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**Systematic optimisation of ethyl glucuronide extraction conditions from scalp hair by design of experiments and its potential effect on cut-off values appraisal**

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**Systematic optimization of ethyl glucuronide extraction conditions from scalp hair by design of experiments and its potential effect on cut-off values appraisal**

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Abstract:	The quantitative determination of ethyl glucuronide (EtG) in hair samples is consistently used throughout the world to assess chronic excessive alcohol consumption. For administrative and legal purposes, the analytical results are compared with cut-off values recognized by regulatory authorities and scientific societies. However, it has been recently recognized that the analytical results depend on the hair sample pretreatment procedures, including the crumbling and extraction conditions. A systematic evaluation of the EtG extraction conditions from pulverized scalp hair was conducted by design of experiments (DoE) considering the extraction time, temperature, pH, and solvent composition as potential influencing factors. It was concluded that an overnight extraction at 60°C with pure water at neutral pH represents the most effective conditions to achieve high extraction yields. The absence of differential degradation of the internal standard (isotopically-labeled EtG) under such conditions was confirmed and the overall analytical method was validated according to SGWTOX and ISO17025 criteria. Twenty real hair samples with different EtG content were analyzed with three commonly accepted procedures: (a) hair manually cut in snippets and extracted at room temperature; (b) pulverized hair extracted at room temperature; (c) hair treated with the optimized method. Average increments of EtG concentration around 69% (from a to c) and 29% (from b to c) were recorded. In light of these results, the authors urge the scientific community to undertake an inter-laboratory study with the aim of defining more in detail the optimal hair EtG detection method and verifying the corresponding cut-off level for legal

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3 1 **Systematic optimization of ethyl glucuronide extraction conditions from scalp**  
4 **hair by design of experiments and its potential effect on cut-off values appraisal**

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9 4 Eugenio Alladio<sup>a,b</sup>, Giulia Biosa<sup>b</sup>, Fabrizio Seganti<sup>a</sup>, Daniele Di Corcia<sup>a</sup>, Alberto Salomone<sup>a</sup>,  
10  
11 5 Marco Vincenti<sup>a,b,\*</sup>, Markus R. Baumgartner<sup>c</sup>

12  
13 6 *a Centro Regionale Antidoping "A. Bertinaria", Regione Gonzole 10, 10043 Orbassano, Turin,*  
14 *Italy*

15  
16 8 *b Dipartimento di Chimica, Università degli Studi di Torino, via P. Giuria 7, 10125 Turin, Italy*

17  
18 9 *c Zurich Institute of Forensic Medicine, University of Zurich, Kurvenstrasse 17, 8006 Zurich,*  
19 *Switzerland*

20  
21  
22  
23 12 **Corresponding author:**

24 13 Prof. Marco Vincenti

25  
26 14 Dipartimento di Chimica, Università degli Studi di Torino,

27  
28 15 Via Pietro Giuria, 7 – 10125 Torino, Italy

29  
30 16 Phone: +390116705264 – Mobile: +393474198878

31  
32 17 E-mail: marco.vincenti@unito.it

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38 21 **Short title:** Dependence of hair EtG cut-off appraisal on the extraction conditions

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**Abstract**

The quantitative determination of ethyl glucuronide (EtG) in hair samples is consistently used throughout the world to assess chronic excessive alcohol consumption. For administrative and legal purposes, the analytical results are compared with cut-off values recognized by regulatory authorities and scientific societies. However, it has been recently recognized that the analytical results depend on the hair sample pretreatment procedures, including the crumbling and extraction conditions. A systematic evaluation of the EtG extraction conditions from pulverized scalp hair was conducted by design of experiments (DoE) considering the extraction time, temperature, pH, and solvent composition as potential influencing factors. It was concluded that an overnight extraction at 60°C with pure water at neutral pH represents the most effective conditions to achieve high extraction yields. The absence of differential degradation of the internal standard (isotopically-labeled EtG) under such conditions was confirmed and the overall analytical method was validated according to SGWTOX and ISO17025 criteria. Twenty real hair samples with different EtG content were analyzed with three commonly accepted procedures: (a) hair manually cut in snippets and extracted at room temperature; (b) pulverized hair extracted at room temperature; (c) hair treated with the optimized method. Average increments of EtG concentration around 69% (from a to c) and 29% (from b to c) were recorded. In light of these results, the authors urge the scientific community to undertake an inter-laboratory study with the aim of defining more in detail the optimal hair EtG detection method and verifying the corresponding cut-off level for legal enforcements.

## 49 Introduction

50 Ethyl glucuronide (EtG) is a minor phase II metabolite of ethanol normally produced after  
51 consumption of alcoholic beverages. It can be analytically detected in urine, blood, and oral fluid in  
52 order to ascertain recent alcohol intake<sup>[1]</sup>. Moreover, EtG is nowadays extensively used as an  
53 effective biomarker to assess prolonged abstinence or chronic excessive alcohol consumption,  
54 provided that its determination is made on a keratin matrix, typically scalp hair<sup>[2]</sup>. The applications  
55 of EtG determination in hair range from compliance to driving regulation<sup>[3]</sup> to workplace testing<sup>[4,5]</sup>  
56 and many other usages of clinical and forensic interest<sup>[6]</sup>. Hair samples different from scalp hair can  
57 cautiously be analysed, in case scalp hair is not available or is degraded<sup>[7-9]</sup>. In practice, the  
58 effectiveness of EtG determination in hair as a biomarker for chronic excessive alcohol  
59 consumption outperforms all the other alcohol biomarkers<sup>[10-12]</sup> to the extent that is frequently used  
60 as a unique laboratory testing. However, several studies have shown that bias can be induced by  
61 cosmetic treatments<sup>[13]</sup> exposure to chlorinated water<sup>[14]</sup>, external contamination by EtG-containing  
62 lotions<sup>[15]</sup> and many others<sup>[16]</sup>. The practical convenience of hair EtG as a biomarker explains its  
63 widespread use, which account for its hundreds of thousands of yearly determinations worldwide,  
64 mainly used for driving license renewal and rehabilitation, and workplace testing.

65 The Society of Hair Testing (SoHT) established cut-off values for hair EtG concentration that  
66 supports judgments of chronic excessive alcohol consumption (30 pg/mg) and non-contradiction  
67 with self-reported abstinence (7 pg/mg). These cut-off values were originally determined on the  
68 basis of several prevalence and observational studies, meta-analyses, and prudential  
69 considerations<sup>[2]</sup>. Previous SoHT consensus documents<sup>[17]</sup> are regularly updated and, in the most  
70 recent issue, the SoHT also recommends to “powder hair prior to the extraction of EtG”<sup>[18,19]</sup>.  
71 Several studies supported the conclusion that higher EtG extraction yields are obtained if the hair  
72 aliquot is pulverized in a mill instead of being manually cut into small snippets<sup>[20-23]</sup>. Recently,  
73 another study proved that also the choice of the extraction solvent and temperature significantly  
74 affected the EtG extraction yield from hair and its detected concentration<sup>[24]</sup>. In particular, the study  
75 demonstrated that EtG extraction with water is more effective than with methanol and conducting  
76 the extraction at 60°C provides more exhaustive recovery than at ambient temperature<sup>[24]</sup>. Notably,  
77 previous SoHT consensus documents prescribed well-defined cut-off values and criteria for their  
78 interpretation, but provided very little hints about the analytical and instrumental methods, leaving  
79 to the specialist the choice and demonstration of equivalence with the best practices. Clearly, if the  
80 extraction yield, and consequently the EtG detected concentration, depend on the hair sample  
81 pretreatment, then also the cut-off values becomes questionable<sup>[23]</sup>.

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3 82 In the present study, we took up the proposal of Mueller and coworkers<sup>[24]</sup> of using multifactorial  
4 83 experimental design to investigate the dependence of EtG extraction on several experimental factors  
5 84 and expanded it further, with closer sampling of the experimental domain and consecutive  
6 85 modelling. In general, Design of Experiment (DoE) strategies allows to reduce the experimental  
7 86 effort and simultaneously increase the quality of obtained information<sup>[25–27]</sup>. In our case, DoE was  
8 87 exploited to obtain robust interpretation of the factors that impact on EtG extraction yield and their  
9 88 reciprocal interactions. Then, the optimized analytical method was validated with a stepwise,  
10 89 analyst-independent protocol. Lastly, we measured the combined effect of hair milling and optimal  
11 90 extraction conditions with respect to previous analytical procedures on a series of real hair samples  
12 91 and discuss the consequences in the forensic toxicology context.  
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## 93 **Materials and Methods**

### 94 Analytical Method

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26 95 The determination of EtG in hair samples was initially performed via a UHPLC–MS/MS method  
27 96 (a) that was validated according ISO/IEC 17025 criteria<sup>[28]</sup>, accredited in 2013<sup>[29]</sup>, and subsequently  
28 97 revised in 2016<sup>[23]</sup> (subsequently referred to as method (b)), when the hair sample pre-treatment  
29 98 procedure was modified according to the superior efficiency of the milling technique<sup>[23]</sup> with  
30 99 respect to the previous method (a) of cutting hair into small segments before the extraction step<sup>[29]</sup>.  
31 100 In summary, our initial analytical method (b) – which was submitted to systematic optimization in  
32 101 the present study - applied the following steps:  
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- 38 102 1. the collected hair samples (about 40-50 mg) corresponding to the proximal 0–3 cm segments  
39 103 were weighted and then washed twice using methylene chloride and methanol in sequence;
  - 40 104 2. the dried hair were pulverized in a Polypropylen Co-Polymer (PPC) tube using a metal  
41 105 beads mill Precellys 24 Tubes Homogenizer (Bertin Pharma, France), equipped with six 2.8  
42 106 mm metal beads;
  - 43 107 3. internal standard (IS, EtG-D<sub>5</sub> at 100 pg/mg final concentration) was added;
  - 44 108 4. EtG extraction was performed overnight at room temperature (ca. 20°C) with a 35:1  
45 109 water:methanol (v/v) mixture;
  - 46 110 5. lastly, the sample was sonicated and an aliquot of the liquid phase was transferred into a vial  
47 111 for UHPLC–MS/MS analysis, performed by injecting 3 µL of hair extract into a Shimadzu  
48 112 Nexera 30 UHPLC-system (Shimadzu, Duisburg, Germany) interfaced to an AB Sciex API  
49 113 5500 triple quadrupole mass spectrometer (AB Sciex, Darmstadt, Germany).
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3 114 At the end of the optimization work, the extraction conditions were modified as follows (method (c)  
4 115 – as subsequently referred to):

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6 116 4. EtG extraction was performed with 500  $\mu$ L of pure water, overnight at 60°C;

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9 117 In the present study, each hair batch was extensively mixed, homogenized, decontaminated, and  
10 118 dried, following the procedure described above. The milling procedure was executed on about 50  
11 119 mg of sample. Then, 500  $\mu$ L of different extraction solvents/mixtures (according to the planned  
12 120 DoE) and 5  $\mu$ L of internal standard EtG-D<sub>5</sub> (using a working solution of 1 ng/ $\mu$ L in methanol) were  
13 121 added. A short centrifugation (1.5 min, 13300 rpm, 17000 x g – VWR Micro Star, Leuven,  
14 122 Belgium) was executed to completely submerge the hair material within the extraction solvent and  
15 123 to remove air bubbles. The extractions were performed within a laboratory stove at different  
16 124 temperatures and extraction times in accordance with the drafted DoE plans. The stove temperature  
17 125 was controlled immediately before and after the extraction period. Lastly, an ultra-sonication of 1.5  
18 126 hours was performed and 100  $\mu$ L of the liquid phase was transferred into a clean vial to be analysed  
19 127 by UHPLC-MS/MS. Further details about instrumental conditions are available in our previous  
20 128 publications<sup>[23,29]</sup>.

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31 130 Hair specimens

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33 131 The hair samples used in this study were collected from anonymized residual specimens, stored and  
34 132 available in our laboratory, formerly belonging to individuals who underwent hair analysis before  
35 133 October 2015, with resulting EtG values higher than the validated limit of quantitation (LOQ) of the  
36 134 UHPLC-MS/MS method (i.e., 1 pg/mg). Samples with EtG values higher than 12 pg/mg were  
37 135 specifically selected, in order to avoid any misinterpretation of DoE results due to the higher  
38 136 uncertainty of the lower points of the calibration curves. In particular, DoE was performed on two  
39 137 large batches of hair samples: the first one (A) contained only hair locks with EtG concentrations  
40 138 comprised between 13 pg/mg and 20 pg/mg, while the second one (B) contained the specimens with  
41 139 EtG values comprised between 40 pg/mg and 100 pg/mg (namely, the ones collected from subjects  
42 140 identified as excessive alcohol drinkers). The reason to consider two batches at different EtG  
43 141 concentrations in the DoE plans was to evaluate the results at EtG levels considered respectively  
44 142 above and below the 30 pg/mg cut-off suggested by the Society of Hair Testing<sup>[17,18]</sup>. All hair  
45 143 samples used in the present study had been originally analysed before the introduction of the  
46 144 milling protocol in our laboratory (i.e., October 2015). The concentration intervals indicated above  
47 145 refer to the original pre-treatment procedure<sup>[29]</sup>.



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147 Design of Experiments (DoE)

148 According to a previous study<sup>[24]</sup>, the choice of the extraction solvent and temperature turned out as  
149 the most significant factors that influence the EtG extraction yield from hair. The first DoE (i.e.  
150 Preliminary DoE) set-up examined in detail these two parameters. A second DoE (i.e. Optimization  
151 DoE) set-up also considered the pH of the solvent and the extraction time as valuable factors to be  
152 examined. In detail, the first DoE consisted in a 2-factors full-factorial design performed by varying  
153 the extraction temperature (the first factor, T) and the composition of the extraction mixture (the  
154 second factor). Four levels were selected for the first factor, namely 20°C, 32°C, 45°C, and 57°C,  
155 and three compositions (levels) for the second factor, respectively distilled water, water/methanol  
156 35:1 (v/v) and water/methanol 17:1 (v/v). Since four and three levels were evaluated for extraction  
157 temperature and extraction solvent, respectively, and each experiment was replicated three times, a  
158 total number of 36 experiments ( $4 \times 3 \times 3 = 36$ ) was executed on each batch (A and B), and 72  
159 experiments overall. A geometric representation of the Preliminary DoE is shown in Figure 1a,  
160 where each point on the square represents one experiment. The levels of the evaluated extraction  
161 temperatures were coded from -1.5 up to +1.5 (i.e., -1.5, -0.5, +0.5, +1.5 for 20°C, 32°C, 45°C,  
162 and 57°C, respectively), while the codes for the different extraction mixture were -1, 0, +1 for pure  
163 distilled water, water/methanol 17:1, and water/methanol 35:1, respectively. The experiments were  
164 executed in random order, and coded as reported in Table S1 of the Supplementary Material.

165 The Optimization DoE was planned with a face-centred central composite design (corresponding to  
166 15 different experimental conditions), where extraction temperature, extraction time and solvent pH  
167 (distilled water) were selected as variable factors. Three levels were chosen for all factors following  
168 the results obtained from the first DoE: (i) 45°C, 54°C and 63°C for the extraction temperature (T),  
169 (ii) 1 h, 8 h and 16 h (overnight) hours for the extraction time (t), and (iii) 5.5, 7.0 and 8.5 for the  
170 pH of the extraction solvent (water). Acidic and basic pH values were obtained by adding HCl and  
171 NaOH 0.1 M, respectively, and the pH constancy at the end of the extraction was positively  
172 verified. Since each experiment was performed in triplicate for both batch A and B, a total number  
173 of 90 experiments ( $15 \times 3 \times 2 = 90$ ) was executed in the second DoE. All levels were coded from  
174 -1 to +1. A geometric representation of the performed DoE is shown in Figure 1b. Again, the  
175 experiments were performed in random order and the respective codes are reported in Table S2 of  
176 the Supplementary Material. For validation purposes, 10 replicates were completed for both batches  
177 at the end of the study, at the experimental conditions identified as optimal according to the  
178 response surfaces of a Multiple Linear Regression (MLR) model.

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### 180 Differential degradation of the internal standard

181 Since the second DoE tested extraction conditions at relatively high temperatures (i.e., much higher  
182 than the traditional room temperature), the occurrence of differential degradation of the internal  
183 standard (EtG-D<sub>5</sub>) was evaluated at the new experimental settings. The experiments were carried  
184 out by adding 5 µL of EtG-D<sub>5</sub> to 500 µL of pure water (the novel extraction solvent). Afterwards,  
185 different combinations of five extraction temperatures (room temperature, 35°C, 45°C, 55°C and  
186 65°C) and three extraction time (1, 8 and 16 hours) were tested in triplicate, for an overall of 45  
187 experiments. Boxplots and Kernel Density Estimation (KDE) curves were calculated to interpret the  
188 results.

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### 190 Method validation

191 The new analytical method was validated using a stepwise, analyst-independent protocol that  
192 required the preparation of seven independent calibration curves<sup>[30,31]</sup>, prepared in three different  
193 days, at seven calibration levels: 2, 5, 10, 30, 50, 100, and 300 pg/mg. Most validation parameters  
194 were determined from these data, including linearity range, limit of detection (LOD), limit of  
195 quantification (LOQ), selectivity, specificity, trueness, accuracy, repeatability and carry-over effect,  
196 in accordance with ISO/IEC 17025 and SWGTOX requirements<sup>[28,32]</sup>. At first, the linearity  
197 parameter was investigated by initially evaluating the homo-/heteroscedasticity of the data,  
198 followed by the estimation of the order (linear or quadratic), and weight (1, 1/x or 1/x<sup>2</sup>) of the  
199 calibration curve. The linearity was checked by lack-of-fit and Mandel tests<sup>[33,34]</sup>. Determination  
200 coefficient (R<sup>2</sup>), relative standard deviation of the slope, normality of the standardized residuals,  
201 and deviation from back-calculated concentrations were also evaluated using in-house spreadsheets,  
202 package mvtnorm<sup>[35,36]</sup>, and the routines developed by B. Desharnais et al.<sup>[30,31]</sup>. LOD and LOQ  
203 were estimated by the Hubaux-Vos algorithm<sup>[37]</sup>.

204 Specificity was assessed by analysing seven blank head samples from acknowledged teetotaller  
205 individuals; in particular, the presence/absence of interfering ions on each single-ion  
206 chromatograms was evaluated, with reference to EtG pure standard. Then, the data collected for the  
207 preparation of the seven calibration curves were used to evaluate selectivity, accuracy, trueness,  
208 intra-assay precision, and repeatability. In particular, the data collected for a specific calibration  
209 curve were quantified by using a different calibration curve, prepared the same day or in a previous  
210 day (i.e., simulating our routine approach to test the method prior of a working session). This

211 procedure allowed us to manage each set of data as independent. Therefore, 7 samples (from 7  
212 batches) per each calibration level were utilized to evaluate the validation parameters previously  
213 cited. The consistency of EtG retention time was successfully verified for all 49 (7×7) samples, as  
214 well as the relative intensities of the characteristic ions. Trueness, and intra-assay precision were  
215 estimated as percent bias and CV%, respectively. Satisfactory results were expected to be within  
216 ±15% for the 1<sup>st</sup> and 4<sup>th</sup> calibration levels (i.e., 2 and 30 pg/mg) and within ±20% for the the 7<sup>th</sup>  
217 calibrator (i.e., 300 pg/mg). The repeatability was determined at the 1<sup>st</sup>, 4<sup>th</sup> and 7<sup>th</sup> calibration levels;  
218 moreover, Shapiro-Wilk, Dixon, and Grubbs tests were performed to investigate the Gaussian  
219 distribution of the data and the occurrence of outliers. Finally, the occurrence of carry-over effect  
220 was tested by injecting one distilled water sample after the highest point of each calibration curve  
221 (i.e., 300 pg/mg), for seven times; the appearance of unintended EtG signal was supposed not to  
222 exceed the 10% of the signal of the lowest calibrator (i.e., 2 pg/mg).

223

#### 224 Matrix effect

225 A final investigation was conducted on matrix effects in order to evaluate possible differences  
226 between manual cutting and mill-pulverisation of the hair specimen. Matrix effect was evaluated  
227 from six replicates by comparing the experimental results from neat aqueous solutions spiked with  
228 EtG at three concentration levels (low level = 10 pg/mg, mid-level = 50 pg/mg, and high-level =  
229 300 pg/mg), with the data obtained from negative hair samples (collected from 1-3 years old  
230 children) that underwent milling or manual cutting procedures, then spiked at the same levels after  
231 the extraction step. The matrix effect for each pre-treatment procedure was expressed as the  
232 percentage ratio between the measured concentrations.

233

#### 234 Comparison of real samples

235 At the end of the optimization process, the new protocol was compared with the previous validated  
236 method<sup>[23]</sup>, involving the manual cutting of hair locks into 2-3 mm snippets on a set of real hair  
237 samples. Two batches of 10 hair samples each were analysed: the first batch (C) contained only  
238 samples with EtG values originally detected in the range between 20 pg/mg and 31 pg/mg, while  
239 batch D included specimens with EtG values above 60 pg/mg. Afterwards, boxplots, t-test and  
240 ANOVA test were performed to compare the analytical results.

241

## 242 Software

243 All statistical analyses were conducted using the software R Studio version 1.0.153<sup>[38]</sup>, while DoE  
244 interpretation was performed with an R package developed by the Italian Group of Chemometrics  
245 of the Italian Society of Chemistry (SCI), freely available on internet<sup>[39]</sup>, in the 3.1.0 version<sup>[40]</sup>.

## 247 **Results and Discussion**

### 248 Design of Experiment – preliminary plan

249 The goal of the first DoE plan was to test our validated analytical method in the light of the results  
250 obtained by Mueller et al.<sup>[24]</sup>, who suggested to carry out the EtG extraction on pulverised hair at 60  
251 °C (instead of room temperature) with pure water as the extraction solvent. Taking advantage of  
252 their differentiation between influencing and non-influencing experimental factors<sup>[24]</sup>, we decided to  
253 focus the study on the significant ones (temperature and solvent) and develop a DoE based on a  
254 more detailed tuning of these selected parameters. In the same time, we verified the inter-laboratory  
255 repeatability of their conclusions. In particular, four temperature levels were checked (from ambient  
256 to 57 °C) and three solvent compositions, in which methanol is used at low percentages (0%, 2.7%,  
257 and 5.6%) as a modifier of the prevalent aqueous constituent.

258 From the  $2 \times 36$  experiments carried out within the first DoE, the ratio of the target analyte area to  
259 the IS provided the corresponding “extracted” EtG concentrations, assuming that the recovery of the  
260 analyte from the real matrix was variable while that of the spiked EtG-D<sub>5</sub> was complete. All the  
261 analytical results are reported in Table S1 of the Supplementary Material for both batches A and B.  
262 According to the full-factorial design, a multiple linear regression (MLR) model was calculated  
263 with reference to the following equation:

$$y = b_0 + b_1x_1 + b_2x_2 + b_{12}x_1x_2 + b_{11}x_1^2 + b_{22}x_2^2$$

264 where  $y$  represents the concentration of the extracted EtG (pg/mg),  $b_i$  represent the regression  
265 coefficient (0 = intercept, 1 = coefficient relative to the extraction temperature, 2 = coefficient  
266 relative to the extraction solvent) and  $x_i$  stands for the evaluated parameters (1 = the extraction  
267 temperature, 2 = the extraction solvent). The quadratic terms for both temperature ( $x_1^2$ ) and solvent  
268 composition ( $x_2^2$ ) were evaluated, too. From the model regression, Figure 2 displays the values of  $b$   
269 coefficients and the relative significance of each factor, as determined by a t-test. The coefficient  
270 plot reported in Figure 2 is relative to the batch A (13-20 pg/mg) and shows that the extraction  
271 temperature is the most significant factor that affects the EtG extraction (p-value < 0.001), with a

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2  
3 272 positive trend (i.e., the higher the temperature, the higher the extraction yield). Analogous  
4 273 conclusions were drawn from the coefficient plot relative to the batch B (40-100 pg/mg) reported in  
5 274 Figure 1 of the Supplementary Material), although the significance level of the temperature factor is  
6 275 lower (p-value < 0.05). The latter diagram also shows a significant negative influence of the  
7 276 quadratic term relative to the extraction temperature ( $x_{11}$ ), which suggests, at the current stage, to  
8 277 regulate the extraction temperature at a relatively high but not extreme value. The two-dimensional  
9 278 response surfaces reported in Figure 2 (Batch A) and Figure S1 of the Supplementary Material  
10 279 (Batch B) confirms the conclusions of Mueller and co-workers<sup>[24]</sup>: the maximum response value for  
11 280 both batches A and B was observed in the lower-right sections of the graph, indicating that the  
12 281 highest extraction yield was reached when (i) only water was employed as the extraction solvent  
13 282 (coded as -1.0 on the y-axis), along with (ii) an extraction temperature setting in the range between  
14 283 45°C and 57°C (coded as 0.5 and 1.5 on the x-axis).

284

#### 285 Design of Experiment – optimization plan

286 The need of accurate temperature adjustment together with the opportunity to reduce the extraction  
287 time inspired the second DoE scheme, which also investigated the solvent pH as a potential  
288 influencing factor. According to the conclusions reported above, the second DoE was planned using  
289 water as the extraction solvent, and a restricted interval of extraction temperatures was tested,  
290 ranging from 45°C to 63°C, with a 9°C interval step. The pH of the aqueous solvent was varied  
291 from slightly acidic to slightly basic (5.5, 7.0, and 8.5) while the extraction time tested both day-  
292 time and overnight conditions (1 h, 8 h, and 16 h) for practical reasons. In the present case, a face-  
293 centred central composite design was selected because its experimental space covered a wide range  
294 of useful setting, including the extreme conditions for all parameters. To interpret the data,  
295 optimization of a MLR model with the following formula is suggested:

$$y = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_{12}x_1x_2 + b_{13}x_1x_3 + b_{23}x_2x_3 + b_{11}x_1^2 + b_{22}x_2^2 + b_{33}x_3^2$$

296 where  $y$  and  $b_i$  have the same meaning as in the preceding section, and  $x_i$  stands for the evaluated  
297 parameters (1 = extraction temperature, 2 = extraction time, 3 = solvent pH). The quadratic terms  
298 for all factors, namely the extraction temperature ( $x_1^2$ ), time ( $x_2^2$ ) and pH ( $x_3^2$ ) were also evaluated.  
299 Figure 3 reports the histogram values of  $b$  coefficients and their significance: in the present case,  
300 both the extraction temperature and time proved highly significant for the extraction efficiency of  
301 EtG (p-value < 0.001), with positive trends, while the pH of the solvent does not appear to have an  
302 influence, at least within the tested range. Likewise, the interaction and quadratic terms of the

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3 303 model show relatively large variance and consequently no significance. The overall conclusion is  
4 304 that increasing both extraction temperature and time - independently from one another - results into  
5 305 an increase of the extraction yield. Analogous deductions were drawn from the coefficient plot  
6 306 relative to the batch B, reported in Figure S2 of the Supplementary Material. It must be noted that a  
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8 307 significant, positive contribution of the quadratic  $x_{11}$  term (i.e. the extraction temperature) has been  
9 308 observed for batch B. Nevertheless, we do not accredit substantial importance to this positive  
10 309 contribution (partly evident also for batch A) because quite large variance is associated to both the  
11 310 interaction and quadratic terms and an opposite - yet not significant - negative contribution was  
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13 311 detected in the preliminary DoE.

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18 312 Maximum response value for both batches A and B was observed in the upper-right parts of the  
19 313 response surface graphs, at the point encoded as [1, 1, 0] (see Figure 3 for batch A, and Figure S2 of  
20 314 the Supplementary Material for batch B). This means that the highest extraction yield for EtG was  
21 315 reached when simultaneously (i) the extraction temperature was set at 63°C (coded as 1.0 on the x-  
22 316 axis), and (ii) the extraction time was set at 16 hours/overnight (coded as 1.0 on the y-axis). In  
23 317 contrast, all the response surfaces provided similar behaviour at any pH value tested (all the  
24 318 response surfaces are reported in Figure S3 of the Supplementary Material), confirming pH as a  
25 319 non-significant factor. In the subsequent experiments, neutral pH of the aqueous solvent was  
26 320 consistently used, for simplicity.

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31 321 At the end of the second DoE plan, both the observed and estimated results indicated that the  
32 322 optimization of the extraction conditions produced a significantly higher EtG extraction yield.  
33 323 Consequently, the original method was modified by substituting pure distilled water as the  
34 324 extraction solvent in place of a 35:1 water:methanol (v/v) mixture, and employing an overnight  
35 325 extraction temperature of 60°C, instead of room temperature. Nevertheless, the final experimental  
36 326 setting had not been directly tested within the DoE plan, but rather high extraction time and  
37 327 temperature were tested at both acidic and basic conditions. Therefore, ten replicates were executed  
38 328 at the point encoded [1, 1, 0] (i.e., T = 63°C, t = 16 hours and pH = 7) for both batches in order to  
39 329 validate the model. The ten replicates for batch A gave an estimated average value of 42 pg/mg,  
40 330 with an estimate standard deviation of 5 pg/mg and a coefficient of variation (CV%) of 12%. Then,  
41 331 the estimate of the experimental response at the tested point was calculated via the following  
42 332 formula:

$$\bar{y} \pm \frac{t \cdot s}{\sqrt{n}}$$



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3 333 where  $\bar{y}$  represents the estimated average of the ten replicates,  $t$  is the tabulated Student's t-value at  
4 334 the 0.05 significance level (95%, d.f. = 9,  $t = 2.262$ ),  $s$  is the estimate of the experimental standard  
5 335 deviation, and  $n$  is the number of replicates. The resulting EtG concentration in batch A is  
6 336 calculated as  $42 \pm 4$  pg/mg. On the other hand, the estimated EtG value that was calculated by the  
7 337 MLR model at the same experimental point [1, 1, 0] is equal to 48 pg/mg, with an experimental  
8 338 uncertainty of 10 % ( $48 \pm 5$  pg/mg). Similarly for batch B (that provided an estimated average value  
9 339 of 154 pg/mg, with an estimate standard deviation of 25 pg/mg and a coefficient of variation (CV%)  
10 340 of 16%), the extracted EtG concentration was equal to  $154 \pm 18$  pg/mg, while the MLR model  
11 341 estimate was calculated as  $140 \pm 14$  pg/mg with positive overlapping of the intervals. Since the  
12 342 experimental values were not significantly different from the predicted concentrations for both  
13 343 batches A and B, the model was validated and could be applied in the entire experimental domain.  
14 344 Notably, both average experimental concentrations for batches A and B largely exceed the values  
15 345 originally determined for the single hair samples that form the batches.  
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#### 27 347 Test on the internal standard

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29 348 The optimization of the analytical method led to increase the extraction temperature from ambient  
30 349 to 63 °C, while keeping the extraction time fixed at 16 h (overnight). While it was experimentally  
31 350 verified that no solvent evaporation occurred from the sealed vial used for the extraction, one can  
32 351 doubt that the apparent increase of the extracted EtG concentration may actually arise from partial  
33 352 degradation of the EtG-D<sub>5</sub> internal standard, whose chromatographic peak area is used as the  
34 353 measurement unit for the analyte concentration computation. In practice, an artificial decrease of  
35 354 the EtG-D<sub>5</sub> concentration would result in an over-estimation of the extracted EtG, leading to  
36 355 artificially increased concentrations. To check the absence of differential degradation of the EtG-D<sub>5</sub>  
37 356 internal standard, 45 experiments were carried out at five temperatures (room temperature, 35°C,  
38 357 45°C, 55°C, and 65°C) and three extraction time (1 h, 8 h, and 16 h). Figure 4 shows the results in  
39 358 the form of boxplots for the time variable (a) and the temperature variable (b). No significant  
40 359 variations of the EtG-D<sub>5</sub> areas were observed at the different levels of both extraction temperature  
41 360 and time. Possibly, a slight non-significant increase of the EtG-D<sub>5</sub> extraction is observed by  
42 361 increasing the extraction time from 1 h to 16 h. These results, together with the limited number of  
43 362 outliers in the boxplots – with the exception of T = 35°C graph – confirm the absence of any  
44 363 differential degradation of EtG-D<sub>5</sub> over the entire experimental domain. Incidentally, also the  
45 364 robustness of EtG under the tested experimental conditions is confirmed. It can be concluded that  
46 365 the changes of the measured EtG concentration observed under different experimental settings in  
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3 366 DoE experiments are actually due to the different extraction yields of the analyte. Despite the  
4 367 response surfaces of the Optimization DoE (Figure 3 and S2) suggested the possibility to raise the  
5 368 extraction temperature even more, no further DoE were performed in order not to damage the hair  
6 369 matrix during the extraction process. In our opinion, the current extraction temperature of 63°C  
7 370 represent a robust compromise between the extraction yield of EtG and the feasibility of the  
8 371 analytical methodology.

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### 15 373 Method validation

17 374 The stepwise systematic method proposed by Desharnais et al.<sup>[30,31]</sup> was used to select the most  
18 375 appropriate calibration model and validate the choice. The first step of the procedure involved the  
19 376 evaluation of data heteroscedasticity by means of F-test; then different statistical tests were  
20 377 executed, including lack-of-fit and normality testing, in order to choose the model order, either  
21 378 linear or quadratic, that best fitted the experimental calibration points (7 levels × 7 replicates), and  
22 379 the corresponding weighting. According to this procedure, the data proved to be heteroscedastic,  
23 380 and a linear model involving the use of  $1/x^2$  weighting turned to be the most appropriate for  
24 381 calibration purposes. All the results of significance tests are reported in the Supplementary Material,  
25 382 together with the information about the the slope and the intercept of the tested calibration model,  
26 383 and its determination coefficient, in the output format provided by the R codes developed and made  
27 384 available by Desharnais and coworkers<sup>[30,31]</sup>. The whole procedure was repeatedly tested on a lower  
28 385 number of the already prepared calibration curves (i.e., including 4 or 5 replicates only) to test the  
29 386 model robustness and similar results were obtained.

31 387 From the final calibration model, LOD and LOQ values were calculated following the Hubaux-Vos'  
32 388 algorithm<sup>[37]</sup>, which yielded the following values: LOD = 0.8 pg/mg and LOQ = 1.7 pg/mg. The  
33 389 latter concentration is lower than the first calibration level, which was experimentally verified (see  
34 390 below). Selectivity and specificity of the method were confirmed, as no interfering signals were  
35 391 detected at the retention times of the target analytes, and the retention time precision proved  
36 392 satisfactory, as the deviations from the expected retention times were largely below 1%. The  
37 393 relative abundancies of the characteristic ions of EtG were positively evaluated. Trueness and  
38 394 accuracy data at the 1<sup>st</sup>, 4<sup>th</sup> and 7<sup>th</sup> calibration levels (2, 30, and 300 pg/mg) turned out adequate, as  
39 395 the percent bias and CV% values were lower than 15% at all concentration levels. Moreover,  
40 396 repeatability was tested at the 1<sup>st</sup>, 4<sup>th</sup> and 7<sup>th</sup> calibration levels giving satisfactory results since all  
41 397 the performed significance tests were passed (i.e., Shapiro-Wilk, Dixon, and Grubbs tests). Lastly,  
42 398 no carry-over effect was observed.



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400 Matrix effect

401 In our previous study<sup>[23]</sup>, it was concluded that – on average – significantly higher EtG extraction  
402 yields were obtained if the hair samples were primarily pulverized with a ball mill, instead of  
403 cutting them manually in small snippets. However, the differences for individual samples were  
404 highly variable and, for a few hair samples, even higher EtG concentrations were measured after  
405 applying the cutting pre-treatment than after milling. These rare events could be explained by  
406 considering that relatively high random variability is generally associated to incomplete extraction  
407 yields. An alternative explanation, that we intended to verify in the present study, was that different  
408 matrix effects may be produced by the two pre-treatment procedures, as a consequence of dissimilar  
409 abundance of interfering substances.

410 Comparison of the matrix effect induced by the two procedures was made by analysing three neat  
411 aqueous solutions spiked with EtG at 3 concentration levels and comparing their results with those  
412 obtained from negative hair samples that underwent milling or manual cutting procedures and  
413 spiked after the extraction step. The use of childhood hair as negative samples may limit the general  
414 legitimacy of the comparison, since it implies that the matrix components are the same in the  
415 childhood and adult age, but represented a practical way to obtain a mixed batch of several  
416 unquestionably negative hair samples. The six replicated determinations at three concentration  
417 levels produced very limited variability (CV% = 7-9 for milling and CV% = 4-7 for cutting  
418 experiments). The measured average matrix effect was equal to -6.9%, -6.6%, and -6.4% at the  
419 three concentration level when the milling procedure was applied, while was equal to -7.4%,  
420 -6.1%, and -7.2% when the hair was manually cut into snippets. For all these data reporting the  
421 signal decrease due to matrix effects, the t-test yielded statistical significance at 90% confidence  
422 level or above. The differences between the milling and the manual cutting procedures was never  
423 statistically significant. If the data were corrected by the contribution of the internal standard, the  
424 measured matrix effect was calculated as +2.0%, +1.4, +2.6% and -1.8%, +3.4, +7.0, respectively.  
425 The latter results are affected by larger uncertainty as a result of the added contribution of the  
426 internal standard variability.

427 The overall results confirm that limited and substantially equal matrix effect is observed no matter  
428 what pre-treatment procedure is used to crumble the hair samples and assures the compliance of  
429 both pre-treatment strategies with respect to the modest impact of matrix component on EtG  
430 quantitation.

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### 432 Comparison of real samples

433 20 hair samples belonging to batches C and D (see Experimental), originally analysed with method  
434 (a) involving manual cutting of the hair lock<sup>[29]</sup>, were analysed again using method (b), namely the  
435 accredited method presently in use in our laboratory<sup>[23]</sup> that involves the pulverisation of the hair  
436 lock, and also with the new method (c) optimized by DoE involving extraction with pure water at a  
437 temperature of 63°C. The summary and detailed results are reported in Table 1 and Figure 5.

438 The data confirm our previous conclusion<sup>[23]</sup> that the average increase of the extraction efficiency  
439 when the hair matrix is pulverized with a mill rather than manually cut into snippets exceeds 30%.  
440 Remarkably, seven samples out of ten (batch C) exhibited EtG concentrations above the 30 pg/mg  
441 cut-off when they were analysed with method (b), whereas this occurred with only one out of ten  
442 samples with method (a). Of course, it should be reminded that specific selection of samples with  
443 EtG close to the cut-off was performed. All ten samples exceeded the cut-off when they were  
444 analysed with method (c). By comparing method (b) with method (c), another 30% average increase  
445 of extraction efficiency is inferred from the data. The comparison made on parallel determinations  
446 on aliquots of the same real hair samples unequivocally shows that modifying the extraction  
447 temperature from ambient to 63 °C increased the detected EtG concentration for all 20 samples,  
448 most likely because more exhaustive extraction is achieved. This improvement is recorded in both  
449 the medium and high EtG concentration levels to a comparable extent. The boxplots represented in  
450 Figure 5 gives a clear graphical evidence of the dependence of the quantitative results from the  
451 experimental conditions adopted for sample treatment.

452 ANOVA and unpaired (two-sided) t-test expressed in a quantitative way the significance level of  
453 the differences observed between the data obtained from the novel and the old conditions of EtG  
454 extraction. In the comparison between methods (b) and (c), the t-test yielded p-values of  $1.5 \times 10^{-5}$   
455 and  $9.7 \times 10^{-5}$  for batches C and D, respectively, rejecting the null hypothesis of no difference. In  
456 the same comparison, ANOVA test gave p-values of  $1.3 \times 10^{-4}$  and  $2.6 \times 10^{-3}$  for batches C and D,  
457 respectively, rejecting the null hypothesis (samples are not different).

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### 459 **Conclusions**

460 The present study supports the conclusions of Mueller and coworkers, with more detailed  
461 investigation of the experimental domain, that (i) pure water represents the best solvent to extract

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3 462 EtG from scalp hair, (ii) an extraction temperature above 60 °C achieves more exhaustive EtG  
4 463 recovery. We also verified that extending the extraction time overnight allows safe and reproducible  
5 464 recovery conditions without implying any risk of analyte and internal standard decomposition.  
6 465 Furthermore, it was observed that limited changes of the pH (from slightly acidic to slightly basic)  
7 466 had no impact on the extraction yield.  
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11 467 The major achievement of the present study is the unequivocal demonstration that several  
12 468 experimental parameters strongly influence the results of the analysis on each tested hair sample.  
13 469 These include particularly the hair crumbling method and the extraction conditions. It is highly  
14 470 plausible that the different analytical results are due to a dissimilar extraction efficiency. Moreover,  
15 471 it is well known that the recovery variability generally depends on its absolute value and that  
16 472 maximizing the extraction yield reduces its variability<sup>[41–43]</sup>. It can be deduced that achieving the  
17 473 most exhaustive extraction of EtG represents a valuable goal in order to obtain stable and reliable  
18 474 analytical results.  
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25 475 In most clinical determinations, each laboratory has its own reference population on which  
26 476 normality ranges are calculated, that depend on the experimental method. On the other hand, in  
27 477 forensic toxicology, fixed cut-off values are commonly defined for general use worldwide, due to  
28 478 the legal consequences of the analytical determinations. Unlike most drugs of abuse, one has to put  
29 479 high requirements on the quantitative determination of EtG in hair samples in order to discriminate  
30 480 the different consumption profiles, which in turn are defined by the use of cut-off values. Therefore,  
31 481 a significant effort should be made in the future to define cut-off values based on the most effective  
32 482 operating conditions and possibly on controlled administration studies, although within the inherent  
33 483 ethical constrains<sup>[44]</sup>. The authors strongly suggest that several institutions should collaborate within  
34 484 an inter-laboratory comparison in order to amend the forthcoming issues of consensus documents.  
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3 621 **Figure captions**  
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5 622 **Figure 1.** (a) Geometric representation of the first DoE (full-factorial design) showing the  
6 623 extraction temperature on the x-axis and the solvents on the y-axis. The levels of extraction  
7 624 temperatures were coded from -1.5 up to +1.5 (i.e., -1.5, -0.5, +0.5, +1.5 for 20°C, 32°C, 45°C, and  
8 625 57°C, respectively), while the codes for the different extraction mixture were -1, 0, +1 for pure  
9 626 distilled water, water/methanol 35:1, and water/methanol 17:1, respectively. (b) Geometric  
10 627 representation of the second DoE (face-centred central composite design) showing the extraction  
11 628 temperature on the x-axis, the extraction time on the y-axis and the pH on the z-axis. The levels  
12 629 were coded from -1 to +1 (i.e. -1, 0, +1) representing (i) 45°C, 54°C and 63°C for the extraction  
13 630 temperature, (ii) 1, 8 and 16 hours for the extraction time, and (iii) 5.5, 7.0 and 8.5 for the pH of the  
14 631 extraction solvent (water).

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21 632 **Figure 2.** Graphs of the full-factorial DoE related to batch A. (left) Coefficients plot showing that  
22 633 the extraction temperature (i.e. x1) was the most significant factor influencing the EtG extraction  
23 634 (p-value < 0.001). In particular, the significance level is indicated in the plot according to the  
24 635 following convention: \* = p-value < 0.05, \*\* = p-value < 0.01, \*\*\* = p-value < 0.001. (right)  
25 636 Bidimensional response surface plot with contour lines outlining equal EtG concentrations  
26 637 (numerical values, pg/mg) showing that the highest EtG concentrations were observed between the  
27 638 temperatures encoded as +0.5 and +1.5 (i.e. 45°C and 57°C) and using the solvent encoded as -1.0  
28 639 (i.e. only pure water). The values reported within the blue lines represent the EtG concentration for  
29 640 each response surface (i.e., the higher the value, the better the extraction yield).

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35 641 **Figure 3.** Graphs of the face-centred central composite DoE related to batch A. (Left): coefficients  
36 642 plot showing that the extraction temperature (x1) and time (x2) were the most significant factors  
37 643 that affected the EtG extraction (p-value < 0.001). In particular, the significance level is indicated in  
38 644 the plot according to the following convention: \* = p-value < 0.05, \*\* = p-value < 0.01, \*\*\* = p-  
39 645 value < 0.001. (Right): two-dimensional response surface plot with Y=EtG concentration (pg/mg)  
40 646 showing that the highest EtG concentration was observed at the temperature encoded as +1.0 (63°C)  
41 647 and at the extraction time encoded as +1.0 (16 hours). The graph was calculated at pH 7.0 of the  
42 648 extraction solvent (only water).

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47 649 **Figure 4.** (a-b) Boxplots of the areas of the EtG-D<sub>5</sub> at the different tested levels of extraction time  
48 650 (left) and temperature (right).

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51 651 **Figure 5.** Boxplots of the extracted EtG concentration for 10 hair samples (left = batch C; right =  
52 652 batch D) analysed by the three tested methods. The red boxes represent the EtG concentration  
53 653 values provided by method (a) that involved manual hair cutting, the yellow boxes indicate the EtG  
54 654 results from the method (b) that employed hair milling, while the green boxes show the EtG values  
55 655 provided by the new method optimized by DoE.



**Table 1.** EtG concentration values (pg/mg) of 10 hair samples for batches C (i.e., with EtG values originally detected in the range between 20 pg/mg and 31 pg/mg) and D (i.e. with EtG values originally detected above 60 pg/mg). The hair specimens were analysed by three method, as follows: (a) involving manual cutting only; (b) using the pulverisation of the hair lock; (c) using pulverisation of the hair lock and the new extraction protocol optimized by DoE. Positive percentage differences among the average results provided by each method are reported.

Batch C	Method (EtG concentration in pg/mg)			Batch D	Method (EtG concentration in pg/mg)		
Sample n°	(a)	(b)	(c)	Sample n°	(a)	(b)	(c)
1	25	33	39	11	71	95	141
2	23	30	41	12	109	140	196
3	26	36	43	13	111	146	192
4	23	32	45	14	100	134	144
5	24	30	37	15	103	131	181
6	25	32	38	16	93	129	139
7	20	27	52	17	84	105	133
8	25	33	44	18	81	104	136
9	31	40	43	19	91	117	142
10	26	34	36	20	65	87	138
Average	24.8	32.7	41.8	Average	90.8	118.8	154.2
Positive differences	(b-a)/a	(c-b)/b	(c-a)/a	Positive differences	(b-a)/a	(c-b)/b	(c-a)/a
	+31.9%	+27.8%	+68.5%		+30.8%	+29.8%	+69.8

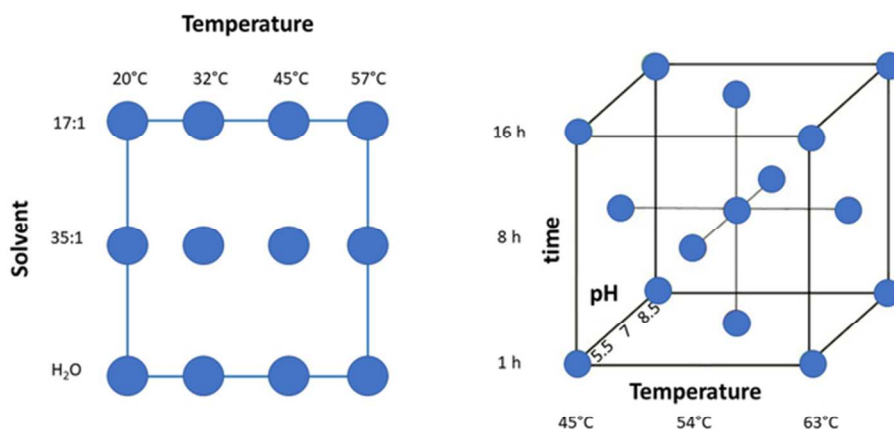


Figure 1. (a) Geometric representation of the first DoE (full-factorial design) showing the extraction temperature on the x-axis and the solvents on the y-axis. The levels of extraction temperatures were coded from -1.5 up to +1.5 (i.e., -1.5, -0.5, +0.5, +1.5 for 20°C, 32°C, 45°C, and 57°C, respectively), while the codes for the different extraction mixture were -1, 0, +1 for pure distilled water, water/methanol 35:1, and water/methanol 17:1, respectively. (b) Geometric representation of the second DoE (face-centred central composite design) showing the extraction temperature on the x-axis, the extraction time on the y-axis and the pH on the z-axis. The levels were coded from -1 to +1 (i.e. -1, 0, +1) representing (i) 45°C, 54°C and 63°C for the extraction temperature, (ii) 1, 8 and 16 hours for the extraction time, and (iii) 5.5, 7.0 and 8.5 for the pH of the extraction solvent (water).

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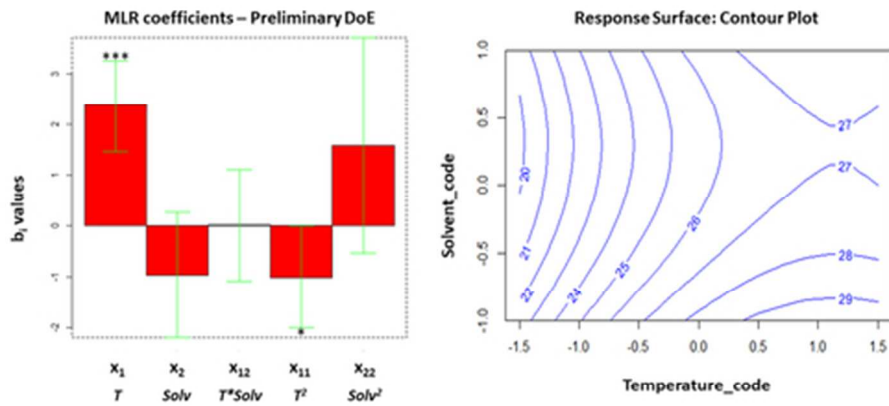


Figure 2. Graphs of the full-factorial DoE related to batch A. (left) Coefficients plot showing that the extraction temperature (i.e.  $x_1$ ) was the most significant factor influencing the EtG extraction (p-value < 0.001). In particular, the significance level is indicated in the plot according to the following convention: \* = p-value < 0.05, \*\* = p-value < 0.01, \*\*\* = p-value < 0.001. (right) Bidimensional response surface plot with contour lines outlining equal EtG concentrations (numerical values, pg/mg) showing that the highest EtG concentrations were observed between the temperatures encoded as +0.5 and +1.5 (i.e. 45°C and 57°C) and using the solvent encoded as -1.0 (i.e. only pure water). The values reported within the blue lines represent the EtG concentration for each response surface (i.e., the higher the value, the better the extraction yield).

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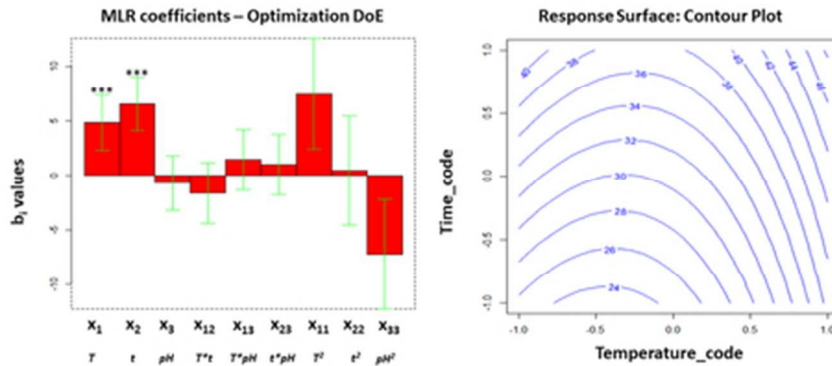


Figure 3. Graphs of the face-centred central composite DoE related to batch A. (Left): coefficients plot showing that the extraction temperature ( $x_1$ ) and time ( $x_2$ ) were the most significant factors that affected the EtG extraction (p-value < 0.001). In particular, the significance level is indicated in the plot according to the following convention: \* = p-value < 0.05, \*\* = p-value < 0.01, \*\*\* = p-value < 0.001. (Right): two-dimensional response surface plot with Y=EtG concentration (pg/mg) showing that the highest EtG concentration was observed at the temperature encoded as +1.0 (63°C) and at the extraction time encoded as +1.0 (16 hours). The graph was calculated at pH 7.0 of the extraction solvent (only water).

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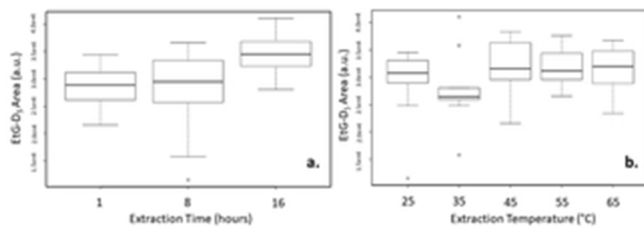


Figure 4. (a-b) Boxplots of the areas of the EtG-D5 at the different tested levels of extraction time (left) and temperature (right).

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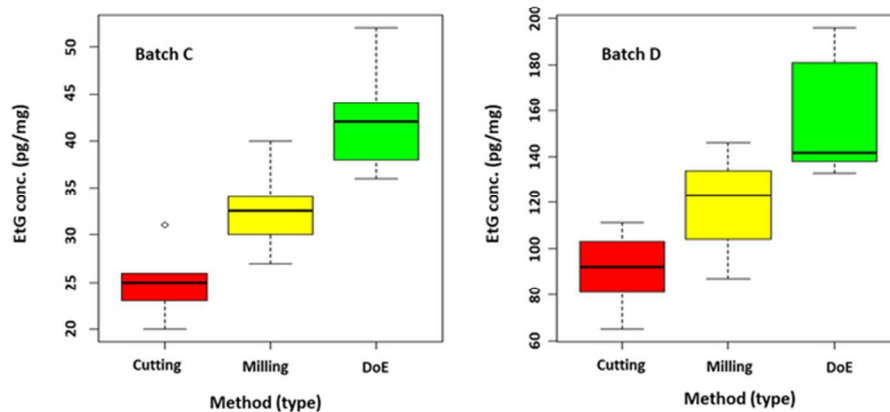


Figure 5. Boxplots of the extracted EtG concentration for 10 hair samples (left = batch C; right = batch D) analysed by the three tested methods. The red boxes represent the EtG concentration values provided by method (a) that involved manual hair cutting, the yellow boxes indicate the EtG results from the method (b) that employed hair milling, while the green boxes show the EtG values provided by the new method optimized by DoE.

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