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# Determination of endogenous GHB levels in chest and pubic hair

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ABSTRACT

Endogenous nature of GHB represents a critical issue for forensic toxicologists, especially in alleged sexual assaults. Therefore, discrimination between physiologically and additional amounts from exogenous sources of such a substance must be effective and reliable in order to avoid severe misinterpretation. This study aimed to quantify the GHB baseline concentrations in chest and pubic hairs collected from 105 healthy volunteers, non-consumers of any drugs of abuse. The final scope was to investigate if these keratin matrices could represent valid alternative to scalp hair when not available. Moreover, we also evaluated the age and gender influences on the GHB baseline levels. 25 mg of hair were incubated overnight with NaOH at 56 °C. After acidification with $H_2SO_4$, the solution was liquid-liquid extracted with ethyl acetate and a trimethylsilyl derivatization was then achieved. Analysis was performed in gas chromatography-mass spectrometry in single ion monitoring mode (*m/z* 233, 234, 147 for GHB; *m/z* 239, 240 and 147 for GHB-d6). The endogenous amount in "blank" hair was estimated by the standard addition method (0.301 for chest hair and 0.235 ng/mg for pubic hair). GHB concentration ranged from 0.205 to 1.511 ng/mg for chest hair and from 0.310 to 1.913 ng/mg for pubic hair. These values were consistent with previous studies on scalp hair and on pubic hair. Unfortunately, research on chest hair is not available in literature. T-Test and Linear Regression highlighted no statistically significant differences for the two matrices and for all age/gender sub-groups. However, further studies are required to estimate a reliable cut-off value for these keratin matrices. For the first time, we demonstrated the suitability of chest and pubic hair to detect endogenous levels of GHB.



## 1. Introduction

γ-Hydroxybutyric acid (GHB) still represents an open issue for forensic toxicologists. Several studies are published each year on various topics related to this compound, such as development of new analytical methods [1–3] and data interpretation in case reports [4–7]. The greatest interest in GHB is due to its multiple nature as an endogenous substance, a medication, a drug of abuse as well as being associated with drug facilitate sexual assault (DFSA). GHB is commonly found in several mammalian tissues and, in particular, within the central nervous system (CNS), as precursor and/or metabolite of the main inhibitory neurotransmitter γ-aminobutyric acid (GABA) [8]. GHB is able to mimic the depressant effects of GABA by the interaction with its receptors (mainly with the b sub-types). Moreover, GHB can also act on the dopaminergic system inducing disinhibitory and excitant effects [9].

Since its synthesis in 1960, GHB has been used as anaesthetic agent and later it found application in treatment of narcolepsy with cataplexy, alcohol withdrawal syndrome and alcohol dependence [10,11]. GHB is also used out of medical prescription as enhancer of muscle mass in body building practices and as psychoactive drug for recreational purposes [12–14]. Besides these uses, GHB can be also employed as date rape drug in DFSA for its organoleptic features and psychoactive effects [12,15,16]. GHB is soluble in water, colourless and odourless, thus it can be easily and surreptitiously added to beverages of unsuspecting victims, who quickly manifest increased sex drive, disinhibition, amnesia, drowsiness and dizziness [17–19]. The effects are dose-dependent with a threshold of 0.5 g able to induce relaxation and disinhibition (this is slightly below the therapeutic dose of 10/mg/Kg for a 60 Kg adult), 1 g induces euphoric effects whereas deep sleep has been observed at doses exceeding 3–5 g [20,21]. Higher doses GHB are usually associated to respiratory depression, coma and death [22]. All the above effects may be

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intensified by the co-consumption of additional psychoactive substances, especially CNS depressant agents such as the ethanol [23]. This could be considered the classic scenario of a DFSA involving GHB, as this substance is usually mixed with alcoholic beverages. GHB is subject to interconversion to its lactonic form (GBL) in aqueous solutions in a pH-depending manner: at pH 2, GHB/GBL ratio is about 1:2; at pH 12, GBL is almost totally converted to GHB [24]. For this reason, GBL can be found on the illegal market as "precursor" of GHB [14]. Given the high health risk associated with GHB/GBL misuse, these substances have been listed within the Schedule IV of the Convention on Psychotropic Substances of 1971 and then upgraded to Schedule II. Currently USA, Australia and most of the European Countries have included GHB and GBL among the substances subjected to strict control.

In literature, many analytical methods have been published for its detection in various biological matrices and gas chromatography–mass spectrometry (GC-MS) is the technique of choice [25,26]. However, procedures involving liquid chromatography–tandem mass spectrometry (LC–MS/MS) systems have also been described with lower sensitivities due to weak ionization efficiency of GHB [2,27]. Although effective technologies and procedures have been developed, even with excellent sensitivities, endogenous nature of GHB entails interpretative issues, making essential an actual and reliable discrimination between basal and exogenous concentrations. Several studies on the baseline levels of GHB have been performed and many cut-off values have been proposed [28]. For scalp hair, adoption of fixed cut-offs to distinguish between endogenous/exogenous nature seems to be outdated by now, preferring a "personalized" approach. This strategy is based on the evaluation of the ratio between the GHB baseline concentrations (estimated by segmental hair analysis) and the GHB concentration in the segment related to the time of alleged exposure. Limitations may be due to the need for two presentations of a victim and thus raise compliance issues. In its "Guidelines for the forensic analysis of drugs facilitating sexual assault and other criminal acts", UNODC suggests a 1:10 ratio threshold for scalp hair, but several studies proved that even higher ratios are effective and reliable enough to assess the endogenous/exogenous nature of GHB [29–31]. This approach has the great advantage of overcoming the high inter-individual variability of endogenous GHB levels that can also be affected by drug interactions and rare genetic disorders (i.e. GHB aciduria) [32–34]. Recently, Strickland et al. [35] proposed a new approach based on adjacent segment concentration differences. This new strategy promises to be more discriminating in subjects with a wide intra-individual variation.

With this research, we aimed to estimate the endogenous levels of GHB in chest and pubic hair in order to evaluate their suitable as alternative keratin matrices when head hair is not available (i.e., due to physio-pathological conditions). In addition, we also verified if age and gender can influence the GHB concentrations in those specimens.

GHB endogenous levels in scalp hair have been reported by many studies and in some cases, age and gender effects have been also investigated with mixed results [27,32,36,37]. In 2004, Duhem et al. [38] published a study on 14 pubic hair samples from volunteers, 7 males (M) and 7 females (F). The authors assessed that this keratin matrix can be used to establish a chronic exposure to GHB and to confirm the scalp hair outcomes. However, no studies are available for chest hair yet.

Thus, to the best of our knowledge, our research was the first one to be carried out on these hair matrices involving a large number of subjects (n = 105). Estimation of baseline levels could be useful for the assessment of conscious/unconscious GHB consumption, especially in DFSA cases. Indeed, hair matrices may store this molecule longer than conventional matrices (blood and urine) [24], thus even a single exposure may be detected after several months. This peculiarity is very helpful in suspected DFSA cases, where the victims may report the rape long time later, when conventional matrices can not be use.

## 2. Materials and methods

### 2.1. Chemicals and reagents

Dichloromethane (DCM), N,O-bis trimethylsilyl-trifluoroacetamide (BSTFA) with 1% trimethylchlorosilane (TMCS) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Sodium hydroxide (NaOH), sulfuric acid ($H_2SO_4$) and ethyl acetate (AcOEt) were purchased from J.T.Baker (Deventen, Holland). GHB and GHB-d6 (internal standard, IS) were purchased from Chemical Research 2000 sr.l. (Rome, Italy).

### 2.2. Enrolled population

In this study, 105 healthy volunteers belonging to a general population (students, colleagues and friends of the authors) were enrolled. The M participants (n = 90) were divided in two groups (n = 45) on the basis of hair sample provided (chest or pubic); 15 F subjects were asked to give pubic hair. The donors declared to be non-consumers of any drug of abuse and medicaments. Studied population was also classified by three age categories (≤35 years; 36–49, years; ≥50 years). Each age range consisted of 30 M (15 chest hair and 15 pubic hair) and 5 F (only pubic hair).

Axillary hair was not included in this study because it was barely available among the enrolled population. Unfortunately, enrol limitations did not allow us to collect more than a sample from the same participant. Moreover, we were not able to study the influence of colour on the endogenous GHB concentrations since the collected samples were mainly brown.

### 2.3. Sample pre-treatment

The body hair samples were washed twice with 5 mL of DCM for 2 min, dried and cut in small fragments (1–2 mm). An aliquot of 25 mg was then used for the analysis. GHB was always present in all our chest and pubic "blank" samples. Thus, since GHB-free body hair were not available, its concentrations in chest and pubic "blank" samples were respectively estimated in 0.301 and 0.235 ng/mg by means of standard addition method.

### 2.4. Sample treatment

Analysis was performed following a previously published method [39]. Briefly, 25 mg of body hair were digested with 100 µL of 1 M NaOH solution in presence of IS (15 ng) at 90 °C for 20 min. After cooling at room temperature, 200 µL of 1 M $H_2SO_4$ and 3 mL of AcOEt were added to the sample which is immediately agitated (1 min) and then centrifugated (2500 G, 5 min). The organic layer was dried under nitrogen stream and the residue was derivatized adding 50 µL of AcOEt and 25 µL of BSTFA-1% TMCS in AcOEt (20 min at 65 °C). 1 µL was injected in the GC–MS system. Limit of detection (LOD) and limit of quantification (LOQ) were 0.05 and 0.19 ng/mg, respectively.

### 2.5. GC–MS

The GC–MS instrument consisted in an Agilent 7890 A GC system equipped with an Agilent 7683B series autosampler (Agilent Technologies, Palo Alto, CA, USA) and interfaced to a single quadrupole Agilent 5975 C mass spectrometer (Agilent Technologies). The column was an Agilent HP-5MS, 30 m length, 0.25 mm i.d. and 0.25 µm film thickness (Agilent Technologies). The gas carrier (He) flow was constant at 1 mL/min. The oven temperature was set initially at 60 °C for 0.5 min, and programmed to 130 °C at 10 °C/min, and 300 °C at 8 °C/min. Injector and transfer line temperatures were

respectively 300 °C and 230 °C. Electron ionization and selected ion monitoring (SIM) acquisition mode were used and the ions of interest were: 233, 234, 147 *m/z* for GHB; 239, 240 and 147 *m/z* for GHB-d6.

### 2.6. Statistical analysis

Data followed a normal distribution as resulted by the Pearson chi-square test for normality. T-test and Linear Regression was used to evaluate potential differences in GHB concentrations between M and F populations checking for pubic hair and chest hair only for M explained by age. Median, standard deviation (SD) and interquartile ranges were calculated to describe the GHB concentrations outcomes. For all analyses, significance was set to $\alpha$=0.05%: we rejected Null Hypothesis if P < 0.05. Statistical analysis was performed by Stata/SE 17.0.

## 3. Results and discussion

GHB natural presence has been widely demonstrated in biological matrices but also in food, meat and beverages [40–42]. For this reason, interpretation of GHB levels in forensic caseworks is still a thorny topic for forensic toxicologists who are called to be cautious in order to avoid any misinterpretation. Several studies aiming to the assessment of a cut-off value for scalp hair have been performed [29–31]. However, research on body hair is limited to the Duhem et al.'s one on pubic hair [38].

In this study, GHB was detected in the following ranges: 0.205–1.511 ng/mg (median: 0.732 ng/mg; SD: 0.294 ng/mg) for chest hair (Table 1); 0.273–1.913 ng/mg (median: 0.768 ng/mg; SD: 0.302 ng/mg) for pubic hair (Table 2). For this latter matrix, a "strongly anomalous" GHB concentration (8.406 ng/mg) was not included in the statistics. It must be underlined that if we had evaluated this sample adopting the fixed cut-off value of 2 ng/mg, as suggested by the Duhem et al.'s study, we would have considered him as a consumer of GHB. Besides this extremely high value, other outliers could be considered as outliers by STATA/SE 17.0 data analysis; nevertheless, they were always < 2 ng/mg and within the ranges described in literature for pubic and scalp hair and so we included them in the statistical analyses. Indeed, we can not exclude they were due to a higher physiological production than the most of the subjects, therefore they may be a further evidence of the great inter-individual variability in endogenous GHB concentration.

Baseline values were in line with the ones previously published for scalp hair. In particular, two recent papers reported ranges of 0.3–2.0 ng/mg (mean: 1.1 ± 0.6 ng/mg) and 0.4–5.47 ng/mg (median: 0.72 ng/mg) [37,43]. To best of our knowledge, no studies have been previously published for GHB concentration in chest hair.

Regarding pubic hair, in their study on 14 samples, Duhem et al. detected GHB in the range 0.36–1.55 ng/mg (mean: 0.77 ng/mg; SD: 0.409 ng/mg) [38]. These values are highly comparable with our outcomes. T-test analysis showed no statistical significance in GHB content of the two types of body hair (p = 0.4672). The substantial matching of concentration ranges among the scalp, chest and pubic hair constitutes an important finding. Indeed, these matrices present specific characteristics and furthermore are subject to different hygienic treatments affecting the detectable levels of many substances [44,45]. First of all, the growth rate for body hair is lower than scalp hair (anagen and telogen phases are considered to be of equal length) which may yield to higher concentrations; moreover, pubic hair is exposed to urinary contamination. This phenomenon may entail an overestimation of substance's levels in this keratin matrix. Overall, urine presents higher concentrations and can release high amount of substance on the pubic hair surface [45]. For this reason, the pre-analytical washing step plays a key role when this matrix is analysed and it should be deeply optimized during the method validation; an effective procedure should be able to remove the contaminant avoiding the extraction of the analytes from the sample. Urinary contamination is a well-known issue and in specific case makes the pubic hair unsuitable for forensic purposes (i.e. evaluation of chronic alcohol consumption by ethyl-glucuronide quantification [46]).

In the case of GHB, endogenous concentrations in urine are even higher than in blood and are reported in the range 0.34–5.7 mg/L [47]. Even if we observed similar ranges for pubic and scalp hair, we cannot definitely exclude that GHB levels in pubic hair are not affected by urinary contamination as it depends by the individual hygiene habits; thus, further study should be achieved. Even more so that external contamination occurs also for scalp hair by the sweat. For this reason, many papers suggest to not analyse the first centimetre of hair sample [39,43,48].

### 3.1. Age influence on endogenous GHB levels in chest hair

Male population was equally divided in three age ranges (≤35 years; 36–49 years; ≥50 years) in order to evaluate the influence of age (Fig. 1). GHB concentrations' ranges were: 0.487–1.229 ng/mg (median: 0.810 ng/mg; SD: 0.268 ng/mg) for ≤ 35 years (mean: 32.4 years); 0.205–1.511 ng/mg (median: 0.621 ng/mg; SD: 0.331 ng/mg) for 36–49 years (mean: 42.2 years); 0.424–1.425 ng/mg (median: 0.854 ng/mg; SD: 0.280 ng/mg) for ≥ 50 years (mean: 57.4 years, Table 1). Linear Regression demonstrated no significant differences among the age groups (36–49 years vs ≤35 years, p = 0.232; ≥50 years vs ≤35 years, p = 0.815). Unfortunately, any evaluation of our data is strongly limited by the lack of available study on this keratin matrix. Indeed, age influence on endogenous GHB levels have been only investigated for scalp hair [27,30,32,49]. No statistically significative variation has been observed, except in our previous study on 150 volunteers non-consumers of any drugs [39]. In this research,

**Table 1**
Range, mean, median and SD of endogenous GHB in chest hair and distribution by age.

| Gender | Range (ng/mg) | Mean (ng/mg) | Median (ng/mg) | SD (ng/mg) | Age (y) | Range (ng/mg) | Mean (ng/mg) | Median (ng/mg) | SD (ng/mg) |
|---|---|---|---|---|---|---|---|---|---|
| M | 0.205–1.511 | 0.768 | 0.732 | 0.294 | ≤35 | 0.487–1.229 | 0.809 | 0.810 | 0.268 |
| | | | | | 36–49 | 0.205–1.511 | 0.680 | 0.621 | 0.331 |
| | | | | | ≥50 | 0.424–1.425 | 0.813 | 0.854 | 0.280 |

**Table 2**
Range, mean, median and SD of endogenous GHB in pubic hair and distribution by gender and age.

| Gender | Range (ng/mg) | Mean (ng/mg) | Median (ng/mg) | SD (ng/mg) | Age (y) | Range (ng/mg) | Mean (ng/mg) | Median (ng/mg) | SD (ng/mg) |
|---|---|---|---|---|---|---|---|---|---|
| M | 0.353–1.913 | 0.816 | 0.781 | 0.292 | ≤35 | 0.353–1.258 | 0.814 | 0.723 | 0.278 |
| | | | | | 36–49 | 0.393–1.125 | 0.733 | 0.627 | 0.243 |
| | | | | | ≥50 | 0.544–1.913 | 0.901 | 0.858 | 0.342 |
| | | | | | ≤35 | 0.529–1.244 | 0.834 | 0.728 | 0.283 |
| F | 0.310–1.244 | 0.792 | 0.766 | 0.340 | 36–49 | 0.310–1.233 | 0.862 | 1.042 | 0.420 |
| | | | | | ≥50 | 0.334–1.174 | 0.678 | 0.766 | 0.350 |

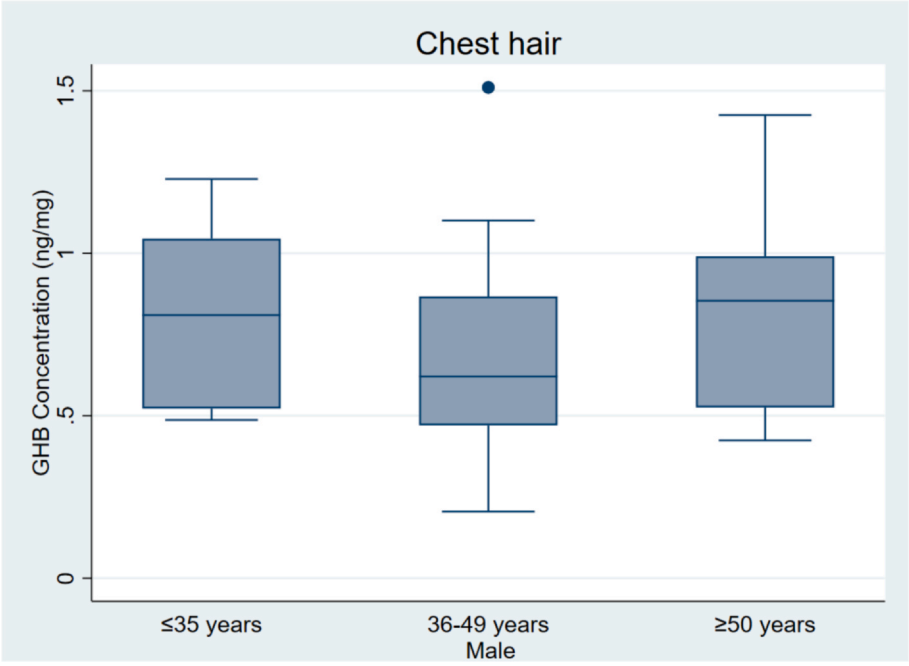


**Fig. 1.** Box plot of GHB concentrations in chest hair and their distribution among the age groups.

significative differences were registered among the age groups both for M and F populations, but with opposite results: in M, the highest concentration was found in < 30 years-old subjects (mean: 1.008 ng/mg); on the contrary, in F the baseline level was highest over 50 years (mean: 0.691 ng/mg). Thomas et al. [43] have recently reported a research on endogenous GHB concentrations in segmented hair from 214 subjects (2074 samples). The authors observed statistical differences only in segment 2 from F population, while for segments 3 and 4 the p-values (Kruskal-Wallis one-way test) were 0.052 and 0.054. No statistically significative difference was observed in M.

### *3.2. Gender and age influence on endogenous GHB levels in pubic hair*

Besides the evaluation of age influence, for pubic hair we were able to investigate also the effect of gender on the concentrations. GHB was quantified in the following ranges: 0.353–1.913 ng/mg (median: 0.781 ng/mg; SD 0.292 ng/mg) for M and 0.310–1.244 ng/mg (median: 0.766 ng/mg; SD: 0.340 ng/mg) for F (Table 2). No significative differences were highlighted by *t*-test ($p = 0.7915$). As seen above for chest hair, we can not compare our outcomes with other studies. The only research on GHB levels in pubic hair has been published by Duhem et al. [38], but potential variations due to the gender were not investigated. However, the gender influences were studied for scalp hair with conflicting results [27,30,32]. In the same paper mentioned before, Thomas et al. [43] concluded that the median GHB concentrations in M were significantly higher ($p < 0.001$) beyond the first 2 cm of hair. These findings were consistent with the Shi et al.'s study and our previous work [36,39].

Regarding age-to-gender sub-groups, the following concentrations were observed for M population (Fig. 2): 0.353–1.258 ng/mg (median: 0.723 ng/mg; SD: 0.278 ng/mg) for ≤ 35 years (mean: 26.3 years); 0.393–1.125 ng/mg (median: 0.627 ng/mg; SD: 0.243 ng/mg) for 36–49 years (mean: 42.8 years); 0.544–1.913 ng/mg (median: 0.858 ng/mg; SD: 0.342 ng/mg) for ≥ 50 years (mean: 57.7 years,

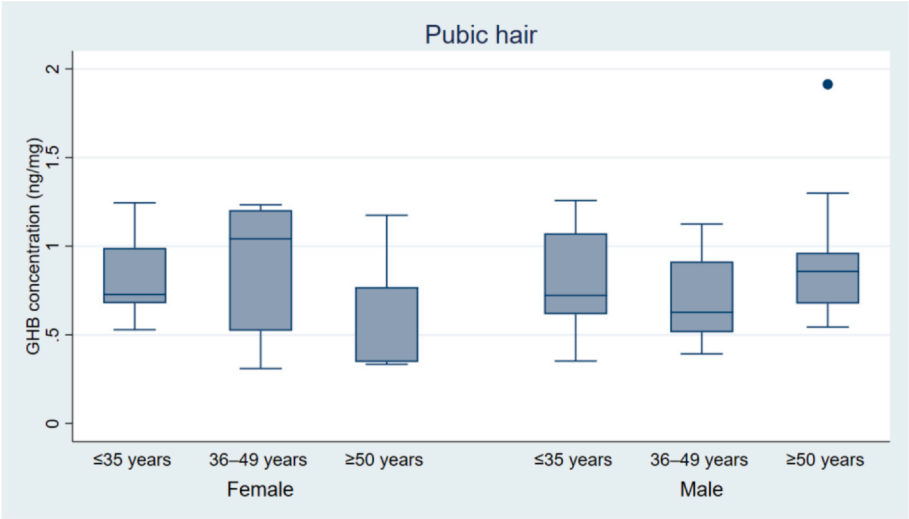


**Fig. 2.** Box plot of GHB concentrations in pubic hair from M and F donors and their distribution among the age groups.

Table 2). In F subjects, the ranges were (Fig. 2): 0.529–1.244 ng/mg (median: 0.728 ng/mg; SD: 0.283 ng/mg) for ≤ 35 years (mean: 26.8 years); 0.310–1.233 ng/mg (median: 1.042 ng/mg; SD: 0.420 ng/mg) for 36–49 years (mean: 42.8 years); 0.334–1.174 ng/mg (median: 0.766 ng/mg; SD: 0.350 ng/mg) for ≥ 50 years (mean: 56.4 years, Table 2). No statistically significant differences were attributable to age neither for M (36–49 years vs ≤35 years, p = 0.514; ≥50 years vs ≤35 years, p = 0.326) and for F (≤36–49 years vs ≤35 years, p = 0.880; 50 years vs ≤35 years, p = 0.411) donors. Also, for pubic hair, the highest concentrations in M were observed in ≥ 50 years-old subjects (median: 0.950 ng/mg). GHB amounts were higher in F aged 36–49 years (median: 1.042 ng/mg). These data are worthy to be further investigated as they are opposite to the evidences from our previous study on scalp hair.

## 4. Conclusions

In this paper we presented the first study on endogenous GHB concentrations in chest and pubic hair involving a large population. GHB was found in all the body hair samples and amount ranges were consistent with the ones reported for scalp help. This can represent the main finding for this research as it demonstrates that hair collected from chest and pubis region can be suitable to confirm scalp hair's outcomes; moreover, they may represent alternative keratin matrices when head hair is not available. The different growth rates and the urinary contamination seemed to not affect the endogenous GHB levels, but of course further investigations are necessary. We also evaluated the influence of age and gender and no statistically significant variations were observed. Nevertheless, in male population the highest concentrations were observed in ≥50 years-old subjects for both the body hair specimens; on the other hand, the highest levels in pubic hair from F donors were observed in ≤35 years.

It must be underlined that these findings should be considered as a first attempt to investigate the GHB baseline concentrations in body hair and more studies are strongly required to explore their diagnostic value especially for forensic purposes, such in alleged DFSA cases. In particular, intra-individual variability, urinary contamination, structural characteristics and incorporation mechanisms should be the target of future studies in order to suggest a reliable cut-off values for these keratin matrices.

## Compliance with ethical standards

None of the authors have any conflict of interest, including specific financial interests, relationships or affiliations relevant to the manuscript. For this research, no founds were obtained. Ethical approval and informed consent were not necessary for this kind of research.

## CRediT authorship contribution statement

**Elisabetta Bertol, Francesco Mari:** Conceptualization, Supervision, Project administration, Methodology. **Giusy Tespio:** performing experiments. **Fabio Vaiano:** Ideas, Performing experiments, Supervision, Methodology, Writing - original draft, Writing - review & editing, Formal analysis. **Alessio Lachi:** Formal analysis.

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