Effects of various sample pretreatment procedures on ethyl glucuronide quantification in hair samples: Comparison of positivity rates and appraisal of cut-off values

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Abstract: The quantification of ethylglucuronide (EtG) in hair is nowadays recognized as the approach with the highest diagnostic performance to evaluate harmful drinking. A widely accepted cut-off of 30 pg/mg has been selected after several accurate compared studies. While most of the studies that were used to establish the appropriate cut-off value prescribed to cut hair into small segments before their extraction, hair milling has subsequently been identified as the most efficient pretreatment procedure and was therefore recommended in the last Consensus document issued by the Society of Hair Testing. In this study, we initially compared the results obtained with the two sample preparations, namely cutting and milling, both being applied to the same specimens (n=781). Among these, 205 samples produced measurable EtG values with both methods, with differences ranging from -41.7% up to +415% (the mean increase in EtG concentration, switching from cutting to milling, was +62.1% and the median was +42.3%). Among the aforementioned 205 samples, 29 specimens (3.7% of the total 781 samples) produced significantly different outcome, being classified as negative (i.e., below 30 pg/mg) if the cutting procedure is used, but largely positive (above 40 pg/mg) when milling is used. Subsequently, the positivity rates obtained on a large population dataset (> 27,000 samples) with the two procedures, were retrospectively compared using variable cut-offs values. The percentage of head hair samples with EtG concentration exceeding 30 pg/mg upon application of the milling procedure shows a 45% increase (from 10.9% to 15.8%) with respect to cutting procedure, whereas the fraction of hair samples with EtG exceeding 40 pg/mg (10.5%) overlaps the percentage of positive samples obtained after cutting pretreatment and applying a cut-off of 30 pg/mg. On the basis of these results, it would be worth considering the application of cut-off values linked with the pretreatment procedure, taking into account the results of forthcoming inter-laboratory calibrations.

Effects of various sample pretreatment procedures on ethyl glucuronide quantification in hair samples: comparison of positivity rates and appraisal of cut-off values.

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Graphical Abstract (for review)

781 samples

EtG >30 pg/mg

8.8%

15.8%

***Highlights (for review)**

- Hair milling is the most efficient pretreatment procedure for EtG analysis
- Hair sample pulverization by milling leads to higher extraction yields than cutting
- The Etg results obtained from the two procedures were compared for 781 hair samples
- Hair milling produced an average increase of 35-45% of EtG detected concentrations
- The use of different cut-off is suggested, as a function of the sample pre-treatment

ABSTRACT

The quantification of ethylglucuronide (EtG) in hair is nowadays recognized as the approach with the highest diagnostic performance to evaluate harmful drinking. A widely accepted cut-off of 30 pg/mg has been selected after several accurate compared studies. While most of the studies that were used to establish the appropriate cut-off value prescribed to cut hair into small segments before their extraction, hair milling has subsequently been identified as the most efficient pretreatment procedure and was therefore recommended in the last Consensus document issued by the Society of Hair Testing. In this study, we initially compared the results obtained with the two sample preparations, namely cutting and milling, both being applied to the same specimens (n=781). Among these, 205 samples produced measurable EtG values with both methods, with differences ranging from –41.7% up to +415% (the mean increase in EtG concentration, switching from cutting to milling, was +62.1% and the median was +42.3%). Among the aforementioned 205 samples, 29 specimens (3.7% of the total 781 samples) produced significantly different outcome, being classified as negative (i.e., below 30 pg/mg) if the cutting procedure is used, but largely positive (above 40 pg/mg) when milling is used. Subsequently, the positivity rates obtained on a large population dataset (> 27,000 samples) with the two procedures, were retrospectively compared using variable cut-offs values. The percentage of head hair samples with EtG concentration exceeding 30 pg/mg upon application of the milling procedure shows a 45% increase (from 10.9% to 15.8%) with respect to cutting procedure, whereas the fraction of hair samples with EtG exceeding 40 pg/mg (10.5%) overlaps the percentage of positive samples obtained after cutting pretreatment and applying a cut-off of 30 pg/mg. On the basis of these results, it would be worth considering the application of cut-off values linked with the pretreatment procedure, taking into account the results of forthcoming inter-laboratory calibrations.

INTRODUCTION

An important goal of forensic and clinical toxicology is to identify appropriate biological markers of ethanol consumption to evaluate harmful drinking [1–5] or to ascertain alcohol abstinence [6–8]. With these aims, the detection of ethylglucuronide (EtG) in hair represents nowadays the approach with the highest diagnostic performances [9,10]. The Receiver Operation Characteristics (ROC) curve [11] is an efficient statistical method, used to evaluate the discrimination power of a certain biomarker and select a suitable cutoff with optimized sensitivity and specificity. This technique was consistently applied in hair EtG studies to compare the performance of this molecule with that of other excessive alcohol consumption biomarkers [4,9,12–16]. In all these studies, the diagnosis of chronic alcohol abuse by means of hair EtG determination was based on a cut-off ranging from 25 and 30 pg/mg, which resulted highly accurate (AUC>0.9). In five of the aforementioned papers [4,9,13–15], the sample treatment prescribed to cut the hair specimen in 1-2 mm segments, while only two studies [12,16] utilized a mill to pulverize the samples. In the past, the Society of Hair Testing (SoHT) established the cut-off value that supports a diagnosis of chronic excessive alcohol consumption at the level of 30 pg EtG/mg, measured in the 0–3 cm up to 0-6 cm proximal segment [17], but more recently the SoHT also recommended to "powder hair prior to the extraction of EtG" [18]. With this supplement, the pre-analytical protocol is gaining higher attention.

A meta-analysis on the distribution of EtG concentrations in the hair of teetotalers, social-, and heavy drinkers [19] showed that in none of the examined studies (0 out of 13) EtG concentrations in the hair of teetotalers and social drinkers exceeded 30 pg/mg. Only 2 studies out of 13 used to pulverize hair by milling [12,20]. Furthermore, it was highlighted that only in a minority of the analyzed studies (3 out of 13), heavy drinkers (self-declared EDAI>60 g) occasionally exhibited EtG concentrations lower than 30 pg/mg, indicating that this cut-off is affected by a limited false negative effect. In two of the latter studies [21,22], the hair samples were cut into 1-2 mm segments and not milled. A similar pooled and meta-analysis was based on 8 studies which reported paired data for 70 subjects [23]. A median EtG concentration of 51.5 pg/mg was found for heavy drinkers, with 95% CI 38.4-86.2 pg/mg, and interquartile range 30-140 pg/mg. Furthermore, sensitivity and specificity indexes were calculated and plotted against several possible EtG decision limits. The 30 pg/mg cut-off led to the best diagnostic efficiency, with 0.97 specificity and 0.85 sensitivity. The diagnostic performance data for the EtG test derived from the meta-analysis (cut-off fixed at 30 pg/mg) proved even better than those calculated from the pooled analysis, yielding a sensitivity of 0.96 and a specificity of 0.99. Among eight considered studies, hair samples were cut in seven cases and milled only in one.

In the recent SoHT Consensus document [18], it is said that laboratories using hair preparation procedures other than powdering (i.e. cutting) should demonstrate comparable recovery of EtG. Principally, this approach is leading the way as only similar efficiency of extraction protocol and analytic allows the use of a common cut-off value. However, it was demonstrated by different authors that grinding the hair sample leads to higher extraction yields rather than cutting hair into short pieces. In particular, it was shown that grinding or milling hair provides 0.95- to 1.8-fold higher extraction after overnight incubation, and to 1.4- to 2.3-times higher extraction yields after 2 days incubation with water [24,25]. A more recent study on two authentic positive hair samples from two alcohol consumers also showed that an extensive pulverization of hair samples leads to a significantly higher amount of detectable EtG [26].

In the study presented hereby, we initially compared the results obtained with the two sample preparations, namely cutting and milling, both being applied to the same specimens. Our aim was to evaluate the difference in the extraction yield between the two procedures on a large number of samples. Subsequently, the positivity rates obtained on different groups of samples with the two procedures, namely cutting and milling, were retrospectively compared using variable cut-offs values.

MATERIALS AND METHODS

Reproducibility of the analytical results after the cutting and milling procedures

A pooled hair sample from 15 donors (approximately 2 grams) was accurately homogenized and analyzed 10 times after extraction with the cutting preparation procedure, and 10 times after extraction with the milling preparation procedure. Each aliquot weighted approximately 40 milligrams. The variability was expressed by means of CV%.

Study protocol for the direct comparison of the two crumbling treatments executed on the same hair samples

The direct comparison of the two hair crumbling procedures was executed on 781 samples (either head or chest hair) randomly selected. All samples were collected during year 2015 from subjects who underwent medical examination either within alcohol abuser's rehabilitation programs or for driving relicensing protocols. Several medical committee located in Piedmont, northern Italy, commissioned these analyses. All hair samples were cut as close as possible to the scalp or the skin surface, using freshly disinfected scissors. The samples were stored at room temperature and

analyzed within 10 working days. Only the proximal 0–3 cm segment was analyzed whenever longer head hair samples were collected. When more than one lock of hair from one subject was available, the material was pooled and homogenized. These hair samples were washed twice using methylene chloride and methanol in sequence [27]. Dried hair was separated into two aliquots of similar weight. One aliquot was cut into small snippets (about 1 mm) with freshly cleaned scissors, whereas the second aliquot was pulverized using a metal beads mill. A Precellys 24 Tubes Homogenizer (Bertin Pharma, France) equipped with six 2.8 mm metal beads was used for hair milling. EtG extraction was carried out overnight at room temperature with a 35:1 water:methanol mixture. Then, the samples were sonicated and an aliquot of liquid phase was transferred into a vial for UHPLC–MS/MS analysis. The two aliquots were extracted simultaneously and analyzed in the same batch (single injection). The comparison of the procedures was made on the EtG concentrations found in the two hair aliquots.

Determination of EtG

Analyses were performed using a Shimadzu Nexera UHPLC-system (Shimadzu, Duisburg, Germany) interfaced to an AB Sciex API 5500 triple quadrupole mass spectrometer (AB Sciex, Darmstadt, Germany) with an electrospray (ESI) source operating in the negative ion mode. The limits of detection (LOD) and quantification (LOQ) were 3 and 10 pg/mg respectively. The method was internally validated and accredited in accordance with ISO/IEC 17025:2005 rules. The laboratory performances in hair EtG analysis were constantly monitored through regular participation to inter-laboratory proficiency tests organized by SoHT and the Gesellschaft für Toxikologische und Forensische Chemie (GTFCh).

Study protocol for comparative evaluation of the percentage of positive samples according to different cut-offs

The comparison of EtG positive samples distributions according to the hair crumbling procedure (cutting or milling) was executed using both prior results and more recent (year 2015) data. These include i) 24771 samples (22825 head hair and 1946 chest hair) analyzed after cutting and presented in a previously published study [27], ii) 5118 samples (4632 head hair and 486 chest hair) analyzed after cutting, and iii) 2730 samples (2461 head hair and 269 chest hair) analyzed after milling. Groups i and ii were united in order to obtain a single group of hair prepared by cutting, for a total of 27457 head hair samples and 2432 chest hair samples. For the large statistical population investigated, the different cohorts of subjects can be viewed as global distributions with specific properties, rather than collections of independent individuals [27,28]..

Statistical analysis

A significant level of 0.05 (CI = 95%) was chosen for the statistical evaluations (two sample t-test and paired t-test), in order to verify the occurrence of statistically significant differences between the two populations divided by the method of extraction.

All statistical analyses were conducted using the software packaging R (The R Project for Statistical Computing, Vienna, Austria), version 3.2.3 for Windows

RESULTS AND DISCUSSION

Reproducibility of the analytical results after the cutting and milling procedures

For the samples undergoing the cutting procedure, the average value was 13.4 pg/mg (range 12-14), with a variability of 4.7 %. For pulverized samples, the average value was 28.4 pg/mg (range 25-31), with a variability of 6.1 %. Therefore, we concluded that each pre-analytical procedure showed a satisfactory reproducibility (CV% <10% in both cases).

Direct comparison of paired hair EtG results upon applying the two crumbling treatments

The selected 781 samples analyzed with the two methods yielded 475 negative results (i.e. <10 pg/mg) with both procedures, while 306 hair samples (39.2%) gave measurable EtG concentration (>10 pg/mg) after milling pretreatment, and only 205 (26.2%) produced results above the LOQ when the cutting pretreatment was used (Figure 1). The percentage of positive samples (>30 pg/mg) was 8.8% with cutting and 15.9% with milling. In 96 out of 101 samples of the group tested negative after cutting pretreatment but positive after pulverization, the EtG content was below 30 pg/mg (i.e., negative to excessive alcohol consumption testing), while in remaining 5 cases the concentration passed from <10 pg/mg after cutting up to >30 pg/mg after milling. These cases represent particularly critical situations, because radically different classification of alcohol consumption arises for the tested subjects.

A more accurate comparison between the results (and possibly the extraction yields) obtained with the two procedures is achieved by considering only the 205 samples that gave measurable EtG values by both methods. Among them, the EtG concentration measured for pulverized samples turned out to be lower than for cut samples in 33 cases, while it was higher for the remaining 172 samples. The correlation between the EtG level measured after cutting (EtG_C) and milling (EtG_M),

for each of these 205 samples, is showed in Figure 2. The slope of the correlation line is equal to 1.31, suggesting an average increase of 31% if the milling technique is used. The observed difference for each sample ranged from –41.7% up to +414.8% if referred to EtG_C [$100\times(EtG_M - EtG_C)/ EtG_C$]. The mean increase in EtG concentration, switching from cutting to milling, was +62.1% and the median was +42.3%. The mean values for cut and milled hair were, respectively, 36.1 pg/mg and 53.9 pg/mg. The median values for cut and milled hair were, respectively, 21.0 pg/mg and 35.5 pg/mg. The results are summarized in Table 1. The corresponding box-plots is shown in Figure 3. After the appropriate statistical tests, the null hypothesis H_0 is rejected (p-value for t-test = 1.5×10^{-8}; p-value for paired t-test = 2.2×10^{-16}), confirming that the two procedures (and the corresponding extraction yields) produce significantly different results.

Among the aforementioned 205 samples with EtG_M and EtG_C > 10 pg/mg, 86 cases tested negative (i.e., below 30 pg/mg) with both procedures and 69 cases tested positive (i.e. above 30 pg/mg) with both cutting and milling procedures. For all the remaining 50 samples, the EtG concentration switched from <30 pg/mg (cutting) to >30 pg/mg (milling). These samples deserve careful evaluation, since they tested negative (i.e. below the SoHT cut-off) when the hair aliquot is subjected to cutting, but positive (i.e. above the SoHT cut-off) if milling is applied to crumble hair. Among these 50 cases, 21 samples yielded limited differences, switching only from <30 pg/mg to <40 pg/mg. Overall, 29 specimens (equivalent to 3.7% of the total 781 samples) produced significantly different outcome, being classified as negative (i.e. below 30 pg/mg) if the cutting procedure is used, but largely positive (above 40 pg/mg) when milling is used. The results are summarized in Table 2.

This direct comparison of the EtG concentrations of corresponding aliquots demonstrates that changing the hair crumbling technique from cutting to milling is likely to produce a more effective extraction of the EtG incorporated into the keratin matrix. This result is in agreement with published data [24–26].

Comparison of hair EtG positive rate of a large dataset having regard to crumbling techniques and cut-off values

In a previous study [27], we investigated a large population dataset (> 20,000) of subjects undergoing medical examination for driving re-licensing during the period 2009-2013, where we demonstrated that randomization on large cohorts of subjects leveled off the individual variability (hair type, color, and length, cosmetic treatments, hygiene habits, and even alcohol consumption), resulting in comparable means, standard deviations, and overall distributions, and enabling the

investigation of separate factors (i.e., age, gender, sampling season, site of hair sampling). In the present study, the previous approach was extended to the new data, collected in 2014 and 2015 for driving re-licensing procedures, partly obtained after using the hair cutting procedure, and partly after we implemented the milling procedure. The distribution of subjects in terms of gender, age and collection season are extremely stable throughout the years, allowing considering the data collectively [27] within the evaluation of the effects of the different hair preparation procedures. One only significant trend was observed during the considered years, namely a progressive smooth reduction of the positive rate from 2009 to 2013 [27].

Following this approach, the differences in EtG concentration distributions and the percentage of positive samples within each group (after cutting and after milling, respectively) were evaluated as a function of the cut-off value used to discriminate positive from negative samples; in addition, scalp and chest hair results are reported separately. The percentages of positive samples when different cut-offs are applied, are presented in Table 3.

The results arising from routine application of the two hair crumbling procedures point out significant differences between the two techniques, but possibly smoother discrepancies with respect to the comparative data discussed in the preceding chapter. In fact, the percentage of head hair samples with EtG concentration exceeding 30 pg/mg upon application of the milling procedure shows a 40% increase (from 11.3% to 15.8%) with respect to cutting procedure, whereas the fraction of hair samples with EtG exceeding 40 pg/mg (10.5%) overlaps the percentage of positive samples obtained when a cutting procedure is used and a 30 pg/mg cut-off is applied (Table 3). In practice, there is no evidence of a significant sample percentage that would turn out negative (i.e., <30 pg/mg) using the old procedure and largely positive (i.e., >40 pg/mg) using the new one. Quite obviously, this represents a general trend, that do not apply to particular cases, where largely different results were infrequently observed using the two sample preparation procedures, as evidenced in the previous paragraph.

It is worth noting that positive rates for chest hair are strictly comparable to those observed for head hair, no matter what crumbling technique is used nor what cut-off value is applied.

In general, the data reported in Table 3 indicate that changing the hair crumbling technique from cutting to milling results in an increased positive rate when a fixed cut-off value is applied. In case of a 30 pg/mg cut-off, the positive rate is 4-5% (absolute scale) and 35-45% (relative scale) higher; similar discrepancies were observed using other cut-offs. Comparable positive rates can only be

observed if the cut-off is maintained at 30 pg/mg when cutting is used as crumbling procedure and an increased value of 40 pg/mg cut-off is adopted when the milling technique is used.

CONCLUSIONS

The results obtained in the present study partly confirm the finding reported in previous investigations [24–26], suggesting that a more exhaustive extraction of EtG is obtained when the hair matrix is pulverized with a metal ball mill rather than cut into small snippets with scissors. The comparison of positive rates as a function of the adopted cut-off value apparently indicates an average increase of 35-45%. However, the direct comparison of the EtG concentrations measured with the two pre-analytical techniques on the same samples, as extensively performed in the present study, highlights that the observed differences are (i) highly variable and (ii) virtually unpredictable. Repeated analysis on different aliquots of the same pooled hair sample proved that these effects cannot be attributed to a scarce reproducibility of the sample preparation methods.

Preliminary attempts to associate the extent of EtG concentration differences, hypothetically related to the inherent extraction efficiency, to specific features of the hair samples under study (i.e., hair color, average diameter, section profile, and subject age) failed [29]. Therefore, much more in-depth investigation is needed to understand the reasons for these differences and single out the factors that determine such a variable extraction efficiency. Similarly, it is necessary to investigate if other modification in the extraction process (e.g. the use of organic solvents in the extraction mixture) would influence the extraction yield. This is particularly critical in the case of alcohol abuse assessment, since the laboratory should accurately quantitate EtG and relate it with the appropriate cut-off to estimate the average intake of a licit compound, namely alcohol. As a matter of fact, it is not unusual to establish different cut-offs or normal ranges with respect to different analytical methods, like in the case of CDT [30], or different hair length, as for fatty acids ethyl esters [18].

The current SoHT consensus document refers at a hair EtG cut-off of 30 pg/mg to verify the condition of chronic excessive drinking. This cut-off was based on a majority of studies that employed the hair cutting procedure rather than milling. Indeed, the newest SoHT document recommends to pulverize hair with a mill and not to cut it into short segments, in order to achieve a more exhaustive EtG extraction from the keratin matrix. The present study indicates that different cut-off values can realistically be applied depending on the crumbling procedure that has been used, cutting or milling, respectively. For example, the traditional cut-off value of 30 pg/mg can be applied in case the hair sample is cut with scissors, whereas an increased value of 40 pg/mg might be more reliable when the hair sample is pulverized by milling. This new cut-off value would be

coherent with (a) the slope of the correlation line observed by applying the two procedures on the same hair samples and (b) the comparable positivity rate obtained by applying a 30 pg/mg cut-off after the cutting procedure and a 40 pg/mg cut-off after the milling procedure. The proposed innovation is immediately applicable in the clinical setting, where the physicians' judgment based on a variety of clues remains decisive. The same does not apply to the forensic context, where changing a cut-off which has been used in court or for administrative procedures for many years has possibly serious practical consequences (appeal, regress, compensation). Further in-depth and cooperative studies, including inter-laboratory comparison and enlarged investigations with different population sub-groups with controlled drinking behaviors, appear to be needed to clearly evaluate the sources of variability, and the most appropriate cut-off values and their associated measurement uncertainty according to the extraction procedure.

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Figure 1

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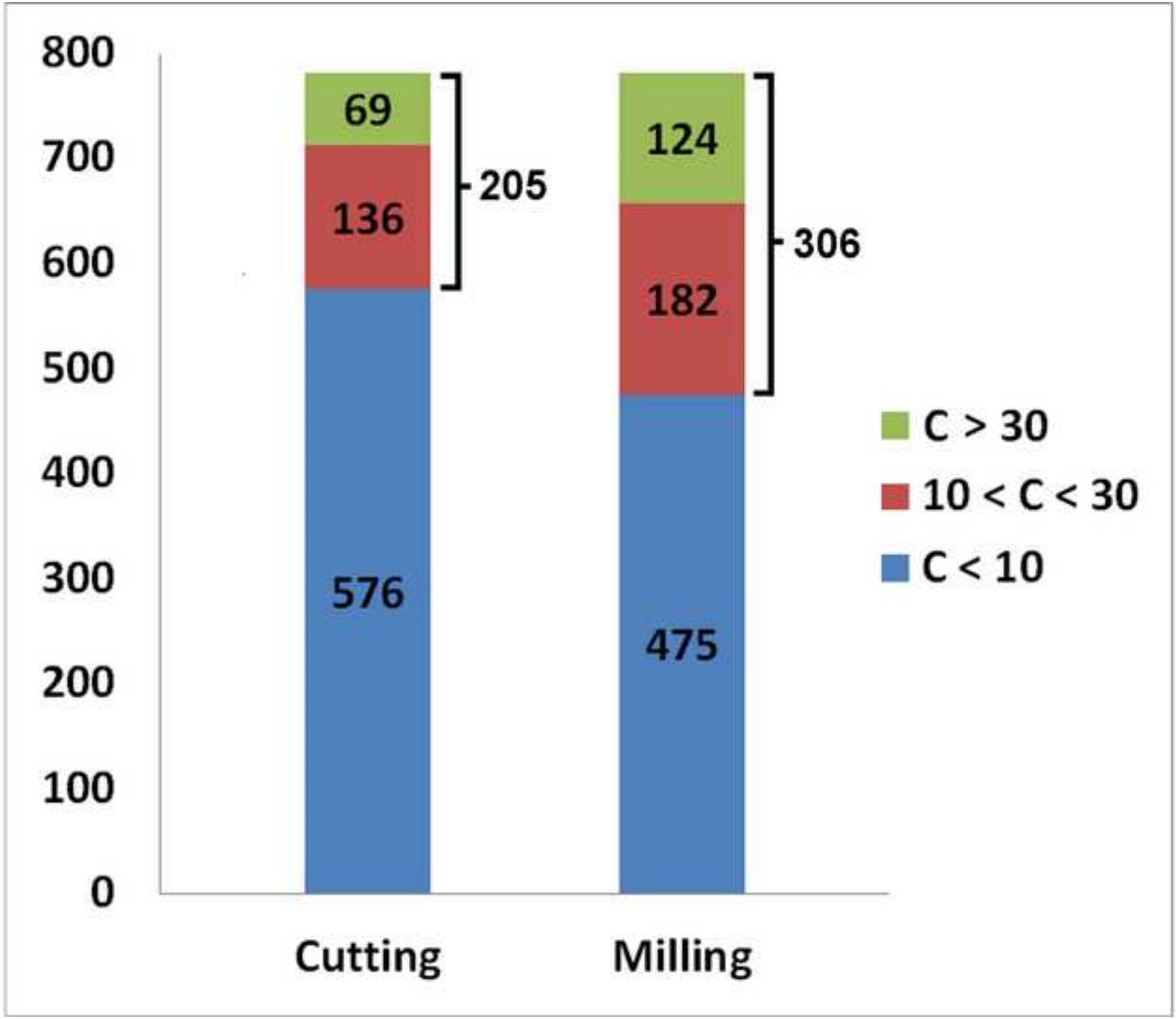

Figure 2

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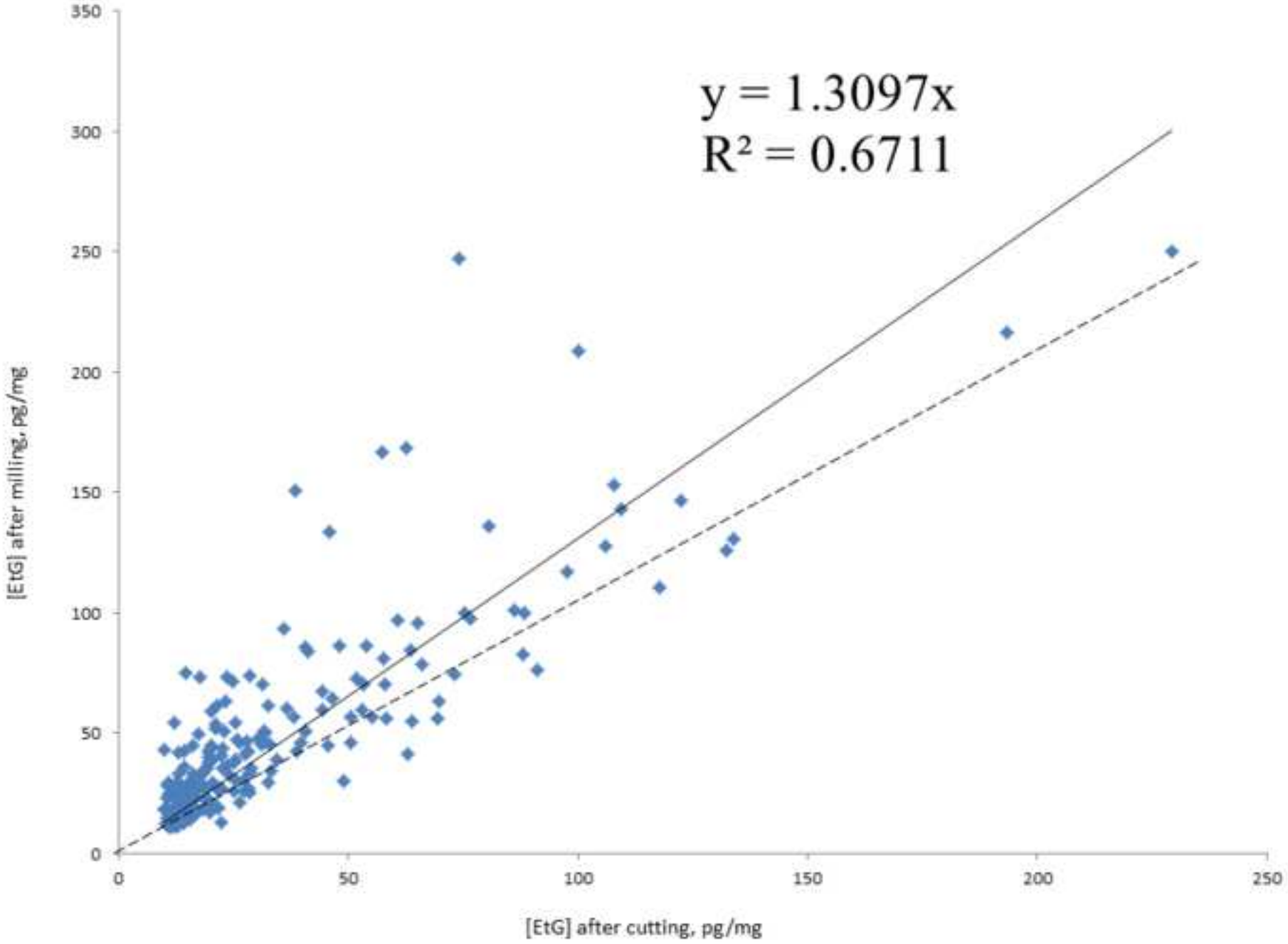

Figure 3

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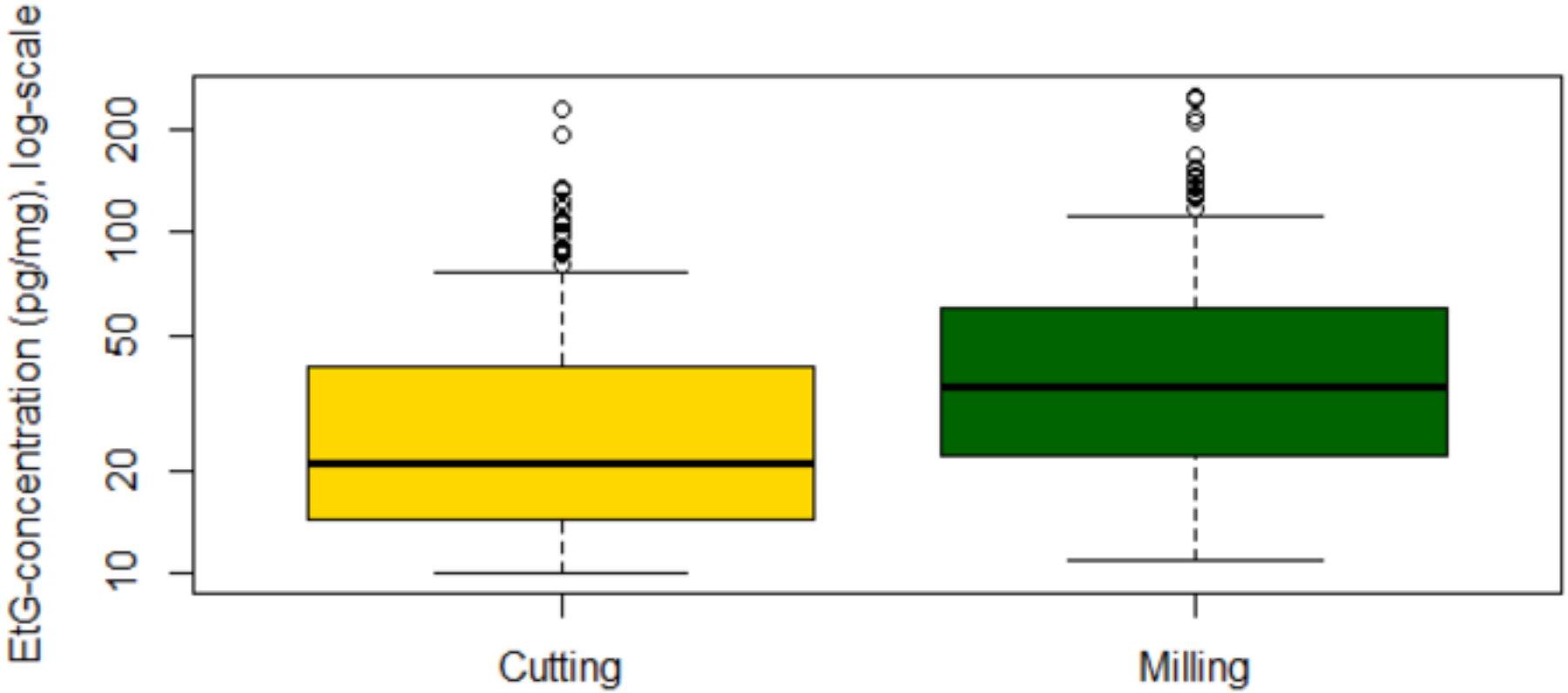

Figure Captions

Figure 1. Number of negative (EtG <10 pg/mg) and measurable (EtG>10 pg/mg) hair samples after cutting and milling treatment (where C stands for EtG concentration), n=781

Figure 2. Correlation between the EtG levels measured after cutting (*x*-axis) and after milling (*y*-axis), for each sample (n=205). The dashed line represents the hypothetical perfect equivalence of the two methods (y = x)

Figure 3. Box plots for the EtG distributions in the two preparation methods (205 samples). Y-axis plotted on a logarithmic scale

Table 1 Comparison of EtG concentrations determined on 205 samples depending on the extraction procedures

	Cutting (pg/mg)	Milling (pg/mg)	Differences (%)
Range	10 - 597	11-1040	-41.7 to + 414.8
Mean	36.1	53.9	62.1
Median	21.0	35.5	42.3

Table 2 Correlation table of the number of samples classified into different ranges of concentration. The EtG-concentration of all 781 samples were determined with both cutting and milling extraction procedures

		Milling				
		< 10 pg/mg	10-30 pg/mg	> 30 pg/mg	30-40 pg/mg	> 40 pg/mg
Cutting	< 10 pg/mg	475	96	5	21	29
	10-30 pg/mg	0	86	50		
	> 30 pg/mg	0	0	69		

Table 3. Distribution of positive results (EtG > cut-off) for two groups of scalp and chest hair samples applying five different cut-off values

Group	**1 – Cutting pretreatment**		**2 – Milling pretreatment**	
	Head hair (n=27457)	**Chest hair (n=2432)**	**Head hair (n=2461)**	**Chest Hair (n=269)**
Cut-off (pg/mg)	**% positive**	**% positive**	**% positive**	**% positive**
30	11.3	10.4	15.8	16.0
40	8.6[§]	7.1[§]	10.5	10.8
50	6.3	5.3	7.7	7.8
100	2.2	1.8	2.6	3.4
200	0.6	0.4	0.5	0.4

[§]This value was not calculated on real samples but estimated using a third degree polinomial fitting. The fitting was performed on the percentage values for Group 1 at cut-offs 30, 50, 100 and 200 pg/mg and verified on Group 2

***Revision Notes (Response to Reviewers)**

Dear **Forensic Science International** editor,

I'm pleased to submit a **revised version** of the manuscript FSI-D-16-00305.

The title was changed, taking into account the reviewers' comments and the discussion that we had during the recent SoHT Expert Meeting in Venice for the revision of Consensus on Alcohol biomarkers.

All the changes requested by reviewers were made to the manuscript and marked using a yellow highlighting. An itemized list of these changes, in response to the referee's observations, is reported below.

Reviewer #1:

1. We agree with the Reviewer that the comparison of different groups alternatively treated with one or two extraction procedures might generate some confusion. Also Reviewer #2 suggests to improve the comprehensibility of Table 3, but he does not suggest to restrict the discussion to the samples treated with both methods only. Since the aim of our comparison was simply to infer the expected percentage of positive subjects in the old population using a large dataset of samples, we decided to maintain Table 3, but in a modified and simplified form. When the expected percentage was set, we cross-evaluated our data in order to identify which combination of sample pre-treatment and cut-off was capable of recognising the same number of positive subjects. In order to clarify this approach, the statistical principle that randomization on large cohorts of subjects levels off the individual variability and equalizes the probability distribution was stressed in the "Materials and method" section. Ref.#28 was also added. In Table 3, former groups 1 and 2 were merged, in order to compare uniquely the percentage of positive samples obtained in our laboratory with the two procedure (cutting and milling), regardless of data being already published or not. Sections "Materials and methods", "Results and discussion" and Table 3 were modified accordingly.
2. Additional experimental work has been added to test the reproducibility of each pre-analytical procedure and further support the method validation. A paragraph was added in the Materials and methods" and "Results and discussion" sections.

Reviewer #2:

- The extraction of EtG from cut hair might be based on other standard hair extraction procedures. Nevertheless, and referring to the results, this extraction procedure has not been tested for the usefulness and efficiency which should be an imperative part of the method validation.

  Response: The laboratory performances in hair EtG analysis have been constantly monitored through regular participation to inter-laboratory proficiency tests organized by SoHT and the Gesellschaft für Toxicologische und Forensische Chemie (GTFCh). The criteria for a successful participation had been always met also when cutting pre-treatment was used. Moreover, additional experimental work has been added to test the reproducibility of each pre-analytical procedure and further support the method validation. A paragraph was added on this subject in the Materials and methods" and "Results and discussion" sections.
- Suggesting different cut-off values depending on the extraction procedure won't be change the problem of strongly varying extraction efficiencies even when the overall data supports this theory. In a single case, it can be completely different as the variation of

EtG concentrations (pulverized to cut hair) from -41.7% to +414.8% shows [table 1].
Response: The reproducibility of each pre-analytical procedure was tested, as mentioned in the preceding response.

- There is no discussion about the possible consequences of changing a cut-off which has been used in court or for administrative procedures for many years (appeal, regress, compensation, etc.).
  Response: The "Conclusions" section was modified to discuss the consequences of changing the cut-off values. Moreover, the title of the manuscript has been changed accordingly.
- Furthermore, at least two studies support the actual SoHT consensus of 30 pg/mg; Appenzeller et al. FSI 173 (2007) 87-92 and Kharbouche et al [10].
  Response: The missing study from Appenzeller was added to the References.
- References: 10 and 12 are identical.
  Response: Ref. 10 was corrected.
- Table 3: The table could gain in comprehensibility if the column headers would directly indicate (instead of asterisks or circle) if cutting or milling was used.
  Response. Table 3 was modified as suggested. Furthemore**,** Groups 1 and 2 were merged, in order to exclusively compare the percentages of positive samples obtained in our laboratory using the two procedures (cutting and milling), regardless of data being already published or not.