Impact of the grinding process on the quantification of ethyl glucuronide in hair using a validated UPLC–ESI–MS-MS method

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Abstract The Society of Hair Testing (SoHT) has provided cut-offs for the quantification of ethyl glucuronide (EtG) in hair to indicate occasional or chronic/excessive alcohol consumption. Although several sensitive methods have been reported, past proficiency tests results show a lack of reproducibility. An ultra-performance liquid chromatographic mass spectrometric method (LLOQ of 10 pg EtG/mg hair) has been validated according to international guidelines, including the successful participation in five proficiency tests. This method was subsequently used to evaluate the impact of different grinding conditions (cut, weakly or extensively pulverized hair samples) on the final measured EtG concentration. Hair from alcohol consumers (n=2) and commercially available quality control samples (QCs)) (n=2) was used. For the QCs, extensive pulverization led to a significantly higher amount of measured EtG. In the hair samples obtained from volunteers, cut or weakly pulverized hair resulted in EtG concentrations below the LLOQ, while mean concentrations of 14 and 40 pg EtG/mg hair were determined after extensive pulverization. Differences in sample preparation could partially explain inter-laboratory variability. As the differences in results can lead to a different interpretation even when applying the SoHT cut-offs, it is of interest to standardize sample preparation techniques in the field of EtG hair testing.

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1 Introduction

Quantification of ethyl glucuronide (EtG) in hair is used to detect excessive/chronic alcohol consumption and to assess alcohol abstinence. As direct quantification of ethanol in hair is not possible (1), specific non-oxidative metabolites of ethanol (2-4), such as ethyl glucuronide (EtG), ethyl sulphate (EtS) and/or fatty acid ethyl esters (FAEE) are targeted. Hair is advantageous over traditional matrices such as blood or urine because of its extended detection window. The Society of Hair Testing (SoHT) has published guidelines (5) concerning the use of EtG and FAEE in hair for the detection of chronic/excessive alcohol consumption. Although these markers can be used independently, the consensus is that in some cases determination of both can prove useful. For EtG, a cut-off value of 30 pg/mg hair, measured in the proximal (3 to 6 cm) segment, has been proposed to strongly suggest excessive/chronic alcohol consumption.

Sample preparation steps, such as decontamination and extraction, have been demonstrated to have an influence on the measured EtG concentration (6-13). Prior to extraction of the analytes, an attempt to improve sample homogeneity is usually made by reducing the hair into smaller pieces, e.g. by cutting or pulverizing. Extraction experiments show that the time required to extract EtG from hair is decreased when the matrix is pulverized to a powder (9) and that the determined concentration in pulverized hair samples was higher than in cut hair samples (6, 12, 14). To our knowledge, to date no consensus has been reached on the sample preparation in hair analysis.

Several validated methods based on liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) (6, 9, 14-26), or gas chromatography coupled to single or tandem mass spectrometry (GC-MS(/MS)) (7, 12, 27-33) have been published. Most of these make use of internal quality control samples (QCs) that have been prepared using either spiked QCs (6, 15, 16, 19, 20, 22-24, 26, 27, 31, 32) and/or authentic hair (7, 9, 15, 17, 21, 34), to estimate the accuracy of the method. Whilst spiked QCs are suitable to estimate bias and precision, they do not take into account the recovery of compounds from the hair matrix; QCs based on authentic hair partially overcome this problem but unfortunately they are not suitable to estimate the bias, because the real concentration is not known. Thus, to estimate the real capability of methods to correctly quantify authentic samples, QCs with a certified reference value and/or proficiency tests are required (25, 33).

Nowadays a number of commercial sources of hair QCs exist; these samples may be supplied either in cut or pulverized form. Furthermore, to assess the

reproducibility, proficiency tests are organized by the German Society of Toxicological and Forensic Chemistry (GTFCh) and by the SoHT in collaboration with Medichem. The SoHT provides samples in both cut and pulverized form while the GTFCh provides pulverized samples. Although several publications report successful participation in these schemes (20, 24, 26, 35), the quantitative results from these proficiency tests show an overall lack of reproducibility for the EtG quantification in hair (25, 35).

The aim of this study was to evaluate the impact of different grinding and extraction conditions on the measured EtG concentrations using a validated UPLC-ESI-MS/MS method. Experiments were based on authentic hair samples collected from two volunteers in addition to two commercial QCs (delivered in cut form). One commercially available QC, based on pulverized hair, had been used during the validation to evaluate the accuracy of the method. The combined uncertainty of measurement was calculated, based on proficiency test results, and was used to illustrate the impact of different parameters (inaccuracy of measurement, uncertainty of the certified value and intermediate precision) on the reproducibility.

2. Materials and methods

2.1 Chemicals

Certified reference standards for ethyl β-glucuronide (EtG) and its penta-deuterated analogue (EtG-d5) were obtained from Sigma-Aldrich (Steinheim, Germany) and were supplied at 1 mg/mL in methanol. Formic acid (mass spectrometric quality i.e. ~98%) was obtained from the same supplier. A separate source of ethyl glucuronide was also purchased from Lipomed (Arlesheim, Switzerland) in powder form and was used for the preparation of internal QCs. UPLC/MS grade acetonitrile, methanol and 0.1% formic acid in water were purchased from Biosolve (Valkenswaard, The Netherlands).

2.2 Validation of the analytical method

2.2.1 Samples

Blank hair samples from children and teetotallers were collected on a voluntary basis and were used for the validation, for the calibrators and for the spiked QC samples.

Hair samples were washed with dichloromethane (vortex 30 sec, sonication 10 min) and methanol (vortex 30 sec, sonication 2 min), and dried overnight at room

temperature. The samples were cut into small pieces with scissors and stored at room temperature.

One external QC sample, EGH 2/12-A from ACQ Science GmbH (Rottenburg-Hailfingen, Germany) was used during validation. This QC sample consists of pulverized authentic human hair with an EtG concentration of 25 pg/mg hair.

Proficiency tests between 2012 and 2014 for EtG in hair were organized by the GTFCh (GTFCh 1/13, GTFCh 2/13, GTFCh 3/13 and GTFCh 1/14) and by the SoHT in collaboration with Medichem (SoHT 2012).

2.2.2 Preparation of standard solutions, calibrators, QC and proficiency test samples

Two stock solutions, one for calibration (Cal-Stock) and one for the preparation of the internal QC (QC-Stock) with EtG at a concentration of 5000 ng/mL were prepared in methanol, using a different source of reference standard. The stock solution containing the internal standard (IS) at a concentration of 50 ng/mL was prepared in methanol. All solutions were stored at -18°C.

Daily calibration working solutions (Cal-W) at 10, 50 and 250 ng/mL were prepared diluting the Cal-Stock solution in water. Calibrators (10, 15, 20, 50, 100, 250 and 500 pg/mg hair) were prepared with 50 mg of pulverized blank hair spiked with 50 µL of the IS solution, an adequate amount (50, 75 or 100 µL) of a Cal-W solution, and water until a total volume of 1.5 mL was reached.

Daily QC working solutions (QC-W) at 10, 25 and 250 ng/mL were prepared diluting the QC-Stock solution in water. Spiked quality controls at 16, 30 and 300 pg/mg hair, were prepared by adding 50 µL of the IS solution, an adequate amount (60 or 80 µL) of a QC-W solution and water until a total volume of 1.5 mL to 50 mg of pulverized blank hair. EGH 2/12-A QC and proficiency test samples were prepared spiking 50 µL of IS solution and 1.45 mL of water to 50 mg of pulverized hair sample.

2.2.3 Sample preparation

Fifty milligrams of hair sample was weighed into a 2 mL Precellys tube containing six 2.8 mm stainless steel beads (Precellys Lysing kit, Hard tissue grinding) and pulverized with the Precellys 24 homogenizer (Bertin Technologies, Montigny-le-Bretonneux, France). For the validated method a pulverization protocol based on

three grinding cycles of 60 sec at 6500 rpm with a cooling time of 2 min between each cycle, was used.

EtG was subsequently extracted from the pulverized hair samples with 1.5 mL water and 50 µL of IS solution using 2 hr of sonication (40°C).

For clean-up, the sample was centrifuged (20, 800 g) for 10 min at 4°C and the supernatant was applied to a BondElut SAX (100 mg, 3 mL) SPE cartridge from Agilent (Diegem, Belgium), conditioned with 2 mL of methanol and 2 mL of water. Special care was paid to ensure the cartridge did not dry out between conditioning steps. The SPE cartridge was washed with 2 mL of water and 2 mL of acetonitrile and was dried under vacuum (-0.3 bar) during 2 min. EtG was eluted with 1 mL of a formic acid/acetonitrile solution (3/97, v/v) in a total recovery glass vial (Waters, Zellik, Belgium). The eluate was evaporated to dryness using a vacuum centrifuge at 38°C for 45 min (Labconco, Kansas City, Missouri, USA). The residue was reconstituted in 100 µL of 0.1 % formic acid in water. Ten microliters was injected onto the UPLC-MS/MS system using the full-loop mode.

2.2.4 Liquid chromatography and mass spectrometry conditions

Analyses were performed on an Aquity UPLC coupled to a Xevo TQ MS tandem mass spectrometer (Waters) equipped with an electrospray ionization source operated in negative mode. Gradient elution was performed on an Acquity UPLC HSS T3 (2.1 x 100 mm, 1.8 µm) column (Waters) with 0.1 % formic acid in water (mobile phase A) and acetonitrile (mobile phase B) at a flow rate of 400 µL/min. The gradient elution started with 99% of mobile phase A, decreasing to 88% mobile phase A, at 2.0 min. The washing step consisted of 100 % of mobile phase B held from 2.1 to 2.5 min. The initial condition was applied from 2.6 min to 5 min. The column temperature was set at 60°C.

MS/MS detection was performed in the multiple reaction monitoring mode (MRM) with a dwell time fixed at 0.078 sec using the following precursor/product ion transitions (cone voltage, collision energy): EtG for quantification 221/85 (30 V, 24 eV), EtG for qualification 221/75 (30 V, 22 eV) and EtG-d5 226/85 (28 V, 30 eV). ESI source parameters used were recently described (36). Briefly, nitrogen was applied as cone gas (40 L/h) and as the desolvation gas (900 L/h at 650°C). Argon was used as collision gas (0.35 mL/min). The capillary voltage and the cone voltage were 1 kV and 30 V, respectively.

2.2.5 Method validation

Selectivity, matrix effect, extraction efficiency, lower limit of quantification, linearity, accuracy, stability and reproducibility were evaluated according to International guidelines (37).

Identification was based on the following criteria: a stable retention time (RSD% < 5) and stable ion ratios for the MRM transitions (within 20% of expected) (5). Two MRM transitions were used for EtG and one transition used for the IS.

Selectivity was determined by the analysis of six blank hair samples from different individuals. To verify that there were no isotope exchange reactions with non-labelled compounds, two samples without hair but with internal standard were analyzed.

Matrix effects were quantified and evaluated using the post-extraction addition technique (38). For the matrix effect, six blank hair samples from different individuals were spiked after the sample preparation and compared with the analytes spiked at the same theoretical concentration in the mobile phase.

Extraction efficiency was evaluated by comparing the responses of six blank hair samples spiked before solid-phase extraction with responses of six blank hair samples spiked after solid-phase extraction. These experiments were performed at LLOQ (10 pg EtG/mg hair), and at low (16 pg EtG/mg hair), medium (30 pg EtG/mg hair) and high (300 pg EtG/mg hair) concentration.

The lower limit of quantification (LLOQ) was defined as the lowest concentration of the analyte with a signal-to-noise ratio calculated as root mean square (RMS) above 10/1 (for both transitions) and for which the bias and precision deviation was below 20%. Other identification criteria, such as a stable (RSD% < 20) ion ratio between the quantifier and the qualifier had to be met.

The calibration model (n=7) and the weighting factor were tested over the range 10 to 500 pg EtG/mg hair and were evaluated via residual plots (37). The goodness of fit was established as the difference between the calculated calibrator value and its nominal value. The coefficient of variation should be lower than 15% except at the LLOQ (< 20%).

Accuracy was measured through the determination of bias and precision (repeatability and intermediate precision). Three internal QCs spiked at low (16 pg

EtG/mg hair), medium (30 pg EtG/mg hair) and high (300 pg EtG/mg hair) concentration and one external QC EGH 2/12-A (25 pg EtG/mg hair) were analysed in replicates on eight different days. A single factor ANOVA test with significance level (α) of 0.05 allows calculating bias, repeatability and intermediate precision with these data. The results are acceptable when they are below 15% (20% at the LLOQ).

The stability of processed samples when stored in the Acquity autosampler was evaluated at low (16 pg EtG/mg hair) and high (300 pg EtG/mg hair) concentrations and using six blank hair samples spiked with EtG. Controls and stability samples (n=6) were prepared at the same time and processed samples stored in the autosampler for up to 72 hr prior the analysis. The mean response of the stability samples should be within 90 – 110% of the mean response of the control samples and the 90% confidence interval of the stability sample responses should be within ± 20% of the control sample responses.

The reproducibility has been evaluated by participation in proficiency tests organized either by the GTFCh or by the SoHT in co-operation with Medichem and the Federal Institute for Materials Research and Testing between 2012 and 2014.

The uncertainty of measurement was calculated using proficiency test results according to the GTFCh guidelines (35, 37, 39).

2.3 Subsequent grinding experiments

Hair from two volunteers (alcohol consumers) and two external QC samples were used. The two volunteers were Caucasian with cosmetically untreated hair; one was a male with brown hair, the other, a female with blond hair. Samples were first washed and cut into small pieces with scissors.

The external QCs were Medidrug ALCM 1/11-C (12 pg/mg hair) and Medidrug ALCM 12-A (39 pg/mg hair) (Steinenbronn, Germany); both were authentic hair reference materials and obtained in a cut (non-pulverized) form.

Approximately fifty mg of cut hair were weighed into a 2 mL Precellys tube and were pulverized with the Precellys 24 homogenizer. The same sample was processed according to four different procedures (4 to 6 replicates for each pulverisation procedure). The first three procedures aimed to compare the determined EtG concentration when using cut hair, weakly pulverized hair or

extensively pulverized hair (which is the standard grinding protocol used for the validation studies). Cut hair samples (process n°1) were initially incubated for 16 hr at 40°C and then sonicated for 2 hr (40°C) with 1.5 mL water and 50 μL of IS solution. Pulverization was performed using the Precellys 24 homogenizer; one cycle of 30 sec (at a speed of 6500 rpm) was used for the weakly pulverized samples (process n°2) and three cycles of 60 sec (with a cooling time of 2 min in between) was used for the extensively pulverized samples (process n°3). Pulverized hair samples were sonicated for 2 hr (40°C) with 1.5 mL water and 50 μL of IS solution. For the extensively pulverized samples, the sonication time was also evaluated (2 hr for process n°3 and 6 hr for process n°4).

The EtG mean calculated concentrations obtained from the four different grinding processes were compared using 1-way ANOVA test ($\alpha = 0.05$) to detect statistical difference.

The impact of the different grinding procedures on the hair was studied using a Phenom G2 pro electron microscope (Phenom-World BV, Eindhoven, The Netherlands).

3. Results

3.1 Method Validation

Data on matrix effect, extraction efficiency, linearity, accuracy and stability are reported in the supplementary information.

The LLOQ was calculated at 10 pg EtG/mg hair (supplementary information). An interfering peak at the same retention time of EtG and with a stable ratio between the two transitions (< 20%) was been detected in some blank hair samples (3/6); the estimated concentrations were around 0.5 pg EtG/mg hair.

An ion suppression of less than 34% (RSD% < 24) was observed for EtG, but the use of EtG-d5 as IS compensated for the matrix effect (MEIS% between 105 and 136, RSD% < 13). The extraction efficiency of EtG was higher than 53% (RSD% < 15). The calibration curve was linear between 10 and 500 pg EtG/mg hair with correlation coefficients above 0.99. A weighted 1/x linear regression was applied. The bias of the method was below 13%. The repeatability (RSDr) and intermediate precision (RSDt) were lower than 16%. No instability was observed for samples staying in the autosampler for 72 hr.

3.2 Grinding experiments

The results of the analyses of hair samples from two volunteers and data on the two QCs (Medidrug ALCM 1/11-C and Medidrug ALCM 12-A) are shown in Figure 1.

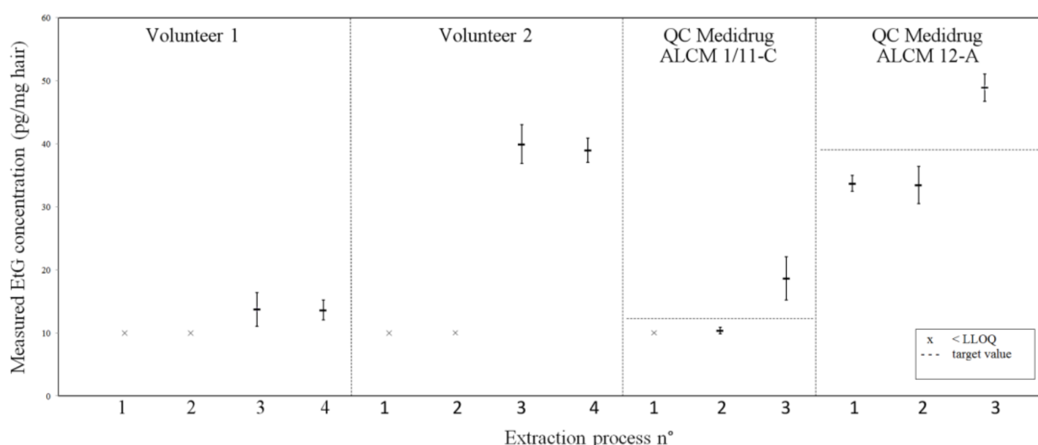


Figure 1: Mean concentration and standard deviation for EtG determined in authentic positive hair samples and two external QC samples applying different extraction processes (cut hair (process n°1), weakly pulverized hair (process n°2), extensively pulverized hair (process n°3) and extensively pulverized hair with longer sonication time (process n°4).

A result below LLOQ (at 10 pg EtG/mg hair) was obtained for EtG using cut hair (process n°1) or weakly pulverized hair (process n°2) for volunteer 1 and 2. After extensive pulverization (process n°3), a mean concentration of 14 (RSD%=20, n=6) and 40 (RSD%=8, n=6) pg EtG/mg hair was determined in the hair of volunteer 1 and 2, respectively.

For the QCs Medidrug ALCM 1/11-C and Medidrug ALCM 12-A, 1-way ANOVA tests demonstrated statistical differences in the determined EtG concentration with regard to the sample preparation. For Medidrug ALCM 1/11-C, which has a target value at 12 pg EtG/mg hair, EtG was not quantifiable using cut hair (process n°1) and a mean concentration of 10 (RSD%=5, n=3) and 19 (RSD%=18, n=4) pg EtG/mg hair were calculated using weakly pulverized hair (process n°2) and extensively pulverized hair (process n°3), respectively. For the Medidrug ALCM 12-A, with a target value at 39 pg EtG/mg hair, a mean EtG concentration of 34 (RSD%=4, n=4) and 33 (RSD%=9, n=4) pg/mg hair were calculated using cut (process n°1) and weakly pulverized hair (process n°2), respectively. The mean concentration measured (n=4) using extensively pulverized hair (process n°3) was significantly higher (49 pg EtG/mg hair, RSD%=4).

Furthermore, experiments on the hair from the two volunteers showed that two hr of sonication (process n°3) resulted in a statistically similar EtG concentration compared to six hr of sonication (process n°4).

The results of the grinding process are visualised in Figure 2. Weak pulverization (process n°2) damaged the external structure of the hair segment (Figure 2–B) while extensive pulverization (process n°3) destroyed the structure of the hair segment (Figure 2–C). The external QC EGH 2/12-A, which consists of pulverized authentic human hair, is presented as a comparison (Figure 2–D).

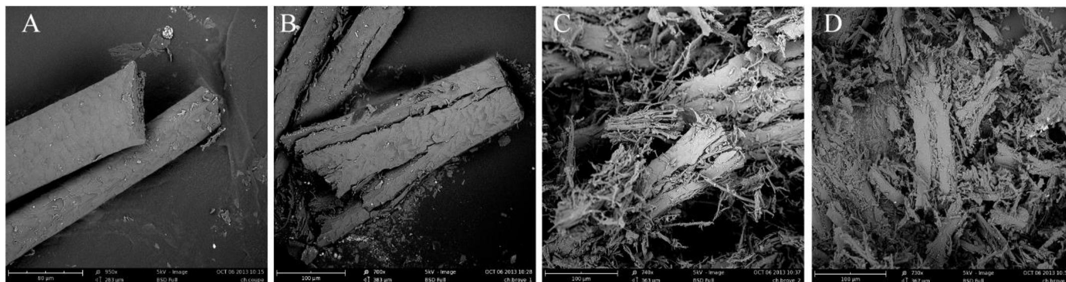


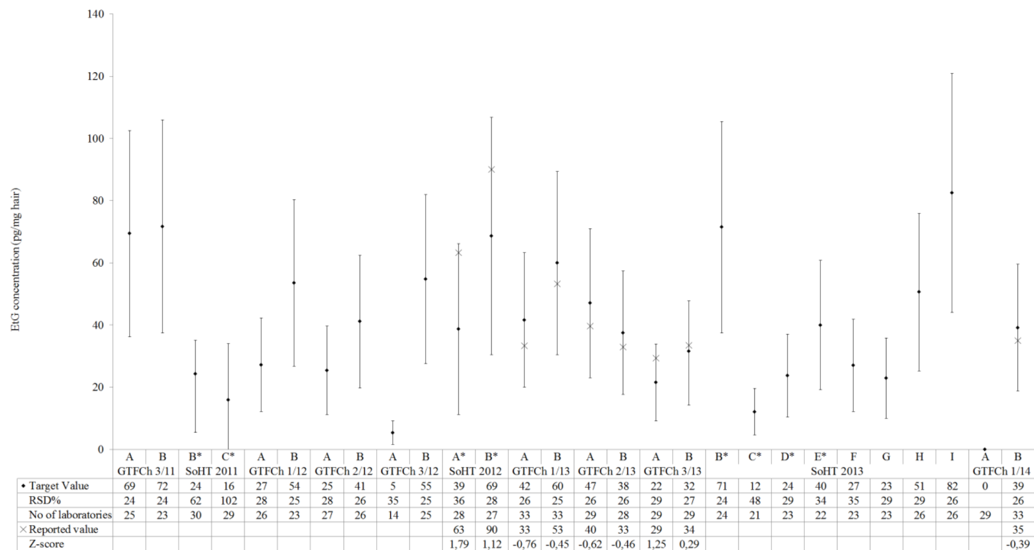
Figure 2: Microscopic observation of A: hair cut into small pieces, B: hair cut into small pieces weakly pulverized, C: hair cut into small pieces extensively pulverized, D: external QC EGH 2/12-A.

3.3 Proficiency tests results between 2011 and 2014

Quantitative results from the proficiency tests, organized either by the GTFCh or by the SoHT, in co-operation with Medichem and the Federal Institute for Materials Research and Testing, are presented in Figure 3.

The tests, based on authentic hair samples that had been cut into small pieces (organized by the SoHT), show a variation in the quantification between laboratories (RSD) between 24 and 102%. The reported variation (RSD) for the quantification of EtG in pulverized hair from proficiency tests organized by the GTFCh and the SoHT in 2013 is between 24 and 35%.

The reproducibility of the presented method was evaluated by means of participation in five proficiency tests: SoHT 2012, GTFCh 1/13, GTFCh 2/13, GTFCh 3/13 and GTFCh 1/14. The reported values and the obtained z-scores are also presented in Figure 3.



	GTFCh 3/11		SoHT 2011		GTFCh 1/12		GTFCh 2/12		GTFCh 3/12		SoHT 2012		GTFCh 1/13		GTFCh 2/13		GTFCh 3/13		SoHT 2013								GTFCh 1/14	
	A	B	B*	C*	A	B	A	B	A	B	A*	B*	A	B	A	B	A	B	B*	C*	D*	E*	F	G	H	I	A	B
◆ Target Value	69	72	24	16	27	54	25	41	5	55	39	69	42	60	47	38	22	32	71	12	24	40	27	23	51	82	0	39
RSD%	24	24	62	102	28	25	28	26	35	25	36	28	26	25	26	26	29	27	24	48	29	34	35	29	29	26		26
No of laboratories	25	23	30	29	26	23	27	26	14	25	28	27	33	33	29	28	29	29	24	21	23	22	23	23	26	26	29	33
× Reported value											63	90	33	53	40	33	29	34										35
Z-score											1,79	1,12	-0,76	-0,45	-0,62	-0,46	1,25	0,29										-0,39

Figure 3: Target values (RSD%), accepted ranges, number of laboratories with a successful participation, reported values and obtained z-score from our laboratory, for proficiency tests organized by the GTFCh and the SoHT between 2011 and 2014 (with permission of the GTFCh and the SoHT). Samples delivered in the cut form are indicated with an asterisk.

4 Discussion

4.1 Analytical method

The presented method is based on pulverized hair samples. Pulverization was performed in disposable tubes containing six stainless steel beads, placed on a Precellys 24 homogenizer. The multi-directional motion gives a high energy level to the beads that grind up to a total 24 samples simultaneously. The motion speed, the number of cycles, the time of cycles and the pause between cycles are variable parameters, which could potentially be optimized to improve the grinding of samples. That system allows a rapid pulverization of samples without a risk of contamination. As the samples are directly weighted and pulverized in the same vial that is used for the extraction, loss of sample is minimized.

4.2 Validation

The method has been validated according to international guidelines. Variation in the ratio between the two transitions (RSD > 20%) at concentrations below 10 pg EtG/mg hair limited the sensitivity of the presented method. Nevertheless, the cut-off used to detect abuse and excessive alcohol consumption, defined as 30 pg EtG/mg hair by the SoHT, exceeds by far the LLOQ of 10 pg EtG/mg hair of the presented method. Furthermore, this LLOQ is suitable for the purpose of further evaluating the grinding process. An interfering peak was observed in some blank samples, but with an estimated concentration 20 times lower than the LLOQ. The origin of the peak was not determined. Interfering peaks have also been reported

in other publications (6, 16) and have been separated from EtG using a 100% porous graphitic carbon column instead of a more conventional silica-based bonded phase column. We opted for an Acquity UPLC HSS T3 column, because of enhanced retention of polar compounds on this column. Other published methods based on liquid chromatography (15, 16, 19, 23) did not report on interfering peaks. However, these research groups used hair samples in the cut form and, as demonstrated recently (6, 12, 14), grinding of hair samples increases the extraction efficiency from the matrix.

Bias, repeatability and intermediate precision obtained from analysis of spiked QC samples were below 14%, which is comparable to other studies (6, 25, 27, 31, 32). The measured precision (<16%) and the calculated bias (<13%) for an external QC (EGH 2/12-A), consisting of authentic hair in pulverized form, demonstrate the accuracy of this method. Comparable precision (<13%) has been reported by other groups (7, 34) using authentic hair samples. No reports on bias, based on the analysis of external QC samples with a certified reference value, have been published. In one published study, cross-validation including several laboratories has been used to determine the bias (<13%) (7). The reference value was determined based on the mean EtG concentration obtained from each laboratory. According to our results, commercially available QC samples, in pulverized form, can be integrated in quality control programs to measure the bias, repeatability and intermediate precision, but do not take into account variations due to the sample preparation.

4.3 Grinding experiments

This paper evaluates the influence of the grinding procedure on the extraction of EtG from hair using a validated UPLC-ESI-MS/MS method. The grinding experiments, performed on two authentic positive hair samples from two alcohol consumers in addition to two QCs, demonstrated the impact of the pulverization process on the quantitative result (Figure 1). An extensive pulverization of hair samples leads to a significantly higher amount of EtG measured, which exceeded the reference value of the commercially available QCs. Indeed, to reach the certified values, these hair samples had to be weakly pulverized or even not pulverized at all. This could partially be explained by the fact that certified values were determined with a mean calculated concentration obtained from different laboratories, working either with cut hair samples or with pulverized hair samples. To avoid that bias, we suggest the creation of different QCs with a different certified value for a method based on cut hair and for a method based on pulverized hair.

Electron microscopy has been used to illustrate the impact of different grinding procedures on hair. Extensive pulverization of samples demolished the structure of hair and so increases the surface in contact with the extraction solvent (Figure 2). The microscopic structure of the external quality control EGH 2/12-A (in pulverized form), is similar to the structure obtained after an extensive pulverization of hair with the Precellys 24 homogenizer.

Further experimental studies based on more hair samples, from different ethnicities, with different colours and which have been subject to different cosmetic treatment are required to propose an optimal sample preparation procedure.

4.4 Proficiency tests results and uncertainty of measurement

Analysis of hair samples provided in five proficiency tests, applying our method, revealed a z-score between -2 and 2, which is required for a successful participation. Data analysis of proficiency tests between 2011 and 2014 using cut hair samples (organized by the SoHT) shows an overall lack of reproducibility (RSD values from 24 up to 102%). Less variation was observed between reported EtG concentrations from different laboratories (RSD from 24 to 35%) for proficiency tests delivered in pulverized form (organized by the GTFCh and the SoHT). Variations can partially be explained by a lack of homogeneity within samples (33), but also illustrate the influence of the grinding process on the quantification of EtG in hair (6).

In agreement with data published in 2011 (35), the combined uncertainty (u(x) = 31%) calculated on results from proficiency tests (37, 39) is mainly due to the uncertainty of the inaccuracy of measurement (RMSbias = 28%) and partially due to the uncertainty of the certified value (u(Cref) = 5%) and intermediate precision of the method (RSDt = 13%). The inaccuracy of measurement (RMSbias = 20%) and so the combined uncertainty (u(x) = 25%) decrease when proficiency tests based on cut hair samples, thus with variable sample preparation, are not taken into account. These results tend to suggest that recommendations or guidelines concerning the sample preparation protocol are to date crucial to lower the observed variation between laboratories in the determination of EtG from cut hair samples.

5 Conclusion

The influence of the preparation of the hair on the quantitative result has been studied. An extensive pulverization of hair samples leads to a significantly higher amount of EtG measured. Pulverization of hair is essential to assure a high recovery of EtG from hair and consequently a correct quantification of authentic samples. The developed method for the quantification of EtG in pulverized hair by UPLC-ESI--MS/MS has been validated. The accuracy of the method has been demonstrated with spiked QC samples and with one external QC sample, consisting of authentic pulverized hair, with a reference concentration value. Participation to proficiency tests was successful. However, recommendations or guidelines concerning the sample preparation protocol are crucial to lower the observed variations between laboratories in the determination of EtG in hair.

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Supplementary information

Number of pages: 2

Number of tables: 1

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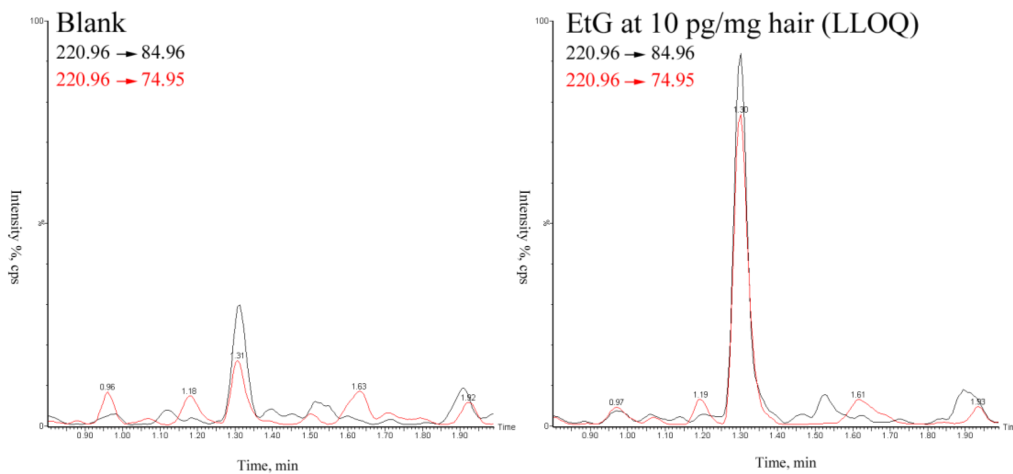
Suppl. Table 1: Dilution table for the whole blood method and for the V-DBS method.

	LLOQ	Low	Medium	High	EGH 2/12-A
Concentration (ng/mg hair)	10	16	30	300	25.4
ME calculated as % recovery (RSD%)	76 (6)	66 (24)	69 (16)	68 (21)	
ME_{IS} % (RSD%)	136 (6)	116 (13)	112 (13)	105 (8)	
Extraction efficiency % (RSD%)	71 (13)	57 (15)	56 (10)	53 (6)	
Mean calibration curve (r^2)	Y = 1.918x + 10.823 (0.997)				
Accuracy					
Bias %	1.3	-0.2	-0.6	1.2	-12.2
Repeatability % (RSD_r)	10.6	8.6	7.2	6.6	15.6
Intermediate precision % (RSDt)	13.4	12.3	12.9	8.6	14.6
Autosampler stability after 72h					
Mean stability samples %		109		104	
90% confidence interval of stability sample responses		38-41		521-550	
± 20% of control sample responses		29-44		410-615	



Suppl. Figure 3: MRM Chromatogram for EtG (m/z 221→85 and m/z 221→75) in a 'blank' sample and spiked at the LLOQ (10 pg/mg hair).