Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Determination of ethyl glucuronide in hair samples of Chinese people by protein precipitation (PPT) and large volume injection–gas chromatography–tandem mass spectrometry (LVI–GC/MS/MS)

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ARTICLE INFO

ABSTRACT

Ethyl glucuronide (EtG) has been shown to be a suitable marker of excessive alcohol consumption. Determination of EtG in hair samples may help to differentiate social drinkers from alcoholics, and this testing can be widely used in forensic science, treatment programs, workplaces, military bases as well as driving ability test to provide legal proof of drinking. A method for determination of EtG in hair samples using large volume injection–gas chromatography–tandem mass spectrometry (LVI–GC/MS/MS) was developed and validated. Hair samples (in 1 mL deionized water) were ultrasonicated for 1 h and incubated overnight; these samples were then deproteinated to remove impurities and derivatisated with 15 μL of pyridine and 30 μL of BSTFA. EtG was detected using GC/MS/MS in multiple-reaction monitoring mode. This method exhibited good linearity: $y = 0.0036x + 0.0437$, $R^2 = 0.9993$, the limit of detection and the limit of quantification were 5 pg/mg and 10 pg/mg, respectively. The extraction recoveries were more than 60%, and the inter-day and intra-day relative standard deviations (RSD) were less than 15%. This method has been applied to the analysis of EtG in hair samples from 21 Chinese subjects. The results for samples obtained from all of those who were teetotallers were negative, and the results for the other 15 samples ranged from 10 to 78 pg/mg, except for one negative sample. These data are the basis for interpretation of alcohol abuse.

1. Introduction

Alcohol abuse is one of the most serious social problems throughout the world. Chronic alcohol consumption not only is bad for health but also easily leads to mental illnesses and abnormal behavior, which increases the rate of traffic accidents, industrial accidents and criminal behavior [1]. For this reason, scientists have focused their studies in search of suitable markers of alcohol abuse for many years.

Ethyl glucuronide (EtG) is a phase II metabolite of alcohol; about 90–95% of alcohol is eliminated by oxidation, primarily in the liver, whereas biotransformation of ethanol to EtG represents only 0.02–0.06% of complete alcohol elimination [2]. Similar to metabolites of other abused drugs, EtG can be incorporated in hair fibers as a consequence of alcohol consumption, and hair analysis can provide a greater retrospective window of detection than bodily fluids. So EtG in hair is gaining increasing importance as a marker of alcohol abuse [3–6]. The first report of the detection of EtG in hair

was made by Sachs in 1993, and progressively more subsequent studies have shown that EtG could be used to confirm chronic, excessive alcohol consumption over a long period of time. Recently, some of the accomplished research has been successfully applied to the fields of clinical and forensic science [7]. When compared with those of foreigners, the drinking habits, alcohol absorption and metabolism of the Chinese population are quite different; thus, establishing a method to detect EtG in the hair of Chinese individuals and studying the relationship between alcohol consumption and hair EtG concentrations are very important. These results can be used to classify social drinkers as well as alcoholism among Chinese people, which has considerable potential applications in medical settings, with military recruitment, in hiring situations and with tests for driving ability.

Because of its high polarity, EtG is incorporated into hair only in very small amounts by a mechanism that is not completely understood yet [6]. Thus, EtG analysis requires analytical methods capable of the highest sensitivity; the concentrations of EtG measured in hair are usually in the pg/mg range. Analytical methods based on GC–EI-MS [1,8], GC–NCI-MS [9], GC–EI-MS–MS [10], GC–NCI-MS–MS [11] or LC–MS–MS [12–16] were developed and the limits of detection (LOD) and quantification (LOQ) have been

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improving over time. The protein precipitation (PPT) method is a new technique for sample preparation that is quick, easy, low-cost and efficient. Recently, PPT has been widely used in urine and blood preparation, but has not yet been used for hair samples. Large volume injection (LVI) by programmable temperature vaporization (PTV) can be used for solvent emptying. When optimizing the streaming emptying volume, purge time, PTV temperature, the injection volume and other parameters, this method can improve the sensitivity by at least 1–2 orders of magnitude compared to traditional 1 μL sample injection [17]. Gas chromatography–tandem mass spectrometry (GC–MS/MS) is uniquely able to eliminate background interference and perform ion-selective analysis, so it is particularly suitable when the sample has a low target concentration and high matrix background [18]. The objective of the present study is to develop and validate a sensitive, precise and specific analytical method for the determination of EtG in hair samples. Based on the advantages of both LVI and GC–MS/MS, the present study developed a LVI–GC/MS/MS method to detect EtG in hair, and applied it in our laboratory for the analysis of EtG in hair samples from 21 Chinese subjects with different drinking behaviors.

2. Materials and methods

2.1. Chemicals and reagents

EtG and deuterated EtG (EtG-D$_5$) standards as well as *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA), which was used for derivatization, were obtained from Cerilliant (Texas, USA). HPLC grade methanol, acetonitrile and pyridine were purchased from Sigma–Aldrich (Missouri, USA). Ammonium hydroxide solution (25%) and formic acid (98%) were purchased from Fluka (Buchs, Switzerland). All other chemicals were of analytical grade. Deionized water was purified using a Milli-Q system (Millipore, Massachusetts, USA).

2.2. Hair sample preparation

Twenty-one hair samples without dyeing, bleaching or any other cosmetic treatment were donated by relatives and friends of laboratory workers, including six from children who have no alcohol consumption history and fifteen from healthy adults possessing clear alcohol consumption history.

Hair samples were rinsed in deionized water, dichloromethane and methanol, consecutively. After air-drying, hair samples were cut into 1 mm lengths with clean scissors, wrapped with clean paper and preserved at room temperature.

Hair segments of 20 mg were precisely weighed and transferred into 2 mL Eppendorf tubes. A volume of 1 mL of deionized water and 5 ng of EtG-D$_5$ (IS) were added to the tubes. After ultrasonication for 1 h, the solutions were allowed to stand overnight at room temperature (approximately 25 °C). The following day, the incubated sample was centrifuged, and the supernatant was transferred into a glass tube and evaporated to dryness under N$_2$ flow at 60 °C. The residue was reconstituted with 700 μL of acetonitrile and 100 μL of deionized water. After vortexing thoroughly, the solutions were filtered through a Sirocco^{TM} Protein Precipitation Plate (Waters, USA) under reduced pressure. The purified solutions were dried again, and 15 μL of pyridine and 30 μL of BSTFA were added to the residue. The derivatization with BSTFA was performed at 90 °C for 30 min. After cooling the solution at room temperature for 10 min, 20 μL of the solution was injected into the LVI–GC/MS/MS.

2.3. LVI–GC/MS/MS procedure

EtG analysis was performed in an LVI–GS/MS/MS system, which consisted of an Agilent 6890 gas chromatograph (California, USA)

equipped with an OPTIC 3 large volume injector (ATAS Inc., Netherlands) and coupled to a Quatro Micro tandem mass spectrometer (Waters Inc., USA). Large volume injection was performed by an OPTIC 3 high performance gas chromatograph injector, which was equipped with a pkg-5 injection liner. The programmable temperature vaporization (PTV) method was initiated at 70 °C with a rate of 16 °C/s and stopped at 270 °C. The sample sweep column flow was 1.0 mL/min, and the transfer column flow was 2.0 mL/min. The sample transfer time was 90 s.

The analytical column was an Agilent HP1 capillary column (30 m × 250 μm × 0.1 μm). Helium was used as the carrier gas with a flow rate of 1 mL/min. The programmed oven temperature was initially held at 100 °C for 1 min, then increased to 205 °C at a rate of 8 °C/min, held for 1 min at 205 °C, increased to 280 °C at a rate of 30 °C/min and held for 3 min at 280 °C. The total run time was 20.63 min.

The mass detector was operated at 70 eV in the electron impact (EI) ionization mode. The ion source and transfer line temperature were 200 °C and 300 °C, respectively. The collision gas was argon. Multiple reaction monitoring (MRM) mode was used. The observed precursor ions for EtG and EtG-D$_5$ were at m/z 261 and m/z 266, respectively, and the product ions were at m/z 73 and m/z 143, respectively. The ion pairs at m/z 261/143 and m/z 266/143 were used for quantification. The retention times were 14.01 min for EtG and 13.97 min for EtG-D$_5$.

2.4. Validation

Method validation was carried out according to the recommendation of Peter and Drummer [19]. The validation included an evaluation of method selectivity by analyzing blank hair samples from six children who have no alcohol consumption history. Blank samples were analyzed to check for the absence of analyte ions for the respective peaks of the internal standards (*n* = 2). For calibration, blank hair samples were spiked with analytes in equivalent concentrations. For the determination of analytical limits (limit of detection (LOD) and limit of quantification (LOQ)) blank hair samples were spiked with 6 calibration standard mixtures near the expected LOD. The intra-day and inter-day precisions were determined at four controlled concentrations by analysis of six aliquots of sample. A full set of calibration standards and a blank standard were run with each analysis.

3. Results and discussion

3.1. Optimization of the method

3.1.1. Optimization of the extraction technique

To increase the extraction yield, the protein precipitation (PPT) method was used, which is a quick and easy method of sample preparation. This study is the first to apply PPT to hair sample pretreatment. Hair samples were washed, dried and cut into pieces as described in Section 2.2. Subsequently, extraction of EtG out of the hair matrix was performed using ultrasonication in water for 1 h and incubated overnight at room temperature. After drying, the residue was reconstituted with 700 μL of acetonitrile and 100 μL of deionized water. The solutions were filtered through the Sirocco^{TM} 96-well plate (Waters) under reduced pressure. The purified solutions were dried for derivatization. Solid-phase extraction (SPE) was used for comparison with PPT. The Oasis MAX cartridge (3 mL, 30 mg) was used for SPE (Waters Inc.), and EtG was eluted with a methanol/2% formic acid mixture. The eluate was then transferred into a 10 mL vial and evaporated to dryness [20]. Extraction recovery, cost and extraction time were used as evaluation parameters of the extraction technique (Table 1). Obvi-

Table 1
Evaluation parameters of PPT and SPE extraction methods.

	PPT	SPE
Extraction time (min)	5	30
Sample size (piece)	96	16
Cost	Low	High
Procedure	Easy	Complex
Extraction recoveries (%)	>60	30–50
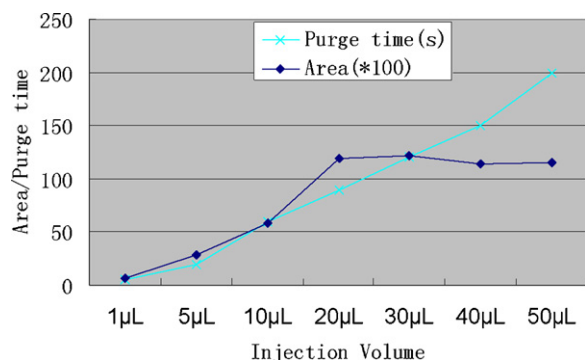
Fig. 1. Optimization of the injection volume and purge time of LVI.

ously, PPT is likely to enhance sample recovery for EtG detection in hair compared with SPE. Although most of the recent papers were using SPE for cleaning-up [11,21–23], it could process only 4 samples at one time, thus costing a lot of time for extraction. However, PPT could prepare 96 samples at one time with low cost. It is better to use PPT for purification when handling with large-scale samples.

3.1.2. Optimization of the LVI technique

This study is the first to determine EtG in the hair of Chinese individuals using LVI–GC/MS/MS. When the streaming emptying volume, purge time, the PTV temperature, the injection volume and other parameters are optimized, this can improve the sensitivity by at least 1–2 orders of magnitude compared to traditional 1 μL sample injection. Among these parameters, the injection volume and purge time were very important. We compared different injection volumes (5 μL, 10 μL, 20 μL, 30 μL, 40 μL and 50 μL) and their purge time, and we found that 20 μL was the optimal injection volume that yielded the highest response and relatively short purge time (Fig. 1). The sensitivity of this method was also greatly improved after applying LVI for the determination of EtG, the response of the 20 μL injection was almost 10 times more than that of the 1 μL injection, as shown in Fig. 2.

3.2. Validation results

3.2.1. Selectivity

Hair samples collected from six children with no alcohol consumption history were regarded as blank samples for analysis. Blank hair samples from these six sources were analyzed, and there were no interfering peaks at retention times characteristic of EtG and IS in the hair matrix. The chromatograms of blank hair samples and a sample spiked with EtG (10 pg/mg) are shown in Fig. 3.

3.2.2. Linearity and limits of detection and quantification

Standard EtG solutions were spiked into blank hair samples to yield final concentrations of EtG at 10 pg/mg, 25 pg/mg, 50 pg/mg, 100 pg/mg, 250 pg/mg, 500 pg/mg and 1000 pg/mg ($n = 2$); 5 ng of EtG-D$_5$ (IS) was also added. The hair samples were prepared according to Section 2.2. A calibration curve was constructed by plotting the area ratio of EtG and IS against the spiked concentrations. The resulting calibration curve for EtG was $y = 0.0036x + 0.0437$ ($R^2 = 0.9993$), which showed good linearity of EtG with this method.

The limit of detection (LOD) was defined as the spiked concentration at which the signal of analyte was at least three times the baseline. The limit of quantification (LOQ) was defined as the lowest concentration in the calibration curve at which the signal of analyte was ten times the baseline. The LOD and LOQ were 5 pg/mg and 10 pg/mg, respectively.
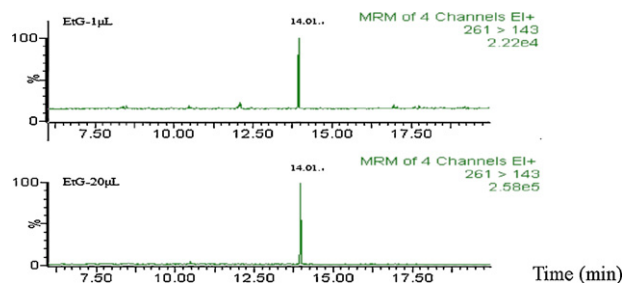
3.2.3. Accuracy and precision

The intra-day and inter-day precisions were determined at four controlled concentrations: 10 pg/mg, 25 pg/mg, 250 pg/mg and 800 pg/mg. The intra-day precision was determined by assaying six spiked hair samples at each concentration level on the same day, and inter-day precision was assayed for 24 replicates on four days (six replicates/day). The precision was expressed as the relative standard deviation (%RSD).

Accuracy was established by assaying quality control samples ($n = 6$) at four concentrations, and the accuracy was expressed as the percentage of the determined concentration against the spiked concentration. Both the intra-day and inter-day precisions were less than 15%, and the accuracy ranged from 90.1% to 106.5%. Detailed results are listed in Table 2.

3.2.4. Extraction recovery

The extraction recovery was determined by comparing the peak areas of the extracted standards with those of neat standards ($n = 6$) at concentrations of 10 pg/mg, 25 pg/mg, 250 pg/mg and 800 pg/mg. The extraction recoveries of EtG at these concentrations were 61.8%, 64.1%, 70.1% and 81.4%, respectively.

Fig. 2. Comparison of MRM chromatograms for 1 μL split injection and 20 μL large volume injection (250 pg/mg).

Table 2
Accuracy and precision data for the utilized method.

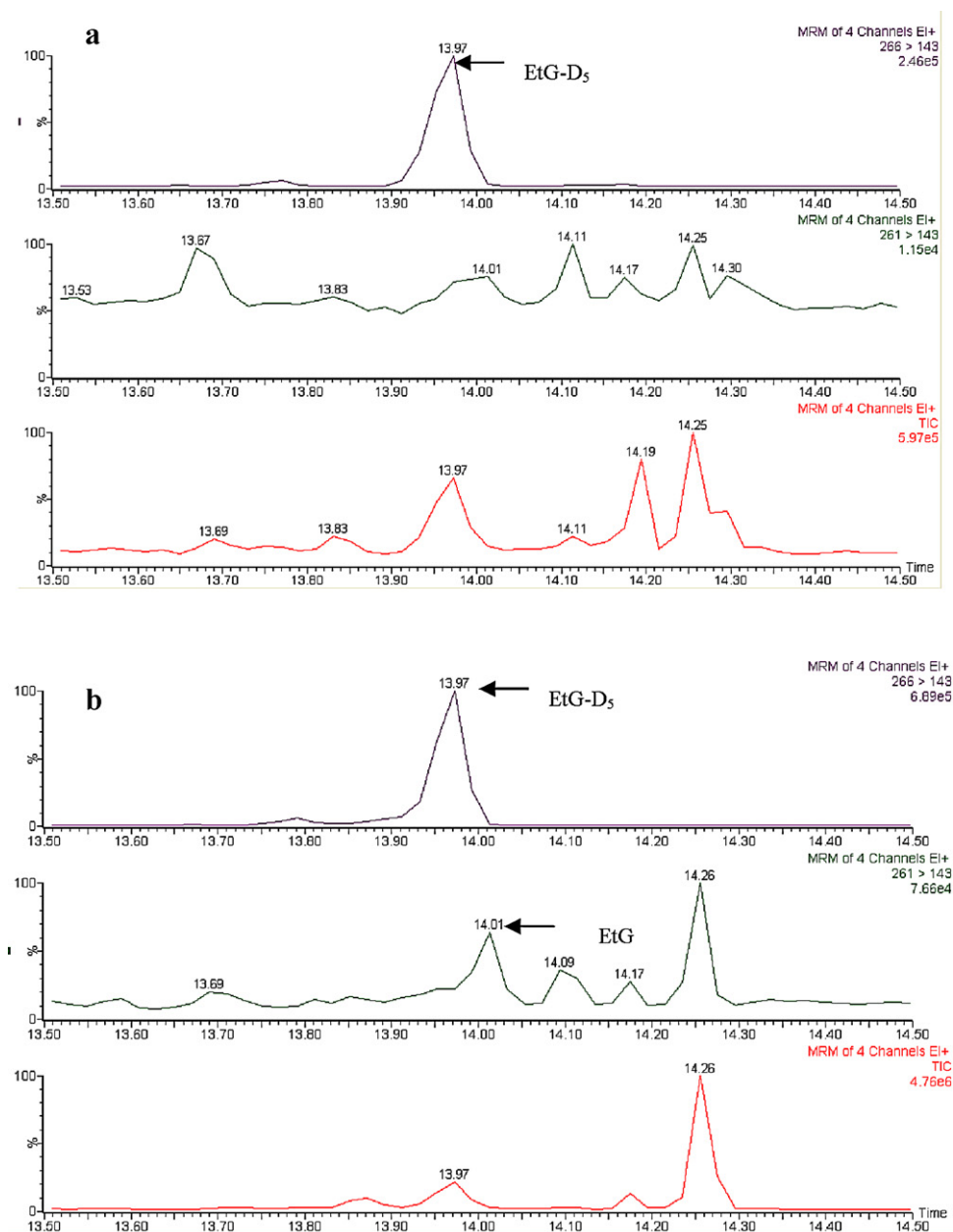| Analyte	Spiked concentration (pg/mg)	Accuracy (%) ($n = 6$)	Precision (RSD%) Intra-day ($n = 6$)	Inter-day ($n = 24$)
EtG	10	90.1	13.9	14.2
EtG	25	91.7	12.6	11.8
EtG	250	102.2	13.1	13.3
EtG	800	106.5	5.2	4.9

Fig. 3. MRM chromatograms of a blank sample (a) and the blank spiked with a standard at 10 pg/mg (b).

3.3. Concentrations of EtG in human hair samples

The described LVI–GC/MS/MS method is being applied in our laboratory for the analysis of EtG in human hair samples. Table 3 shows the results obtained for 21 samples from individuals with different drinking behaviors. The amount of alcohol intake was calculated by the formula: Alcohol intake (g) = Alcohol consumption (mL) * Alcohol concentration (%) * 0.8 (g/mL). Six hair samples obtained from children with no prior consumption of alcohol were found to be negative for EtG, and 15 samples obtained from alcohol-drinking subjects were found to be positive for EtG, except for one negative sample that may have been due to a low amount of alcohol consumption. The concentration of EtG in positive samples ranged from 10 to 78 pg/mg in hair. With an increase in average daily alcohol consumption, the concentration of EtG in hair also exhibited a corresponding increase, confirming the results of recent studies [23–25].

3.4. Hair color

It remains to be elucidated why EtG was low in six cases (subjects D04, D05, D07, D09, D10 and D13) despite a known history of alcohol abuse. The color of hair could be significant in this issue. Our study suggested that EtG determination in hair should consider hair color for correct interpretation of hair testing results. When alcohol consumption was the same, the EtG concentration of hair was quite different when the hair samples were of different colors (Fig. 4). In different studies performed on humans and animals, differences in drug concentration between white and pigmented hair were observed [26–28]. Drugs were often combined with melanin, so black hair usually combined more drugs than hair of other colors. However, the study by Appenzeller et al. demonstrated that the concentration of EtG in hair did not seem to be affected by its melanin content [24]. That result may due to differences in race, age, gender and metabolism that need further study.

Table 3
Results for EtG analysis in hair samples from people with different drinking behaviors (according to alcohol consumption).

Subject number	Gender	Age	Hair color	Drinking behaviors	Average daily alcohol consumption (g/d)	EtG concentration (pg/mg)
N01	F	13	Black	Children	0	ND
N02	F	7	Black	Children	0	ND
N03	F	7	Black	Children	0	ND
N04	M	9	Black	Children	0	ND
N05	F	8	Black	Children	0	ND
N06	F	12	Black	Children	0	ND
D01	M	28	Black	Social drinkers	4	ND
D02	M	43	Black	Social drinkers	20	<LOQ
D03	M	24	Black	Social drinkers	25	11
D04	M	52	Black streaked with gray	Social drinkers	30	<LOQ
D05	M	45	Black streaked with gray	Alcoholics	50	10
D06	M	50	Black	Alcoholics	63	14
D07	F	51	Black streaked with gray	Alcoholics	100	12
D08	M	40	Black	Alcoholics	100	22
D09	M	46	Black streaked with gray	Alcoholics	125	15
D10	M	52	Black streaked with gray	Alcoholics	125	11
D11	M	38	Black	Alcoholics	150	48
D12	M	46	Black	Alcoholics	150	40
D13	M	56	Black streaked with gray	Alcoholics	150	29
D14	M	56	Black	Alcoholics	175	55
D15	M	53	Black	Alcoholics	200	78

Abbreviations: N, never drink; D, have drinking behaviors; M, male; F, female; ND, not detectable.

3.5. Cut-off value

In interpreting the results, external sources for EtG, e.g. sweat, must be taken into consideration in every case. Alcohol consumption cannot be excluded from cases in which EtG is not detected; however, if the analysis for EtG in hair is positive, alcohol consumption has to be strongly assumed. The Society of Hair Testing (SoHT) has recently published new guidelines for the detection of EtG in hair. They recommend a cut-off value of 30 pg/mg scalp hair measured in the 0–3 cm proximal segment to distinguish between moderate and heavy consumption of alcohol [29]. However, we used the 0–5 cm proximal segment for determination because the history of drinking behavior was usually required more than 3 months. Our preliminary data indicate that discrimination between social drinkers (up to 30 g/d) and alcoholics might be possible by monitoring EtG levels in hair. A cut-off of 20 pg/mg of EtG in hair is proposed to differentiate between social drinkers and heavy drinkers, which is slightly different from that which was proposed by SoHT. Due to EtG's high polarity hydrophilicity, a strong hair treatment followed by a shampooing may lead to removal/degradation of this molecule from hair matrix. By frequent hair washing, EtG is believed to elute out of the hair matrix and therefore, our results measured in 0–5 cm hair segment is lower than that of SoHT. Besides, different experimental standards, gender, age and metabolic differences between Chinese Han and Caucasian individuals may be the reason for results that lead to

different cut-off values. Nevertheless, variability of EtG concentrations due to sampling region and biological differences suggest to responsibly consider a general surveillance policy for the subjects evaluation before any sanction is assigned.

Cosmetic treatment is also a critical issue, other studies suggest that EtG is easily removed from hair matrix after cosmetic treatment like dyeing and bleaching [9,30], it is advisable to collect hair sample from a different region of the body, except from the pubic area. EtG in pubic hair in fact does not correlate with EtG in hair from the other body regions as observed by Kintz et al. [16]. Currently, the mechanism of incorporation of EtG into hair remains unclear, and differences in race and metabolic rate may lead to different EtG concentrations in hair. Metabolomics, genomics and the elucidation of the mechanisms of the distribution of EtG in hair are areas for further study.

4. Conclusion

In this study, a selective and sensitive identification and quantification method for EtG in hair was developed and validated using PPT and LVI–GC/MS/MS. The method has been validated as accurate and reliable, and it was used in our laboratory for the analysis of EtG in hair samples from several Chinese individuals with a known history of alcohol consumption. The EtG concentrations of positive samples were 10–78 pg/mg hair. With an increase in average daily alcohol consumption, the concentration of EtG in hair also increases correspondingly. Our study suggests that 20 pg/mg of hair is suitable to differentiate between social drinkers and heavy drinkers, but when interpreting results of EtG concentrations from hair testing, hair color and cosmetic treatment had to take into account for the correct interpretation.

Fig. 4. EtG concentrations of different hair colors.

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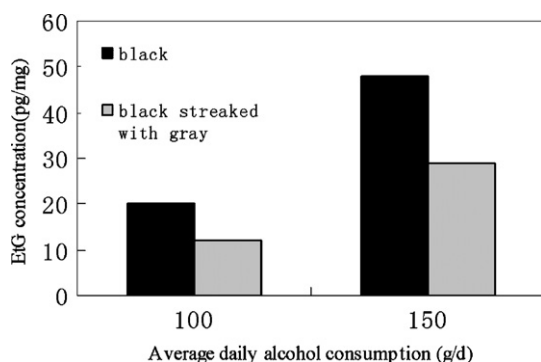[3] M.E. Albermann, F. Musshoff, B. Madea, Anal. Bioanal. Chem. 396 (2010) 2441.

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