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

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 ± 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.

Keywords

- UHPLC–MS/MS;
- Ethyl glucuronide;
- Hair;
- Alcohol abstinence

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], [2], [3] and [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],[8] and [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], [14], [15], [16], [17] and [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], [20], [21], [22], [23],[24], [25], [26], [27] and [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 ≤3 pg/mg is recommended, since “a concentration ≥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 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] and [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] and [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] and [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] and [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.

Table 1.

Description of main characteristics for each subject included in the study protocol.

Case	Gender	Age (years)	Provenience	Hair color	Head hair (cm)	Analyzed hair (cm)	Category[a]	Note
1	M	5	Morocco	Black	1.5	1.5	T	Elementary school student
2	F	7	Morocco	Black	2.0	2.0	T	Elementary school student
3	M	6	Morocco	Black	4.5	4.5	T	Elementary school student
4	M	6	Romania	Black	3.0	3.0	T	Elementary school student
5	F	5	Italy	Brown	5.5	5.5	T	Elementary school student
6	M	7	Morocco	Black	2.0	2.0	T	Elementary school student
7	M	6	Romania	Brown	5.5	5.5	T	Elementary school student
8	F	7	Morocco	Black	5.5	5.5	T	Elementary school student
9	F	5	Italy	Blond	5.0	5.0	T	Elementary school student
10	M	6	Italy	Blond	6.5	6.0	T	Elementary school student
11	M	2	China	Black	3.0	3.0	T	Nursery school student
12	F	2	Italy	Black	2.5	2.5	T	Nursery school student
13	F	3	Italy	Blond	4.5	4.5	T	Nursery school student
14	F	1	Italy	Blond	3.0	3.0	T	–
15	M	5	Italy	Black	3.0	3.0	T	Elementary school student
16	F	4	Italy	Brown	6.0	6.0	T	Nursery school student
17	M	4	Italy	Blond	6.0	6.0	T	Nursery school student

Case	Gender	Age (years)	Provenience	Hair color	Head hair (cm)	Analyzed hair (cm)	Category[a]	Note
18	M	4	Italy	Black	2.0	2.0	T	Nursery school student
19	F	n.d.	Italy	Red	20	3.0	A (1 year)	Alcohol Abuse Treatment Service
20	M	45	Italy	Gray	4.0	3.0	T	Worker at a specialized industry of electronic devices
21	M	57	Italy	Black	5.0	3.0	A (1 year)	Alcohol Abuse Treatment Service
22	F	23	Italy	Blond	16.0	3.0	T	Bachelor student at Turin University
23	F	22	Italy	Black	20	3.0	T	Bachelor student at Turin University
24	M	21	Italy	Brown	23	3.0	T	Bachelor student at Turin University
25	F	22	Italy	Brown	20	3.0	T	Bachelor student at Turin University
26	F	27	Italy	Brown	25	3.0	T	Research assistant at Turin University
27	M	28	Italy	Black	8.0	3.0	T	Research assistant at Turin University
28	F	25	Italy	Black	17	3.0	T	Master student at Turin University
29	M	26	Italy	Brown	3.5	3.0	T	Master student at Turin University
30	F	27	Italy	Brown	28	3.0	T	Ph.D. student at Turin University
31	F	22	Italy	Blond	11.5	3.0	T	Bachelor student at Turin University
32	F	28	Italy	Red	38	3.0	T	Research assistant at

Case	Gender	Age (years)	Provenience	Hair color	Head hair (cm)	Analyzed hair (cm)	Category[a]	Note
								Turin University
33	F	80	Italy	Gray	3.0	3.0	T	–
34	F	n.d.	Italy	Gray	2.0	2.0	T	–
35	F	24	Italy	Red	23	3.0	T	Bachelor student at Turin University
36	M	12	Italy	Black	8.0	6.0	T	Junior high school student
37	M	6	Italy	Black	5.0	5.0	T	Elementary school student
38	M	4	Morocco	Black	1.0	1.0	T	Nursery school student
39	F	5	Morocco	Black	1.0	1.0	T	Elementary school student
40	F	7	Romania	Black	1.5	1.5	T	Elementary school student
41	M	5	Italy	Brown	2.0	2.0	T	Nursery school student
42	F	1	Italy	Brown	5.5	5.5	T	–
43	F	4	Italy	Blond	4.5	4.5	T	Nursery school student
44	M	–	Italy	Brown	2.0	2.0	A (2 years)	Alcohol Abuse Treatment Service

n.d. = data not available.

[a]T = teetotaler; A = abstainer form alcohol consumption.

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 freshly disinfected (with alcohol-free disinfectant) scissors. The samples were stored in closed containers at room temperature until analysis. Only the proximal segments 0–6 cm and 0–3 cm for children and adults, respectively, were analyzed whenever a longer head hair sample was collected. The amount of hair that could be collected from most children hair was limited. Therefore, it was necessary to analyze the segment 0–6 cm in order to obtain a sufficient weight of specimen. Unlike adults, children are not likely to take cosmetic treatments or consume alcoholic beverages, making their

hair samples hypothetically homogenous for longer length (6 cm rather than 3). Shorter head hair samples were analyzed in their full length.

The study protocol was approved by the recognized Ethics Committee at the San Luigi Gonzaga University Hospital (Turin, Italy). All adults and children's parents/tutors gave a written informed consent before attending the study, voluntarily.

2.6. EtG quantitative determination

For seven subjects providing a limited amount of hair, only one aliquot was prepared and analyzed, just by adding EtG-d_5 as the internal standard (10 pg/mg). For the other 37 subjects, the not-spiked hair sample (about 50 mg) plus other 3–5 aliquots (according to the available quantity of hair) spiked at different HEtG final concentrations (1.0, 2.5, 5.0, 7.5 and 10 pg/mg) were analyzed and quantified by the standard addition method (SAM). This quantification method represents the standard procedure whenever a true blank cannot be obtained (i.e., when a physiological level has to be determined) and generally allows to extrapolate extremely low concentrations, even below the nominal LOD/LOQ. Moreover, the standard addition quantification method has the ability to overcome the problem of matrix effects, that modify the analytical sensitivity (i.e., the slope of the calibration curve) and consequently bias the quantitative results. Noteworthy, the quantification made by extrapolation (usually adopted for SAM) is less precise than interpolation, as largely remarked in the scientific literature [40]. In our study, the SAM was used as the best approach to extrapolate hypothetically low concentrations and tentatively estimate a value for basal EtG hair concentration. From this value and its range of variability, it is possible to deduce an appropriate cut-off value for alcohol abstinence assessment.

Unweighted linear regression lines were determined. The ratio of the chromatographic peak areas for the SRM transitions m/z 221 > 75 and 226 > 75, respectively, were plotted on the Y-axis with the ratio between EtG and EtG-d_5 concentrations on the X-axis. The X-intercept of the standard addition plot represents the extrapolated concentration of EtG present in each sample. The standard error for the predicted $x_0$concentration was calculated according to Miller et al. [41].

The precision (expressed as CV%) of the regression line slopes was compared in different subjects as an indicator of the relative matrix effect. CV% smaller than 3–4% indicated that the method can be considered free from relative matrix effect liability. The slope difference (expressed as percentage difference between the highest and the lowest slope values, divided by the lowest value) was also calculated [42].

For case 14, quantitative results by the standard addition method are not reported in Table 2, since HEtG concentration proved to be higher than 10 pg/mg. Thus, the UHPLC–MS/MS protocol used in our routine laboratory work (validated in accordance with the ISO/IEC 17025:2005 criteria and periodically verified in its quantitative performances by regular participation to international

laboratory proficiency tests) for the identification of excessive chronic alcohol consumption was used to definitively quantify HEtG in hair (results presented in Table 2), as specified in Section 3.4.

Table 2. Linear equations, correlation coefficients and EtG hair concentration results (with relative standard error) from standard addition quantification method.

Case	Number of aliquots[a]	Average hair weight (mg)	Linear equation	R^2	Extrapolated EtG concentration (pg/mg)
1	6	51.24	$y = 0.855x + 0.058$	0.9986	0.7 ± 0.3
2	6	49.37	$y = 0.854x + 0.075$	0.9954	0.9 ± 0.6
3	4	50.41	$y = 0.814x + 0.087$	0.9979	1.1 ± 0.8
4	6	50.04	$y = 0.820x + 0.084$	0.9961	1.0 ± 0.6
5	6	50.15	$y = 0.874x + 0.044$	0.9984	0.5 ± 0.3
6	6	51.58	$y = 0.860x + 0.054$	0.9992	0.7 ± 0.3
7	6	49.87	$y = 0.852x + 0.060$	0.9991	0.7 ± 0.3
8	4	50.44	$y = 0.925x + 0.046$	0.9999	0.5 ± 0.2
9	4	50.77	$y = 1.020x + 0.027$	0.9995	0.3 ± 0.4
10	6	51.34	$y = 0.877x + 0.042$	0.9982	0.4 ± 0.4
11	6	50.44	$y = 0.819x + 0.086$	0.9960	1.0 ± 0.6
12	6	49.58	$y = 0.857x + 0.057$	0.9960	0.7 ± 0.5
13	4	50.30	$y = 0.966x + 0.043$	0.9995	0.5 ± 0.4
14	1	50.84	See Section 3.4		17
15	4	50.28	$y = 0.655x + 0.141$	0.9962	2.1 ± 1.3
16	6	51.20	$y = 0.847x + 0.062$	0.9984	0.7 ± 0.3
17	6	50.17	$y = 0.809x + 0.149$	0.9930	1.8 ± 0.8
18	5	50.39	$y = 0.974x + 0.098$	0.9995	1.0 ± 0.2
19	6	48.13	$y = 0.840x + 0.066$	0.9980	0.8 ± 0.4
20	4	49.85	$y = 0.748x + 0.136$	0.9997	1.8 ± 0.3
21	6	47.84	$y = 0.866x + 0.053$	0.9979	0.6 ± 0.4
22	6	49.59	$y = 0.887x + 0.028$	0.9997	0.3 ± 0.1
23	6	50.32	$y = 0.888x + 0.028$	0.9995	0.3 ± 0.2
24	6	50.12	$y = 0.877x + 0.036$	0.9995	0.4 ± 0.2
25	6	51.36	$y = 0.888x + 0.028$	0.9995	0.3 ± 0.2
26	6	49.90	$y = 0.861x + 0.048$	0.9982	0.6 ± 0.4
27	6	47.79	$y = 0.810x + 0.085$	0.9801	1.1 ± 1.3
28	6	50.19	$y = 0.880x + 0.043$	0.9995	0.5 ± 0.2
29	6	49.55	$y = 0.844x + 0.060$	0.9943	0.7 ± 0.6
30	6	50.38	$y = 0.867x + 0.049$	0.9997	0.6 ± 0.2

Case	Number of aliquots[a]	Average hair weight (mg)	Linear equation	R^2	Extrapolated EtG concentration (pg/mg)
31	6	47.63	$y = 0.854x + 0.062$	0.9992	0.7 ± 0.2
32	6	50.25	$y = 0.822x + 0.087$	0.9954	1.1 ± 0.6
33	6	50.49	$y = 0.882x + 0.055$	0.9971	0.6 ± 0.5
34	6	49.17	$y = 0.920x + 0.073$	0.9989	0.8 ± 0.3
35	6	50.18	$y = 0.604x + 0.031$	0.9825	0.5 ± 1.1
36	6	49.83	$y = 0.776x + 0.072$	0.9967	0.9 ± 0.5
37	6	51.09	$y = 0.950x + 0.099$	0.9970	1.0 ± 0.4

[a]In case of 4 aliquots, spiked samples with final EtG concentrations of 5.0 and 7.5 pg/mg were not prepared. In case of 5 aliquots, the EtG spiking at 7.5 pg/mg final concentration was skipped.

3. Results

3.1. UHPLC–MS/MS method validation

Linear calibration was observed for EtG in the range from 1.0 pg/mg to 10 pg/mg, with a squared correlation coefficient (R^2) of 0.999. All the back calculations of standards were within 5% at each calibration level. Selectivity and specificity tests proved successful. SRM chromatograms from both positive and negative head hair samples showed no interfering signals at the EtG retention time. Moreover, no peaks in the SRM profiles for EtG were observed in blank samples spiked with EtG-d_5. Trueness, intra- and inter-assay precision were satisfied, as the percent bias and the CV% were lower than 5.0% at 1.0, 2.5 and 5.0 pg/mg (Table 3). On real blank samples, LOD and LOQ values were 0.5 pg/mg and 1.0 pg/mg respectively (Table 3), in agreement with SoHT consensus for alcohol abstinence assessment which recently fixed the minimum quantitative performances for such an investigation (LOQ values ≤3 pg/mg) [11]. The same experimental LOD and LOQ values were also found for EtG-d_5, whose endogenic occurrence in blank samples is excluded. Fig. 1 shows the SRM chromatograms of a blank hair sample spiked with EtG at the LOQ value (Fig. 1a) and a blank hair samples with undetectable EtG concentration (i.e., <LOD, S/N < 3) (Fig. 1b). The signal of EtG-d_5 at 10 pg/mg is also shown (Fig. 1c).

Table 3. Method validation: intra- and inter-assay precision, trueness.

Compound	LOD (pg/mg)	LOQ (pg/mg)	Matrix effect[a]	Conc. (pg/mg)	Intra-assay PR (CV%)	Inter-assay PR (CV%)	TR (bias %)
EtG	0.5	1.0	9.0%	1.0	3.5	3.0	+3.7
				2.5	2.8	3.5	+4.1
				5.0	2.1	5.0	−1.1

[a]Coefficient of variation of the regression line slopes for the standard addition quantification method.

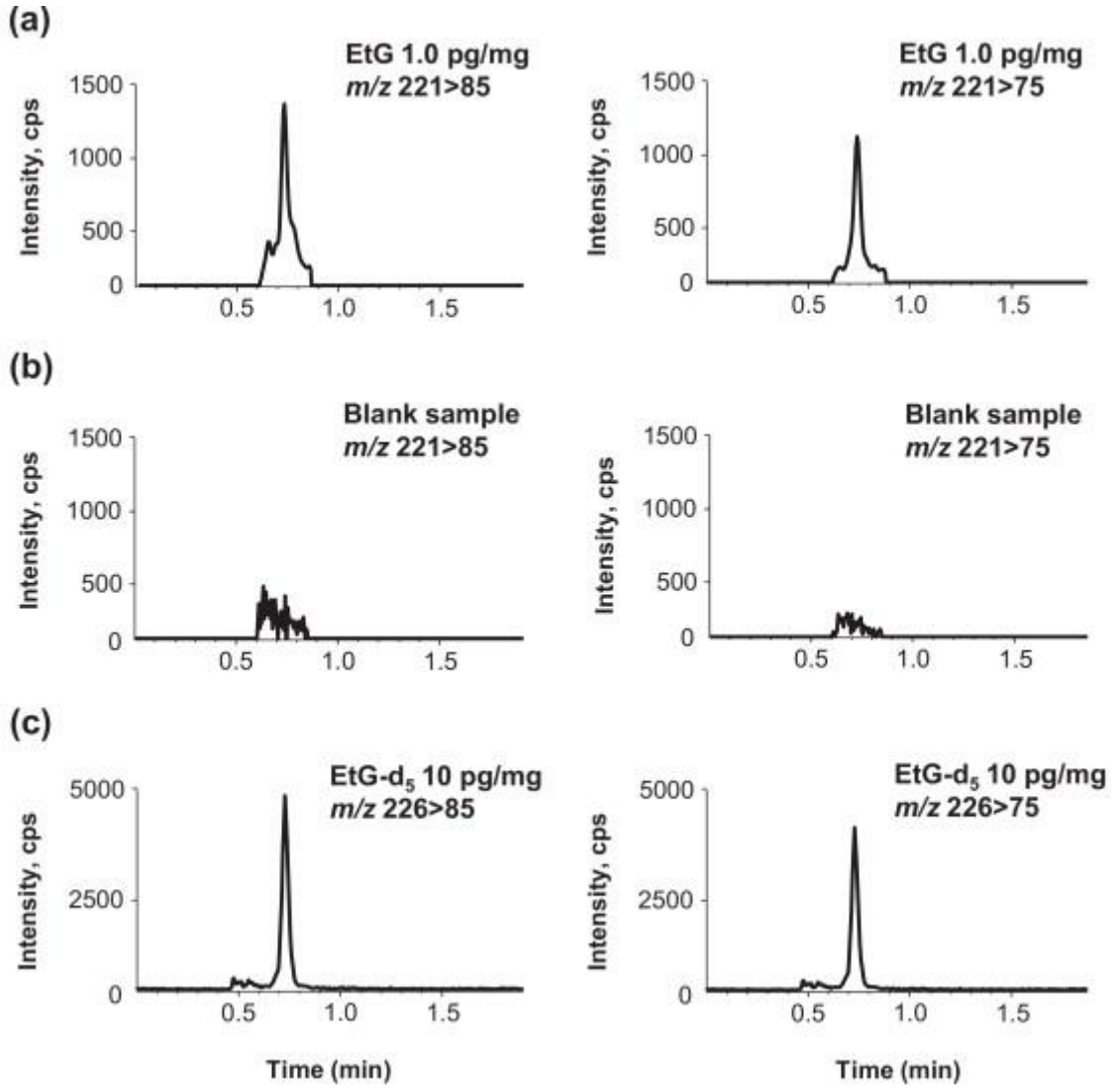

Fig. 1. (a) SRM chromatograms from a hair blank sample spiked with EtG at 1.0 pg/mg concentration: transitions *m*/*z* 221 > 85 (left) and *m*/*z* 221 > 75 (right). (b) SRM chromatograms from a hair blank sample with undetectable (S/N < 3) concentration of background EtG concentration: *m*/*z* 221 > 85 (left) and *m*/*z* 221 > 75 (right). (c) SRM

chromatogram from a hair blank sample spiked with EtG-d_5 at 10 pg/mg: transitions *m/z* 226 > 85 (left) and *m/z* 226 > 75 (right).

Remarkably, the developed method provides LOD and LOQ values comparable with those of other highly sensitive GC–MS methods [4], [31], [43] and [44], with the advantage of greatly simplified sample pre-treatment steps.

3.2. EtG concentrations in abstainers and teetotalers

Table 2 reports the quantitative EtG results obtained from the standard addition method, the number of aliquots analyzed and their average weight. Cases reported as 38–44 yielded EtG results below the LOD. Tentative quantification with the standard addition method was not attempted because the available quantity of hair was not sufficient (therefore, the cases are not reported in Table 2). Fig. 2 shows the standard addition plots from two selected subjects (cases 4 and 17), yielding concentrations slightly above the LOD and around the LOQ value, respectively.

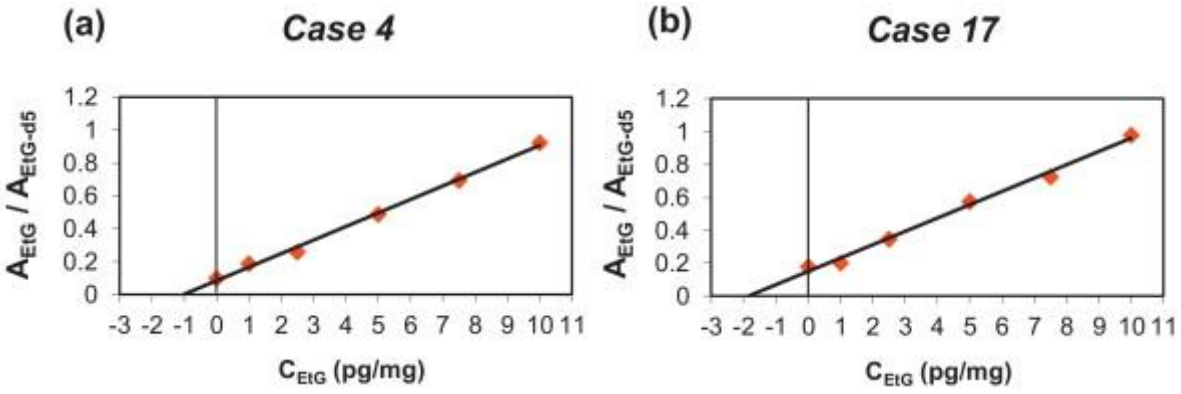

Fig. 2. Experimental points and SAM regression lines for the EtG quantification of hair samples relative to (a) case 4 and (b) case 17.

Among the 44 subjects studied, 13 (30%) proved to have EtG hair concentration lower than the LOD value (S/N < 3), most of which have an extrapolated EtG concentration of about 0.2–0.4 pg/mg. The remaining 31 cases (70%) presented an EtG concentration equal to or greater than the LOD value (S/N ≥ 3); among them, 3 cases presented EtG concentrations equal or higher than the LOQ, even after considering the standard error associated with the results. Among children, case 17 presented an EtG concentration of 1.8 ± 0.8 pg/mg. The hair sample was collected from a male of 4 years (Table 1), from which no information on possible alcohol exposure could be drawn. A striking result relates to case 14, described extensively below, that has to be considered as an “outlier” in this context. Among adults, the highest EtG value was 1.8 ± 0.3 pg/mg, obtained from

case 20, namely a 45-year-old worker in charge of electronic device cleaning in an electronics industry. He declared a daily professional use of both ethanol and isopropyl alcohol.

Linearity was verified in the 1.0–10 pg/mg EtG concentration range by means of the internal standard calibration, but the last aliquots prepared for the SAM (i.e., samples spiked with EtG at 10 pg/mg, without considering the initial concentration) slightly exceeded the higher quantitation limit (10 pg/mg). Therefore, the appropriateness of the linear regression model used to extrapolate the basal EtG concentration was verified again for each sample (as reported in Section 2.4), and double-checked by means of the internal standard calibration.

For all the samples (excluded cases 38–44), the average extrapolated EtG concentration was 0.7 ± 0.3 pg/mg (mean ± SD), calculated without considering the subjects with EtG concentrations ≥LOQ (cases 14, 17 and 20), or 0.8 ± 0.4 pg/mg, if only the case 14 is excluded. Remarkably, 95% of abstainers and teetotalers (41 out of 43 – excluding case 14) presented an EtG concentration equal or lower than 1.0 pg/mg (LOQ value), i.e., significantly lower than 7 pg/mg. These results partially support other independent experimental results. For example, Imbert et al. reported HEtG concentrations lower than 1 pg/mg (LOD value) for 10 selected teetotalers [28]. Kharbouche et al. reported extrapolated HEtG concentrations of 0.6 ± 2.1 (mean ± SD; range: 0–10 pg/mg) pg/mg versus 4.9 ± 6.2 pg/mg for a population of teetotalers and low-risk drinkers, who reported an alcohol consumption lower than 20/30 g/day (women/men), respectively. Even in this case, some low-risk drinkers had HEtG concentrations lower than the LOD (2 pg/mg), meaning that an ultimate cut-off value unequivocally separating teetotalers from low-risk drinkers, cannot be reasonably fixed, but values much lower than 7 pg/mg can surely be suggested [44].

3.3. Evaluation of relative matrix effect

For all subjects, with the exception of 14 and 38–44, the standard addition method was used for quantification. Extrapolated EtG concentrations and relative standard errors are reported in Table 2, as well as slopes, intercepts and R^2 values of the regression lines. In theory, all slopes should be alike, which is not the case: regression line slopes show a variation coefficient of 9.0% (Table 3), even if 26 slopes out of 36 fall inside a restricted 0.850 ± 5% interval. This variability can plausibly be attributed to a significant matrix effect, partly counterbalanced by the utilization of the isotopically-labeled internal standard [39] and [42]. In fact, the use of isotope-labeled internal standard should reduce (or eliminate) matrix effect liability, within the assumption that isotopic purity and stability issues are addressed. The occurrence of some matrix effect in the present UHPLC–ESI-MS/MS protocol was expected, because its optimization was focused on high speed and productivity, although maintaining high sensitivity and detection capability.

3.4. Case 14

A three-cm-long strain was collected from the hair (never cut after birth) of a one-year-old female in April 18, 2012. She weighed 10 kg at sampling time and was about 81 cm tall. An EtG hair concentration of 17 pg/mg was found. This result was subsequently confirmed with a routine protocol for EtG quantification, working in the linearity range 10–300 pg/mg. The extremely young age of this child, in comparison to other children involved in this study, offered possible explanation for this anomalous analytical outcome: (i) breast-feeding after maternal ethanol consumption, (ii) pre-natal alcohol exposure, and (iii) intensive use of specific hygiene ethanol-containing products for newborns, such as lotions or products for teething pain. Eventually, the mother reported that a therapy against asthenia with an L-arginine supplement was prescribed to her child, which required daily administration for one month. The therapy started 2 months before hair sampling. Each oral administration of a 20 mL glass bottle contained 1.66 g of L-arginine, plus excipients: sucrose, 5 g; citric acid, 0.65 g; methyl *p*-hydroxybenzoate, 18 mg; propil *p*-hydroxybenzoate, 2 mg; ethanol 96°, 0.5 mL; strawberry flavor, 20 mg; in distilled water. Thus, the child consumed an absolute ethanol volume of 15 mL in one month. This intake might justify the experimental evidence of 17 pg/mg hair EtG concentration, although no literature data exist on correlation between alcohol intake and EtG levels in small weight children.

4. Discussion

The determination of EtG in hair is commonly employed to ascertain both alcohol abuse and abstinence, using different cut-off values. However, cut-off values for abstinence have been fixed on the basis of the analytical method's detection capability, rather than the assessment of endogenous levels of EtG in hair. As a matter of fact, the basal concentration of HEtG in teetotalers and abstainers has never been determined, since its value generally lies below the LOD of the adopted analytical protocols. This indefinite EtG basal concentration can hypothetically arise from metabolic biotransformation of food or minute presence of ethanol itself in food other than alcoholic beverages. For example, a small amount of ethanol is added in many packaged snacks as a preservative and softener ingredient.

Whenever the LOD value was used as the decision threshold to support alcohol abstinence assessment, negative results were likely to overestimate the number of abstainers, as was confirmed by studies of controlled alcohol consumption [27].

In the present study, a specific UHPLC–MS/MS method was used for the determination of basal HEtG concentration, and the consequent highly-reliable recognition of alcohol abstinence. The LOD value of 0.5 pg/mg appeared to be sufficiently low to detect the basal EtG level in the hair samples from most certain abstainers and teetotalers, even if 30% of the studied population (13/44) still provided results below this LOD (S/N ≤ 3). An alternative approach to evaluate the LOD was utilized, based on the quantitative determination of isotopically-marked EtG, spiked on

"blank" samples at various concentration levels. EtG-d_5 has almost identical chromatographic behavior than EtG and, consequently, is likely to be subjected to similar matrix effects. On the other hand, its SRM transitions are corresponding but not identical to those of EtG, presumably resulting in similar response factors (signal), but potentially different background noise. As a matter of fact, LOD and LOQ values for EtG-d_5 turned out to be the same as for EtG.

In this study, we used the conservative standard addition method to experimentally verify the LOD value and extrapolate a hypothetical basal EtG concentration in real samples. It is evident that the application of the standard addition method for quantification is highly time-consuming and a significant quantity of hair is required. Therefore, this procedure can hardly be used in routine to ascertain alcohol abstinence, although it proved to be essential for the specific purpose of this study. Once a tentative cut-off for abstinence is established, the classical quantification method using deuterated EtG as the internal standard needs to be stressed, in order to gain the requested detection capability.

5. Conclusions

Outcomes of the present study encourage the adoption of highly sensitive analytical protocols, at least capable to break down the 1.0 pg/mg detection limit, in order to accurately determine HEtG for abstinence assessment. In spite of the small number of participants, the preliminary conclusions of the present study open a debate on the possible existence of a basal HEtG concentration in the sub-ppb range and the possible sources of bias due to ethanol-containing food, prescriptions, cosmetic and body-care products. These results should be confirmed on a larger number of abstainers and teetotalers.

For the alcohol abstinence assessment, our experimental data suggest that a revision of the existing cut-off value is advisable and that a cut-off as low as 1.0 pg/mg can reliably be proposed, at least for clinical purposes. Meanwhile, prudently higher values (about 2.0 pg/mg) can be maintained for legal and forensic controversies, until consistent independent results will be collected to support the selection of a cut-off value at such a low concentration.

Remarkably, EtG should be the first choice among direct biomarkers in abstinence assessment [11]. Whenever the cut-off value is exceeded, careful case history has to be collected from the subject under investigation, before making the diagnosis. In doubtful cases, for mutual confirmation and exclusion of false positive and false negative results, the determination of other parameters such as fatty acid ethyl esters (FAEE) can be useful [11]. This implies also that the subjects requested to prove alcohol abstinence should be warned about the use of daily products containing ethanol, since their consumption could actually interfere with the analysis.

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