Accelerated Extraction and Analysis of Ethyl Glucuronide in Hair by Means of Pressurized Liquid Extraction Followed by Liquid Chromatography-Tandem Mass Spectrometry Determination

Accelerated extraction and analysis of ethyl-glucuronide in hair by means of pressurized liquid extraction followed by liquid chromatography-tandem mass spectrometry determination

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Abstract

The measurement of ethyl glucuronide (EtG) in hair is an established practice to evaluate alcohol consumption habits of the donors, nevertheless analytical variability has shown to be an important factor to be considered: measured EtG values can vary significantly as a consequence of analyte wash-out during decontamination, pulverization of samples, extraction solvent and incubation temperature. In the present study we described a new method for automated hair decontamination and EtG extraction from the inner core of the hair by using pressurized liquid extraction (PLE), followed by solid phase extraction (SPE) clean-up; validation was performed according to SWGTOX guidelines. The extraction efficiency of the new method was evaluated by comparing the results with those obtained by a validated and ISO/IEC 17025:2005 accredited method; an average positive difference of +32% was observed when the extraction was performed by PLE. The effect of hair pulverization was also studied and a good correlation between cut and milled hair was observed, implying that PLE allowed a highly efficient extraction of EtG from the inner keratin core of the hair, no matter if it has been cut or pulverized. Finally, to verify the results, paired aliquots of 27 real hair samples were analysed with both PLE and a protocol optimized by design-of-experiment strategies planned to maximize the extraction yield; in this case a comparable

efficiency was observed, suggesting that exhaustive EtG extraction was obtained with both approaches. This finding opens new perspectives in the eligible protocols devoted to hair EtG analysis, in terms of speed, automation, and reproducibility.

Keywords: Ethyl Glucuronide, Hair; PLE, HPLC-MS/MS

Introduction

The evaluation of chronic excessive alcohol consumption through the measurement of ethyl glucuronide (EtG) in 3–6 cm hair samples is nowadays an established practice for a wide range of clinical and forensic applications (1-2], and it is routinely used for the toxicological evaluations connected to driving license withdrawal and rehabilitation procedures. EtG is a minor non-oxidative ethanol metabolite produced after consumption of alcoholic beverages. This metabolite possesses hydrophilic and acidic properties which limits its incorporation into the hair shaft making the resulting concentration in hair generally low, but independent from its melanin content (3]. Another challenge of EtG analysis in hair - linked to the legal status of alcohol intake in most countries - is that the analytical results are not aimed to merely verify teetotalism but rather to evaluate the individual consumption habits. For this reason, current consensus guidance issued by the Society of Hair Testing (Revision 2019) has established different cut-off values which aim to distinguish between presumable abstinence (< 5 pg/mg), social/moderate drinking (>5 pg/mg and < 30 pg/mg) and chronic excessive alcohol consumption (>30 pg/mg) (4-5). The use of these values to make categorical determinations is widely diffused in the scientific community. However, it is worth to point out that the alternative use of statistical decision theories is progressively recommended (6-10). Indeed, the use of cut-off values within a decision procedure is prone to the "fall-off-cliff" problem (11) according to which close experimental values slightly above or below the cut-off limit respectively lead to opposite decisions. In this context, the accuracy associated to quantitative results of hair EtG appears particularly critical with respect to other toxicological determinations, including those for illicit drugs. This criticality is apparent when the literature related to EtG hair analysis is examined, as most of the papers are dedicated to the study of the numerous analytical factors that may contribute to false negative/positive results (1,12,13), rather than the description of radically new analytical methodologies. Among the non-analytical factors that may affect inter-individual hair EtG, specific studies have been devoted to investigate the influence of body mass-

index (BMI) (14), seasonality (15), kidney disease (16), hair hygiene (17) and regular use of cosmetic chemical treatments (18) and alcohol-based hair products (19). Wash out effects have also shown to represent a potential source of variation (20) which, together with other factors, must be considered in interpreting the results. Other sources of analytical variability to be considered include wash-out during decontamination, hair pulverization vs. scissors mincing, choice of extraction solvent and incubation temperature, all leading to inter- and intra-laboratory result variability (6,21,22).

A typical protocol for EtG extraction involves the following steps: (i) decontamination by multiple washing using water, methanol and/or acetone as solvents; (ii) sample cutting/grinding possibly with a ball mill; (iii) extraction, usually performed with water for a period ranging from about 30 min to overnight; (iv) discretional clean-up and (v) instrumental analysis, which is mainly performed by LC–MS, due to the polar and hydrophilic character of EtG. Comprehensive reviews discussing the features and issues of the analytical methods reported in the literature have been recently published (1,23). An unanswered critical issue is still represented by the duration and complexity of the extraction step, involving extensive manual handling for workloads frequently exceeding thousand hair samples per month (15).

In the present study, the development of a fast and highly efficient new method is described for hair decontamination and EtG extraction from the inner core of hair by using pressurized liquid extraction (PLE) followed by clean-up with solid phase extraction (SPE). This technique proved to be extremely versatile for hair extraction leading to a number of published procedures for the detection of illicit drugs (24,25), cannabinoids (26) and novel psychoactive substances (NPS) (27) in human hair. Taking into account the issues related to EtG hair analysis previously mentioned, the use of a fast and automated technique performing both decontamination and extraction may be beneficial, leading to high throughput combined with reproducible and accurate results. The accuracy achieved by the new method was tested by comparing the results with those obtained by a validated and ISO/IEC 17025:2005 accredited method (13), performed on separate aliquots of the

same hair samples. The effect of hair pulverization was also studied in combination with the new method to verify the degree of correlation between the results obtained from milled and finely cut hair samples. This comparison proved that PLE allows a highly efficient extraction of EtG from the inner keratin core of the hair.

Materials and methods

Chemicals

Standard solutions of EtG and EtG-d5 were purchased from Sigma-Aldrich (Milwaukee, WI, USA) at the concentration of 100 μg mL^{-1}. Individual working solutions were prepared in methanol at the concentration of 1 μg mL^{-1} and stored at -20°C.

Methanol, acetone, acetonitrile, water and formic acid, all LC-MS grade, as well as monobasic sodium phosphate salt, dibasic sodium phosphate salt, and diatomaceous earth were obtained from Sigma-Aldrich.

1.1. Sample preparation

Hair decontamination and extraction were sequentially performed by means of a PLE instrument (ASE 200 from Dionex - Sunnyvale, CA, USA). Sample preparation was performed as follows: 50 mg of hair were cut into 1-2 mm segments with scissors and mixed with diatomaceous earth to fill a stainless-steel PLE cell; two cellulose filters were used to seal the two ends of the cell, which was then loaded into the PLE instrument. To perform a complete and reliable decontamination, three different solvents were sequentially used: phosphate buffer 0.25 mM at pH 6.5, 2-propanol and acetone. PLE operating conditions for decontamination were set as follows: T= 50°C, P= 50 bar, preheat time= 1 min, heat time= 3 min, static time= 5 min, flush volume= 0%, purge time= 1 min. The three washing solutions were collected and kept for further analysis. At the end of the decontamination procedure, the cell was opened and 25 μL of IS solution at the concentration of

500 ng mL^{-1} was added in order to obtain a concentration of 25 ng mL^{-1}in the extract. The cell was then reloaded in the PLE instrument for the extraction step.

Hair extraction was performed in a single cycle using a mixture of water/methanol, 80/20 (v/v) using the following conditions: temperature 150°C; pressure 100 bar; preheat time 1 min; heat time 7 min; static time 5 min; flush volume 0%; purge time 1 min. The extract was collected in a 25 mL glass vial closed with cap equipped with solvent resistant PTFE septa. The PLE extract was transferred into a 15 mL Falcon tube and centrifuged at 4500 rpm for 10 min at 2°C.

Clean-up: SPE procedure

The PLE extract was purified by SPE on a strong anion-exchange cartridge (Strata SAX 55 µm, 70 Å, 100 mg/1 mL from Phenomenex, Torrance, CA, USA). The cartridge was installed on a Visiprep™ SPE Vacuum Manifold (Merck KGaA, Darmstadt, Germany) and initially conditioned with 1 mL of methanol, then 1 mL of water and finally with 1 mL of a mixture of water/methanol 80/20 (v/v). The PLE extract was filtered by means of a 0.45 µm PTFE membrane filter (Phenomenex) and fully loaded in the SPE cartridge. The cartridge was then washed with 1 mL of water/acetone 50/50 (v/v) and finally eluted with 500 µL of water 2% formic acid. Three µL of the eluate was injected in the UHPLC-MS/MS system.

UHPLC-MS/MS analysis

The UHPLC equipment consisted of a Nexera LC20AD XR system, with autosampler, vacuum degasser and column oven, from Shimadzu (Tokyo, Japan) coupled with a 4500 Qtrap from Sciex (Toronto, ON, Canada) equipped with a Turbo V ESI source. The column used was a EC 100/2 NUCLEODUR C18 Gravity-SB (1.8 µm 100mm × 2 mm ID). The mobile phases were H_2O 5 mM HCOOH (phase A) and MeOH:AcN (50:50) 5 mM HCOOH (phase B); the flow rate was 0.3 mL min^{-1}. The injection volume was set at 3 µL. Chromatographic separation was performed using a

gradient elution as follows: starting conditions of 0% of phase B held for 0.1 min, then phase B was increased to 25% in 0.4 min, then increased to 30% in 4.5 min, finally phase B was increased to 100% in 0.5 min and held in these conditions for 1 min. Initial conditions were re-established in 0.5 min and held for 2 min. The column oven temperature was set at 40°C. The total run-time including column equilibration was 9 min.

Negative ionization mode was used for detection; ion spray voltage was set at -4500 volt, source temperature was 600 °C, Ion Source Gas 1 and 2 were set respectively at 50 and 60 unit, nitrogen was used as curtain gas and set at 30 unit, nitrogen was used as collision gas and was set at medium level. Two selected reaction monitoring (SRM) transitions were chosen for EtG, viz. the ones that produced the best S/N. The source and instrument parameters were optimized by infusing a standard methanolic solution of EtG (10 ng mL^{-1}). Peak areas for the selected ions were determined using MultiQuant Software from Sciex. Table 1 gives an overview of the selected SRM transitions and UHPLC–MS/MS parameters.

Validation

For method validation, the following parameters were investigated: LOD, LOQ, linearity, selectivity, specificity, accuracy, intra and inter-assay precision, matrix effect and recoveries in accordance with SWGTOX international guidelines (28). Blank hair samples from ten acknowledged teetotalers were used for method validation; five samples were pooled for Quality Controls (QC) preparation and used individually for LOD, LOQ, specificity and matrix effect evaluation.

LOD was evaluated by fortifying five drug-free hair samples with a solution containing EtG at decreasing concentrations. Every sample was analyzed in duplicate over five separate batch-runs. LODs were determined as the concentrations which provided signals-to-noise values equal or higher than 3 for the less intense fragment.

LOQ was estimated likewise, verifying the presence of a signal-to-noise values exceeding 10, for at least two fragments. LOQ was then confirmed by verifying compliance with the identification criteria, RSD% and accuracy tolerance which was required to fall within ± 20%. Identification criteria included a retention time (t_R) tolerance below 2% for calibrators and controls, and fragment ion ratio (quantifier/qualifier intensity) acceptability within ±20% with respect to the control.

To evaluate linearity, calibrator EtG solutions were prepared in 2% formic acid in water. Selected concentrations were 2, 5, 25, 50, 100, 250, 400 pg mg^{-1}. Every calibrator sample was analyzed over five runs. The calibration curve was derived by plotting the ratio of the area of EtG to the area of internal standard versus the concentration using least squares regression model. Lack-of-fit and Mandel tests were used to verify the appropriateness of the linear model (99% significance level). Homoscedasticity was checked by using an F-test on the lowest and highest calibration levels (95% significance level). A weighing factor (1/x) was chosen to minimize the sum of the residuals.

Precision and accuracy were calculated at three concentrations, LOQ, 50 and 400 pg mg^{-1}, respectively. Five QCs samples were prepared for each concentration on five different days by spiking hair with EtG in the PLE cells after the decontamination procedure. Percent accuracy was calculated at each concentration by subtracting the grand mean of the calculated concentration (V_O) from the nominal value (V_D), and dividing by V_D, as follows: $Acc\% = (V_D - V_O)/V_D \times 100$. Within-run- precision was calculated separately for each day by calculating the CV% of the calculated values for each concentration. Between-run precision was estimated for each concentration over the five days, by using the combined data from all the replicates at each concentration.

Recoveries (R%) were calculated from the analysis of five different hair samples fortified with EtG at LOQ, 50 and 400 pg mg^{-1} concentrations. The peak areas obtained from the analysis of the QCs (X^I) were compared with the areas obtained from the same pooled samples fortified after extraction (X^{II}), as follows: $R\% = (X^I / X^{II}) \times 100$.

Matrix effect was evaluated from the same fortified hair samples. The peak areas from the samples fortified after extraction (X^{II}) were compared with the ones obtained from the analysis of the EtG methanol solutions at the same concentration (X^{III}). Matrix effect (ME%) was calculated as follows: ME%=(X^{II}/ X^{III})×100; ME% variability was expressed as CV%.

Selectivity was tested by analyzing five different samples of blank hair and verifying the absence of interfering ions on each SRM transition. The possible interference arising from the IS was assessed by adding EtG-d5 to five different cells filled with the same blank hair and processed as previously described.

Carry-over was evaluated by injecting blank samples before and after the highest calibrator sample and verifying that EtG signal did not exceed 10% of the signal of the lowest calibrator (i.e., 2 pg mg^{-1}).

Results and Discussions

HPLC-MS/MS optimization

Due to the hydrophilic characteristics of EtG, a stationary phase featuring polar functional groups was selected. A polar RP column, namely NUCLEODUR C18 Gravity-SB, was preferred over hydrophilic interaction (HILIC) columns because of the longer re-equilibration times of HILIC columns (29). The chosen column provided a satisfying t_R for EtG (3.2 min) which corresponded to k'≈5 and reduced risk of interference from highly polar components. The optimized gradient resulted in a total run time of only 9 min, including re-equilibration, making the method compatible with high workload. The choice of a ternary mobile phase that includes an acetonitrile:methanol 50:50 mixture as phase B assured improved S/N and peak shape.

PLE extraction

On the basis of previous experiences with PLE for hair extraction, different solvents, temperatures and pressures were tested. The first experiments were conducted without hair, mainly to evaluate the stability of EtG in the conditions used for extraction. Considering the hydrophilic nature of EtG, water was initially tested as the elution solvent. Experiments conducted at 150°C and 100 bar proved the stability of EtG under these conditions, leading to a recovery constantly above 90%. The following experiments were carried out with spiked hair and tested the effect of adding low amounts of methanol to water. The results, shown in Figure 1, suggested that the use of a water:methanol mixture (80:20, v:v) in the presence of the matrix yielded higher and more complete EtG recovery than with pure water. No further additional solvents were tested.

In a subsequent stage, the performance of PLE extraction was evaluated on real hair samples to verify if the procedure was suitable to release EtG from the inner core of hair. To this aim 11 hair specimens previously analyzed at the Turin laboratory - Centro Regionale Antidoping e di Tossicologia “A. Bertinaria” - (CAD) with a validated and ISO/IEC 17025:2005 accredited method (13) were re-analyzed with the present method. Relatively high sample amounts (50 mg) were typically used, when available, in order not to introduce excessive aliquot variability in the quantitative data, even if lower amounts (30-40 mg) were occasionally sampled. Each of the 11 real hair samples was decontaminated and separated into two aliquots; one was cut into small snippets (about 1 mm), whereas the second aliquot was pulverized using a metal beads mill. Both cut and milled samples were extracted by PLE, making it possible to evaluate the effect of hair pulverization and at the same time the recovery of PLE with respect to the quantitative results obtained at CAD.

The effect of hair pulverization vs. cutting was of special interest, because this initial sample treatment was shown in several studies to represent a major source of variability. Salomone et al. (13) tested the impact of cutting and grinding on 781 samples and observed an average increase of 31% when the sample was pulverized, with differences up to +414.8%. Similarly, in another study taking into account 7 samples, an extraction improvement ranging from 137 to 230% was observed

(30) when hair was pulverized rather than cut with scissors. Other authors observed lower average increases (≈20%) (31,32), but the effect of pulverization was consistently demonstrated as statistically relevant (22,33). In further studies (12,34) the dependence of hair extraction efficiency on the initial milling vs. cutting treatment was confirmed, but the extent of this factor's effect was proved to depend also on other experimental factors, including the extraction time and the incubation temperature, whereas sonication apparently had no significant effect (21). Actually, extensive hair pulverization destroys the structure of the hair segment (33), increasing the sample surface in contact with the extraction solvent, and makes the extraction efficiency higher even in a reduced time. The results obtained by Kronstrand et al. (34) showed that the differences between cut and pulverized hair became less extreme if the extraction time was increased up to 24 hours. Similarly, Mueller et al (12) did not observe significant improvement of hair EtG extraction produced by pulverization when incubation was performed at 60°C.

The results obtained in our study, that compare the EtG concentrations detected upon PLE on cut and pulverized hair aliquots, are shown in Table 2. The corresponding correlation plot reported in Figure 2 shows a coefficient of determination (R^2) of 0.99. The slope of the correlation line is 1.075, suggesting an average increment of 7.5% when hair is pulverized rather than cut with scissors. This increment is substantially lower than that recorded in the literature and discussed above. This observation provides a first demonstration of the PLE potential for hair extraction, as it suggests that the high pressure and temperature used in PLE promote exhaustive extraction of EtG from the inner core of hair, no matter if it has been cut or pulverized. The slight enhancement of extraction recovery produced by pulverization parallels what was observed in previous studies when extended extraction time and/or extreme incubation temperatures were used (12,34).

Another interesting clue concerning the efficiency of PLE recovery arises from the comparison (Table 2) of hair EtG concentration results measured with the present method with those originally determined by CAD using overnight extraction at ambient temperature on pulverized hair, as was formerly adopted (13). Interestingly, an average positive difference of +32% was observed when

the extraction was performed by PLE. It is noteworthy that a similar difference (+28%) was observed when the extraction method used in CAD was optimized by a design of experiment (DoE) strategy (22). It was shown that increased recoveries were obtained when hair was incubated overnight with water at 60°C, rather than at room temperature.

A final verification of the extraction efficiency achieved by PLE was provided by the direct comparison of the results obtained by the present method and the DoE optimized one (22) on separate aliquots of the same hair samples. To this purpose N=27 recently cut hair specimen were collected and separated into three aliquots. For each sample, one aliquot (a) was decontaminated, extracted by PLE and analyzed as described in the present method, while the other two aliquots were decontaminated, pulverized and extracted overnight with water (atmospheric pressure) at 60 °C (b) (22) and room temperature (c), respectively. The results are reported in Table 3 and Figure 3. The differences observed between the results from (a) and (b) obtained with the two optimized methods range from -39% to +34, with an average difference of 2% and a standard deviation of 19%. In particular, ten differences are below 10%, eight of them range between 10% and 20%, while the remaining nine differences are between 20% and 40%. No differences exceeded 40%.

Taking into account that the paired aliquots were extracted with completely different methods and the EtG concentrations were determined in different laboratories with different instrumentation, the diversity of the quantitative results appears to be modest and considerably lower than those observed in most inter-laboratory proficiency testing organized for EtG hair analysis. The comparison with the results differences observed between the aliquots (c) and (b) extracted at room temperature and 60 °C, respectively, is quite striking. Despite EtG in the corresponding extracts was determined in the same laboratory with the same instrument, the differences produced by the dissimilar extraction temperature combined with the random variability typical of hair analysis span from -33% up to +69%, with a mean difference of +8.6% and a standard deviation of 27%. It can be concluded that the present method based on PLE extraction for 12 min. and the previously

optimized method based on overnight extraction with water at 60 °C (22) provide comparable results and can be alternatively used to determine EtG in hair specimen.

The whole set of experiments conducted in the present study together with observations made in the studies previously cited suggest that the different experimental factors limiting the extraction recovery of EtG from hair, i.e. (i) hair cutting with scissors, (ii) short extraction time, (iii) extraction at room temperature, are overcome when more radical experimental conditions are adopted, including high temperature, high pressure, long extraction time. At the same time, a leveling appears in the data, indicating that further improvements of the extraction yield cannot be achieved. It is extremely likely that this leveling corresponds to the accomplishment of exhaustive EtG extraction from the inner hair core. If this hypothesis is true and further detailed studies will further confirm it, then an important success has been obtained, because any new technology that will be proposed in the future to speed up the analysis, improve throughput, or introduce automation in the hair processing has – for the first time - target conditions to compare with, that produce a "true" EtG concentration from real hair samples, within the experimental uncertainty.

In this context, the high recovery obtained with PLE provide clear evidence of its potential in hair EtG analysis; the results suggest that the use of PLE exceeds traditional solvent extraction methods, providing increased recoveries in a reduced extraction time. The time required for hand cutting and mixing the sample with diatomaceous earth is about 3 minutes, the decontamination time is 5 minutes while the time required for extraction is 7 minutes. Then in about 20 minutes, including instrumental equilibration times, a sample is decontaminated and extracted and after centrifugation it is ready for the clean-up phase which is carried out in about 5 minutes. Overall, a sample is ready for analysis in 30 minutes. Thanks to the automation of the decontamination and extraction procedure, time required for a set of samples is not a sum of the time required for a single sample, but it is definitively less.. In addition the combination of the decontamination procedure with extraction within the same apparatus contributes to the robustness and automation of the entire procedure and opens new perspectives in the hair EtG data reproducibility and inter-laboratory

comparison. Clearly, these are preliminary results that need additional testing on large sample sets and participation to inter-laboratory proficiency test to confirm the performance of PLE for EtG analysis.

SPE clean-up

The high extractive power of PLE makes the resulting extract solutions potentially rich of impurities. A clean-up step of the extract was prudently introduced in the sample treatment procedure to reduce ionization suppression/enhancement phenomena. In addition, purification by SPE has also the advantage of providing a tenfold enrichment factor by reducing the solvent volume (5 mL are loaded while 0.5 mL are used for elution). SPE was performed with a strong anion exchange (SAX) cartridge which is well-suited for EtG due to its acidic characteristics; a suitable protocol was obtained by testing different solvents especially for washing and elution. The eluent from PLE, which was constituted by water:methanol (80:20, v/v), was directly processed by SPE with no extra dilution steps.

Elution was performed with water and methanol containing formic acid, which promoted the protonation of EtG with the consequent disruption of the interaction with the quaternary ammonium bonded SPE phase.

Hair decontamination

As already reported (24), the PLE apparatus allows to execute automated hair decontamination prior of extraction. Three different solvents sequentially used to wash hair provided a satisfactory decontamination of the sample. The mild conditions (T=50°C, P=50 bar) adopted avoided EtG extraction from the inner core of hair. To verify that the decontamination was suitable to efficiently wash the hair without extracting the EtG incorporated into the hair segment, a pool of authentic positive hair, previously decontaminated with a standard procedure (three sequential washes with

pH 6.8 phosphate buffer, isopropanol and dichloromethane), was externally contaminated with a solution of EtG-d5 in acetone. An aliquot was sequentially decontaminated and analysed by PLE. The three washing solutions were analysed to verify the complete removal of the contaminating EtG-d5 and absence of EtG. The results proved the complete recovery of EtG-d_5 in the solvent wash and its absence in the extract (Figure 4). At the same time, only a small fraction (≈3%) of the hair-incorporated EtG was detected in the solvent wash, assuring that the incorporated EtG was almost quantitatively detected in the extract.

Validation

Linearity was assessed over two orders of magnitude, from 5 to 400 pg mg^{-1} using lack-of-fit and Mendel tests, as suggested by SWGTOX guidelines criteria. The selected range allow to monitor both moderate drinkers and excessive consumers. The data-points collected for calculating the calibration model showed heteroscedastic distribution, suggesting a 1/x weighting factor in the least squares regression. Experimental value for LOD and LOQ were obtained from the analysis of spiked hair with EtG at progressively decreased concentration. By extrapolation at S/N=3 and 10, LOD and LOQ were calculated, yielding 1.9 and 4.5 pg mg^{-1} concentrations, respectively, that were further tested for accuracy and precision within the limits. The practical LOQ value of 4.5 pg mg^{-1} is below the cut-off of 5 pg mg^{-1} recommended to estimate abstinence and is similar (35) or slightly greater (22) than the values reported in recent literature studies. The present conditions proves adequate within the objectives of this study, where samples were purposely selected so as to span over a wide EtG concentration range and cover a continuous set of EtG values, making the comparison between different analytical methods especially robust. In routine workload, most hair samples exhibit low EtG values (< 50 pg/mg) and particularly critical concentrations are the ones close to the 5 and 30 pg/mg cut-off values. Therefore, in routine conditions it would be advisable to

split the present calibration range into two subsets, for example 5-50 pg/mg and 50-500 pg/mg, and choose the IS concentrations accordingly.

The selectivity of the method was confirmed from the absence of interfering signals at the retention time of EtG when blank hair was analyzed; also, the ISs did not show any relevant signals for the corresponding non-deuterated analytes.

Good precision and accuracy at 5, 50 and 400 pg mg^{-1} was ascertained, as reported in Table 4. The average recovery of spiked EtG, also reported in Table 4, is 77%. Matrix effect is not significant at all the concentration tested proving the suitability of the SPE clean-up procedure after PLE.

Conclusions

A radically new approach has been proposed and optimized to perform the preliminary treatment of hair samples within the procedures devoted to quantitative EtG determination and aimed to detect chronic excessive alcohol intake by the donors. The new approach involves an automated hair decontamination step followed by fast extraction under high-pressure and high-temperature conditions achieved by a commercial PLE device.

While the extraction procedures most widely used involve prolonged hair soaking in order to allow the keratin structure to swell up and the solvent to penetrate in it, the present procedure achieves a comparable outcome by the combined effect of temperature and pressure. This finding opens new perspectives in the eligible protocols devoted to hair EtG analysis, in terms of speed, automation, and reproducibility. These potential improvements may have a significant impact in the workloads entailing hundreds of hair samples to be processed within each analytical session, as is the case in the laboratories serving the medical commissions in charge of the driving licence withdrawal and rehabilitation examinations.

The new method proved to achieve an extraction efficiency equivalent to that obtained by a traditional protocol optimized by design-of-experiment strategies and planned to maximize the extraction yield, as was demonstrated by the comparison of the quantitative EtG results obtained on paired aliquots of the same hair samples set. Moreover, the experiments conducted within the present study positively match with literature data in supporting the hypothesis that a maximum extraction yield is reached whenever hair extraction is made in protracted time intervals, at higher temperatures, or with supporting devices, such as ultrasonic shakers. This limit is likely to represent the achievement of exhaustive EtG extraction from the keratin matrix, which is an important target in an analytical determination not having certified standards of known concentration available.

In conclusion, the comparison between the analytical method proposed in the present study with a more traditional approach - historically used on several thousand hair samples - suggest that the improvements achieved by PLE in terms of speed and automation do not implicate a lower recovery of the target analyte, even if more extensive comparison on a variety of hair typologies and EtG concentrations is needed to further support a widespread technological transition. Further confirmation is expected by the repeated participation to inter-laboratory proficiency tests.

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	Q1	DP	FP	EP	t_R	Q3	CE	CXP
EtG	221.0	-27	-380	-9	3.2	75.1	-23	-19
						85.1	-22	-17
EtG-d5	226.1	-27	-380	-10	3.2	84.9	-24	-18

Table 1 Liquid chromatography- tandem mass spectrometry parameters for EtG and EtG-d5 (t_R: retention time; Q1: precursor ion mass; DP: declustering potential; EP: entrance potential; Q3: product ion mass; CE: collision energy; CXP: cell exit potential)

Sample code	PLE - cut hair (pg/mg) (a)	PLE - milled hair (pg/mg) (b)	Room temperature incubation - milled hair (pg/mg) (c)	Difference (b)-(a) %	Difference (b)-(c) %
15496	31	42	35	26%	19%
15168	58	65	34	12%	92%
15728	75	92	78	19%	18%
15564	78	85	69	8%	23%
15697	91	113	69	19%	63%
15446	94	107	84	12%	27%
15368	110	113	65	2%	73%
14730	120	121	129	1%	-6%
15581	126	135	94	7%	44%
15510	156	159	137	2%	16%
15088	325	349	239	7%	46%
Mean of differences %				10%	32%
Standard deviation of differences %				8%	29%

Table 2 EtG concentration values (pg mg^{-1}) of 11 hair samples analysed following different hair pretreatment and/or extraction methods (a) involving manual cutting and PLE extraction; (b) using pulverisation and PLE extraction (c) using pulverisation and overnight incubation at room temperature. Percentage differences among the average results provided by each method are reported

Extraction conditions				PLE	60° C	Room Temperature	
Sample code	**Gender**	**Age**	**Hair color**	**Aliquot (a) (pg/mg)**	**Aliquot (b) (pg/mg)**	**Aliquot (c) (pg/mg)**	**Difference (b)-(a) %**
1	M	69	Gray	178.8	232.7	168.3	23.2%
2	M	54	Dark brown	122.2	96.6	119.2	-26.5%
3	M	41	Dark brown	80.7	67.8	43.4	-18.9%
4	M	57	Gray	357.0	349.1	181.9	-2.3%
5	M	49	Dark brown	142.9	116.1	151.0	-23.0%
6	M	46	Dark brown	133.9	116.2	86.2	-15.2%
7	M	41	Light brown	68.3	69.6	67.0	1.8%
8	M	39	Dark brown	29.5	44.7	35.8	34.2%
9	M	26	Light brown	96.8	69.5	64.9	-39.4%
10	M	46	Dark brown	512.6*	538.5	471.7	4.8%
11	M	45	Dark brown	58.2	68.0	60.6	14.3%
12	M	28	Dark brown	17.6	19.5	24.7	9.8%
13	F	44	Light brown	45.0	47.2	36.8	4.6%
14	M	67	Gray	132.0	121.9	144.8	-8.3%
15	M	45	Gray	31.2	37.6	50.0	17.0%
16	F	26	Light brown	50.2	48.0	40.7	-4.6%
17	M	45	Gray	190.3	150.2	173.1	-26.7%
19	M	53	Dark brown	64.0	77.0	67.3	16.8%
20	M	27	Dark brown	79.0	106.6	120.0	25.9%
21	M	40	Dark brown	48.2	59.9	45.1	19.5%
22	M	23	Dark brown	36.2	47.6	34.1	23.9%
23	M	53	Gray	124.4	113.3	96.4	-9.7%
24	M	46	Dark brown	72.8	83.1	92.0	12.4%
25	M	45	Gray	139.0	133.4	142.0	-4.2%
26	M	49	Gray	110.1	144.6	65.6	23.9%
28	M	59	Gray	234.0	215.1	268.2	-8.8%
30	F	32	Light brown	371.1	418.3	129.5	11.3%
Mean of differences %							2.1%
Standard deviation of differences %							19.0%

Table 3 EtG concentration values (pg mg^{-1}) of 27 hair samples analysed by 3 methods (a) PLE (b) overnight incubation at 60°C (c) overnight incubation at room temperature. Percentage differences among the average results provided by each method are reported. Samples coded 18, 27, and 29 were available in two aliquots only and were not considered in the present study.
*A lower sample amount was weighted for sample 10 (i.e., 36.5 mg) so that it did not exceed the calibration range.

	Level - Concentration (pg mg^{-1})	Accuracy (A%)	Precision (RSD%)	Matrix Effect (ME%)	Recovery (R%)
EtG	5	13	8	91	82
	50	5	13	86	79
	400	0	4	87	69

Table 4. Validation parameters, precision expressed as RSD%, accuracy, matrix effect and recovery.

Figure captions

Figure 1 Recovery performances related to different PLE extracting phases

Figure 2 Correlation between the EtG levels measured after cutting (x-axis) and after milling (y-axis), for each sample (n = 11).

Figure 3 Comparison between EtG levels measured with PLE (aliquots (a)) and overnight extraction performed at 60°C (aliquots (b)). *A lower amount of sample was weighted for sample 10 (i.e. 36.5 mg) so that it did not exceed the calibration range.

Figure 4 Recovery of EtG-D_5 spiked on a pool of positive hair samples in the three washes of the decontamination procedure and the subsequent extraction. The amount of extracted EtG is also shown.

Figure 1

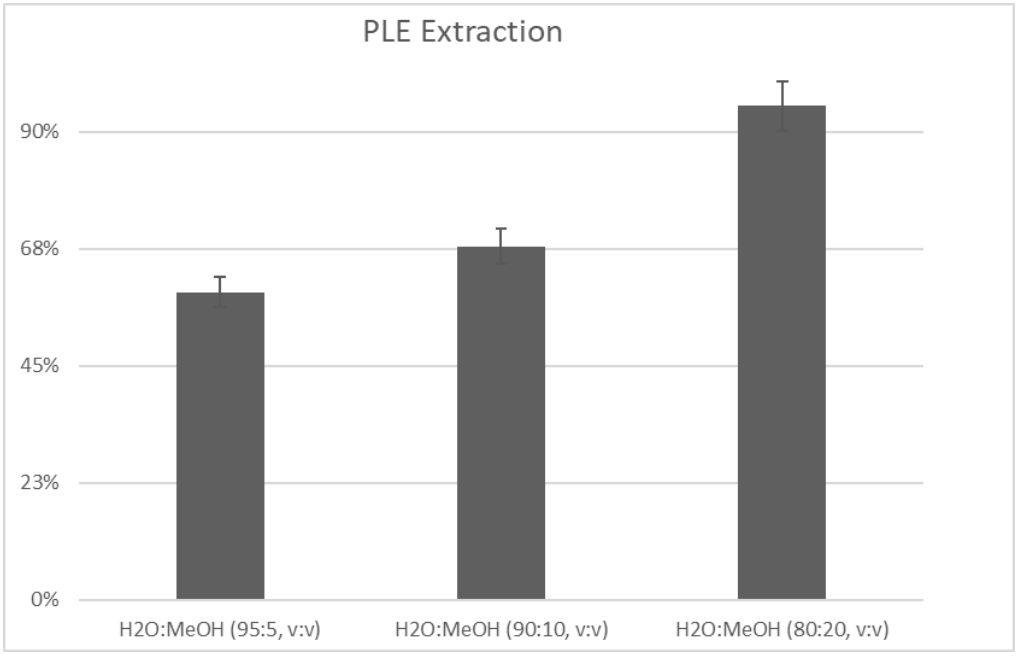

PLE Extraction
90%
68%
45%
23%
0%
H2O:MeOH (95:5, v:v)
H2O:MeOH (90:10, v:v)
H2O:MeOH (80:20, v:v)

Figure 2

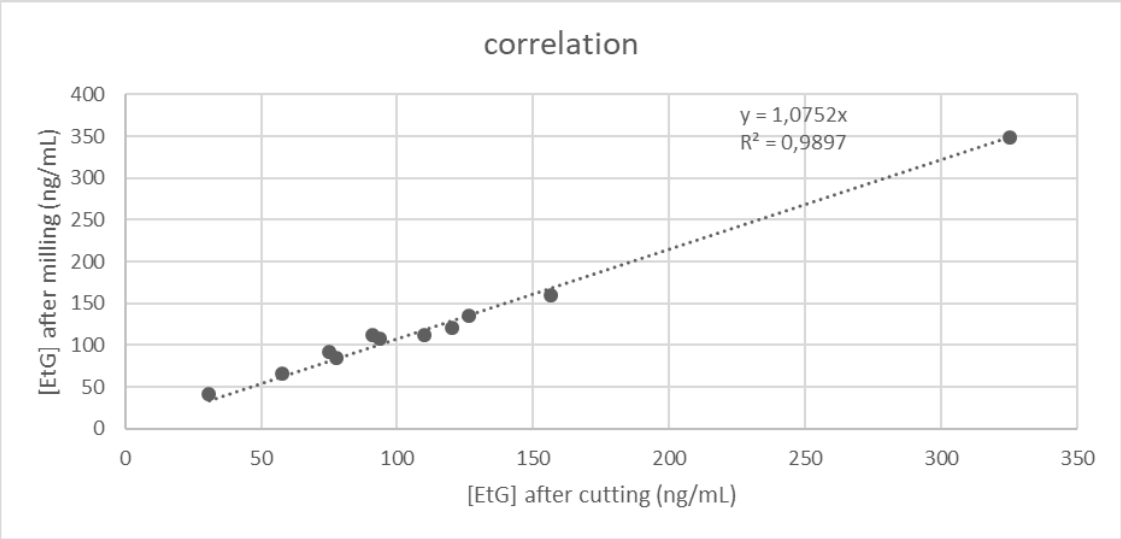

correlation
y = 1,0752x
R² = 0,9897
[EtG] after milling (ng/mL)
[EtG] after cutting (ng/mL)
0
50
100
150
200
250
300
350
400

Figure 3

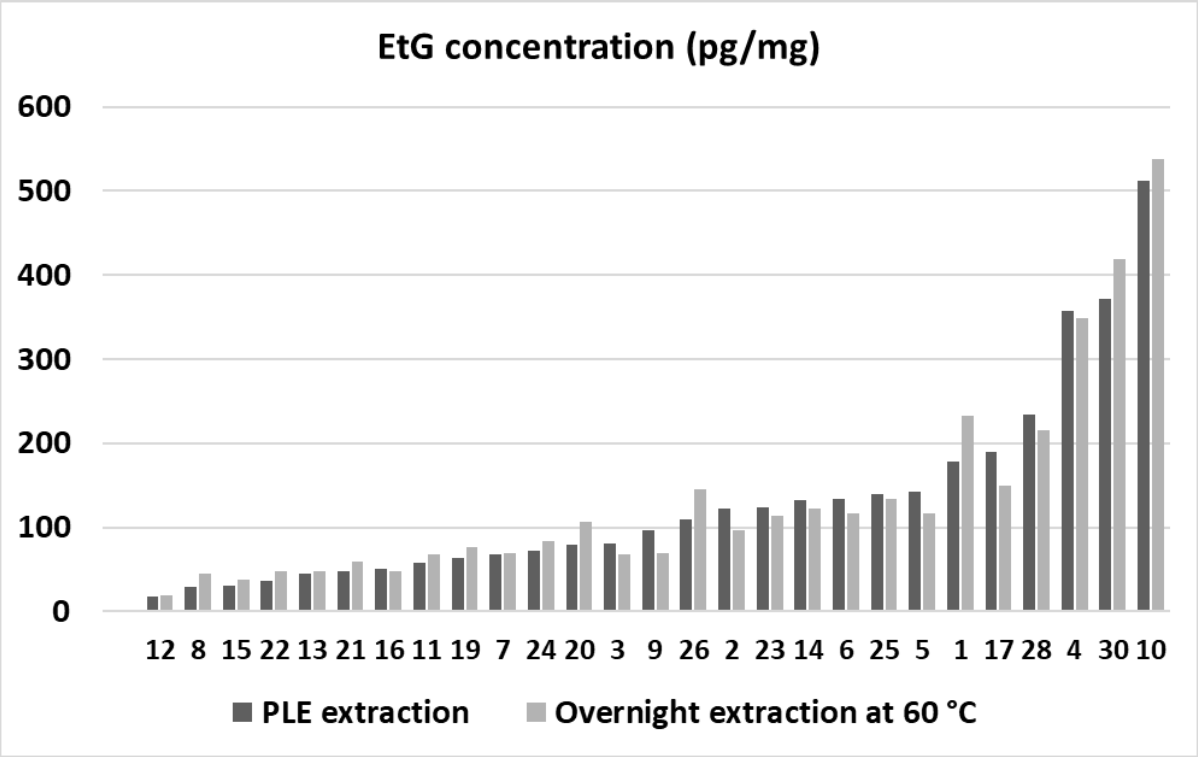
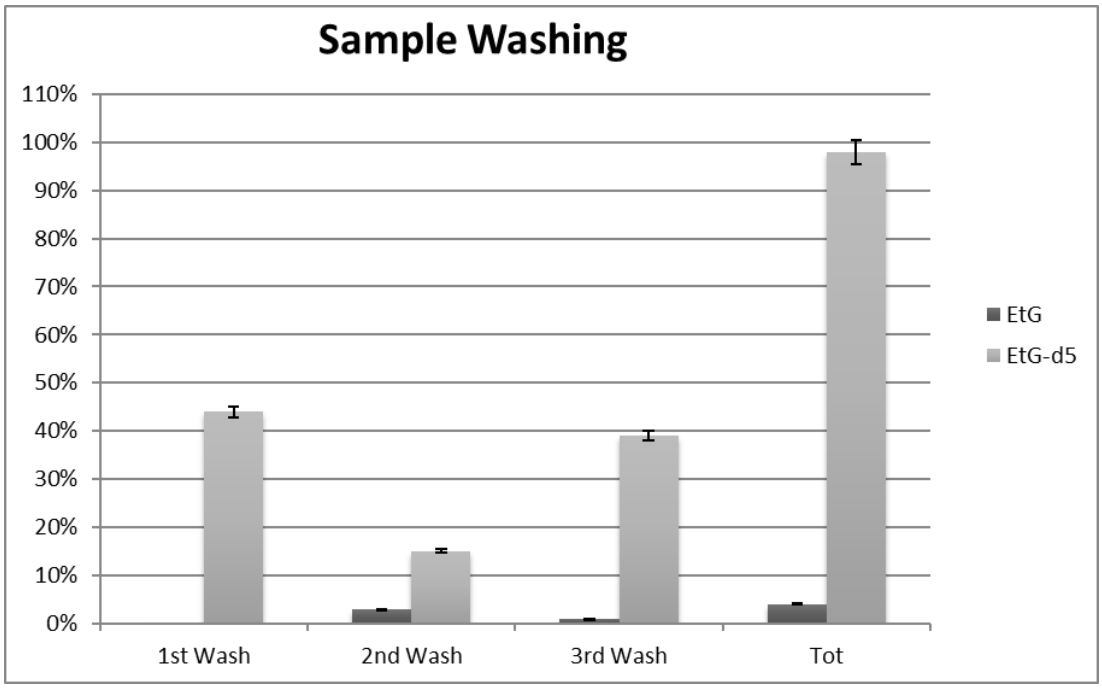

EtG concentration (pg/mg)
600
500
400
300
200
100
0
12 8 15 22 13 21 16 11 19 7 24 20 3 9 26 2 23 14 6 25 5 1 17 28 4 30 10
PLE extraction
Overnight extraction at 60 °C

Figure 4


Sample Washing
110%
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
1st Wash
2nd Wash
3rd Wash
Tot
EtG
EtG-d5