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# Determination of ethyl glucuronide levels in hair for the assessment of alcohol abstinence

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ABSTRACT

This study examined the potential of a highly sensitive LC–MS/MS method for the determination of EtG in head hair (i) to ascertain alcohol abstinence, (ii) to estimate the basal level of EtG (sub-ppb concentrations) in head hair in a population of alcohol abstainers and (iii) to suggest a revision of cut-off values for assessing alcohol abstinence. An UHPLC–MS/MS protocol previously developed was modified and validated again to detect low EtG levels in head hair samples from a population of 44 certain abstainers and teetotalers. Basal level of EtG in hair was determined by a standard addition quantification method. The validated UHPLC–MS/MS method allowed detecting and quantifying 0.5 and 1.0 pg/mg of EtG in hair, respectively. EtG concentrations lower than 1.0 pg/mg were determined for 95% of abstainers; 30% of them had non-detectable (<0.5 pg/mg) EtG values. Two samples evidenced EtG concentrations higher than 1.0 pg/mg that were subsequently explained by unintentional ethanol exposure. The method's feature of high analytical sensitivity makes it particularly suitable for alcohol abstinence ascertainment and, in the same time, allows to tentatively estimate basal EtG concentrations in hair around $0.8 \pm 0.4$ pg/mg. This finding opens a discussion on the possible origin of basal EtG concentration and potential sources of bias in the evaluation of alcohol abstinence. Cut-off value in the range of 1.0–2.0 pg/mg can be reliably proposed to support alcohol abstinence.

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## 1. Introduction

The concentration of ethyl glucuronide (EtG), a direct alcohol metabolite, in the keratin matrix proved to be a powerful meter of alcohol consumption [1–4]. Its analytical determination is consistently requested within clinical and forensic investigations to monitor chronic excessive alcohol consumption [5] or, conversely, to ascertain alcohol abstinence, for several purposes: workplace testing, driving license reissue/renewal, child custody, divorce proceeding [6], post-mortem or pre-natal alcohol exposure [7–9], withdrawal treatment [10], liver transplantation [11].

The quantitative determination of EtG in hair (HEtG) provides long-term retrospective information, either to differentiate acceptable ethanol intake from hazardous or harmful misuse, or to prove total abstinence. Therefore, analytical protocols should provide high sensitivity, extended quantitation capability, precision, and accuracy. Many research efforts have been addressed to refine existing methods for EtG determination in hair [12], so as to (i) improve validation protocols and consider all potential sources of bias and uncertainty, (ii) propose simpler and faster analytical protocols, with reduced analysis-time and sample preparation steps, and (iii) enhance the analytical performances to accurately quantify minimal EtG hair concentrations, taking advantage of the continuous improvement of chromatographic and tandem mass spectrometric (MS/MS) instrumentation.

Both GC–MS/MS and LC–MS/MS are suitable techniques for EtG determination: background interferences are minimized by the double mass-selection stages and optimal detection capability is provided, generally in the pg/mg range. Even though the GC–MS and GC–MS/MS procedures proved to be accurate and highly sensitive [13–18], most toxicology laboratories currently prefer LC–MS/MS methods, because they are faster and no derivatization is needed. Among these, different sample preparation steps, hair amounts and chromatographic conditions were adopted [19–28].

Currently, ever-decreasing limits of detection and quantification (LOD and LOQ) are requested to reliably support the ascertainment of alcohol abstinence through the analysis of EtG in hair. Precisely, the Society of Hair Testing (SoHT) proposed that EtG should be the first choice among direct biomarkers for this purpose [11]. Furthermore, the adoption of methods with LOQ values $\leq$3 pg/mg is recommended, since "a concentration $\geq$7 pg/mg in the 0–3 up to 0–6 cm proximal scalp hair segment strongly suggests repeated alcohol consumption. A lower concentration is

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not in contradiction to the self-reported abstinence of a person during the corresponding time period before sampling" [11]. As a matter of fact, a HEtG concentration of 7 pg/mg was proposed by the German Society of Toxicological and Forensic Chemistry (GTFCh) as first tentative cut-off to verify abstinence [29,30]. It is worth noting that German drivers whose license has been suspended are requested to completely abstain from alcohol consumption in order to reissue/renew their driving license. Nevertheless, HEtG concentrations lower than 7 pg/mg were still found in hair from subjects who consumed 16 g ethanol/day for 3 months, and concentrations ranging from 5 to 11 pg/mg were found in subjects who consumed 32 g alcohol per day [27]. Another study reported EtG concentrations in hair samples <8 pg/mg after an average consumption of 3.1 g alcohol/day (IQR: 0.7–13.6 g alcohol/day) [31]. According to these results, much lower hair EtG concentrations are likely to be expected for real abstinent rather than 7 pg/mg [11]. However, a basal HEtG concentration is not to be excluded, since small amounts of ethanol are possibly produced by metabolic biotransformation of food, or may arise from exogenous sources, other than alcoholic beverages [4,32]. Remarkably, several studies confirmed that the presence of alcohol itself, for example in cosmetic or hygiene products, does not cause external contamination, since EtG is not formed in vitro; conversely, specific products containing EtG can significantly contaminate negative hair samples, as it has been recently verified [16]. Therefore, it would be helpful to estimate this basal hair EtG level, in order to suggest a cut-off value which might be reliably used to verify alcohol abstinence.

Aim of the present work was to modify and validate again a previously developed UHPLC–MS/MS protocol [33], for detection of low EtG levels in hair, so that it could be adopted for alcohol abstinence ascertainment. Hair samples from a selected population of abstainers and teetotalers were analyzed to investigate if traces of EtG not deriving from alcoholic beverages could be detected or even quantified, so as to propose a valuable cut-off value to test alcohol abstinence by hair analysis.

## 2. Materials and methods

### 2.1. Chemical, reagents and standard solutions

EtG and ethyl glucuronide-$d_5$ (EtG-$d_5$), used as internal standard (IS), were acquired from Medichem (Stuttgart, Germany). Standard solutions of EtG and EtG-$d_5$ were prepared in methanol at 10 mg/mL concentration and stored at −20 °C; working solutions were prepared by progressive dilution. CHROMASOLV® acetonitrile, methylene chloride, and methanol, and formic acid were obtained from Sigma–Aldrich (Milan, Italy). All chemicals and reagents were of analytical purity grade. Ultra-pure water was obtained using a Milli-Q UF-Plus apparatus (Millipore, Bedford, MA, USA).

### 2.2. Hair samples treatment

The treatment of hair samples was performed using a standard procedure with only minor modifications [33]. Briefly, all hair samples were washed twice using methylene chloride and methanol (3 mL, 3 min) in sequence and then dried. Each sample was cut into small pieces (1–2 mm length) and weighted. About 50 mg of hair was added with EtG-$d_5$ (10 pg/mg final concentration) and 500 μL of a 35:1 (v/v) water/methanol mixture. Then, the samples were centrifuged (4000 rpm, 5 min) and incubated overnight at room temperature. Fifteen h later, after ultra-sonication with an UCI-150 Ultrasonic Cleaning Bath (Raypa®, Ankara, Turkey) for 90 min, 100 μL of the liquid phase was transferred into a clean vial for UHPLC–MS/MS analysis.

The chance of inducing partial EtG degradation by sonolysis (potentially critical at low EtG concentrations) was verified by exposing EtG solutions to ultrasonic treatment for 0, 30, 60 and 90 min, followed by the addition of the internal standard and quantification. From these experiments, sonolytic degradation of EtG could be excluded.

### 2.3. UHPLC–MS/MS method

Analyses were performed by injecting 3 μL of hair extract into a Shimadzu Nexera 30 UHPLC-system (Shimadzu, Duisburg, Germany) interfaced to an AB Sciex API 5500 triple quadrupole mass spectrometer (AB Sciex, Darmstadt, Germany) with an electrospray source operating in the negative (ESI−) ion mode. An Acquity UPLC® BEH C18 column 100 mm × 2.1 mm i.d. × 1.7 μm (Agilent Technologies, Italy), protected by a C18 guard column, was used for UHPLC separation. The column oven was maintained at +50 °C. Elution solvents were water/formic acid 5 mM (solvent A) and acetonitrile/formic acid 5 mM (solvent B). The mobile phase eluted under the following linear gradient conditions (A:B; v/v): from 97:3 to 96:4 in 1.0 min, then to 30:70 in 2.0 min, followed by isocratic elution at 70% B for 1.0 min. The flow rate was 0.5 mL/min and total run time was 5.5 min, including re-equilibration at the initial conditions between consecutive injections. EtG and EtG-$d_5$ were eluted in about 1 min. Data were recorded at unit mass resolution in the selected reaction monitoring (SRM) mode, using nitrogen as the collision gas. Mass transitions for EtG and EtG-$d_5$ were previously selected and described [33]. All the other mass spectrometer parameters, namely, declustering potential (DP), entrance potential (EP), collision offset voltage (CE), cell exit potentials (CXP), and scan time for ion acquisition, source block temperature and ion-spray voltage were specifically optimized.

### 2.4. Method validation

The following parameters were investigated: selectivity, specificity, linearity range, LOD, LOQ, matrix effect, trueness, intra- and inter-assay precision. Identification criteria for the analyte were established according to national [34] and international guidelines [35,36]. Blank head hair from certain teetotalers (two employees from our laboratory) was used to prepare all validation experiments and calibration samples. Retention time ($t_R$) precision was determined at 1.0, 2.5, and 5 pg/mg EtG concentrations. Deviations of 1–2% from calibrators and controls are acceptable for LC-based assays. One qualifying transition was monitored ($m/z$ 221 > 85), in addition to the primary fragmentation ($m/z$ 221 > 75). Variations of relative transition intensities were considered acceptable within ±20%, with respect to the control. Their repeatability was determined on five spiked blank head hair samples at the same concentration levels.

Selectivity was determined on ten blank head hair samples spiked with 1.0, 2.5 and 5.0 pg/mg EtG concentrations. The signal-to-noise ratio (S/N > 3) was measured on both mass transition at the expected EtG retention time. Analogous check was made on blank samples spiked with EtG-$d_5$, at 10 pg/mg concentration to verify that this isotopically-labeled standard did not contain a significant concentration of the non-labeled EtG as an impurity, which could bias quantitative determinations of EtG, including LOD evaluation.

Linearity was checked in 1.0–10.0 pg/mg EtG concentration range (1.0, 2.5, 5.0, 7.5 and 10.0 pg/mg), using EtG-$d_5$ as the internal standard. The linear calibration parameters were obtained using the least squares regression method. The squared correlation coefficient ($R^2$) was utilized to roughly estimate linearity. The appropriateness of the linear model was assessed by defining residuals and examining residual plots. The assumption of homoscedasticity, as well as the significance of slope and intercept of the regression line, was successfully verified.

LOD was estimated as the analyte concentration whose response provided S/N = 3, as determined by the least abundant ion. LOD numerical value was extrapolated from S/N value of the lowest concentration level (LCL) using the calibration curve. The noise was measured from ±0.05 min before the peak onset till the beginning of the peak. The LOD was indirectly confirmed within the standard addition quantification method, by verifying the presence of a peak with S/N > 3 in the non-spiked aliquot, for samples with extrapolated EtG concentration almost equal to the LOD. LOQ was set as the double of LOD [37,38] and was confirmed from the low-concentration aliquots spiked at 1.0 pg/mg and re-analyzed to verify the concentration increment. Moreover, validation experiments at 1.0 pg/mg for intra-assay precision and trueness evaluation confirmed the reliability of this LOQ value.

Trueness, intra- and inter-assay precision were evaluated on ten blank head hair samples spiked with 1.0, 2.5 and 5.0 pg/mg EtG concentrations and expressed as CV% and percent bias, respectively. Satisfactory intra- and inter-assay precision and trueness are expected to lie within ±15%.

Due to the low concentration range investigated, the carry-over phenomenon was not evaluated in the present study. Likewise, the extraction recovery from blank hair samples spiked at 30 pg/mg EtG concentration was determined in our previous study [33]. At very low EtG concentration, spiking experiments are meaningless, since the unknown EtG content inside the hair matrix should have to be added to the spiked amount, resulting in biased extraction recovery results. Considerations about the matrix effect [39] are reported below.

### 2.5. Study protocol

The 44 subjects involved in this study are listed in Table 1. Almost half of them were children (mean age ± SD: 5 ± 2 years, range: 1–12 years) representing a population of teetotalers. The other subjects were adults (mean age ± SD: 32 ± 17 years, range: 21–80 years) either teetotalers or abstainers who at least refrained from consuming alcohol for the last 12 months. Selection of subjects was made very carefully to involve only those with a perfectly known alcohol abstinence history. Personal interview declarations and medical history data were also collected, as well as information about frequent use of products containing ethanol.

Only the head hair samples were collected from each subject, none of which had undergone cosmetic treatments. Hair was cut as close as possible to the scalp, using

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