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An evaluation of washing and extraction techniques in the analysis of ethyl glucuronide and fatty acid ethyl esters from hair samples

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ABSTRACT

Ethyl glucuronide (EtG) and fatty acid ethyl esters (FAEEs) are alcohol metabolites measured in hair and are after a decade of research thought to be the best markers in hair to indicate alcoholism and abstinence *Forensic Sci. Int*. 218 (2012) 2. A great body of work concerning EtG and FAEEs detection in hair has been performed. However, no recent extensive comparison has been made concerning washing and extraction procedures. This work shows that the washing procedure of dichloromethane followed by a methanol rinse of the hair sample removes more than 16% of the FAEEs and 50% of the total EtG that is present in and on the hair. A review of ten washing protocols (where the removal is categorised: high, medium or low) showed that a relatively high percentage of FAEEs was removed and "medium" amount of EtG compared to the other washing protocols. This work shows promising results for the extraction of the FAEEs and the combined extraction of FAEEs and EtG by using 30 min of sonication with methanol. More FAEEs were recovered from hair with methanol than with any other extraction solvent including the commonly used dimethyl sulfoxide/heptane mixture. When the sonication time was increased a higher percentage of transesterification of the FAEEs was observed, the extraction was "dirtier" as solids and a colour change was observed whereas the extraction efficiency did not increase. Therefore, washing the hair sample with dichloromethane and methanol followed by an addition of 1 ml of methanol and sonication for 30 min to extract the FAEEs and EtG from hair is recommended for FAEEs as well as for the combined analysis of EtG and FAEEs. A linear calibration curve ($r^2 > 0.99$) was obtained for all analytes.

1. Introduction

Though alcohol consumption is not illegal in many parts of the world, establishing someone's alcohol consumption can be useful in the legal and medical field. In child custody cases, for instance, it is important to determine if allegations of chronic heavy drinking are true. Knowledge about someone's drinking behaviour is required when diseases occur, like fetal alcohol spectrum disorder, that can only be diagnosed and treated when it is known that someone has been drinking. Alcohol hair testing can aid in the confirmation of someone's drinking behaviour in such cases [1]. Currently, the consensus of the Society of Hair Testing (SoHT) [1,2] describes that concentrations of the minor metabolites EtG (ethyl glucuronide) and FAEEs (fatty acid ethyl esters) in hair are expected to be above a cut-off value (30 pg/mg and 0.5 ng/mg) for chronic heavy drinkers.

Several washing procedures and solutions are currently used for hair prior to the EtG and FAEEs analysis. Most FAEEs methods include a non-polar solvent as was suggested by Politi et al. [3] to remove the greasy layer from hair, which may otherwise hinder the extraction. However, it is clear that no general consensus is reached especially not for EtG analysis. The washing protocol described for EtG analysis differ in (i) the polarity of the solvents that are used, (ii) the volume of the solvents, (iii) the amount of washes and (iv) the application of sonication. Kronstrand et al. [4],

Abbreviations: EtG, ethyl glucuronide; FAEE, fatty acid ethyl esters; SoHT, Society of Hair Testing; BSTFA, *NO*-bis(trimethylsilyl)trifluoroacetamide; DMSO, dimethyl sulfoxide; DCM, dichloromethane; GC, gas chromatography; MS, mass spectrometry; HS SPME, head space solid phase micro extraction; PDMS/DVB, polydimethylsiloxane/divinylbenzene; SIM, selected ion monitoring; SPE, solid phase extraction; E1, an ester formed from ethanol and a saturated (0 double bonds) 14 carbon long acid; 14:0, an ester formed from ethanol and a saturated (0 double bonds) 14 carbon long acid; E16:0, an ester formed from ethanol and a saturated (0 double bonds) 16 carbon long acid; E18, 1an ester formed from ethanol and a unsaturated (1 double bonds) 18 carbon long acid; E18:0, an ester formed from ethanol and a saturated (0 double bonds) 18 carbon long acid.

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for instance, used ultrasonication and 3 ml per wash whereas Albermann et al. [5] uses 1 ml and Morini et al. [6] did not use sonication. In our study solvents are used with a range of polarities and distinct hair swelling properties; for compounds that are protic are said to have the ability to swell hair and thereby facilitate the diffusion and thus extraction of the analytes from hair [7]. In this study the polarity and protic ability of the solvent washes were varied to investigate the influence of these characteristics on the wash and extraction efficiency of EtG and FAEEs. It is also interesting to see the effect on the two alcohol markers since their chemical and physical properties are different. EtG is polar and non-volatile whereas FAEEs are non-polar and semi-volatile. As stated before non-polar solvents can remove a greasy layer that may hinder the extraction of the analytes and protic solvents cause hair swelling and facilitate extraction. In our research is looked at these effects and the occurrence in the analysis of both markers. To our knowledge this has not yet been presented in another study whereas it is important because a difference in washing procedure can influence the reported concentration of the alcohol markers in hair. Hence, using the same cut-off values to come to a negative or positive test result for methods with different washing procedures may not be appropriate.

Table 1
Summary of washing solvents used in this research.

Washing solvents	Analytes	References
De-ionised water and dichloromethane	FAEEs	[10]
n-Heptane	FAEEs	[3,11–17]
De-ionised water and *n*-heptane	EtG and FAEEs	[15,18]
Dichloromethane 2×	EtG	[19]
Diethyl ether and acetone	EtG	[20] Used a similar protocol, but with ether
De-ionised water, acetone and methanol	EtG	[21]
Methanol	EtG:	[9]
	FAEEs:	[22]
Methanol and acetone	EtG	[23,24]
Dichloromethane and methanol	EtG	[3–6,25–28]
De-ionised water and acetone	EtG	[14,18,28–36]
De-ionised water	EtG:	Used as part of the decontamination protocol by [8,14,15,18,21,28–37]
	FAEEs:	Used as part of the decontamination protocol by [10,15,18]

2. Material and methods

2.1. Solvents and reagents

FAEEs-D5 and EtG-D5 were purchased from LGC Standards (Teddington, UK). *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) was purchased from Stratlab Ltd (Macherey-Nagel, Germany). Lab reagent grade dimethyl sulfoxide (DMSO), acetone, ethyl acetate, and dichloromethane (DCM); technical grade reagent sodium chloride; analytical grade acetic acid and diethyl ether; HPLC grade heptane, hexane were purchased from Fisher. HPLC grade methanol and toluene were bought from Sigma-Aldrich (Gillingham, UK). Methane of a purity of 99.9995%, Argon of a purity of 99.9999% and Helium of a purity of 99.999% were supplied by Air Liquide (Birmingham, UK). Ultra-pure water was prepared with a Purite Neptune system equipped with NCP 8 cartridges (Thame Oxon, UK).

2.2. Instrumentation and analysis

Sample preparation was required before analysis. Various extraction solvents were used of which those with DMSO required an additional step to separate the DMSO from the extraction solvent heptane or hexane. These samples containing DMSO were frozen and the alkane layer was decanted to be analyzed. All samples were divided in two equal aliquots one for the determination of EtG and one for the analysis of FAEEs. The EtG samples were evaporated in a GC vial, derivatised with 10 μl BSTFA in the presence of 10 μl ethyl acetate at 80 °C. The samples were subsequently injected directly on the gas chromatography mass spectrometry (GCMS) system. The FAEEs samples were evaporated in a 10 ml head space vial, extracted and injected onto the GCMS system by head space solid phase micro-extraction (HS SPME) with a polydimethylsiloxane/divinylbenzene (PDMS/DVB) fibre as was done by Pragst et al. [8].

Analysis was performed on a Varian CP3800 gas chromatograph equipped with a 320 series triple quadrupole mass spectrometer. The column used was a Varian Factor IV 5-MS (30 m × 0.25 mm × 0.25 μm film thickness). For the FAEEs, the injector and ion source temperatures were set to 250 °C and 150 °C, respectively. The column temperature was initially set at 50 °C for 1 min before increasing to 140 °C at a rate of 20 °C/min. The temperature was then increased to 220 °C at 5 °C/min before a final temperature gradient was applied of 30 °C/min to 300 °C, taking the total run time to 25.17 min. A chemical ionisation selected ion monitoring (SIM) program was used (88, 101, M + 1 for the FAEEs and for FAEEs-D5 93, 106 and M + 1) and the sample was introduced via HS SPME as described by Pragst et al. [8]. For EtG a shorter temperature program was used for this analysis and tandem MS was used as described by Paul et al. [9]. However, in this research no solid phase extraction (SPE) was used. Analysis for both EtG and FAEEs were performed with the helium carrier gas flow at 1 ml/min and the data was acquired and analysed using the MS workstation 6.9.2.

2.3. Obtaining hair samples from alcoholics

Studies were performed on hair obtained from alcohol abusers via collaboration with a local clinic. These volunteers admitted to have been drinking more than 60 g of alcohol per day by answering the first authors questions concerning frequency of drinking and quantity per day, history of their consumption how often they washed their hair and what kind of hair care products were used. A bundle of hair of about a pencil thickness was then cut close to the scalp at the posterior vertex (back of the head). The hair of each subject was cut with a pair of scissors in 1 cm segments and mixed prior the washing step, dried and cut in mm pieces prior to the extraction. Either the 1 cm segments or the mm pieces were weighed in the washing or extraction vial to obtain 30 mg that was used as sample size in these experiments.

2.4. Comparison of washing solvents

1 ml of a washing solvent was applied to 30 mg hair; the mixture was shaken briefly after which the solvent was removed. 5 μl of 200 ng/ml EtG-D5 and 20 μl of a mixture of 2000 ng/ml ethyl oleate-D5 and 1000 ng/ml of the other three deuterated FAEEs were then added to the washing solvent which were subsequently analysed for EtG and FAEEs. 1 ml methanol and the same amounts of deuterated standards were added to each of the washed hair samples for extraction. All experiments were performed in duplicate.

The washing procedures that were investigated (from Refs. [3–6,8–37]) are listed in Table 1. One millilitre of each washing solvent was used and when more than one washing solvent was used in a washing procedure the solvents were used after each other.