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Research Article

Measurement Uncertainty of Ethanol Concentration in Venous Whole Blood Determined By a HS-GC-MS Method

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Abstract

Since the alcohol intake strongly affects the driving capability and the Blood Alcohol Concentration (BAC) is related to the car crash risk, many countries define threshold values of BAC for drivers. In this work the measurement uncertainty of the BAC was estimated in view of the use of the BAC value in the legal procedures. Moreover, the in-house validation of the HS-GC-MS analytical method, used for the determination of the BAC, was carried out to ensure a suitable level of quality of the measurement. The method employed uses matrix-matched references for both calibration and quality control procedures. The parameters examined during the validation procedure were: sensitivity, linearity and uncertainty of the calibration, limit parameters, carry over, precision and trueness. Both intra-assay repeatability and intermediate precision were evaluated at 0.5 and 0.8 g L^{-1} of ethanol. In the second case, the variability of three factors, i.e. operator, liquid dispenser and time, was considered. The limits of detection and quantitation are $5.8 \cdot 10^{-4}$ and $1.8 \cdot 10^{-3}$ g L^{-1}, respectively. The values of intra-assay precision at 0.5 and 0.8 g L^{-1} are 1.7% and 2.2% g L^{-1}, respectively. Those of intermediate precision are 6.7% and 5.6%. The method provides unbiased results.

Various contributions were taken into account to assess the uncertainty budget by the bottom up approach. Relative combined standard uncertainty, for the two concentration levels (in bracket), are: u^c_r (0.5)=3.4% and u^c_r (0.8) = 3.1%, using intra-assay repeatability, and u^c_r (0.5) = 7.3% and u^c_r (0.8) = 6.0% with intermediate precision.

Keywords: HS-GC-MS; BAC; Validation; Measurement uncertainty; Ethanol

Introduction

Ethanol is a well-known psychoactive depressant drug consumed worldwide in food and beverages and it is also one of the most widely used substances of abuse. High doses of ethanol cause changes in perception and motor incoordination up to stupor, unconsciousness and coma. Long-term immoderate consumption of ethanol produces toxic effects leading to abuse up to physical dependence (chronic alcoholism). Long-term ethanol misuse is associated with liver and cardiovascular diseases, cancer and nervous system damage as well as psychiatric problems such as depression, anxiety and antisocial personality disorder [1]. Data on ethanol consumption all over the world are available on the World Health Organization periodic document "Global status report on alcohol and health 2018" [2].

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Many countries have laws regulating the production, sale and consumption of alcoholic beverages. Moreover, since the alcohol intake strongly affects the driving capability and the Blood Alcohol Concentration (henceforth: BAC) is related to the car crash risk, many countries define threshold values of BAC for drivers. The Italian regulation identifies three threshold values: 0.5, 0.8 and 1.5 g L^{-1}. Driving having BAC higher than 0.5 g L^{-1} is forbidden and to have a BAC greater than 0.8 or 1.5 g L^{-1} leads to harsher penalties.

The analytical measurement of BAC is performed on venous whole blood by way of i) an enzymatic method based on the biochemical oxidation with the enzyme ADH (alcohol dehydrogenase); [3] or ii) a gas-chromatographic (GC) method, with the headspace (HS) sampling technique (HS-GC) [4]. The first one is used as screening method, while the second one is considered as a reference method and provides results having forensic validity. HS-GC-FID (Flame Ionization Detector) or -MS (Mass Spectrometry) methods were used for the routine determination of ethanol concentration on whole blood [4-7], specifically, for the determination of BAC in suspected drunk drivers.

Goal of this paper is the evaluation of the measurement uncertainty of BAC measured by the HS-GC-MS method. The method was previously in-house validated to ensure a suitable level of quality in view of a forensic application.

Validation implies the evaluation of the performances of a measuring system according to a given measurement procedure [8-10]. Parameters of validation here examined were: sensitivity,

range of linearity and uncertainty of the calibration, limit parameters, carry over, precision (as intra-assay repeatability and intermediate precision, at two ethanol levels – 0.5 and 0.8 g L^{-1}), trueness and accuracy. The evaluation of the measurement uncertainty is important to ensure the selection of a BAC value suitable to express the judgment of compliance or disconformity requested by the law [11], and the uncertainty has to be expressed according to the guidelines of the forensic associations (in Italy: Group of Italian Forensic Toxicologists) and to a metrological (or bottom up) approach.

Some papers were found in the literature regarding the measurement uncertainty of the BAC [7,11-15]. Gullberg presented the application of the bottom up approach to a hypothetical example of a forensic blood alcohol analysis assuming to use a HS-GC method. The refs. 7, 12-15 evaluated the measurement uncertainty of the BAC on real systems and an overview on the results obtained in the different works is shown in Table 1. The most of these works reported data about measurements conducted by GC-FID and/or in which water-based ethanol references were used for calibration and/or quality controls. The estimated relative combined standard uncertainties are in the range 2 - 3%.

In this work, according to the bottom up approach, the value of the combined standard uncertainty, at two concentration levels – 0.5 and 0.8 g L^{-1} – was evaluated for results coming from a HS-GC-MS method that uses matrix-matched references for both calibration and quality control procedures. The uncertainty budget was carried out on the basis of EURACHEM guide lines [16], and takes into account various contributions of dispersion of the measurement, derived from the validation procedure expressly conducted, namely: intra-assay and intermediate precision, dispensed volumes, calibration straight line and the recovery uncertainty.

Material and Methods

Materials

Chemicals: Distilled water was purchased by Broun, ethanol (purity >99.8%) and 1-propanol (purity >99.5% - GC) were purchased by Sigma Aldrich (Saint Louis, Missouri, US).

Reference materials: Commercial reference materials bringing the analyte of interest directly in its real matrices were used to overcome those problems of inaccuracy coming from the handling of commercial ethanol (very volatile).

The standard solutions for the calibration procedure were prepared by dilution of certified reference materials (CRMs), made of ethanol in human whole blood, with an aqueous solution of 1-propanol (1.0 g L^{-1}), where 1-propanol was used as internal standard (IS). Medidrug Ethanol VB 080, 200 and 300 (Medichem, Steinenbronn Germany) were used. The nominal concentration of ethanol (g L^{-1} ± forensic confidence interval) in these solutions were 0.808 ± 0.062, 1.995 ± 0.100 and 2.972 ± 0.149, respectively.

The CRMs (ACQ Science GmbH, Germany) with 0.5 and 0.8 g L^{-1} of ethanol in whole blood, were used for the quality control during the measurement and 44 BAC values obtained on CRMs in a period of about 1 year, with one measure per week, were employed for the trueness evaluation. The stabilized CRMs are stored at 4°C.

Sample collection and preparation: Whole blood from driving persons suspected to drive at high levels of BAC was collected into specific tubes (Vacutainers, total volume 10 mL) containing 100 mg of sodium fluoride as preservative. The same protocol was applied to the whole blood levied for a non-drinker patient – a healthy abstaining subjects, woman – and used as blank. 80 µL of sample were then mixed with 200 µL of the aqueous solution of IS (1-propanol 1.0 g L^{-1}) in 20 mL capacity Supelco vials (dimension of 75.5 × 22.5 mm) and brought to the analysis.

Apparatuses: The gas-chromatograph Autosystem XL GC, equipped with TurboMass mass spectrometer and a TurboMatrix headspace autosampler, was from Perkin Elmer (Waltham, MA, USA). A capillary column Perkin Elmer Elite Volatiles 60 m × 0.25 mm ID (internal diameter) and film thickness 1.4 µm was used.

Pipettes 10-100 µL (model Eppendorf 100) and 20-200 µL (model Eppendorf 200) capacities were used.

Methods

Analytical methods: The automated headspace system of the HS-GC-MS system worked at 80°C and the equilibration time was of 16 minutes. Helium carrier gas was settled at 18 psi (pounds per square inch). Oven temperature was maintained at 120°C; elution program was isothermal. The GC cycle, thermo-stating, pressurization, injection and withdrawal times were 7.7, 16, 0.3 and 0.5 min, respectively. As to mass analysis, monitored ions were 45 m/z ($CH_3CH_2O^+$) and 46 m/z ($CH_3CH_2OH^{\cdot+}$, the quantifier ion) for ethanol and 59 m/z for 1-propanol ($CH_3CH_2CH_2O^+$). The signals were collected in SIM mode (Selected Ion Monitoring) because more suitable for quantitative determinations. The ratio between the signals of ions 45 m/z and 46 m/z was used to identify the analyte. The results, expressed in grams of ethanol per liter (g L^{-1}), were calculated by the calibration straight-line. Calibration straight-line was built using the ratio between ethanol area and IS area plotted *vs* ethanol concentration (g L^{-1}). Internal standard (IS) used to quantify was 1-propanol because it is the alcohol that shows the most similar chemical behavior with respect the analyte and, therefore, assures a high quality response. 1-propanol could be observed at low concentration in severely decomposed corpses in case of post-mortem determination, consequently, only in these cases, it is not the ideal IS [17].

Kristoffersen et al. [18], reported the risk of ethanol oxidation to acetaldehyde during the sample heating in the range 50 – 70°C in the headspace sampling system, but the concentration of acetaldehyde detected by Kristoffersen et al. [18], is so low to be negligible in the concentration range of ethanol here considered (g L^{-1}). Monitoring the ions with m/z 44, 43 and 29 ($CH_3CHO^{\cdot+}$, CH_3CO^+ and HCO^+, respectively) we can exclude the presence of such a criticism in our procedure. Finally, in case of a little amount of ethanol oxidized to acetaldehyde, the same loss of analyte would manifest itself in both the sample and standards

and the interference would be thus removed (according to the principles of comparative methods of quantification).

Software: All data obtained were presented using the software Origin 6.1. (by OriginLab) and analyzed using XlStat 2013.2.04 software package and SPSS Statistics 17.0 (by SPSS).

Statistical evaluation: Cochran test to verify the homoscedasticity (variance homogeneity) among set of data at different concentration was used (P = 0.95). Consequently, weighted or not weighted linear regression model to fit data of calibration was used. In addition, the formula used to estimate the calibration uncertainty was chosen on the basic of homoscedasticity (homogeneity of variances) or heteroscedasticity of data.

The value of the correlation coefficient calculated for a linear regression model is considered inadequate to estimate carefully the linearity of a dataset [19], therefore, the Mandel test to verify the linearity of the calibration points, at both working and low concentration ranges, was applied. Shapiro and Wilk test to verify the normality of a set of repeated data was used.

Significance of the intercept of a straight line was verified with a t-test with a specific discriminating function to choose a linear regression model of interpolation forced (y = ax, one parameter) or not (y = a + bx, two parameters) through zero.

To evaluate the precision (both intra-assay and intermediate one), outliers were tested by means of the Huber test. Outliers on calibration data were also identified examining visually the values of the residuals.

Data elaboration: Calibration uncertainty was estimated at two levels of concentration, 0.5 and 0.8 g L^{-1}, to be responder as to forensic requirements of reliability, applying the equation (1):

$$\mathrm{var}(X_O) = \frac{\mathrm{var}(y_{obs})}{b^2} + \frac{S^2}{b^2}\left[\frac{1}{\sum w_i} + \frac{(X_0 - \overline{X})^2}{\sum (w_i X_I^2) - \frac{\sum (w_i X_i)^2}{\sum w_i}}\right] \quad (1)$$

where:

- $\mathrm{var}(y_{obs})$ is the variance of the observed response

$$S = \frac{\sum w_i (y_i - y_{fi})^2}{n-2}$$

$(y_i - y_{fi})^2$ is the residual for the ith point,

- n is the number of data points in the calibration,

- b is the calculated best fit gradient,

- w_i is the weight assigned to y_i,

- $(X_0 - \overline{X})$ is the difference between X_0 and the mean $\overline{X}$ of the n values

w_i values are calculated as $w_i = \frac{s^{-2}\sum s^{-2}}{n}$, where s is the standard deviation of the signals of the ith point [14].

The data collected for the estimation of intermediate-precision were elaborated with multi-factor Analysis of Variance (ANOVA) to detect the significant factors of variability (significance level = 0.05).

The uncertainty budget was built based on the relationship (2) that combines relative standard uncertainties - $u_r(x_i)$ - of the variables x_i. This reckoning is used according to an empirical approach [14]:

$$\frac{u_c(x)}{x} = \sqrt{\sum (u_r(x_i))^2} \quad (2)$$

Results and Discussion

Calibration parameters at working concentrations

Monitoring BAC in a routine clinical/forensic laboratory implies to be ready for daily measurements in a wide concentration range. Specific measurements of BAC were conducted in the working range of concentration – 0.1 - 3.0 g L^{-1} – with five experimental points and three replicates *per* point, to estimate sensitivity, linearity range and uncertainty of calibration for routine application of the method. Tests to verify homoscedasticity and linearity returned the necessity to adopt a weighted linear fitting to model the experimental data of calibration. Parameters calculated for the five calibration curves in the working concentration range (75 experimental points in all) are collected in Table 2. For each level of concentration considered – 0.5, 0.8 g L^{-1} – the value of uncertainty associated to the calibration was estimated (for details see the *Data elaboration* paragraph in the *Material and Methods* section). As averaged result, we have the following values of relative uncertainties (u_r) of calibration: u_r (0.5) = 0.0278, u_r (0.8) = 0.0196; these values will be considered as contributes to the measurement uncertainty.

Calibration at low concentration: limit parameters

Specific measurements were conducted at low concentration – in the range 0.005 - 0.1 g L^{-1}, four experimental points – to estimate the limit of detection (LoD) and the limit of quantification (LoQ). A test to ensure the applicability of a linear model to the experimental data of calibration at low concentration was applied. Five replicates, one *per* day, of the calibration procedure were done and the blank signals were measured – ten replicates *per* day – on venous whole blood levied for a non-drinker patient (woman). The standard deviation of the blank signals and the calibration parameters were used to determine LoD and LoQ values applying the formula proposed by Long and Winefordner [20]. The results obtained with the five replicates enable us to estimate a reliable LoD value of $5.8 \cdot 10^{-4}$ g L^{-1} as mean of the five experimental values obtained, comprised between $1.3 \cdot 10^{-4}$ and $1.1 \cdot 10^{-3}$ g L^{-1} of ethanol. The correspondent LoQ value is $1.8 \cdot 10^{-3}$ g L^{-1}.

The limit values were also evaluated using the signal-to-noise ratio (henceforth: S/N), a typical method used in chromatographic analysis. The formula used is LoD = C[F(N/S)] [21], where C is the concentration of the analyte, F is a coverage factor often

assumed equal to 3, S is the magnitude of the instrumental signal (as the height of the analyte peak) and N is the magnitude of the instrumental signal when the analyte is not eluted. In order to estimate the S/N values, the instrumental signals of solutions with $5 \cdot 10^{-3}$ and $1 \cdot 10^{-2}$ g L^{-1} of ethanol were recorded. The values obtained – LoD = $2.5 \cdot 10^{-4}$ g L^{-1} and LoQ = $7.6 \cdot 10^{-4}$ g L^{-1} – are lower than the mean value estimated using the Long and Winefordner [20] formula, but are included in the variability range reported above.

In both cases, the LoQ value is quite lower than the lower limit of the calibration range, therefore the operational LoQ value of the method results to be 0.1 g L^{-1}.

Carry-over

The carry-over, or memory effects or effect of dragging, is characteristic of a separation-based analytical method and it is a typical problem coming from repeated injections, or injection of dirty samples, that overload the injection port. The carry-over was tested by injecting a blank after the injection of the highest concentration of ethanol employed (the CRM with 3 g L^{-1} of ethanol). The experimental procedure was repeated in triplicate. The calculation was performed according to the approach proposed by Haeckel [22,23]. The carry-over (*C.O.*) was expressed as percentage, or as ethanol concentration value, starting from the ratio:

$$C.O. = \frac{y_{b1} - y_{b2}}{y_{std} - y_{b2}} \quad (3)$$

where y_{b1} = signal of the blank injected after the standard, y_{b2} = signal of the blank (injected in sharply favourable conditions), y_{std} = signal of highest concentration of ethanol employed. The mean *C.O.* resulted 0.021% of the concentration of the standard used. It corresponds to a concentration of $6.3 \cdot 10^{-4}$ g L^{-1} of ethanol. This value is lower than the LoQ and this indicates that the memory effect does not affect the measurement outcome.

Precision

Intra-assay precision: Precision was evaluated (P = 0.95) as intra-assay repeatability in the same day (intra-day), with the same operator and apparatus at two concentration levels, 0.45 and 0.70 g L^{-1} ethanol (average values). For each level, eleven replicates were carried out. Two aliquots of a pool of human whole blood were spiked with different volumes of 99.8% ethanol up to reach values of ethanol concentration near to the nominal one (namely, 0.5 and 0.8 g L^{-1}) selected in this paper according to Italian regulation prescriptions. The real concentrations are lower than the nominal ones because of the volatility of the ethanol that strongly affects the handling. For each level of concentration considered, the value of uncertainty associated to the repeatability was estimated. Table 3 reports the results obtained. For the intra-day repeatability it was possible to work with a not stabilised pooled.

Intermediate precision: According to International Standard ISO 5725-3:1994 [24], precision was estimated by studying the repeatability under different experimental conditions. Experiments were planned in order to evaluate the effect related to the variation of three factors: i) the time, ii) the operator and iii) the volume of liquids dispensed by the pipettes used for the sample preparation. Two operators analysed four samples *per* day, each of one using two different pipettes, with a total of 8 replicates *per* day (*n*). Replicates of the measurements were done along 5 days (40 experimental points in all) on solutions with 0.5 and 0.8 g L^{-1} (nominal concentration) of ethanol, prepared by dilution of CRMs, in order to mimic the routine sample preparation. In this case, employing stabilised CRMs it was necessary to avoid coagulation processes often caused by the variation of the thermal conditions between measuring and storage step.

The data were elaborated with multi-factor Analysis of Variance (ANOVA) to detect the significant factors of variability. The outcome of the test shows that the changing of both the operator and the pipettes does not affect significantly the precision of the measurements, while the time is a significant factor. Therefore, the within-day repeatability (S_r) was estimated using the equation reported below (4):

$$S_r^2 = \frac{1}{d}\sum_{j=1}^{d} S_{rj}^2 \quad (4)$$

where *d* is the number of days; S_{rj}^2 is the variance of each group of data. The degrees of freedom (d = 5, n = 8) for S_r^2 are $\nu = d\,(n - 1) = 35$.

The intermediate precision S_I was calculated as reported in ref. 25 and results to be 0.034 and 0.039 g L^{-1} at 0.5 and 0.8 g L^{-1}, respectively.

Trueness and accuracy

The trueness of the measures was evaluated at the two concentration levels (0.5 and 0.8 g L^{-1}) by the comparison of the results of 44 replicated measurements on CRMs and the corresponding reference values. The mean values obtained are 0.5005 ± 0.0047 g L^{-1} and 0.7968 ± 0.0048 g L^{-1} (P = 95%, ν = 43) on CRMs with 0.500 ± 0.020 g L^{-1} and 0.793 ± 0.035 g L^{-1} of ethanol (statistic uncertainty reported, P = 95%), respectively. The method provides unbiased results.

In order to compare method performance including both distortion (trueness) and dispersion (precision) contributions the accuracy can be calculated [8,24], as Mean Squared Error (MSE), therefore as the sum of the squared bias and the observed variance at each level of concentration. MSE (0.5) = $6.3 \cdot 10^{-5}$ (g L^{-1})^2 and MSE (0.8) = $2.5 \cdot 10^{-4}$ (g L^{-1})^2, using the intra-assay repeatability, and MSE (0.5) = $1.2 \cdot 10^{-3}$ (g L^{-1})^2 and MSE (0.8) = $1.5 \cdot 10^{-3}$ (g L^{-1})^2, considering the intermediate precision.

Uncertainty budget

Sources of uncertainty identified were:

– precision of the measurement: the uncertainty contribution derived from the precision of the measurement, $u(s)$, was estimated using both intra-assay and intermediate precision data;

– dispensed liquids: the uncertainty derived from the use of calibrated pipettes, $u(V)$, was estimated from the random error declared by the supplier and considering a triangular distribution. We also considered that the variability of the dispensed whole blood volumes is higher than that declared for non-viscous samples, however its contribution to the uncertainty is included in the calibration process;

– calibration straight line: uncertainty $u(y)$ on the instrumental signal;

– CRM concentration: the uncertainty $u(CRM)$ associated to the concentration of the reference materials used for the calibrating solutions and declared by the supplier. The statistic confidence interval, expressed with a confidence level of 95%, was used;

– Recovery uncertainty: although our method is unbiased, the uncertainty associated to the determination of the bias, $u(rec)$, may be expressed as the uncertainty of the analytical recovery (value observed divided by value expected) [16].

All these sources of variability were considered in the uncertainty budget assessment and the corresponding uncertainties were combined as reported in the paragraph *Data Elaboration* in the *Material and Methods* section. The various contributions to the uncertainty of the ethanol concentration are reported in Table 4, with the combined uncertainty, expressed as relative and absolute values, and with the expanded uncertainty evaluated with a coverage factor k = 2. The results are reported for each concentration level taken into account, using the contribution of the intra-assay repeatability and intermediate precision. The values of absolute combined standard uncertainty are $u^c(0.5)$ = 0.017 g L^{-1}, $u^c(0.8)$ = 0.025 g L^{-1}, using the intra-assay repeatability, and $u^c(0.5)$ = 0.036 g L^{-1}, $u^c(0.8)$ = 0.048 g L^{-1}, considering the intermediate precision. Relative combined standard uncertainty, for the two concentration levels (in bracket), are: intra-assay repeatability $u^c_r(0.5)$=3.4% and $u^c_r(0.8)$=3.1%, intermediate precision $u^c_r(0.5)$=7.3% and $u^c_r(0.8)$=6.0%.

The statistical variability of the analytical results and the variability of the signals of the reference solutions are the main contributions to the measurement uncertainty (Figure 1) considering both the intra-assay repeatability and the intermediate precision.

The uncertainty values could be used to express the result of the forensic analysis, in view of a decision of compliance/non-compliance to a lawful limit, starting from the data of ethanol concentration calculated from the calibration curve. If the analytical result exceeds the limit value plus the expanded uncertainty of the measure, non-compliance is considered clearly demonstrated [16]. The choice of the factor k used to obtain the expanded uncertainty is based on the level of confidence desired and, for an approximate level of confidence of 95%, k is equal to 2. By the use of the expanded uncertainty it is possible to express to calculate the decision limits, i.e. the concentration beyond which the BAC value can be considered, with a certain probability, higher than the threshold values defined by the law [7,11]. Table 4 reports the decision limits calculated with the expanded uncertainty values estimated in this work.

Conclusions

The HS-GC-MS analytical method for ethanolemia measurement in venous whole blood was validated – according to a specific experimental procedure (in-house modality) and statistical approach – to verify those metrological performances useful to establish a reliable medico-legal applicability. The analytical procedure adopted employs matrix-matched references in order to take into account the matrix effect on the analytical result. The use of specific CRMs during both calibration and quality control procedures accounts for the conditions of real samples, and this improves the traceability of the analytical response. Nevertheless, the procedure is easy, practical, safe to use, suitable for routine application of a clinical/forensic laboratory and it ensures a very good level of quality in term of both precision and trueness.

The estimation of the measurement uncertainty was carried out strictly following the guidelines provided by EURACHEM [16], and in accordance with the Gullberg approach [11]. The results show that the variability of replicated measurements and the calibration procedure are the main sources of uncertainty; therefore, in order to increase the quality of the measurement, it is rational to act on these two analytical steps. Moreover, the value

Table 1: Relative combined standard uncertainties estimated for the Blood Alcohol Concentration (BAC) derived from different literature sources.

Analytical method [a]	Matrices of the reference materials used		Relative combined standard uncertainty (concentration level g L^{-1})	Reference
	calibration solutions	*quality control samples*		
HS-GC-FID	water [b]	blood [c]	---	[12]
HS-GC [d]	water	---	2.4% (0.5 g L^{-1}) [e]	[13]
HS-GC-FID	water	water	2.7% (0.8 g L^{-1})	[14]
HS-GC-MS	blood	water/ blood	2.0% (0.5 g L^{-1}); 3.0% (0.8 g L^{-1})	[7]
HS-GC-FID [f]	blood	blood	2.39% (0.01 – 0.4 g L^{-1})	[15]

a HS = headspace; GC = gas chromatography; FID = flame ionization detectors; MS = mass spectrometry;
b Solutions with known amount of ethanol in water;
c Whole blood samples with a known amount of ethanol;
d Five headspace GC systems were used, the detector was not defined;
e In the ref. 13 the relative combined standard uncertainty at other concentration levels is reported;
f In this work, analytes are ethanol, methanol, acetone, and isopropanol, and the final expanded uncertainty includes the contribution of all of them.

Table 2: Parameters related to the five (A-E) calibration curves obtained in the working concentration range.

Straight line	Slope	Slope stand. error	Intercept	Intercept stand. error	RSS [a]	R^2	u_r (0.5) [b]	u_r (0.8) [b]
A	0.544	0.013	-0.0056	0.0014	13.877	0.99816	0.0191	0.0211
B	0.481	0.006	-0.0038	0.0007	1.436	0.99978	0.0069	0.0075
C	0.528	0.008	-0.0030	0.0037	34.651	0.99938	0.0065	0.0064
D	0.514	0.003	-0.0129	0.0015	53.108	0.99869	0.0267	0.0203
E	0.506	0.003	0.0	-	0.0018	0.99941	0.0800	0.0428

a RSS = Residual Sum of Squares.
b $u_r(C)$ means the value of relative standard uncertainty associated to the calibration for the concentration C (g L^{-1}).

Table 3: Intra-assay and intermediate precision (influencing factor: time, 5 days of measurements) of the ethanol quantification in blood.

Intra-assay precision			Intermediate Precision		
Concentration level (g L^{-1})			Concentration level (g L^{-1})		
Theoretical values	0.5	0.8	Theoretical values	0.5	0.8
Mean	0.4536	0.6977	General mean	0.5109	0.6877
Maximum	0.4693	0.7251	Maximum	0.5962	0.8027
Minimum	0.4417	0.6747	Minimum	0.4274	0.609
SD [a]	0.0079	0.0154	SD [a]	0.0333	0.0367
RSD [b]	0.0175	0.0221	RSD [b]	0.0653	0.0534
s c	0.0057	0.0109	S_r [d]	0.0293	0.0259
			$S_{M(t)}$	0.0203	0.0302
			S_I [f]	0.0342	0.0387

a Standard deviation on repeated measurements.
b Relative standard deviation on repeated measurements.
c Repeatability calculated as SD ($t/\sqrt{v}$), where t is the student value (P = 0.95, two-tails distribution), v is the degrees of freedom (number of replicates minus one).
d Within-day repeatability.
e Between-day repeatability.
f Intermediate precision.

Table 4: Combined and expanded standard uncertainty and decision limit.

Experimental setup	Conc. (g L^{-1})	Relative standard uncertainty						Absolute standard uncertainty		Decision limit [e]
		Contributions [a]					Combined [b]	Combined [c]	Expanded [d]	
		$u_r(s)$	$u_r(V)$	$u_r(y)$	u_r(CRM)	u_r(rec)	u_r [b]	u [c]	U	(g L^{-1})
Intra-assay repeatability	0.5	0.0175	0.0008	0.0278	0.0080	0.0046	0.03_{42}	0.01_{71}	0.03_{42}	0.53_{42}
	0.8	0.0221	0.0008	0.0196	0.0080	0.0030	0.03_{08}	0.02_{46}	0.04_{92}	0.84_{92}
Intermediate precision	0.5	0.0669	0.0008	0.0278	0.0080	0.0046	0.07_{30}	0.03_{65}	0.07_{30}	0.57_{30}
	0.8	0.0563	0.0008	0.0196	0.0080	0.0030	0.06_{02}	0.04_{82}	0.09_{64}	0.89_{64}

a Contributions to the uncertainty of the ethanol concentration expressed as relative standard uncertainty and derived from:
- intra-assay repeatability or intermediate precision: $u_r(s)$;
- dispensed volumes: $u_r(V)$;
- calibration straight line (signal uncertainty): $u_r(y)$;
- concentration of the certified reference material: u_r(CRM);
- recovery: u_r(rec).

b Combined relative standard uncertainty calculated by the combination of the contributions (see *Data elaboration* paragraph).
c Combined absolute standard uncertainty.
d Expanded uncertainty calculated with a coverage factor k = 2.
e Concentration beyond which the BAC value can be considered with a probability of 95% higher than the threshold values.

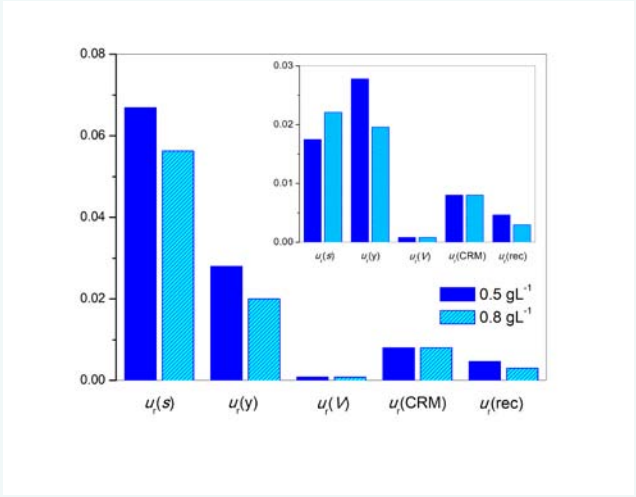

Figure 1 Contributions to the measurement uncertainty.
Contributions to the uncertainty of the ethanol concentration, expressed as relative standard uncertainty, at two concentration levels (0.5 and 0.8 g L^{-1}). $u_r(s)$ in the main graph: intermediate precision, $u_r(s)$ in the inset graph: intra-assay precision, $u_r(y)$: calibration straight-line (signal uncertainty), $u_r(V)$: dispensed volumes, $u_r(CRM)$: concentration of the CRM, $u_r(rec)$: recovery.

of the expanded uncertainty can be used to express a prudential value of BAC suitable to give forensic judgement concerning the exceeding of threshold values.

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