female (n=689)							Male (n=280)						
Last time alcohol was consumed in excess before sampling													
Never (n=248)				<1 month (n=281)				≥1 month; <6 months (n=140)			≥6 months (n=148)		
Reported alcohol consumption													
Abstinent (n=71)				Social drinking (n=672)				Frequent (n=222)			Excessive (n=5)		
Consumption of other substances													
Tobacco (n=357)					Drugs of abuse			Substances sold in "smart shops"					
					Cannabis (n=146)	Cocaine (n=9)	Others (n=8)	(n=126)					
Analysed segment length													
0–1 cm (n=5)		1–2 cm (n=14)		2–3 cm (n=46)		3–4 cm (n=129)		4–5 cm (n=91)			5–6 cm (n=766)		
Concentration of EtG													
n.q. (n=54)				≥3 and <7 pg/mg (n=153)				≥7 and <30 pg/mg (n=671)			≥30 pg/mg (n=97)		

n.q. not quantifiable (below LLOQ)

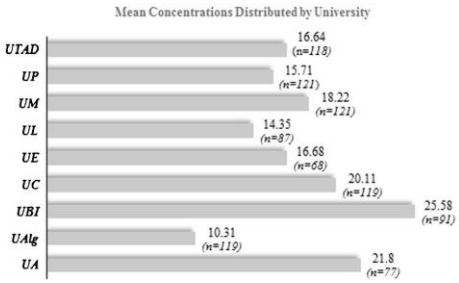

Fig. 1 Mean EtG concentrations (in pg/mg) and total amount of analysed samples for each university

EtG concentration correlated with the quantity of 100 % ingested alcohol (Spearman rho (r)=0.105, p< 0.01), increasing with the ingested quantity. Concerning the alcohol consumption habits reported by the participants, a significant correlation (Spearman rho (r)= 0.217, p<0.01) was found, with lower concentrations corresponding to the answer "abstinence" and higher concentrations with the answer "excessive drinking". The last time alcohol was consumed in excess before hair sampling correlated positively (Spearman rho (r)= 0.108, p<0.01) with the lower concentrations corresponding to no reported excessive consumption and increasing from the longest date before sampling (>6 month) to the date closest to sampling (less than 1 month). Correlation studies were also performed for the use of tobacco, illicit substances and substances sold in smart shops versus the concentration of EtG; however, no significant correlation was found between these variables.

Discussion

The mean alcohol consumption varied across the different universities (Fig. 1), with the highest EtG concentration average obtained in UBI and the lowest average in UAlg. EtG concentrations were higher in males compared to females (median of 16.91 versus 11.88 pg/mg, respectively), which is in agreement with the observations reported in other works [4, 6]. Based on the concentration, most samples fitted in the moderate consumption group (68.8 %), followed by abstinence (21.2 %) and finally excessive consumption (10.0 %). Interestingly, the number of abstinent individuals is much higher based on EtG concentration analysis compared to the answers of the questionnaires; an increase of 13.9 % occurs while the moderate drinkers decrease by almost 23 % (Table 4). While considering that some of these participants may have misclassified their consumption, this situation is partly in agreement with the results published by others [25], supporting that a concentration of EtG below the cut-off of 7 pg/mg does not completely exclude alcohol use.

The results did not appear to be influenced by the number of hair washes per week, but the use of conditioner and hair mask during washes was associated with statistically lower EtG concentrations. The use of shampoo could not be

Table 5 ANOVA statistical analysis on the use of hair washing and cosmetic treatment products

Treatment	n	EtG concentration (pg/mg)		ANOVA			
		Mean	Median	p value	F	F critical	Statistical difference
Washing products							
No reported product[a]	338	19.8	14.9	–	–		–
Conditioner only	278	16.0	13.1	0.004	8.28	3.86	Yes
Hair mask only	87	15.3	11.9	0.031	4.69	3.86	Yes
Conditioner and mask	218	16.6	11.9	0.040	4.24	3.86	Yes
Cosmetic products							
No reported product	611	18.2	13.6	–	–		–
Dyeing[b]	102	14.3	9.9	0.033	4.58	3.85	Yes
Bleaching	30	13.4	8.0	0.137	2.22	3.86	No
Dyeing and/or bleaching	143	13.8	8.9	0.005	8.03	3.85	Yes
Hairspray	34	16.3	14.5	0.520	0.41	3.86	No
Gel	41	19.6	13.5	0.637	0.22	3.86	No
Wax	73	18.8	15.5	0.797	0.07	3.86	No
Others[c]	60	13.9	10.9	0.062	3.51	3.86	No
2 or more treatments	47	14.1	11.0	0.119	2.43	3.86	No

[a] Except for shampoo

[b] Other treatments are excluded

[c] Mostly hair mousse and serum

Table 6 Total counts of true positives, true negatives, false positives, false negatives, sensitivity and specificity for the proposed and determined cut-off values for assessing abstinence and excessive drinking

	Cut-off (pg/mg)	TP	FN	FP	TN	Sensitivity	Specificity
Proposed values	7	734	165	31	40	0.816	0.563
	30	3	2	94	871	0.600	0.903
Values determined in this study	7.3	721	178	29	42	0.802	0.592
	29.8	4	1	96	869	0.800	0.903

TP true positives, *FN* false negatives, *FP* false positives, *TN* true negatives

evaluated since of all participants only 11 did not report its use. Regarding cosmetic treatments, dyeing alone and dyeing and/or bleaching were associated with statistically lower EtG concentrations. This is in agreement with the results of other works [4–7] and supports the need to register any cosmetic treatment during sampling and consider it during EtG data analysis [5, 7, 11, 15–17]. Lower mean EtG concentrations were also observed for bleaching, other treatments (as hair mousse or hair serum) and two or more combined treatments; however, these differences were not significant. Even without significance, lower concentrations associated with bleaching have been reported by others [5–7, 18], and our results follow the same trend. The EtG concentrations did not appear to be influenced by the use of hairspray, gel or wax, supporting the findings of others [3].

Alcohol consumption patterns vary between different populations, since different consumption habits exist. To date, no studies were performed on a student population to assess alcohol consumption by means of hair analysis; therefore, we initially assumed the possibility that the currently proposed cut-off values might not completely adapt to our study population, due to possible differences between populations. Therefore, we intended to verify if the proposed cut-off values were adequate for our study population by determining the optimal cut-off values using the obtained results and compare by means of specificity and sensitivity. Comparing with the proposed cut-off values, a cut-off value of 7.3 pg/mg resulted in 222 abstinent or rare occasional drinkers (versus 207), raising specificity by almost 3 %, while slightly lowering sensitivity (1.4 %). These small variations strongly indicate the close agreement of the determined cut-off value with that proposed by the SoHT. Using the cut-off value of 29.8 pg/mg, the number of excessive consumption cases increased to 100 (versus 97), the specificity was not affected (difference of 0.1 %), but a severe increase in sensitivity of 20 % occurred. This increase should, however, not be considered significant due to the low number of self-reported excessive drinkers (n=5). Although the SoHT has well established the cut-off values for assessment of both abstinence or rare occasional drinking and excessive consumption, the results herein presented contribute to the knowledge that the proposed cut-off values are indeed

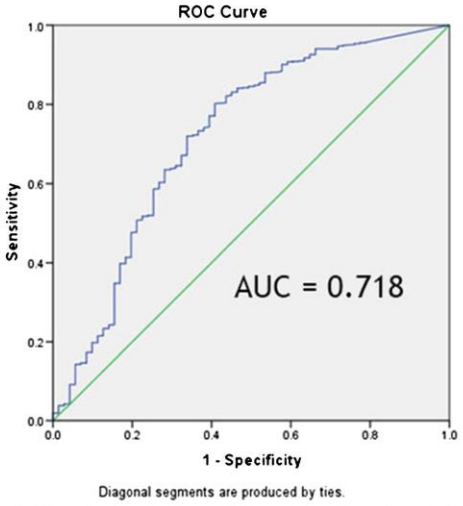

Fig. 2 Receiver operating characteristic plot obtained for abstinence. The area under the curve (*AUC*) is 0.718 (acceptable accuracy)

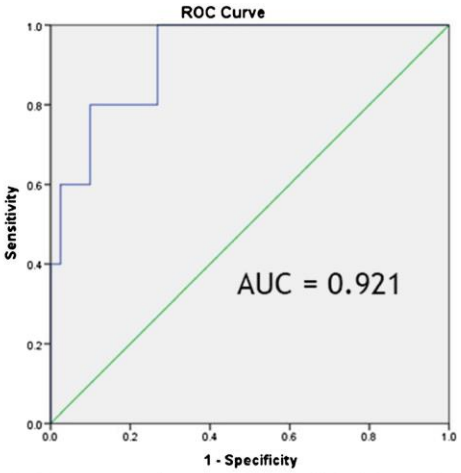

Fig. 3 Receiver operating characteristic plot obtained for excessive drinking. The area under the curve (*AUC*) is 0.921 (highly accurate)

adequate to be used on a study population as particular as university students.

The number of excessive consumption cases determined through EtG analysis was much higher than the number of self-reported excessive consumption (n=5); this allows us to conclude that participants may tend to underestimate their consumption, considering it as moderate or frequent, instead of excessive. This was further confirmed by evaluating the sensitivity and specificity of the answers provided in the questionnaire. As can be seen, at the cut-off of 30 pg/mg, a very low sensitivity was obtained (3.1 %) which clearly indicates that a great amount of participants (n=94) had in fact falsely reported their consumption as abstinent, moderate or frequent, whereas in fact the results suggest excessive drinking (false negative). As a consequence, the total number of participants reporting excessive consumption is very low, which is the most likely explanation of the great increase in sensitivity observed at the cut-off of excessive consumption. One should note, however, that the number of cases showing good agreement between self-report and hair concentrations of EtG (for excessive consumption) was too small, and further studies with a bigger number of such cases are needed. Interestingly, at the cut-off value of 7 pg/mg, a low specificity (19.5 %) was obtained for the answers obtained in the questionnaire. This indicates that a good number of participants classified their consumption as moderate, frequent or excessive, but the analytical results suggested abstinence or rare occasional drinking. This highlights the fact that a concentration below 7 pg/mg does not completely exclude alcohol use [25]; however, those individuals might have presented an abstinence or occasional drinking behaviour only in the timeline relative to the analysed segment length.

Apart from the situation observed at excessive consumption, participants are generally aware of their consumption habits, which can be seen by the close agreement between the determined and proposed cut-offs. This is also noticeable, since EtG concentrations correlate with the reported consumption habits and with the last time alcohol was consumed in excess; indeed, higher concentrations result when excessive consumption occurred more close to sampling. Similarly to other works [8], we obtained a correlation of the concentrations with the amount of alcohol intake. The use of tobacco, illicit drugs and substances sold in smart shops did not appear to be associated with increased EtG concentrations, based on the lack of correlation of these variables. Several individuals did report the use of these substances, but their use is independent from alcohol consumption. This is most probably due to the fact that the analysed population is in its majority composed of moderate/social drinkers, with occasional excessive consumption during certain academic events, and because the use of those substances is uniformly distributed, with individuals reporting their consumption in the abstinence, moderate and excessive drinking groups.

Our study presents some limitations; the time of sample collection could not consider the different academic schedules, which means that collection at some universities coincided closer to academic events where higher doses of alcohol are consumed, while at other universities, the opposite may have occurred. In any case, to try to minimize this factor, the total proximal 6 cm was analysed, accounting for a wider time frame. It would be interesting for this effect to conduct a study where hair samples are collected more often from the same individuals during a certain time period. Another limitation is inherent to the process of cut-off value determination, which is dependent on the answers provided by the participants. Although for most of the samples EtG concentration matched the self-reported answers, some of these may not correspond to the exact consumption habit; as mentioned before, participants tend to underestimate their excessive consumption. Nevertheless, similar cut-off values to the ones proposed by the SoHT were obtained.

Conclusion

The majority of the studied individuals were classified as social drinkers based on both the EtG concentration results and self-reported consumption in the questionnaires. Abstinence or rare occasional drinking and excessive consumption occurred at a lesser extent. The mean of EtG concentrations was significantly lower for females than males. The use of hair conditioner, mask and dyeing products was associated with significant lower EtG concentration, confirming the finding of other authors that the use of cosmetic treatments should be registered and considered during sample analysis. Classification of the results as abstinence or rare occasional drinking, moderate or excessive drinking was performed according to cut-off values proposed by the SoHT. For the situation of the studied population, the optimal cut-off values were determined based on the results and found to be 7.3 and 29.8 pg/mg for abstinence or rare occasional drinking and excessive drinking, respectively. These values are similar to the ones suggested by the SoHT (7 and 30 pg/mg, respectively), reinforcing the adequacy of the proposed values to our study population. EtG analysis in hair proved to be an efficient and non-invasive tool for monitoring alcohol consumption, correlating with the self-reported volume of alcohol and consumption habits. It would be interesting as future work to analyse the studied samples for other substances, for example cannabis, which seems to have a significant incidence on the studied individuals.

Acknowledgments The authors acknowledge the Fundação Calouste Gulbenkian for the financing of this project (Investigação e Saúde—reference: 125895), the programme COMPETE and the Fundação para a Ciência e Tecnologia (PEst-OE/SAU/UI0709/2014). D. Oppolzer

acknowledges the Programa Operacional Regional do Centro 2007–2013 QREN (Programa "Mais Centro") in the form of a fellowship (CENTRO-07-ST24-FEDER-002014, reference TPCR-2-004).

Compliance with ethical standards All the participants were informed about the objectives of the study and have provided a signed "Informed consent" form, which was approved by the ethical committee of Universidade da Beira Interior.

Conflict of interest The authors declare that they have no competing interests.

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Submitted to the Journal Addiction, 2016

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Abstract

The alcohol consumption in a university student population was assessed through combined hair sample analysis of the alcohol biomarkers ethyl glucuronide (EtG) and fatty acid ethyl esters (FAEEs). A total of 975 hair samples were analysed for EtG using liquid chromatography coupled to mass spectrometry (LC-MS/MS) and for FAEEs using gas chromatography coupled to mass spectrometry (GC-MS/MS). The results were analysed using the cut-offs proposed by the Society of Hair Testing. Receiver operating characteristic (ROC) curve analysis was performed to verify the adequacy of the proposed values for the study population. Good sensitivity and specificity were obtained for both biomarkers, especially for EtG, and a correlation was found with the self-reported alcohol consumption habit. In around 60-70 % of the cases, self-reported alcohol consumption could be confirmed by combined alcohol biomarker analysis. Combined analysis of EtG and FAEEs in hair samples proved to be an important tool for the monitoring of alcohol consumption in a student population. For a feasible result interpretation, it is very important to document the use of hair products, cosmetic treatments and washing frequency, and for these to be considered during interpretation. Overall the participants were aware of their

consumption pattern, however for doubtful cases and to account for academic calendars, repeated analysis of samples collected in different time frames would be advisable.

Keywords: Student population; Alcohol consumption; Hair; Ethyl glucuronide; Fatty acid ethyl esters

Introduction

Hair analysis for alcohol biomarkers is an effective tool for forensic and clinical purposes. By collecting, appropriate hair segments, alcohol consumption can be traced back up to 6 month before sampling. Hair analysis of the direct alcohol biomarkers ethyl glucuronide (EtG) and the four fatty acid ethyl esters (FAEEs), ethyl myristate, palmitate, oleate and stereate, is an effective approach to determine alcohol exposure and has been widely applied in several studies [1–8]. In 2014 the Society of Hair Testing (SoHT) published cut-off values for EtG and FAEEs for distinguishing abstinent, moderate and excessive drinking [9]. Since FAEEs concentrations tend to increase with the hair segment length [1], different cut-off values are proposed at 0 – 3 cm and 0 – 6 cm proximal segments; a sum of the four FAEEs of 0.2 ng/mg in 0 – 3 cm segments, or 0.4 ng/mg in 0 – 6 cm segments is used to discriminate abstinent from moderate drinking and concentrations above 0.5 ng/mg (0 – 3 cm) or 1.0 ng/mg (0 – 6 cm) is strongly suggestive of excessive alcohol consumption [9]. For EtG the cut-off values for abstinence and excessive drinking are 7 pg/mg and 30 pg/mg, respectively and are valid for both 0 – 3 cm and 0 – 6 cm segment length. Result interpretation of these values is however complex, and every case has to be analysed with caution. EtG is a polar compound, and therefore wash-out effects due to frequent hair washing can occur, originating false-negative results [4, 7]. Additionally the use of cosmetic treatments may also lower EtG concentrations [5, 10–13], while the use of alcohol containing hair products may increase FAEEs concentrations leading to false-positive results [1, 14]. It is therefore evident that determining cut-off values that exclude false-positive and false-negative results is a challenging task. One way to minimize these situations is by the combined measurement and of EtG and FAEEs, which has been recommended to improve discrimination between abstinence, moderate and excessive drinking [3, 5, 7, 8]. The combined analysis of both markers was performed in several studies [3–5, 7,

8, 12, 15], however no studies have so far been made on the student population where alcohol consumption is very common [16, 17]. We have previously reported results from a study on a university student population based on the analysis of EtG [17]. In this paper we intend to perform a more complete study with added reliability based on the combined analysis of EtG and FAEEs of the same samples, for assessment of alcohol consumption habits and consumption incidence. For this purpose, validated methods for the analysis of EtG by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) and FAEEs by gas chromatography coupled to tandem mass spectrometry (GC-MS/MS) were used. Additionally, we intended to confirm the proposed cut-off values for our study and evaluate the congruence of the participants self-reported alcohol consumption with the measured alcohol biomarkers results.

Experimental

Reagents and standards

Methanol, acetonitrile, water and isopropanol (MS-grade) from Fisher were acquired from Enzymatic (Santo Antão do Tojal, Portugal). Methanol and heptane (HPLC-grade) were acquired from VWR Internacional (Carnaxide, Portugal). Formic acid (MS-grade) was acquired from Sigma-Aldrich (Sintra, Portugal). Acetone, dichloromethane and ammonium hydroxide from Fisher were purchased from Enzymatic (Santo Antão do Tojal, Portugal). Oasis MAX 3cc SPE cartridges were acquired from Waters (Lisboa, Portugal), Phenomenex Strata NH_2 (100 mg/1mL) SPE cartridges were purchased from Tecnocroma (Caldas da Rainha, Portugal). Ethyl glucuronide, its deuterated analogue (EtG-d5, internal standard - IS) at 100 µg/mL in methanol were purchased from LCG Promochem (Barcelona, Spain). The FAEEs ethyl myristate (E 14:0), ethyl palmitate (E 16:0), ethyl oleate (E 18:1) and ethyl stearate (E 18:0) and respective penta-deuterated analogues (IS) were purchased from LCG Promochem (Barcelona, Spain). All stock and working solutions of EtG were prepared in methanol, solutions of FAEEs were prepared in heptane, and stored in amber glass vials, below -20 ºC.

Biological specimens

Calibration curves were prepared using a homogenized pool of human hair free of alcohol biomarkers (EtG and FAEEs) obtained from children aged 3-6 years. Hair samples were collected at 9 Universities of Portugal: Universidade do Algarve (UALG), Universidade de Aveiro (UA), Universidade da Beira Interior (UBI), Universidade de Coimbra (UC), Universidade de Évora (UE), Universidade de Lisboa (UL), Universidade do Minho (UM), Universidade do Porto (UP) and Universidade de Trás-os-Montes e Alto Douro (UTAD). A self-completion questionnaire was provided to each participant, which included information on genre, alcohol consumption habits, amounts of alcohol consumed per occasion, hair washing products and the use of cosmetic treatments, as dyeing or bleaching, amongst others. From each participant a hair sample was collected, cut as close as possible to the scalp and stored in paper envelopes until analysis. Hair samples were washed with water, methanol, acetone and dichloromethane, left to dry at room temperature and then cut with scissors to fragments smaller than 1 mm. Approximately 30 mg of cut hair was weighted for each analysis.

Analysis of EtG and FAEEs

Two methods, previously developed and validated in our laboratory, were used for EtG and FAEEs analysis. The methods were based on LC-MS/MS for EtG and GC-MS/MS for the four FAEEs species (ethyl myristate, ethyl palmitate, ethyl oleate and ethyl stearate). The detailed methods description and validation have already been described in detail [18].

Statistical analysis

Statistical analysis was performed using the IBM® SPSS® Statistics software (version 22) and Microsoft® Excel 2013 (version 15). Analysis of variance (ANOVA) was performed on Excel, with significance attributed to *p*-values less or equal to 0.05. Receiver operating characteristic (ROC) analysis and its respective plots, normality tests and correlation studies were performed on SPSS.

Results and discussion

Hair samples

From a total of 1192 collected hair samples, 217 were excluded due to insufficient sample amount for both analyses (at least 2 x 30 mg). From the remaining 975, the proximal 6 cm were analysed, shorter samples were completely processed and the segment length recorded. For EtG analysis results were interpreted independently of the segment length, however for the FAEEs analysis samples were grouped in 0-3 cm and 0-6 cm proximal segments, due to the different cut-off values proposed for each hair shaft length [9]. A total of 66 samples had segment lengths within 0-3 cm and 909 samples had lengths within 0-6 cm. Ethyl palmitate and oleate were the most occurring FAEE species, which is in agreement with the reports of several authors [2, 6, 19]. For result interpretation, the four FAEEs were considered as a sum of concentrations ($\sum FAEEs$). A total of 54 samples had EtG concentrations below the LLOQ (3 pg/mg) and 46 samples had all of the four FAEEs below the LLOQ (0.03 ng/mg).

EtG and FAEEs results based on sex and region

The concentration of EtG and $\sum FAEEs$ were compared for the male and female populations in terms of mean and median. Additionally, as shown in Table 1, ANOVA analysis was performed for both markers in both sexes, with statistical significance attributed to a *p*-value < 0.05. Results showed statistical significant differences based on sex for both EtG and FAEEs. For EtG significantly higher concentrations were obtained for males (median of 16.91 pg/mg, range 3.09 – 147.39 pg/mg) when compared to females (median of 11.88 pg/mg, range 3.15 pg/mg - 153.41 pg/mg). For the FAEEs a similar statistical difference was observed, a median of 0.203 ng/mg was obtained for males (range 0.031 – 3.045 ng/mg) compared to 0.172 ng/mg for females (range 0.030 – 3.170). The significant higher concentrations of EtG in males are in agreement with the results of other studies [5, 12]. Crunelle and co-workers have shown that in alcohol-dependent patients, the correlation between EtG concentrations and amount of consumed alcohol is not influenced by genre [20]. In our study, the higher concentrations found

in males could be mostly explained by the fact that statistically males ingest higher quantities of alcohol when compared to females [21]. However, for FAEEs the sex-dependent incorporation in hair might also be a possible explanation for the differences between males and females observed for this biomarker [1].

Table 1. Alcohol biomarkers concentration comparison between sexes.

Biomarker	Sex	*N*	Mean	Median	ANOVA			
					p-value	*F*	*F-critical*	Statistical difference
EtG	**Female**	639	15.33 pg/mg	11.88 pg/mg	0.000	34.40	10.90	Yes
	Male	276	21.93 pg/mg	16.91 pg/mg				
FAEEs	**Female**	654	0.281 ng/mg	0.172 ng/mg	0.026	4.99	3.85	Yes
	Male	269	0.339 ng/mg	0.203 ng/mg				

The mean and median concentrations of both markers were distributed according to the universities where samples were collected (Figure 1). EtG mean and median concentrations were higher in UBI, UA and UC, and the lowest obtained in UALG. For the FAEEs the highest mean and median values were obtained for UBI, UC, UP and UTAD, while the 5 remaining universities showed only small variations. Overall, both biomarkers seem to be in agreement in the incidence of the highest obtained concentrations.

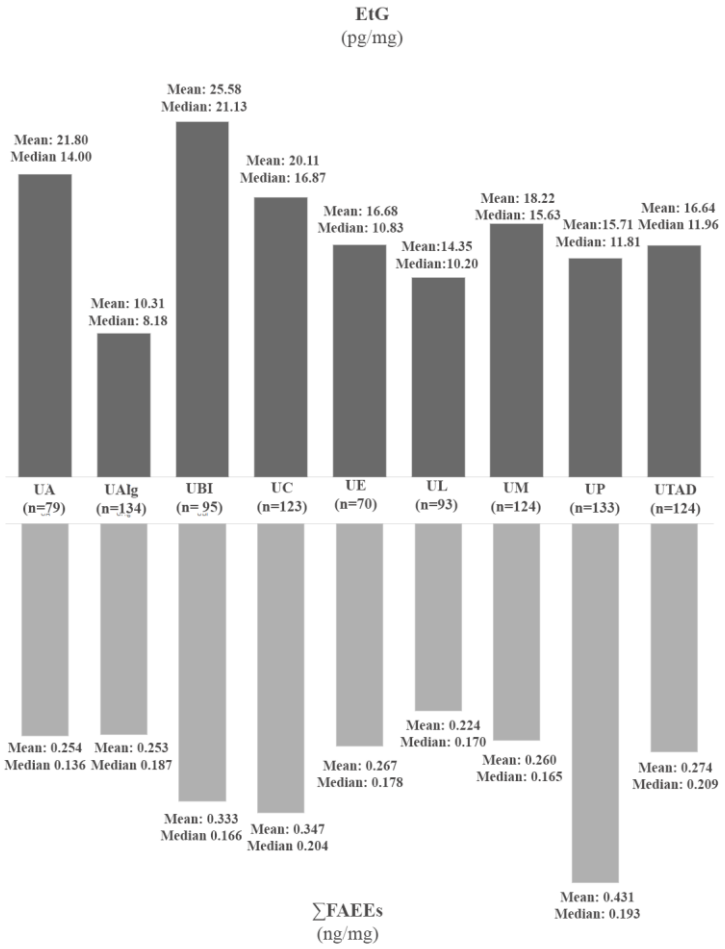

Figure. 1. Mean and median concentrations of EtG and FAEEs distributed according to University.

Specificity and sensitivity using the proposed cut-off values

Samples were grouped as belonging to abstinent, moderate or excessive drinkers according to the cut-off values suggested in the guidelines of the SoHT. For EtG, a concentration of 7 pg/mg was used to distinguish abstinence from moderate drinking. For the FAEEs, a sum of concentrations of 0.2 ng/mg in a 0-3 cm proximal segment or 0.4 ng/mg in a 0-6 cm proximal segment was used. For distinguishing moderate from excessive drinking a cut-off concentration of 30 pg/mg, and a sum of concentrations of 0.5 ng/mg in the 0-3 proximal segment or 1 ng/mg in a 0-6 cm proximal segment was used for EtG and FAEEs, respectively [9]. Based on the self-reported consumption habits obtained from the questionnaires, the total number of true positives (TP), true negatives (TN), false positives (FP) and false negatives (FN) were counted, and the specificity and sensitivity were calculated ($specificity = \frac{TN}{TN+FP}$, $sensitivity = \frac{TP}{TP+FN}$) at

the proposed cut-off values, this information is presented in Table 2 (the data for EtG was already reported in a previous work [17] but were included in the table for comparison). Overall the sensitivity varied from 60.0% to 81.6%, except at the FAEEs cut-off value of moderate drinking (0.4 ng/mg) for 0-6 cm segments were the sensitivity was very low (30.5%). Therefore, in order to attempt to improve the obtained sensitivities and specificities and overall verify the adequacy of the proposed cut-off values, the optimal cut-off values for the obtained results were determined.

Table 2. Sensitivity and specificity of EtG and FAEEs at the proposed cut-off values.

Segment length	EtG			FAEEs		
	Cut-off (pg/mg)	Sensitivity	Specificity	Cut-off (ng/mg)	Sensitivity	Specificity
Abstinence						
0-3 cm	7	**0.816**	**0.563**	0.2	**0.655**	**0.875**
0-6 cm				0.4	**0.305**	**1.000**
Excessive						
0-3 cm	30	**0.600**	**0.903**	0.5	**1.000**	**0.828**
0-6 cm				1.0	**0.666**	**0.970**

Cut-off value estimation

Optimal cut-off values for the studied population were determined by tracing receiver operating characteristic (ROC) plots. For this, the concentrations of EtG and $\sum FAEEs$ were set as test variable and the answer provided by the participants concerning their consumption habit (abstinent, moderate or excessive) was set as state variable. Curves were individually traced for each cut-off value for both biomarkers, in the case of the FAEEs cut-off values were estimated both on the 0-3 cm proximal segments and on the 0-6 cm proximal segment. The coordinates of the ROC curve were used to find the best relation between specificity and sensitivity; for this the Youden's index (*J*) was calculated as: $J = specificity + sensitivity - 1$, and the concentration with the highest *J* value selected as optimal cut-off [22].

For EtG, the optimal cut-off values were already reported in a previous work, and found to be 7.30 pg/mg and 29.85 pg/mg for identifying abstinence and excessive drinking, respectively [17]. These values, were situated very close to the ones proposed by the SoHT at 7 pg/mg and 30 pg/mg, and therefore we found the proposed values to be adequate for our study population.

For the FAEEs, the optimal cut-off values were determined as 0.185 and 0.817 ng/mg (in 0-3 cm proximal segments), and 0.378 ng/mg and 0.889 ng/mg (in 0-6 cm proximal segments), for identifying abstinence and excessive drinking, respectively. As an example, the obtained ROC plots ($\sum FAEEs$) for abstinence and excessive drinking at 0-3 cm and 0-6 cm proximal segments are presented in Figures 2 and 3, respectively.

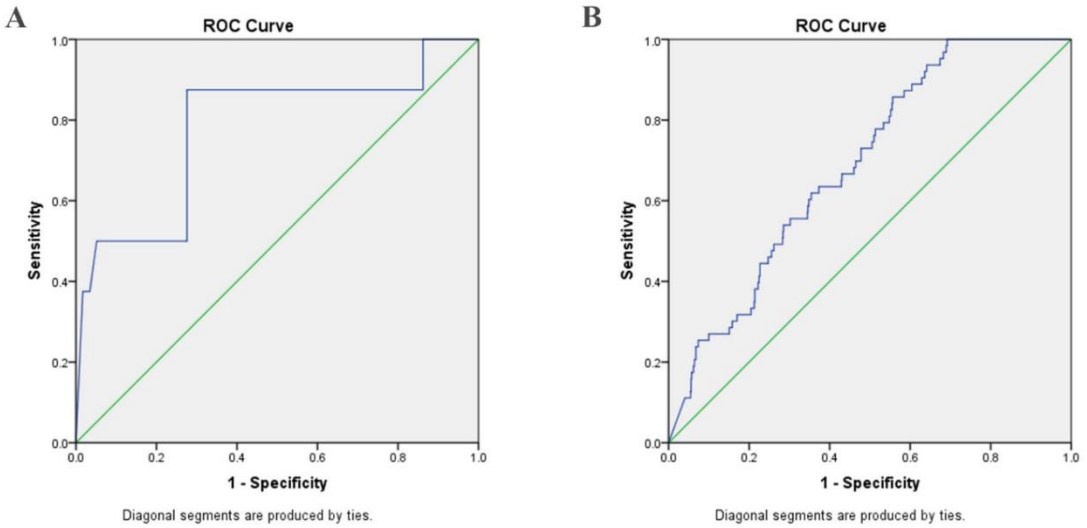

Figure 2. FAEEs receiver operating characteristic plots for optimal cut-off value estimation for abstinence at 0 – 3 cm (A) and 0 – 6 cm (B) proximal segments.

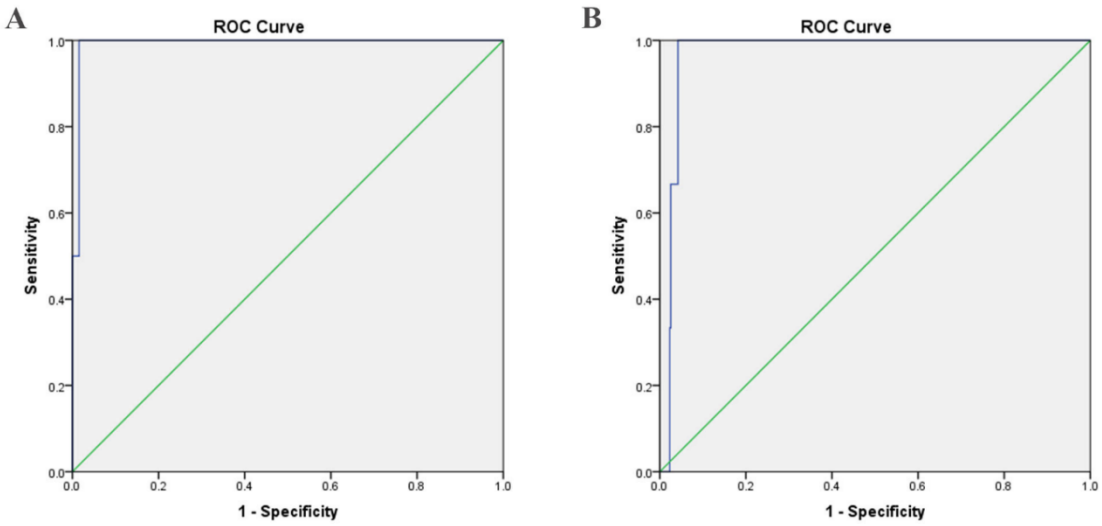

Figure 3. FAEEs receiver operating characteristic plots for optimal cut-off value estimation for excessive drinking at 0 – 3 cm (A) and 0 – 6 cm (B) proximal segments.

The area under the curve, highest calculated Youden's index value (*J*), respective cut-off concentration, selectivity and specificity for FAEEs are presented in Table 3. Regarding the cut-off for distinguishing abstinence from moderate drinking, using 0.185 ng/mg compared to 0.2 ng/mg in 0-3 cm proximal segments increased the sensitivity by 6.9% (from 66.5% to 72.4%) maintaining the specificity at 87.5%. Considering the sample size in the 0-3 cm segment group (n=66), we did not consider this increase of sensitivity significant and considered the proposed cut-off value (0.2 ng/mg) adequate for our study. For 0-6 cm proximal segments, the number of false negatives was only slightly lowered (from 584 to 582) by using 0.378 ng/mg compared to 0.4 ng/mg. Consequently, no significant improvement on the sensitivity was obtained. This, in addition to the closeness of both cut-offs, demonstrates the adequacy of the proposed value.

Table 3. Receiver operating characteristic analysis for the optimal cut-off values of FAEEs based on the studied population.

Consumption	Concentration (ng/mg)	Sensitivity	Specificity	*J* value maximum	AUC[1]	AUC accuracy
Abstinence						
0-3 cm	0.185	0.724	0.875	0.599	0.780	Good
0-6 cm	0.378	0.308	1.000	0.308	0.690	Acceptable
Excessive						
0-3 cm	0.817	1.000	0.984	0.984	0.992	High
0-6 cm	0.889	1.000	0.958	0.958	0.970	High

[1]**AUC:** Area under the curve

For the cut-off value for identifying excessive drinking, 0.817 and 0.889 ng/mg were respectively determined for 0-3 cm and 0-6 cm proximal segments. Using 0.817 ng/mg, the number of false positives decreased from 11 to 1, raising the specificity from 82.8% to 98.4%. For 0.889 ng/mg a great increase in sensitivity occurs (of 33.3%). One must consider however, that the number of participants classifying their consumption as excessive was too low (n=5) to an appropriate estimation of the cut-off values for excessive consumption. Moreover, from those 5 samples, 2 were situated within 0-3 cm segments and 3 within 0-6 cm segments. On 0-3 cm segments, both excessive samples were identified using the proposed and determined cut-off values therefore sensitivity is not affected, the optimal cut-off value was only estimated based on a better specificity. This is not the case at 0-6 cm segments, where 2 of the 3 self-

reported excessive samples were identified using the proposed cut-off value and all 3 were identified using the estimated value. This is the reason why such a great difference in sensitivity is observed. Additionally, the sample in question showed an EtG concentration slightly below the cut-off for excessive drinking. For this reasons and based on the differences of sensitivity and specificity we considered the cut-off values proposed by the SoHT, and were used for further result interpretation.

Interestingly both estimated cut-off values for excessive drinking are situated within 0.8-0.9 ng/mg, supporting a recent report of a possible FAEEs cut-off harmonization for both hair segments (0-3 cm and 0-6 cm) at about 0.3 ng/mg (abstinence) and about 0.8 ng/mg (excessive drinking) [15]. However, as stated above, the number of self-reported excessive drinkers is too low to conclude the use of those values. Additionally, we performed ROC curve analysis combining both 0-3 cm and 0-6 cm segments in order to verify if the value of 0.3 ng/mg (to distinguish abstinence from moderate drinking) mentioned above would adapt, this was not the case since two candidate optimal cut-off values were found, and these were close to the values determined for both segments in separate (data not shown).

Results interpretation based on combined EtG and FAEEs analysis

In Table 4 the total amount of samples is grouped based on the combined results of EtG and FAEEs analysis. For comparison, samples were also grouped according to the determined cut-off values. A total of 185 abstinent and 15 excessive drinkers were identified using the proposed cut-offs for EtG and FAEEs. Abstinence was excluded in 274 cases, and excessive drinking was excluded in 851 cases. However 49 cases occurred where a negative EtG concentration and a positive $\sum FAEEs$ was obtained. Possible explanations for this situation can be the decrease of EtG concentrations due to its hydrophilic nature by frequent hair washing [4, 7] or the use of cosmetic treatments [5, 10, 11, 13]. Also, FAEEs concentrations may increase with the use of alcohol-containing hair products [1, 14]. This may explain the inconclusive cases with negative EtG and positive FAEEs concentrations; in any case, repeated sampling and analysis of those

cases for both markers would be advisable. It has to be noted that several studies have proven that grinding procedures usually result in higher recoveries of EtG [23–25]; however, this approach was not used in our study. The main reason for this was due to the fact that both the washing and hair cutting procedures were simultaneously performed for EtG and FAEEs analysis, and it has been proven that grinding may result in FAEEs loss, which makes it unsuitable for their analysis [26]. Nevertheless, and according to SoHT's recommendations, we have randomly selected 20 samples, which were pulverized in an independent laboratory and the EtG concentrations were compared to those obtained when the same samples were not grinded. In all the tested samples EtG concentrations were identical (variation of less than ± 15% between both procedures, data not shown).

Table 4. Combined EtG and FAEEs result interpretation, grouped according to both proposed and determined cut-off values.

Cutoff		EtG positive FAEEs positive	EtG negative FAEEs negative	EtG negative FAEEs positive	EtG positive FAEEs negative
EtG (pg/mg)	FAEEs[1] (ng/mg)				
Proposed values					
7	0.2 (0.4)	274	185	22	494
30	0.5 (1.0)	15	851	27	82
Determined values					
7.30	0.185 (0.378)	274	184	28	479
29.85	0.817 (0.889)	13	744	31	87

[1] according to the sample segment length either within 0 – 3 cm or 0 – 6 cm

The situation is much more problematic regarding the high number of samples (494) where a positive EtG concentration (≥ 7pg/mg), combined with a negative $\sum FAEEs$ (< 0.2 ng/mg and < 0.4 ng/mg) was obtained. The percentage of these inconclusive cases is much higher than in studies performed by other authors, which have found this situation only occurring in less than 10% of the samples [5, 7, 8, 15]. There is no simple explanation for this situation, an EtG concentration ≥ 7 pg/mg strongly suggests repeated alcohol consumption and, as seen in other papers [5, 7, 8, 15], situations of negative EtG and positive FAEEs concentration are more likely to occur, by the reasons explained above. Decreased FAEEs concentrations could be caused by aggressive cosmetic treatment [19], and frequent use of lipophilic or alkaline hair treatment [8], but EtG is unlikely to increase due to cosmetics use [27]. Only in 167 of those cases cosmetic

treatment was reported, therefore this fact cannot explain the majority of the results. There is no general agreement in other published papers concerning a possible explanation for these cases, and the high number of cases in our study suggests that other variables are affecting FAEEs concentrations. It also has to be assumed the possibility that these cases occurring at the cut-off for abstinence are individuals with an occasional social drinking habit, since it is frequent for social drinkers to have FAEEs concentrations in the range of those obtained in abstainers [26]. For a combined interpretation of EtG and FAEEs the repeated analysis of the samples for both markers would be advisable, especially in the cases with large deviations. It is however accepted by several authors, and included in the consensus of the SoHT that EtG should be the first choice in abstinence assessment and that a positive FAEEs result confirms a positive EtG result, but a negative FAEEs result cannot overrule an EtG result ≥ 7 pg/mg [3, 5, 7, 9]. Indeed, in 461 of those cases, the participants considered their consumption as moderate which would be confirmed based on EtG analysis. Based on these supporting facts these cases would most probably be considered as moderate drinkers.

Based on the combine analysis of EtG and FAEEs, of the 975 participants, 675 presented moderate drinking (69%), 185 abstinent (19%) and 115 (12%) excessive drinking behaviours.

Self-reported intake compared to combined biomarker analysis

To assess generally how well aware participants are of their consumption pattern, mean and median concentrations of EtG and $\sum FAEEs$ were calculated for self-reported abstinence, moderate and excessive drinking (Figure 4). Additionally, correlation studies were performed between the concentrations of both biomarkers and the self-reported alcohol consumption habit and quantity of ingested alcohol. Significant correlations with the consumption habit were found for EtG (Spearman rho (r) = 0.217, $p < 0.01$) and FAEEs (Spearman rho (r) = 0.137, $p < 0.01$). Regarding the ingested quantities of alcohol, only EtG had a significant correlation (Spearman rho (r) = 0.105, $p < 0.01$), which is in agreement with the observations of others [3, 6, 7, 28–30], the sex and age dependence of FAEEs production [1], biological variability and use of hair treatments [6] might explain the lack of correlation for FAEEs. Overall, these results

show that part of the participants is aware of their consumption habit, and consumed quantities.

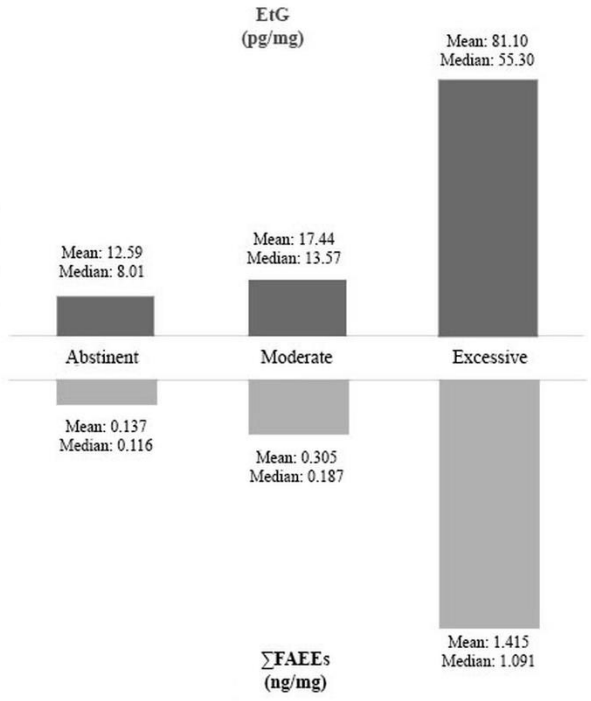

Figure. 4. Mean and median concentrations of EtG and FAEEs according to the self-reported alcohol consumption habit.

Observing the obtained Spearman rank correlation coefficients (rho) we can conclude, however, that the number of disagreeing cases between self-reported consumption and biomarker analysis result is significant. Therefore, the congruence of the self-reported consumption habit with the combined interpretation of both biomarkers was evaluated. This was performed by verifying the number of self-reported abstinent, moderate an excessive drinking cases with the combined interpretation of EtG and FAEEs according to the proposed cut-off values, which are shown in Table 5. In cases with non-agreeing results of EtG and FAEEs, cosmetic treatment and hair products were considered and a suggestive interpretation was performed in a similar manner as performed by other authors [3, 8]. By observing Table 5, it is noticeable that in only around 60% to 70% of the samples cases, the self-reported consumption was confirmed by alcohol biomarker analysis. In the self-reported abstinent and moderate groups, 43.7% and

12.4%, respectively, of those cases the consumption was underestimated by the participants. Interestingly, in 16% of the self-reported moderate drinking groups, abstinence was proven by both biomarkers. One possible explanation for those cases is that the individuals were abstinent towards alcohol consumption only at the time frame relative to the analysed hair segments. Consumption of alcohol among students may be more incident during some occasions, especially in certain academic events, therefore it would be relevant in these cases to perform repeated biomarker analysis on hair segments collected in different time periods to account for this fact.

Table 5. Congruence of the self-reported consumption habit with the combined result interpretation of both biomarkers.

Self-reported consumption	Combined biomarker result interpretation	Cases (*N*)	Percentage of confirmable cases
Abstinence (n = 71)	Confirmation of abstinence	40	56.3%
	Suggestive of moderate drinking	30	
	Excessive drinking	1	
Moderate drinking (n = 894)	Confirmation of moderate drinking	206	71.6%
	Suggestive of moderate drinking	434	
	Abstinence	143	
	Suggestive of excessive or strong moderate drinking	100	
	Excessive drinking	11	
Excessive drinking (n = 5)	Confirmation of excessive drinking	3	60.0%
	Moderate drinking	1	
	Suggestive of moderate drinking	1	

Conclusions

The combined analysis and interpretation of EtG and FAEEs was applied for the first time in a university student population. Alcohol biomarker concentrations were found to be significantly higher in the male population, and both markers were in agreement regarding the incidence of the highest concentrations by university. Both markers showed good sensitivity and specificity according to the cut-off values proposed by the SoHT and self-reported consumption, with exception of the cut-off for abstinence, where a lower specificity was obtained for the FAEEs. Optimal cut-off values were determined and found close to the ones suggested, except at the cut-off for excessive consumption for the FAEEs, most likely due to the low number of self-reported excessive drinkers. With the results of this study, the complexity of hair EtG and FAEEs

analysis and result interpretation is noticeable. A complete documentation of hair products, cosmetic treatments and washing frequency during sampling is of great importance and its consideration during result interpretation can be crucial for the efficient proving of abstinent, moderate or excessive drinking.

Overall, participants were well aware of their consumption, which was shown by the existence of correlations between both markers and the reported alcohol consumption behaviour. A correlation with the reported quantities of ingested alcohol was obtained only for EtG; the lack of correlation for the FAEEs is likely due to sex and age dependence, biological variability or the use of cosmetic treatments. About 60 – 70% of the analysed cases showed congruence between the self-reported consumption and hair biomarker analysis, while in the remaining cases consumption was mostly underestimated. Both, self-reported information and alcohol biomarker analysis show that in the analysed population, moderate drinking is most incident (69%), followed by abstinence (19%) and excessive drinking (12%) as least incident. The combined analysis of EtG and FAEEs in hair proved to be an effective, non-evasive tool for alcohol consumption monitoring. It would be relevant, in future studies, to perform repeated analysis in different time frames to account for different academic schedules between universities, since these may be associated to periods of heavier drinking.

Acknowledgements

The authors acknowledge the Fundação Calouste Gulbenkian for the financing of this project (Investigação e Saúde - reference: 125895), the program COMPETE and the Fundação para a Ciência e Tecnologia (PEst-OE/SAU/UI0709/2014). D. Oppolzer acknowledges the Programa Operacional Regional do Centro 2007-2013 QREN (Programa "Mais Centro") in the form of a fellowship (CENTRO-07-ST24-FEDER-002014, reference TPCR-2-004).

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Chapter III – General Discussion

In this project, alcohol consumption was assessed on a Portuguese university student population and two main approaches were used: gathering of information about alcohol consumption through a questionnaire, and assessment of the alcohol consumption through alcohol biomarker analysis in hair samples. It is a well-known fact that the efficiency of self-report survey analysis for substance consumption, on the general population, is highly affected by individual opinion, interpretation, and whether or not the examinees feel comfortable with the questions. Consequently, consumption underestimation and/or overestimation frequently occurs, not to mention that being a human product these approaches are susceptible to several errors. This is the main reason why in this project, besides using information concerning alcohol consumption collected through questionnaires, additional and complementary information was sought through analytical means. Hair analysis has proven to be very valuable for the analysis of several substances, with wide windows of detection and low chances of adulteration. By using this tool, it is therefore expected that gathered alcohol consumption information can be confirmed and falsely-reported cases identified by combining both information, adding extra reliability.

1192 individuals participated in this study. The analysed population was composed of students attending an academic course with a minimum age of 18 (mean participation age was 21.5 years). Participation was limited to individuals that agreed to provide a hair sample weighting at least 100 mg, the necessary amount to guarantee enough sample to be available for both analytical procedures (EtG and FAEEs analysis). One limitation that can be noted in this study is the fact that in 5 universities the population was mainly composed by females, with a low participation from males. This can actually be associated with the requirement of providing a hair sample for participation, since this will mostly be easier for female individuals. However, in Portugal, a higher number of female individuals are enrolled in academic courses [36].

Each participant in the study self-completed a questionnaire that included questions relative to the age at which alcohol consumption started, how frequent the consumption was, last time alcohol was consumed in excess, drinking alone or in group, places of consumption, preferred drink and ingested quantities per drinking occasion. Additionally, the consumption of other substances, as tobacco and/or illicit drugs, was assessed. The ESPAD survey on *Substance Use Among Students in 36 European Countries* was performed in 2011, to monitor trends within, and between, countries and contains data about alcohol consumption from Portugal and other European countries [35]. The ESPAD study represents therefore a global overview of alcohol consumption amongst students in Europe and Portugal and, for this reason, results obtained from the questionnaire data of this project were mostly compared to those of the mentioned study. Results from this project are useful to observe major differences in consumption habits among students, between the years 2011 and 2014; however, it must be taken into account that our study is based on an older population (mean of 21.5 years) when compared to the ESPAD survey (15/16 years). Nevertheless, other studies performed on Portuguese student

populations were also used to compare our results; namely the ECTAD 2011 (Estudo sobre o Consumo de Alcoól Tabaco e Drogas), an extended version of the ESPAD, containing data on students age 13-18 [37]; INME 2011 (Inquérito Nacional em Meio Escolar), containing data on students from the Portuguese 3rd school cycle (7th-9th grade) [38] and high school (10th-12th grade) [39]; and the Heath Behaviour in School-Aged Children (HBSC) 2014, containing data on students from the Portuguese 6th, 8th and 10th grade [40].

Alcohol consumption started mostly between the ages of 12 and 18, with the majority of students starting at the age of 15, superior to the age of 13 reported in the ESPAD survey of 2011 [35]. The majority of the students admitted to have a moderate or frequent drinking behaviour, with only 7.38% reporting abstinence. Concerning the answers given in the questionnaires, the number of excessive drinkers was incredibly low (0.5% of the total enquiries), which represents a limitation to this study since one of the objectives was to identify excessive drinkers, combining the results of both the questionnaires and hair analysis. Underestimation of excessive alcohol consumption was assumed as the main explanation for this observation. Nevertheless, the existence of such cases is further verified based on the results of hair analysis.

29.36% of the participants admitted to have consumed alcohol excessively in the past 30 days. The 30-day period evaluation of excessive consumption is an indication of drunkenness and occasional intensive consumption. Besides this indication, excessive consumption was also evaluated as occurring within 1 to 6 months (13.93%) or for more than 6 months (14.43%) before sampling, or never (25.92%). This part of information present in the questionnaire is of important analytical value, since it is generally agreed that alcohol biomarker analysis in hair is performed in the proximal 0 - 6 cm segment [10,32]. Assuming the growth rate of hair at 0.35 mm per day, an approximate growth rate of 1 cm per month can be assumed. Therefore, it is relevant to consider if excessive consumption has occurred within the time frame, corresponding to the analysed hair segment length during result interpretation.

The wide majority of students (95.70%) preferred to drink alcoholic beverages accompanied, in group, and the most common place was in coffee bars (44.71%). Most consumed beverages were beer and spirits, or both, accounting for 70.39% of the population. This is in agreement with the results of the ESPAD survey (where both drinks accounted for 70% of the consumed drinks), as well as that beer is the most consumed drink in the total population, and spirits among individuals of the female gender [35], additionaly other studies confirm this tendency [37-40]. The results confirm that higher quantities of alcohol are ingested per occasion, by males (median of 82.5 mL in males and 60 mL in females). However, they also indicate that the ingested quantities by both males and females have increased since 2011. While one must consider that the student population of both studies are not exactly similar (differing on age and level of education), drinking can be classified as risky when 40 to 120 g (approximately 50 – 152 mL) of pure alcohol is ingested per day, and as excessive for quantities higher than 120 g per day [3,5,32,33]. Based on the obtained medians shown above, the population presents a

great incidence of risky and excessive drinking, and therefore further monitoring of alcohol consumption by the university student population would be advisable in the future, particularly to identify developing trends and patterns in excessive alcohol consumption.

Smoking was reported by 36.41% of the students, lower than the European average (54%) for students aged 15 – 16 of 2011 [35], Portuguese students age 13-18 [37], Portuguese 3rd cycle and high school students [38,39], but higher then the results of the 2014 HBSC study [40]. Other substances (illicit drugs of abuse) consumed by students were also assessed through self-report questionnaires. Drug consumption was more prevalent in our study (27%) when compared to the study population of 2011 (18%), and almost doubled for the male gender. Cannabis was the most consumed illicit drug amongst students, the reason can mostly be explained by its ease of availability [35], and this is clearly observable in our study. Drugs were majorly consumed at rare occasions (63.93%) and their use were mostly associated with drinking events (53.37%). This distribution clearly demonstrates that alcohol consumption is often associated with the use of other substances, including drugs of abuse.

The alcohol biomarkers EtG and FAEEs were analysed by two different analytical methods, EtG by LC-MS/MS and FAEEs by GC-MS/MS. Given the high polarity of EtG, liquid chromatography was used, which also presents the advantage that no derivatization procedure is required, oppositely to what would happen if gas chromatography was used for the analysis of this compound. Conversely, FAEEs are suitable for gas chromatography due to their non-polar nature and, although analysis is also possible through LC-MS/MS, GC was chosen to account for less systematic errors by using different analytical equipment, also facilitating the simultaneous operation of both analysis procedures.

Hair was decontaminated using a procedure which was suitable for both biomarkers; therefore, water, methanol, acetone and dichloromethane were used. This procedure was evaluated using authentic hair samples positive for these compounds, and no loss of EtG or FAEEs was observed comparing to a non-decontaminated hair sample. For the analytical determination of substances present in hair samples (incorporated within the solid hair matrix), their extraction needs to be performed prior to any sample clean-up procedure. The choice of solvents used for extraction is highly based on the polarity of the compounds and their affinity for hydrophilic or hydrophobic solvents. Therefore, water was used for EtG extraction due to its polar nature and affinity for aqueous solvents. On the other hand, FAEEs are non-polar compounds with affinity for non-polar solvents as hexane or heptane. Heptane was chosen, since during optimization higher extraction yields were obtained in authentic hair samples. For EtG, an ultra-sonication procedure was applied for 2h, in order to facilitate EtG extraction from the matrix; this choice was based on the available scientific literature, since it has been shown that with this procedure extraction is complete after 2h [42]. For FAEEs the extraction was performed by incubation during 15h at 45ºC, the conditions that yielded the highest extraction efficiency during optimization. It is important to mention that, although ethyl heptadecanoate was used in several scientific papers as internal standard, this compound is not suitable for hair analysis

since it is present in the sebum and hair after alcohol consumption [10]. Additionally, the use of deuterium-labelled internal standards (for both EtG and FAEEs) has the advantage that an IS can be attributed to each analyte, which can be unequivocally identified by mass spectrometry, accounting for less errors related to various interferences.

The developed and optimized methods were validated in order to verify and guarantee their reliability and repeatability, following international guidelines from the FDA [43] and ICH [44]. The studied parameters included sensitivity, linearity, limits, precision and accuracy, recovery and stability. The selected calibration intervals were based on the obtained LLOQs, and the highest concentration of these biomarkers found in literature for chronic excessive alcohol drinkers [5,23,34,45]. The selected linearity ranges were of 3 – 500 and 30 – 5000 pg/mg for EtG and each of the FAEEs, respectively. The sensitivity regards analysis of the lowest quantifiable concentrations with a signal-to-noise ratio of at least 5:1, in relation to the analysis of blank hair samples (N = 10). The lower limits of quantification were assured at 3 pg/mg and 30 pg/mg for EtG and each FAEE, respectively.

Precision describes the closeness of individual measures when repeated analysis of different aliquots of a sample is performed, and accuracy describes the closeness of the mean test result obtained by the method and the true concentration of the analyte in the biological specimen. Both parameters were assessed during the same sample run and also between runs. The assessed within- and between-run precision and accuracy were found acceptable according to the guidelines from the FDA and ICH, which establish that the coefficient of variation should not exceed 15%, except at the LLOQ where 20% is found acceptable, and the mean relative error should not exceed ± 15%, or ± 20% at the LLOQ, from the true concentration of the analyte. Additionally, precision and accuracy were evaluated using 5 control samples, prepared separately to the calibration curves. The control samples included blank hair samples spiked at concentrations close to the cut-off proposed for alcohol biomarker analysis in hair [32], in order to assure the reliability of the method at those critical concentrations. Three control samples were also prepared using three authentic hair sample pools positive for both biomarkers, obtained from frequent consumers. Three pools were prepared with significant different concentrations in order to cover the linearity range of each method. The use of authentic hair as control samples adds extra reliability for method validation; in our opinion this is mandatory because a spiked sample does not mimic adequately an authentic sample, because hair is a solid and non-homogenous specimen. In addition, this approach also takes into account the alcohol biomarker extraction step and assures that the method is reliable towards real hair samples.

Absolute recovery is an indication of the analyte loss during the sample preparation procedure and was evaluated using three levels of concentration for each compound, repeated in triplicate. At each concentration level, spiked samples were extracted and compared with blank sample extracts spiked only after SPE clean-up. In both cases, the IS was only added after

clean-up to allow for comparison of both sample sets. The recovery ranged from 74.79 - 82.48% for EtG, and 77.44 - 97.90% for each of the four FAEEs.

The stability of a substance in a biological sample depends on the sample storage conditions, chemical properties of the substance and matrix, and the container systems that the analyte enters into contact with. It is therefore important to assess the stability at conditions that are likely to be encountered during actual sample handling. These usually include freeze and thaw stability, short- and long-term stability and processed sample stability. When working with hair, freezing and thawing is not necessary for sample storage, and stability of compounds in the hair matrix is difficult to perform since the incorporation rate of substances in hair is not known and is not possible to reproduce adequately. For this reason, only the stability of processed samples was evaluated. This was performed by analyzing the control samples that were left at least 24h in the auto sampler and comparing to freshly prepared control samples in terms of mean relative error and coefficient of variation. Stability was evaluated in periods of 24h, up to 96h for EtG (Attachment 3); for FAEEs however, only 24h-stability was possible (Attachment 4), since the heptane present in the auto sampler vials evaporated mostly within 24 - 48h after injection. The longer period of stability evaluation for EtG was considered of great importance, since it guarantees that samples are stable when for example these are processed at the end of a week and only analyzed after the weekend. Overall the samples were found to be stable during at least 96h for EtG and 24h for FAEEs when left in the injector.

During authentic hair sample analysis, a calibration curve was prepared daily using the same calibrators that were used for validation. Following this procedure ensures that between day variability in detector response is accounted for, since sample data sets are quantified according to calibration curve prepared and analyzed during the same run.

Other sample components, other than the analyte of interest, may have an effect on the analytical method [46]. This effect, termed as matrix effect, has a higher incidence in LC-MS/MS techniques [46], and therefore was evaluated in this work for EtG. This was done using a method proposed by Matuszewski *et al.* [47], comparing samples spiked only after extraction of a blank hair sample, with the corresponding amount of standard prepared in methanol, both evaporated and re-suspended in mobile phase. The matrix effect (%) is calculated by the ratio of both sample sets; a value close to 100% indicates no significant effect, while at values above 100%, the effect is positive and at values below 100%, the effect is negative. The calculated ME % was above 98%, and therefore the matrix effect was found not significant.

In hair segments over 6 cm in length, only the proximal 6 cm are used for alcohol biomarker analysis [32], and in these cases the length above 6 cm was not analysed. Therefore, although at least 100 mg of hair were collected from each participant, there were cases where the proximal 6 cm had not sufficient weight. 217 samples were excluded due not having at least 2 x 30 mg, the quantity necessary to perform both analysis (EtG and FAEEs). Length measurement and cutting of hair segments was carefully performed by aligning the site of cut of each hair,

in order to assure equal length in the entire segment. The use of plastic recipients and materials, including gloves, was avoided, or used at minimum, during all hair handling steps due to the potential interference of such materials on FAEEs analysis [10].

From the 975 analysed samples, 54 were not quantifiable for EtG, and 46 for FAEEs, since the obtained signal was below the limit of quantification. In the case of the FAEEs, the 46 samples represent only cases where all the four FAEEs (ethyl myristate, palmitate, oleate and stearate) were below the LLOQ. It is important to mention that the four FAEEs used in hair analysis as alcohol biomarkers are interpreted as a sum of their concentrations. Since the individual species present a high variability of concentrations, the use of isolated species of FAEEs as biomarkers is not recommended, and their analysis only proves value when interpreted as a sum.

The Society of Hair Testing (SoHT) organizes regular meetings to show the latest findings and results on hair analysis and to standardize analytical performance and interpretation. One important result of those meetings, which is relevant to our work is the consensus for the use of alcohol markers in hair for assessment of both abstinence and chronic excessive alcohol consumption, adopted by the SoHT during the meeting in Bordeaux, France, in 2014 [32]. Important procedures for the analysis of EtG and FAEEs in hair are provided in this document, including normalization of sample preparation procedures, considerations during result interpretation and parameters for assessment of abstinence and excessive alcohol consumption (cut-off values). It should therefore be clear why this consensus was adopted as a guideline, during the entire sample analysis procedure and data interpretation of the current study.

In order to evaluate if alcohol consumption was significantly different between genders, ANOVA was performed on the total concentrations of both biomarkers. Statistically significant differences were found for EtG and FAEEs between both genders, with higher concentrations in the male population. The median concentration of EtG was 16.91 pg/mg in males and 11.88 pg/mg in females, for FAEEs the value was 0.203 ng/mg in males and 0.172 ng/mg in females. Relating to information already discussed above, these results confirm that consumption is more incident on the male population. The higher incidence of excessive drinking, heavy drinking in the past 30 days and ingestion of quantities of alcohol per occasion in males contribute to higher quantities of both biomarkers in hair. However, it has also to be considered that other authors also reported higher concentrations of EtG on male populations [23,25], and that sex differences may play a role in the incorporation of FAEEs in hair [34]. Nevertheless, this confirms the findings of questionnaire analysis, that higher quantities of ethanol are ingested per drinking occasion in the male population.

Both biomarkers were in relative agreement when distributed according to university, with only one clear exception (UP). It has to be noted that several authors didn't find correlation between EtG and FAEEs, suggesting differences in formation and incorporation as the main reason for this fact [28,29,33], therefore this situation is not unusual. Both markers' concentrations were however in agreement with the fact that higher levels were obtained at UBI and UC.

For the interpretation of the analytical results in terms of consumption frequency, results were grouped according to the classifications provided by the SoHT, as abstinent, moderate (or social drinker), and excessive (or chronic) drinker. For this, the self-reported answer regarding consumption frequency was used, "frequent" consumers were included in the moderate or social drinker group, since the classification of excessive drinking would not be adequate in these cases. Interpretation of the analytical results, in terms of this classification, relies on the use of cut-off values that delineate the limit of a positive or negative result, while false results (false-negative or false-positive) are less occurring. However, as already highlighted and discussed in this work, the establishment of cut-off values for alcohol biomarkers in hair is a challenging and complex task, with constant adaptations and adjustments due to emerging research. This can be observed in the different consensus published by the SoHT over the past years (2009, 2011 and 2014), where these values have been continuously revised [32,48,49]. Therefore, we found it relevant that the analytical results of the studied population were used to contribute on the general knowledge on alcohol biomarker cut-off values in hair, by verifying the adequacy of the currently established values proposed by the SoHT for our study population. Two approaches were used, verifying the sensitivity and specificity of the biomarkers on the studied population, and performing a receiver operating characteristic (ROC) analysis in order to find the cut-off values that would be ideal for our results (by optimizing the sensitivity and specificity).

It is important to mention that, while performing ROC analysis and determination of sensitivity and specificity of a test, a test variable (the outcome of a test) is plotted against the state variable (the actual true state), and this state variable would be the information obtained using a "standard" technique. Since in this study we used the participants' self-reported information, this presents a limitation, especially due to the high expected underestimation and overestimation of consumption rates. This approach has, however, also been applied in other studies [23], but it is expected to have an impact on the determined sensitivities and specificities. The sensitivity of a test is the ability of the test to identify correctly positive cases, and is therefore negatively affected by the number of false-negative results. The specificity regards the ability to correctly identify negative cases, and is rather affected by the number of false-positives. On a perfect cut-off value, both parameters would be close to 100%, corresponding to 100% of true positives and 100% of true negatives; however, when false-negatives and false-positives are identified these percentages are obviously lower.

The determination of the sensitivity and specificity for both biomarkers was performed at the cut-off values for abstinence and excessive drinking proposed by the SoHT [32]. The concentration of FAEEs increases along with the hair length [10,34], and therefore for this biomarker it is recommended to interpret results based on cut-off values for either 0 – 3 or 0 – 6 cm segment lengths; the same does not occur for EtG, and analysis is recommended in the 0 – 6 cm proximal segments [32].

For EtG, the sensitivity was 81.6% and 60.0%, and the specificity was 56.3% and 90.3% for the cut-off values of abstinence and excessive drinking, respectively. The relatively low percentage of sensitivity at excessive drinking, and specificity at abstinence cut-off values, could be an indication of self-reported consumption underestimation or overestimation. For FAEEs the specificity (82.8% - 100%) was generally high, while the sensitivity was only high at the cut-off of excessive drinking for 0–3 cm segments (100%). One problem encountered during cut-off value estimation was the fact that only 6 participants in the study reported their consumption as excessive (and thereof only 5 were analysed for biomarkers), and this number was not sufficient to perform a significant cut-off value evaluation. Consequently, the existence of false-negatives greatly decreased sensitivity due the low number of true-positives, when compared for example to the cut-off for abstinence, where a much higher number of samples was available. This is even more valid for FAEEs, where 2 samples were situated within 0-3 cm segments and 3 samples within 0-6 cm segments, accounting for an even lower number of samples.

With the purpose of optimizing the test's sensitivity and specificity for EtG and FAEEs, and also to further confirm the adequacy of the proposed cut-off values, ROC analysis was performed using the same information as for the determination of sensitivity and specificity. Generally, by varying the cut-off value in a specific result set, either sensitivity or specificity increase, while the other decreases, and vice-versa [50]. ROC analysis consists therefore of plotting 1-specificity (x-axis) against sensitivity (y-axis), at a given cut-off value. An ideal test would be represented by a plot where the curve crosses the upper left side of the plot, since this is the point where sensitivity is close to 1 and 1-specificity close to 0 (consequently, the specificity is close to 1). The area under the curve represents the overall accuracy of the test, and in and ideal test would be 1, while 0.5 corresponds to the line of zero discrimination [50]. Cut-off value concentrations, and respective sensitivity and 1-specificity, can be observed on the coordinates of the ROC curve, these concentrations are determined by the averages of two observed consecutive ordered test values. One approach to determine the optimal cut-off value is by finding the best relation between sensitivity and specificity, in other words, the concentration at which the difference between both parameters is minimal. In this approach importance is also given to the ability to identify positive and negative drinking cases; therefore, the occurrence of both false-negative and false-positive cases is likewise tentatively minimized, and this can be done through the use of the Youden's index [51].

For EtG the determined cut-off values were very close to those suggested by the SoHT. Additionally, the sensitivity and specificity using both proposed and determined values, were very similar, except the sensitivity at the cut-off for excessive consumption which suffered a great increase (from 60 to 80%). However, this difference should not be considerable, again due to the low number of self-reported excessive drinkers, since this change of 20% corresponds actually to one sample that was only identified as excessive using the determined cut-off, and not with the proposed concentration. The obtained cut-off values (7.30 and 29.85 pg/mg, for

abstinence and excessive drinking, respectively) were very similar to the ones suggested by the SoHT (7 and 30 pg/mg for abstinence and excessive drinking, respectively). Additionally, it was considered that neither sensitivity nor specificity suffered a significant increase by using the optimal cut-off values determined based on the results. Therefore, we found that the currently proposed cut-off values of the SoHT for EtG were suitable for the assessment of alcohol consumption in the analysed student population, presenting moderate to high sensitivity and specificity.

For the FAEEs, no significant improvement was observed on the sensitivity and specificity of the abstinence cut-off, which was optimal at 0.185 ng/mg (0-3 cm segments) and 0.378 ng/mg (0-6 cm segments). Only a slight increase in sensitivity (0.069%) was observed at 0–3 cm segments, however the number of cases that had this segment length was lower (N = 66) than the number of cases with 0–6 cm segments (N = 909), and this increase was considered not significant. The obtained values were also situated very close to those suggested by the SoHT, therefore these were considered adequate for the studied population. Great discrepancies were however found at the cut-offs for excessive consumption, where both 0–3 cm and 0–6 cm segments values (0.817 and 0.889 ng/mg) were distant of the respective proposed cut-off value (0.5 and 1 ng/mg). Beside this discrepancy, the obtained values for FAEEs concerning excessive drinking were found to be in agreement with a recently proposed cut-off value for the harmonization of both segment lengths (0.8 ng/mg) [26]. This demonstrates that the standardisation of cut-off values for alcohol biomarker analysis, in hair, is still a work in progress. However, as a compromise solution, and due to the low number of self-reported excessive drinkers, the cut-off values suggested by the SoHT were further used for results interpretation.

One aspect that makes result interpretation of alcohol biomarkers in hair a complex and sensible task is the possibility that EtG and FAEEs concentrations may be influenced by hair care products and cosmetic treatments. It should therefore be evident that the use of these products should be documented, and carefully considered during results interpretation. However, to complicate the scenario, only a few hair treatment procedures and products are generally agreed to influence biomarker concentration in a known manner [32]. Therefore, we intended to use the obtained results and contribute to the general knowledge of the effect of hair treatment procedures and products, on the concentrations of alcohol biomarkers in hair. For this reason, the self-completion questionnaire presented to the participants included questions regarding hair treatment habits, as the number of hair washes per week, use of washing products and cosmetic treatments.

Different authors have reported that EtG concentrations in hair may be decreased by abundant hair washing, known as the wash-out effect [28,29]. The possibility of decreasing EtG concentrations due to intensive hair washing was assessed by performing ANOVA analysis, however there were no significant differences on the EtG concentration according to the number of hair washes per week. An attempt was also performed by comparing the EtG

concentration with less than 4 washes per week, 4 to 7 washes per week and more than 7 washes per week by ANOVA analysis, but no significant differences were found. However, this result does not contradict the fact that EtG concentrations are affected by washout effects, it only demonstrates that in the studied population there is no significant association between the number of hair washes and EtG concentration.

Regarding the use of hair washing products, the use of shampoo was reported by the vast majority (N = 964) of the participants, and therefore the effect of its use could not be evaluated. EtG concentrations were significantly lower in participants that reported using hair conditioner, hair mask or both. These findings have not been reported by other authors; however, they represent an indication that the use of hair conditioner and mask during hair washing may negatively influence EtG concentrations. In order to verify this effect with higher reliability it would be advisable to perform analysis on hair pools (treated and non-treated with conditioner or mask) produced from the same sample, and compare the results. This would, however, require a higher sample amount to be collected.

Cosmetic treatments, as bleaching and/or dyeing of hair, has been shown by several authors to decrease EtG concentrations in hair [23,25–27,52], and this information has also been included in the 2014 consensus of the SoHT [32]. In the studied population it was possible to confirm this fact. ANOVA analysis revealed statistically significantly lower concentrations of EtG when dyeing and/or bleaching were used. Lower concentrations were also observed for bleaching, but this difference was not significant. No observable effect was associated to the use of hairspray, gel or wax, which is in agreement with the findings of others [53] and the consensus of the SoHT [32]. This demonstrates the importance of documenting the use of hair washing products and cosmetic treatments during hair sample collection, and the high care that must be taken during results interpretation.

Cosmetic treatments, as bleaching, dyeing, or use of ethanol containing products, may also influence the concentrations of FAEEs. However, in this work ANOVA analysis did not reveal significant differences concerning the use of these hair products or cosmetic treatments (Attachment 5). Again, this does not contradict the fact that these products influence FAEEs concentrations, but in the studied population there is no significant association between the use of these products and FAEEs concentrations.

The analysis of FAEEs alone for monitoring abstinence is not recommended by the SoHT [32], this marker is only recommended as a complimentary result and to confirm false-negative EtG results. Nevertheless, the combined use of both biomarkers is recommended to improve discrimination between abstinence, moderate and excessive drinking [5,23,29,33]. By combining the results of EtG and FAEEs at the cut-off values proposed by the SoHT, 185 participants were identified as abstinent and 15 as excessive. From the remaining samples, abstinence could be excluded in 274 cases, and excessive drinking in 851 cases. However, a significant number of inconclusive cases was obtained at the cut-off values for abstinence and

excessive drinking. For the occurrence of the first group of these inconclusive cases, characterized by a negative EtG result and a positive FAEEs result (N = 49), possible explanations include decrease of EtG concentrations due to washout effects [28,29] or cosmetic treatments (bleaching and dyeing) [23,27,52,54], or increase of FAEEs concentrations due to the use of alcohol-containing hair products [34,53]. The second group of inconclusive cases was significantly higher (n = 494) and was characterized by a positive EtG result and a negative FAEEs result. An explanation for this occurrence is not simple, especially because the incidence of these cases is much higher in this work when compared to other papers [5,23,26,29]. A clear explanation cannot be given, but it can be suggested that FAEEs concentrations are decreased due to the action of aggressive cosmetic treatment [55], and frequent use of lipophilic or alkaline hair treatments [5]. However, EtG is unlikely to increase due to cosmetics use [56], since this has only been associated with the use of herbal hair tonics [10,15,16]. Nevertheless, EtG should be the first choice in assessing abstinence, and a positive EtG result cannot be overruled by a negative FAEEs result [32]. Taking into account the additional fact that in 461 of those cases consumption was self-reported as moderate, these cases should mostly be considered as moderate drinkers. It is however important to mention that several authors suggested repeated analysis, or further monitoring of cases where inconclusive results are obtained between EtG and FAEEs [5,29,33]. Since the majority of the inconclusive cases obtained in this study could be verified by the questionnaires and EtG analysis, we did not find that repeated analysis would be justifiable added to the fact that in most cases no more sample was available, and collecting new hair specimens from the individuals was not possible. Since the inherent costs of each analysis are considerable, repeated analysis of inconclusive cases should be performed in situations of legal nature, where it is crucial to know the positive or negative outcome with high certainty.

Another objective in this work was to assess how well aware students are about their alcohol consumption habits, by comparing the biomarker analysis results with data obtained from the questionnaires. Kolmogorov-Smirnov and Shapiro-Wilk normality tests of the concentrations of EtG or the sum of concentrations of the FAEEs *vs* the self-reported alcohol consumption frequency (abstinent, moderate, frequent or excessive) showed a non-parametric distribution (Attachments 6 and 7). This was also strengthened by the skewness and kurtosis, obtained during descriptive analysis (Attachments 8 and 9). Therefore, correlation studies were performed using a non-parametric test, the Spearman correlation.

Both biomarker concentrations correlated well with the self-reported consumption, in other words, increasing concentrations were obtained along abstinent, moderate and excessive drinkers. This indicates that students are generally aware of their consumption pattern, but based on the low Spearman rank correlation coefficients, a significant incidence of analytical outcomes not in agreement with self-reported consumption is present. Participants also seemed to be aware of the ingested quantities of alcohol per drinking occasion. Only EtG correlated with the ingested quantities of ethanol, while for FAEEs such correlation was not observable.

However, the lack of correlation for FAEEs is expected, as it has been reported in other works [23,34]. Possible explanations for this lack of correlation are the sex and age dependence of FAEEs production, biological variability and use of hair treatments [34,57].

A significant incidence of cases where biomarker analysis was not in agreement with the self-reported consumption habit was suspected during correlation studies. Therefore, we intended to verify the percentage of self-reported abstinent, moderate and excessive drinkers that could be verified by the combined interpretation of EtG and FAEEs. A combinatory interpretation of both biomarkers was performed, and inconclusive cases were interpreted in a similar manner as other authors [5,33], but applying the most recent cut-off values suggested by SoHT, while taking into account the use of hair products. Based on this interpretation the test outcome was classified as abstinence, suggestive of moderate drinking (abstinence excluded), moderate drinking, suggestive of excessive drinking (or heavy moderate drinking) and excessive drinking. It was found that only 56.3% of the abstinent cases could be confirmed by biomarker analysis, with good part of the participants (43.7%) underestimating their consumption. Of the self-reported moderate drinkers, 71.6% of the cases could be confirmed, with a significant incidence of both underestimation and overestimation of consumption. Finally, 60% of the self-reported excessive drinkers could be confirmed, however as stated along this work, this group presented a low number of cases (N = 5), thereof 2 cases could not be confirmed by biomarker analysis, which accounts for 40% of the excessive drinking cases. As mentioned along this work, consumption underestimation, or overestimation, was expected to occur at the studied population. Indeed, 43.7% of the participants that reported their consumption as abstinent, had underestimated their consumption, which according to biomarker analysis showed to be moderate (N = 30) and excessive (N = 1). From the self-reported moderate groups, 12.4% (N = 111) had underestimated their consumption, which was shown as excessive based on the results. Overestimation of alcohol consumption occurred mostly in the self-reported moderate group, of these, 16% were proven as abstinent according to biomarker analysis. However, it has to be noted at this point that it may be possible that those individuals were only abstinent during the time-frame represented by the sample length. Since alcohol consumption amongst students, is more accentuated during certain academic events. It would therefore be relevant to perform a study on the student population, with repeated hair sampling during a 1-year period, to assess an overall alcohol consumption pattern, since it may vary during the academic year.

Overall the combined analysis of EtG and FAEEs showed that the majority of students consumed alcohol moderately (69%), followed by an incidence of abstinence of 19% and excessive drinking as least incident (12%). This tendency is comparable to the obtained from questionnaire analysis, however, and as suspected, the low percentage of self-reported excessive drinkers (0.5%) was mostly due to consumption underestimation, since by biomarker analysis several other cases of excessive consumption were identified.

Chapter IV - Conclusions

Taking into account the obtained and already discussed results, we may conclude that:

- Alcohol consumption was assessed at a university student population by means of self-completion questionnaires and analysis of alcohol biomarkers, EtG and FAEEs, in hair samples.
- Questionnaire analysis showed that alcohol consumption started mostly around the age of 15. The majority of students (67.28%) considered to have a moderate consumption habit, with almost one-third reporting excessive alcohol consumption in the past 30 days.
- The most consumed drinks among students were beer (26.51%), spirits (21.48%) or both (22.40%), and students reported high preference for drinking in group (95.7%), and in public places, mostly coffee bars (44.71%).
- Higher quantities of alcohol are ingested per drinking occasion by male students. However, the reported quantities for both genders are higher than those reported in a student survey of 2011. Additionally, the reported quantities indicate that risky and excessive drinking is present amongst university students.
- About one-third of the students are smokers, and an identical percentage of the individuals reported consumption of illicit substances. Amongst these substances, cannabis was the most common, consumed mostly on rare occasions. The use of illicit substances was associated with alcohol consumption in more than half of the cases.
- Two analytical methods were developed for hair alcohol biomarker analysis, based on LC-MS/MS for EtG determination, and GC-MS/MS for FAEEs determination, using a small sample amount of 30 mg.
- The methods were validated according to international accepted guidelines, in terms of sensitivity, limits, linearity, precision and accuracy, recovery, stability and matrix effect. The methods proved to be selective, at a LLOQ of 3 pg/mg for EtG, and 30 pg/mg for each of the FAEEs. Linearity was proven from 3–500 pg/mg (EtG) and 30–5000 pg/mg (FAEEs). Within- and between-run precision and accuracy were found acceptable according to guidelines. Controls were used during validation, and included authentic hair samples, and adequate precision and accuracy was obtained at these samples. The overall recovery ranged from 74.79% and 97.90%, and in the case of EtG, matrix effects were found inexistent. Processed samples of EtG analysis were stable up to 96h after preparation, and up to 24h for FAEEs analysis, when left in the injector.
- Biomarker analysis confirmed that higher quantities of alcohol are ingested by males, since these where significantly higher, when compared to females. Additionally, higher biomarker concentrations were obtained at the university of Beira Interior and Coimbra.
- For results interpretation the most recently proposed cut-off values by the SoHT were used, at this concentrations good sensitivities and specificities were obtained for EtG.

For FAEEs, high specificities were obtained, however the sensitivity ranged from 30.5% to 100%.

- ROC analysis was used for the estimation of optimal cut-off values for our study population, and these were situated very close to the ones proposed by the SoHT. However, a discrepancy was observed at the cut-off for excessive drinking for FAEEs, which supports the opinion of other authors that a harmonized cut-off value for both hair segment length is possible, using a concentration of around 0.8 ng/mg. Overall, the cut-off values determined in this study, demonstrate that the proposed cut-off values are suitable to be applied in the studied population.
- Concentrations of EtG were found to be associated with the use of hair products as conditioner and hair mask, and with cosmetic treatments, dyeing and/or bleaching. It is therefore of high importance to collect data concerning hair care products and treatments during hair sample collection, and their consideration during result interpretation, as their use can severely affect the analytical outcome.
- Both biomarkers correlated with the self-reported consumption habit, indicating that participants are generally aware of their consumption. But only EtG correlated with the ingested quantity of pure alcohol, per drinking occasion.
- A significant number of inconclusive cases occurred with opposite outcomes of EtG and FAEEs analysis. In these cases, a complex interpretation was performed taken various facts into account, including hair treatment, and it was found that the majority of inconclusive cases, had agreeing results based only on EtG analysis and questionnaire analysis. These findings highlight the fact that EtG should be regarded as the first choice in alcohol consumption assessment, while FAEEs should be used to confirm the results from EtG analysis.
- The congruence of the self-reported consumption habit with the analytical outcome of the combined analysis of EtG and FAEEs was evaluated, and it was found that only 60–70% of the cases could be verified analytically. In the remaining cases, underestimation or overestimation of alcohol consumption was common. Overall, analytical results showed that 69% of university students consume alcohol in moderate amounts, followed by abstinence (19%), and excessive drinking as least incident (12%).
- The analysis of alcohol biomarkers in hair proved to be a powerful tool for the assessment of alcohol consumption in a student population, presenting such advantages as easy and non-evasive collection, low chances of adulteration and relatively easy storage until analysis. This presents an added advantage to survey studies on a population, since a great part of the limitations associated to the use of questionnaires can be completed by information provided from biomarker analysis. Additionally, alcohol biomarker analysis can be of interesting use on the assessment of the efficiency of self-completion questionnaires given to a study population.

Chapter V - Future Perspectives

Alcohol consumption in the student population varies between years, with certain trends prevailing during some periods. Additionally, alcohol consumption varies in a single academic year due to different academic events, where drinking is more accentuate. Therefore, one important future perspective would be to perform a similar study, however with repeated sampling during a single year, in order to assess overall yearly alcohol consumption.

Moreover, it would be relevant to compliment and compare the present study, with similar data from a future study. This would allow identifying emerging drinking trends and evaluating developments in overall drinking behaviour.

Lastly, it would also be relevant to assess consumption of drugs of abuse among the students. One likely candidate would be cannabis, since it is the most consumed illicit substance, and because its use was mostly found to be associated to drinking events.

Chapter VI - References

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Chapter VII - Attachments

Attachment 1. Brief description of the study, applied methodologies and written consent provided to the participants.

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DECLARAÇÃO DE CONSENTIMENTO INFORMADO

(CONFORME "DECLARAÇÃO DE HELSÍNQUIA", DA ASSOCIAÇÃO MÉDICA MUNDIAL, 1964)[1]

INFORMAÇÃO AO PARTICIPANTE

Título projeto de investigação

Avaliação do consumo agudo e crónico de álcool na população estudantil portuguesa – Determinação de marcadores em amostras de cabelo

Objetivo do estudo

O consumo de bebidas alcoólicas aumentou nos últimos anos em Portugal, um dos países onde consumo é mais elevado. No entanto, os padrões de consumo têm vindo a alterar-se, sendo que estas variações não são menos preocupantes, já que se configuram em dois grupos populacionais de particular vulnerabilidade e de tradicional baixo consumo – os jovens e os indivíduos do sexo feminino. Desta forma o presente trabalho tem como intuito a avaliação do consumo agudo e crónico de álcool na população estudantil portuguesa de forma a contribuir para o conhecimento europeu sobre o consumo desta droga licita entre os jovens. Este trabalho vai ser desenvolvido pelo Centro de Investigação em Ciências da Saúde da Universidade da Beira Interior.

Procedimentos

No caso de concordar em participar neste projeto, será necessário o preenchimento de um questionário (que demora aproximadamente 1 minuto a realizar) e ser-lhe-á colhida uma amostra biológica de cabelo. A quantidade máxima de amostra a recolher será 100 mg, uma madeixa de cabelo, da região distal da cabeça (menos aparente esteticamente). Esta amostra será preservada em condições apropriadas e as informações clínicas com ela relacionadas serão introduzidas numa base de dados, passando a sua identificação a estar codificada, e não acessível aos utilizadores das amostras. Serão cumpridas todas as normas éticas aceites internacionalmente.

Identificação das amostras e Confidencialidade

Após colheita, as amostras de cabelo bem como os questionários a estas associados serão identificadas por um código de forma a nunca serão associados à identidade do dador garantindo assim privacidade. Durante o desenvolvimento do projeto, a equipa de investigação poderá ter necessidade de verificar a informação do questionário. O anonimato será, contudo mantido, ou seja os dados do seu questionário serão fornecidos ao investigador, mas sem qualquer identificação relativa a dados pessoais, ou qualquer informação que permita saber a quem pertencem. Os dados serão tratados confidencialmente, de acordo com a Lei, com os regulamentos e de acordo com as normas éticas aprovadas pela Comissão de Ética da Faculdade de Ciências da Saúde. Os dados resultantes dos estudos realizados serão alvo de publicação de uma forma anónima e agregada, em termos de percentagens ou de dados numéricos, nunca individualmente.

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Possíveis benefícios para os participantes

Esta é uma doação altruísta, não havendo por isso qualquer compensação para o dador. Para mais, não se garante que este estudo envolva quaisquer benefícios diretos para o participante. Contudo, a sua participação proporcionará a aquisição de conhecimentos que poderão vir a beneficiá-lo a si ou a terceiros no futuro.

Riscos físicos previsíveis

Os riscos e o desconforto associados ao processo de recolha serão inexistentes.

Equipa de investigação e de trabalho de campo

Prof. Doutora Eugenia Gallardo – UBI (Investigador responsável);
Doutor Mário Barroso – INMLCF;
Prof. Doutor João Queiroz – UBI;
Mestre David Oppolzer – UBI;
Prof. Dra. Ana Martinho – UBI;
Mestre Sara Silva – UBI;
Mestre Tiago Rosado – UBI;
Licenciada Catarina Santos – UBI;
Licenciado David Figueirinha – UBI;

Fontes de financiamento

Este estudo está inserido num projeto de investigação financiado Fundação Calouste Gulbenkian (Programa de investigação de saúde em 2012-referencia 125895)

Conflitos de interesse

Não existem conflitos de interesse a declarar.

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[1] E respetivas alterações: Tokyo, Japan, 1975; Vernice, Italy, 1983; Hong Kong, 1989; Somerset West, South Africa, 1996; Edinburgh, Scotland, October 2000; Seoul, October 2008.

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DECLARAÇÃO DE CONSENTIMENTO INFORMADO

Eu, (nome completo do voluntário)_____

_____

compreendi a explicação que me foi fornecida acerca do projeto e do uso que pretendem dar aos meus dados, tendo-me sido dada a oportunidade de discutir e colocar as questões que julguei necessárias. Por isso, declaro que aceito voluntariamente que me sejam aplicados os métodos propostos no atual estudo.

Data: _____/ _____/_____

Assinatura participante e/ou o seu representante legal:

_____

Discuti este estudo de investigação com o participante e/ou o seu representante legal, utilizando uma linguagem compreensível e apropriada. Informei adequadamente o participante sobre a natureza deste estudo e sobre os seus possíveis benefícios e riscos, considerando que o participante compreendeu a minha explicação.

Data: _____/ _____/_____

Assinatura do Investigador:

_____

Foi entregue um duplicado deste documento ao participante/representante legal.

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Attachment 2. Self-completion questionnaire

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Nº identificação amostra:_____
Data de recolha:_____
Instituição:_____

Inquérito para recolha de dados

Este questionário, de carácter **anónimo e confidencial**, destina-se à recolha de informação no âmbito do projecto de investigação "Avaliação do consumo agudo e crónico de álcool na população estudantil portuguesa – Determinação de marcadores em amostras de cabelo", realizado pelo Centro de Investigação em Ciências da Saúde da Universidade da Beira Interior e financiado pela Fundação Calouste Gulbenkian (referência 125895). Além desta informação, será necessário proceder a recolha de uma amostra de cabelo de 100mg. Todos os dados recolhidos serão tratados estatisticamente, pelo que **as informações obtidas não serão facultadas a quaisquer outras entidades que não estejam relacionadas com o âmbito do projecto**.

Dados

Idade:_____ **Sexo:** ☐ F ☐ M **Localidade:** _____

Habilitações literárias ☐ Ensino Básico ☐ Ensino Secundário ☐ Ensino Superior; Qual? _____ ☐ Curso Profissional

Estado Civil ☐ Solteiro ☐ Casado (nº de filhos_____) ☐ Divorciado ☐ Outro_____

Consumo de álcool

Quando experimentou pela primeira vez?
☐ Nunca experimentei ☐ Não lembro ☐ Tinha___anos

Considera o seu consumo?
☐ Abstinente (Total abstinência pelo menos há 3 meses)
☐ Moderado (Consumo em raras ocasiões e em baixas doses, um copo numa ocasião especial)
☐ Frequente (Consumo de álcool uma ou duas vez por semana)
☐ Excessivo (Consumo de álcool diário e em elevadas doses)

Costuma beber só ou em grupo? ☐ Só ☐ Em grupo

Tipo de bebida que ingere:
(bebida mais frequente ou preferida - uma opção):
☐ Cerveja ☐ Vinho ☐ Bebidas brancas
☐ Espumantes ☐ Licores ☐ Outras_____

Quantidade aproximada/ocasião (litros ou copos): _____

Locais de consumo (mais habitual, uma opção):
☐ Café/Bar ☐ Discoteca ☐ Casa ☐ Outros

Alguma vez consumiu álcool em excesso?
☐ Não ☐ Sim, **Quando foi a última vez?** ----------

Consumo de outras substâncias

Licitas: (Tabaco) ☐ Não ☐ Sim
Em caso afirmativo indique o nº de cigarros/dia_____

Ilícitas: ☐ Cannabis ☐ Ecstasy ☐ Cocaína
☐ Heroína ☐ Outras _____

Já experimentou drogas vendidas nas *smartshops*?
☐ Não ☐ Sim, Quais? _____

Em caso afirmativo, com que frequência consome estas drogas:
☐ Diariamente ☐ Semanalmente
☐ Mensalmente ☐ Raramente

Consume estes compostos concomitantemente com álcool:
☐ Sim ☐ Não

Cabelo

Hábitos de Higiene
(Nº vezes que lava o cabelo/por semana) _____

Que tipos de produtos utiliza:
☐ Champô ☐ Amaciador ☐ Máscara

Tratamentos cosméticos:
☐ Descoloração ☐ Pintura ☐ Laca
☐ Gel ☐ Cera ☐ Outros_____

Quando cortou o cabelo pela última vez:
☐ Até 1 mês ☐ De 1 a 3 meses
☐ Há mais de 3 meses

Quantos centímetros aproximadamente cortou o cabelo:
☐ Total (rapar) ☐ Até 3 cm ☐ Entre 3 a 6 cm
☐ Mais de 6 cm

Informação Adicional

Escolaridade do pai: _____
Profissão do pai: _____
Idade do pai: _____
Nº de membros do agregado familiar (total) _____

Escolaridade da mãe: _____
Profissão da mãe: _____
Idade da mãe: _____

Algum destes familiares tem/teve problemas alcoólicos? ☐ Sim ☐ Não

Muito obrigado pela sua colaboração.

Attachment 3. Processed sample stability of EtG

Control	Measured concentration (pg/mg)	CV (%)	Bias (%)
Stability at 24 Hours (n=3)			
Authentic sample #1	33.01 ± 2.65	7.53	-9.37
Authentic sample #2	67.74 ± 4.05	7.44	10.75
Authentic sample #3	126.84 ± 12.69	7.13	-4.88
Spiked (30pg/mg)	29.24 ± 0.37	4.66	-6.17
Stability at 48 Hours (n=3)			
Authentic sample #1	33.33 ± 1.99	4.72	4.77
Authentic sample #2	54.71 ± 2.97	9.94	-14.65
Authentic sample #3	123.73 ± 14.78	9.91	-10.15
Spiked (30pg/mg)	30.73 ± 1.65	3.50	0.06
Stability at 72 Hours (n=3)			
Authentic sample #1	38.64 ± 3.88	7.56	6.09
Authentic sample #2	55.47 ± 4.73	8.07	-9.31
Authentic sample #3	122.79 ± 8.12	4.80	-2.22
Spiked (30pg/mg)	31.12 ± 1.64	4.41	-0.15
Stability at 96 Hours (n=3)			
Authentic sample #1	33.94 ± 1.43	5.08	-6.80
Authentic sample #2	65.23 ± 8.98	8.99	2.22
Authentic sample #3	132.13 ± 11.89	6.12	-0.92
Spiked (30pg/mg)	33.19 ± 3.49	7.77	5.66

Attachment 4. Processed sample stability of FAEEs

Stability at 24 Hours (n=3)

	Control	Measured concentration (pg/mg)	CV (%)	Bias (%)
Myristate	*Authentic sample #1*	*46.37 ± 3.33*	*8.64*	*10.84*
	Authentic sample #2	*90.24 ± 11.07*	*12.55*	*0.52*
	Authentic sample #3	*137.20 ± 13.30*	*10.49*	*-14.61*
	Spiked (50 pg/mg)	*44.28 ± 2.54*	*7.89*	*-3.59*
	Spiked (200pg/mg)	*171.32 ± 8.30*	*3.09*	*-0.53*
Palmitate	*Authentic sample #1*	*100.96 ± 5.42*	*9.31*	*14.91*
	Authentic sample #2	*170.98 ± 12.55*	*14.78*	*11.53*
	Authentic sample #3	*371.95 ± 19.93*	*8.44*	*14.96*
	Spiked (50 pg/mg)	*51.00 ± 5.16*	*10.97*	*13.38*
	Spiked (200pg/mg)	*188.13 ± 3.79*	*7.36*	*2.14*
Oleate	*Authentic sample #1*	*208.19 ± 17.02*	*7.85*	*-2.83*
	Authentic sample #2	*452.89 ± 45.05*	*10.33*	*8.32*
	Authentic sample #3	*877.71 ± 110.96*	*10.91*	*13.29*
	Spiked (50 pg/mg)	*47.96 ± 6.71*	*13.99*	*-7.74*
	Spiked (200pg/mg)	*191.62 ± 21.38*	*9.63*	*-3.43*
Stearate	*Authentic sample #1*	*68.55 ± 2.79*	*14.51*	*13.89*
	Authentic sample #2	*119.50 ± 20.23*	*14.74*	*-5.72*
	Authentic sample #3	*218.33 ± 23.89*	*8.99*	*-4.89*
	Spiked (50 pg/mg)	*56.07 ± 4.78*	*8.52*	*7.89*
	Spiked (200pg/mg)	*202.32 ± 16.73*	*9.47*	*0.92*

Attachment 5. ANOVA analysis of the effect of washing products and cosmetic treatments on FAEEs concentrations.

Treatment	*N*	ΣFAEEs (ng/mg)		ANOVA			
		Mean	Median	*p-value*	*F*	*F-critical*	Statistical difference
Washing products							
No reported product[a)]	330	0.315	0.177	-	-		-
Conditioner only	283	0.287	0.179	0.373	0.794	3.857	No
Hair mask only	91	0.262	0.161	0.242	1.372	3.864	No
Conditioner and mask	225	0.305	0.198	0.747	0.104	3.858	No
Cosmetic products							
No reported product	569	0.309	0.186	-	-	-	-
Dyeing[b)]	102	0.285	0.150	0.555	0.348	3.855	No
Bleaching	29	0.227	0.164	0.223	1.487	3.857	No
Hairspray	36	0.281	0.179	0.655	0.200	3.857	No
Gel	35	0.241	0.159	0.272	1.210	3.857	No
Wax	62	0.269	0.174	0.405	0.695	3.856	No
Others[c)]	55	0.265	0.139	0.383	0.761	3.856	No
2 or more treatments	46	0.228	0.175	0.135	2.236	3.857	No

a) Except for shampoo. b) Other treatments are excluded. c) Mostly hair mousse and serum.

Attachment 6. Normality tests of the EtG concentrations vs self-reported consumption habit.

Tests of Normality

	Como considera o seu consumo?	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
		Statistic	df	Sig.	Statistic	df	Sig.
EtG concentration in 30 mg of hair	Abstinent	.232	56	.000	.680	56	.000
	Moderate	.173	638	.000	.680	638	.000
	Frequent	.194	217	.000	.663	217	.000
	Excessive	.255	5	.200*	.825	5	.127

*. This is a lower bound of the true significance.

a. Lilliefors Significance Correction

Attachment 7. Normality tests of the FAEEs concentrations *vs* self-reported consumption habit.

Tests of Normality

	Como considera o seu consumo?	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
		Statistic	df	Sig.	Statistic	df	Sig.
FAEEs sum in 30 mg of Hair in pg/mg	Abstinent	.141	61	.004	.868	61	.000
	Moderate	.223	643	.000	.645	643	.000
	Frequent	.204	216	.000	.665	216	.000
	Excessive	.392	5	.011	.692	5	.008

a. Lilliefors Significance Correction

Attachment 8. Descriptive analysis of the EtG concentrations *vs* self-reported consumption habit.

Descriptives

	Como considera o seu consumo?			Statistic	Std. Error
EtG concentration in 30 mg of hair	Abstinent	Mean		12.5911	1.72431
		95% Confidence Interval for Mean	Lower Bound	9.1355	
			Upper Bound	16.0467	
		5% Trimmed Mean		10.6242	
		Median		8.0200	
		Variance		166.502	
		Std. Deviation		12.90355	
		Minimum		3.15	
		Maximum		72.49	
		Range		69.34	
		Interquartile Range		10.09	
		Skewness		2.747	.319
		Kurtosis		9.025	.628
	Moderate	Mean		16.7084	.57194
		95% Confidence Interval for Mean	Lower Bound	15.5853	
			Upper Bound	17.8315	
		5% Trimmed Mean		14.7715	
		Median		12.9400	
		Variance		208.701	
		Std. Deviation		14.44648	
		Minimum		3.09	
		Maximum		127.33	
		Range		124.24	
		Interquartile Range		12.29	
		Skewness		3.581	.097
		Kurtosis		18.733	.193
	Frequent	Mean		19.5788	1.15092
		95% Confidence Interval for Mean	Lower Bound	17.3104	
			Upper Bound	21.8473	
		5% Trimmed Mean		17.1237	
		Median		15.2600	
		Variance		287.443	
		Std. Deviation		16.95416	
		Minimum		3.34	
		Maximum		114.19	
		Range		110.85	
		Interquartile Range		12.48	
		Skewness		3.266	.165
		Kurtosis		13.195	.329
	Excessive	Mean		81.1000	28.90065
		95% Confidence Interval for Mean	Lower Bound	.8589	
			Upper Bound	161.3411	
		5% Trimmed Mean		80.5056	
		Median		55.3000	
		Variance		4176.237	
		Std. Deviation		64.62381	
		Minimum		19.49	
		Maximum		153.41	
		Range		133.92	
		Interquartile Range		125.70	
		Skewness		.439	.913
		Kurtosis		-3.100	2.000

Attachment 9. Descriptive analysis of the FAEEs concentrations *vs* self-reported consumption habit.

Descriptives

	Como considera o seu consumo?			Statistic	Std. Error
FAEEs sum in 30 mg of Hair in pg/mg	Abstinent	Mean		137.1759	11.97269
		95% Confidence Interval for Mean	Lower Bound	113.2270	
			Upper Bound	161.1248	
		5% Trimmed Mean		127.9575	
		Median		116.9100	
		Variance		8744.056	
		Std. Deviation		93.50966	
		Minimum		32.55	
		Maximum		528.55	
		Range		496.00	
		Interquartile Range		105.66	
		Skewness		1.670	.306
		Kurtosis		4.274	.604
	Moderate	Mean		307.8078	14.39513
		95% Confidence Interval for Mean	Lower Bound	279.5406	
			Upper Bound	336.0750	
		5% Trimmed Mean		257.1238	
		Median		186.4900	
		Variance		133242.290	
		Std. Deviation		365.02368	
		Minimum		30.12	
		Maximum		3170.68	
		Range		3140.56	
		Interquartile Range		322.30	
		Skewness		3.639	.096
		Kurtosis		18.981	.192
	Frequent	Mean		297.0342	21.95911
		95% Confidence Interval for Mean	Lower Bound	253.7515	
			Upper Bound	340.3169	
		5% Trimmed Mean		258.2935	
		Median		193.2150	
		Variance		104155.707	
		Std. Deviation		322.73163	
		Minimum		30.03	
		Maximum		2965.17	
		Range		2935.14	
		Interquartile Range		315.50	
		Skewness		3.959	.166
		Kurtosis		25.177	.330
	Excessive	Mean		1415.3320	412.61906
		95% Confidence Interval for Mean	Lower Bound	269.7178	
			Upper Bound	2560.9462	
		5% Trimmed Mean		1356.2867	
		Median		1091.8900	
		Variance		851272.432	
		Std. Deviation		922.64426	
		Minimum		847.94	
		Maximum		3045.54	
		Range		2197.60	
		Interquartile Range		1253.12	
		Skewness		2.102	.913
		Kurtosis		4.514	2.000

Participation in other works

Co-supervision of master theses:

David Carreira Figueirinha, “Deteção de opiáceos em sangue post-mortem por cromatografia líquida de alta eficiência com deteção eletroquímica usando microextração em seringa empacotada.”, Universidade da Beira Interior, Covilhã, academic year of 2013/2014. Supervisor: Maria Eugenia Gallardo Alba. Co-supervisor: David Jerónimo Oppolzer

Victor Barros Almeida, “Optimização de uma metodologia analítica para a determinação de pregabalina em plasma com recurso a microextracção em seringa empacotada”. Universidade da Beira Interior, Covilhã, academic year of 2013/2014. Supervisor: Maria Eugenia Gallardo Alba. Co-supervisor: David Jerónimo Oppolzer

Scholarship:

“Technologies for Purification and controlled release of biopharmaceuticals to be applied in age-related diseases”. Scholarship from the Programa Operacional Regional do Centro 2007-2013 QREN (Programa "Mais Centro"), CENTRO-07-ST24-FEDER-002014 (Reference TPCR-2-004)

Articles:

Augusto Pedro, David Oppolzer, Maria Bonifacio, Claudio Maia, Joao Queiroz, Luís Passarinha, “Evaluation of MutS and Mut+ Pichia pastoris Strains for Membrane-Bound Catechol-O Methyltransferase Biosynthesis.”, Applied biochemistry and Biotechnology, Volume 175, Issue 8, 3840-3855, 2015.

João Marques, Vítor Gaspar, David Markl, Elisabete Costa, Eugenia Gallardo, Ilídio Correia, “Co delivery of Sildenafil (Viagra(®)) and Crizotinib for synergistic and improved anti-tumoral therapy.”, Pharmaceutical Research, Volume 31, Issue 9, 2516-2528, 2014.

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