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Comment

Comments on: Uncertainty of Blood Alcohol Concentration (BAC) Results as Related to Instrumental Conditions: Optimization and Robustness of BAC Analysis Headspace Parameters. *Chromatography* 2015, *2*, 691–708

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The 2015 publication of original research by Boswell and Dorman [1], *Uncertainty of Blood Alcohol Concentration (BAC) Results as Related to Instrumental Conditions: Optimization and Robustness of BAC Analysis Headspace Parameters* described an evaluation of the effect of modifying headspace parameters on ethanol analysis by gas chromatography with dual flame ionization detection (GC-FID). The data reported may be useful when developing a new method for ethanol analysis, however there are several suggestions and considerations that should be made regarding implementing the protocol, as described in the paper.

1. The method was described as an optimized analytical process for the determination of blood ethanol concentration (BAC) although human blood samples, which are primarily the type of forensic evidence received for BAC testing, were not part of the experimental design and since no matrix effect evaluation was completed, this method could not be used on casework evidence in an accredited laboratory.

2. Insufficient empirical data is provided to appropriately estimate the uncertainty of measurement (UOM) for a quantitative measurement, although the authors referenced a publication in which an appropriate approach is described [2]. Historically, an UOM was not routinely calculated and/or reported in a forensic toxicology laboratory as stated by the authors, but currently many laboratories have been accredited to the International Organization for Standardization/International Electrotechnical Commission (ISO/IEC) 17025 and American Society of Crime Laboratory Directors/Laboratory Accreditation Board (ASCLD/LAB) *Supplemental* standards which require an estimation of the UOM to be performed for BAC determinations and weight measurements near statutory limits [3]. Providing an UOM based on data supporting the method described would be a valuable contribution for the laboratories considering implementation on casework evidence.

3. The authors rightfully conclude that "chromatographic resolution and peak shape are vital in determining peak area for quantification of blood alcohol determination". An explanation why chromatographic resolution for one of the internal standards used, *t*-butanol, was not achieved would be insightful for the reader. The resolution mixture presented in Figure 2 did not include *t*-butanol. Examination of the caption of Figure 2 reveals that *t*-butanol would coelute with acetone on the DB-ALC1 column and with acetonitrile on the DB-ALC2 column. Coelution of the internal standard with acetone is of considerable concern for a BAC method as acetone is frequently found in human blood samples [4,5], and if present would interfere with the internal standard quantitation of ethanol by causing a falsely low concentration.

4. In 2013, The Scientific Working Group for Forensic Toxicology (SWGTOX) published standard practices for method validation in forensic toxicology [6]. In order for the method described to be in compliance with the 2013 validation guidelines and for the method to be fit-for-use in whole blood

ethanol analysis casework, the authors should consider conducting these mandatory studies in order for laboratories to consider its use.

The parameters described as optimized for BAC analysis were never applied to blood specimens and no matrix effect evaluation was completed. All of the headspace (HS) oven temperatures studied were above the temperature previously reported to cause the oxidation of ethanol to acetaldehyde in blood and therefore cause falsely low ethanol quantitation [7]. The same phenomenon was not observed in water based standards or plasma [7]. To prevent the oxidative loss of ethanol in whole blood while thermostatting for HS analysis, addition of sodium dithionite as an inhibitor [8] or temperatures less than or equal to 50 °C have been recommended [7]. Only water based standards were used and an inhibitor was not added in the presented experiments. The utility for ethanol analysis in whole blood specimens has not been demonstrated and, due to the recommended temperature of 85 °C, is questionable.

For HS analysis to provide accurate and precise results, the volatile compounds must be equilibrated between the liquid and gas phase in a closed system (the headspace vial). The time to reach equilibration will vary depending on the HS oven temperature. No information was presented that any evaluation of the HS equilibration was performed at any of the conditions studied. The conclusions of the studies could be biased if equilibration was not reached under the conditions evaluated.

Many published methods for BAC analysis require a much lower, more appropriate sample volume of 100 µL [9,10] as compared to the 500 µL reported. In forensic toxicology analysis, samples with limited sample volume are frequently encountered and regularly require the performance of many different procedures due to the presence of multiple drugs along with alcohol. Validation using low sample volumes would have been beneficial as different results may be obtained, especially for the limit of detection (LOD). Sample volume and preparation are parameters that should be evaluated and validated before an optimized method for BAC can be presented.

The referenced Environmental Protection Agency (EPA) method for determining method detection limits (MDL) states that when calculating the MDL "the sample may be used as is for determining the method detection limit if the analyte level does not exceed 10 times the MDL of the analyte in reagent water" [11]. The manuscript published an MDL below 0.002 g/dL using a 0.02 g/dL standard, therefore the EPA method cannot be used. "The variance of the analytical method changes as the analyte concentration increases from the MDL, hence the MDL determined under these circumstances may not truly reflect method variance at lower analyte concentrations" [11]. Therefore, the calculated MDL using the 0.02 g/dL standard reported for the optimized methods of 0.00002 g/dL and 0.00004 g/dL are not valid and the true limit of detection of the method was not determined or estimated.

In conclusion, although an interesting approach to method optimization is described, the data does not support the use of the reported HS parameters for alcohol analysis in whole blood specimens.

Conflicts of Interest: The author declares no conflict of interest.

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