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# Development of Certified Reference Materials of Drug Abuse (Heroin, Etc) for Elimination of Measurement Error in Forensic Drugs

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## Abstract

The need for certified reference materials (CRM) of illicit drug was emphasized by drug detection in the forensic science as a tool to improve comparability, ensuring accuracy and traceability of analytical results. The production, characterization and certification of reference materials (RMs) is a key activity in improving and maintaining a worldwide coherent system of measurements. For the first time, we developed 9 illicit drug CRMs, including methamphetamine, heroin, ketamine and others. This work describes the production of the series of illicit drug CRMs, according to ISO Guides 34 and 35, which comprises the material processing, homogeneity and stability assessment, CRMs' characterization including moisture content, trace metal content. The certified values were assigned by two methods. Homogeneity of the CRMs was determined by an in-house validated liquid chromatographic methodology. Potential degradation during storage was also investigated and a shelf-life based on this value was established. The certified values for all the studied reference materials are traceable to the international system of units (SI). The application of purity CRMs with accurate value could eliminated the measurement error in the forensic labs, which could give the accurate and reliable results for the forensic evidence. NIM will continue to carry out in-depth cooperation with IFS with raising the level of science to provide a greater level of rigor and confidence in forensic evidence used in the Chinese criminal justice system.

Ketamine and methamphetamine have been applied as CRM in the APMP comparison, and gave the good results (Lab(7)). These CRMs studied have been widely used as standards in routine inspection work for the labs affiliated Institute of Forensic Science.

The application of purity CRMs with accurate value could eliminated the measurement error in the forensic labs, which could give the accurate and reliable results for the forensic evidence.

**Keyword:** Illicit drugs; Certified reference material; Liquid chromatography; Gas chromatography; Uncertainty evaluation

## Introduction

The use of drugs of abuse is increasing worldwide and causing serious social problems. Concern over driving under the influence of drugs (DUID) as a risk factor and a cause of road accidents has recently risen, as illicit drugs can influence driving performance in different ways [1-3].

Opiates induce sedation, indifference to external stimuli, and increase reaction time. Stimulating drugs such as cocaine, amphetamines, and designer drugs (methylenedioxymethamphetamine, MDMA; methylenedioxy-N-ethylamphetamine, MDE) modify concentration and attentiveness, produce dilated pupils, which increase sensitivity to blinding by light, and the euphoric phase may lead to increased risk taking. Cannabis can influence perception, psychomotor performance, cognitive and affective functions, and finally hallucinogens produce hallucinations, sleepiness, and psychotic reactions incompatible with most social activities [4-6]. The determination of opiates is, therefore, important for the protection and prevention of the risk to human health, with young people being the most exposed category.

As part of quality control, the need for a reference material (RM) for drug analysis has rapidly increased in forensic and clinical laboratories. This paper describes the production of the series of CRMs composed of 9 illicit drugs, including the material processing, evaluating the homogeneity and stability among its units and within a same unit, and the strategy to assign reference values in the materials. The procedures included qualitative analysis, quantitative analysis, verification, homogeneity study, stability study and evaluation of uncertainty. The qualitative analysis was carried out by IR (infrared spectrometry) and LC-MS. Quantitative analysis was performed by two methods, HPLC-DAD and GC-FID, with a combination of TGA (thermo gravimetric analyzer) for moisture content and ICP-MS (inductively coupled plasma mass spectrometry) for trace level metal content. A homogeneity study between vials and within vial was tested by F-test and t-test. A long-term stability study for storage at 4°C for 1 year was carried out for ensuring that the CRMs are stable before deliver to customers. The uncertainties of CRMs were evaluated extensively.

## Experimental

### Materials

The chemical structures of all the nine illicit drug compounds in this study are listed in Figure 1, including Ketamine Hydrochloride, Pethidine Hydrochloride, Cocaine Hydrochloride, Methadone Hydrochloride, Diazepam, Estazolam, Heroin Hydrochloride, Morphine, Methamphetamine Hydrochloride with purity more than 98% were offered by Institute of Forensic Science Ministry of Public Security) and stored at controlled conditions. Methanol and ethyl

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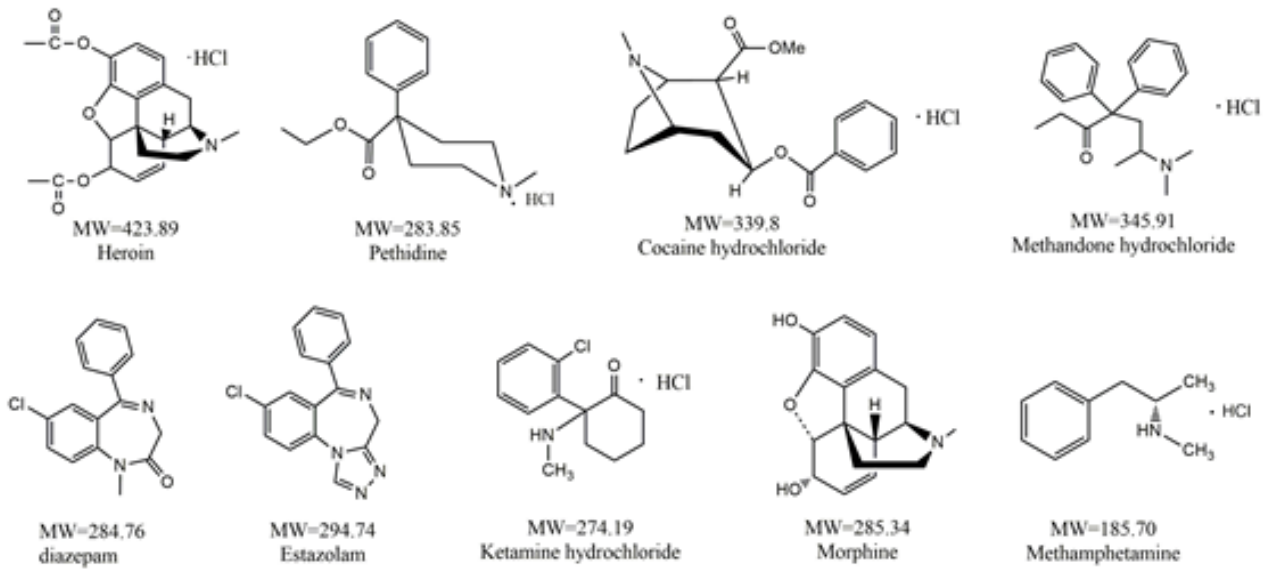


**Figure 1:** Structures of nine illicit drugs in this study.

acetate used as solvent or mobile phase all with HPLC grade were obtained from Merck (TX, USA).

## Preparation of candidate CRM

This research was conducted according to the guideline of the National Institute of Scientific Investigation Ethics Committee [7]. All samples were stored in an airtight container with desiccant after pretreatment. The procedure for the preparation and characterization of the reference material and the homogeneity study has been carefully designed and optimized step by step.

High-purity materials are generally expensive and available in limited amounts. In addition, replicate preparations of solutions are required for estimation of preparation variation and stability. The candidate reference materials were firstly qualitative analyzed by liquid chromatography–mass spectrometry (LC–MS) and Fourier transforminfrared spectroscopy (FT-IR), and then the impurities including moisture and other trace metal residues were measured by the established methods. The value of the CRMs was certified by liquid chromatography (LC) and gas chromatography (GC).

## Characterization of candidate CRM

**Liquid chromatography–mass spectrometry:** All the illicit drugs that were diluted with concentration of 10 ug/mL in methanol was quantified at flow injection analysis mode with a Agilent LC-MS/MS 6410 system, equipped with electro-spray ionization source (ESI) and linear ion trap mass analyzer, the target molecule can be qualified by analysis the mass spectrum peak molecular weight at the positive ion polarity mode at specified MS scan range of 100-400.

**Fourier transform infrared spectroscopy:** Infrared spectra were collected with FT-IR spectrometer Thermo Nicolet iS10 spectrophotometers via KBr pellets technique. Specimens were ground with dried spectroscopic grade KBr powder and the mixture was compressed to pellets for FT-IR measurements. The sample to KBr mass ratio was 1:100. All spectra were collected in the 4000-400 $cm^{-1}$ range at 8 $cm^{-1}$ resolution.

**Determination of moisture content:** The moisture content of all illicit drugs was measured by PerkinElmer Pyris 1 thermo gravimetric analyzer (TGA) (Metrohm, Herisau, Switzerland). For TGA, about 5 mg was heated at 5°C /min between 25 and 1000 °C under an atmosphere of nitrogen. Alumina pans were used. The TGA instrument was calibrated using sodium bicarbonate (Aristar grade, Fisher) before performing the analysis using six samples. The presented values are the means of 10 replicates.

**Determination of trace metal residues:** The Agilent 7500CE ICP-MS was used for analysis of 72 trace metal residues in all the illicit drug samples with optimized sensitivity. A Babington ultrasonic nebulizer and a 100 high solids torch were applied in this study.

**Liquid chromatography-diode array detector:** The purity assessments were performed using high-performance liquid chromatography-diode array. All the illicit drugs' purities were certified by an Agilent 1200 liquid chromatography system equipped with a diode array detector. The HPLC-DAD parameters for all the studied illicit purity determination were listed in Table1.

The linearity of the DAD detector and the repeatability of the HPLC were also studied prior to the purity assessment.

**Gas chromatography analysis:** According to the ISO 35, the value of purity should be certified by two methods. The gas chromatography (GC) was used for the purity assessment of nine illicit drug CRMs. All the illicit drugs' purities were certified by an Agilent 6890, equipped with column ZB-1(30m, 0.32mm, 0.25μm) and FID detector. $H_2$ was used as carrier gas. The GC-FID parameters for all the studied illicit purity determination were listed in Table2.

## Homogeneity study

A homogeneity study was carried out by LC-DAD method. According to the Technical Norm of Primary Reference Material of China [8], 15 bottles, randomly selected from a batch, was assayed for homogeneity between bottles, and seven portions from one bottle was assayed for homogeneity within bottle. The results were examined via F-test and t-test [9]. In F-test, standard deviations were used to examine whether the deviation between bottles is significantly larger

| CRMs | Column | Mobile phase Acetonitrile:water | Flow Rate (mL/min) | Injection volume(uL) | DAD (nm) |
|---|---|---|---|---|---|
| Ketamine hydrochloride | a | 40:60* | 1.5 | 20 | 210 |
| Pethidine | b | 20:80** | 1.0 | 10 | 210 |
| Cocaine hydrochloride | b | 25:75** | 1.0 | 5 | 230 |
| Methandone hydrochloride | c | 80:20** | 1.0 | 5 | 210 |
| Diazepam | b | 30:70*** | 1.0 | 5 | 230 |
| Estazolam | b | 25:75*** | 1.0 | 5 | 220 |
| Heroin | b | 23:77** | 1.0 | 10 | 210 |
| Morphine | c | 15:85**** | 1.0 | 5 | 210 |
| Methamphetamine hydrochloride | a | 30:70* | 1.5 | 20 | 210 |

Note: Column: a: Phenomenex Luna 5μ SCX 100A (250mm×4.6mm, 5μm); b: XTerra RP18 (150×4.6mm, 5μm); c: Platisil ODS (5 μm, 4.6mm, 250mm)

*: $KH_2PO_4$, 50mmol, pH3.0; **: $KH_2PO_4$ (10mmol):K2HPO4 (10mmol)=4:5; ***: $KH_2PO_4$, 10mmol, pH3.0; ****: $KH_2PO_4$ (10mmol): Sodium 1-heptanesulfonate (5mmol):=1:1.

**Table1:** HPLC-DAD conditions for the studied illicit drugs.

| CRMs | Injector Temp. | Column Temperature | Detector Temp. |
|---|---|---|---|
| Ketamine hydrochloride | 180℃ | 150℃(1min) -10min/L -240℃(10min)-30min /L-300℃(5min) | 320℃ |
| Pethidine | 250℃ | 200℃(1min)-4℃/ min -260℃-20℃/ min -300℃(6min) | 320℃ |
| Cocaine hydrochloride | 280℃ | 100℃ (1min)-10℃ /min-180℃ (5min)-30℃ /min-280℃(5min) | 300℃ |
| Methandone hydrochloride | 200℃ | 120℃(1min)-10min/L -180℃-20min/L-250℃(6min) | 300℃ |
| Diazepam | 280℃ | 100℃(1min)-20℃/ min -250℃(5min)-30℃/ min -300℃(5min) | 320℃ |
| Estazolam | 180℃ | 150℃(1min)-15℃/ min-260℃(10min)-30℃/ min -300℃(5min) | 320℃ |
| Heroin | 200℃ | 120℃(1 min)-15℃/min -200℃(15 min) | 300℃ |
| Morphine | 250℃ | 150℃(1min)-15min/L -240℃(4min)-40℃/min-300℃(5min) | 320℃ |
| Methamphetamine hydrochloride | 250℃ | 60℃(1min)-10℃/min-150℃(3min)-20℃/min-250℃(2min) | 315℃ |

**Table2:** GC-FID conditions for the studied illicit drugs.

than the deviation within bottle. In t-test, means were used to examine whether the mean between bottles is significantly different from the mean within bottle [9].

The requirements of F-test and t-test were shown below:

$$F = S_1^2 / S_2^2 < F(n,m)$$

$$t = |P_1 - P_2| < 2\sqrt{\left(\frac{S_1}{\sqrt{n_1}}\right)^2 + \left(\frac{S_2}{\sqrt{n_2}}\right)^2}$$

Where, $P_1$ and $P_2$ are mean values of determined purity between vials and within vial, respectively; $S_1$ and $S_2$ are standard deviations of determined purity between vials and within vial, respectively; n (=15) and m (=7) are numbers of determination between vials and within vial. $F_\alpha$(n, m)= 3.96 from F-test list.

### Stability study

A study of long-term stability for storage at 4°C was carried out by HPLC-DAD method, in which each material was determined at 1, 3, 6 and 12 months after quantitative analysis.

The stability of the pure illicit drug reference standards is critical in achieving good-quality measurement. The stability studies of each illicit drugs were investigated over a period of time (12 months).

### Evaluation of uncertainty and verification of uncertainty by statistics

Absolute expanded uncertainty (U) of this CRM was calculated as followed:

$$U = X \times u_c = X \times k \times \sqrt{u_m^2 + u_{bb}^2 + u_s^2}$$

X is the certified value of the CRM. k is the coverage factor. $U_{rel}$ is the relative expanded uncertainty of the CRM. Where $u_m$, $u_{bb}$ and $u_s$ are the uncertainties from value assignment, homogeneity and stability source.

## Results and discussion

### The moisture content of reference materials

The results of moisture content for all the nine illicit drug by standard operational procedure are indicated in Table 3. The moisture content for methandone, pethidine, diazepam, cocaine, estazolam and methamphetamine were less than 0.1%, while the moisture content of others were more than 0.1%, which should be substrate from 100%. For one crystal water in one heroin molecule, the water content of heroin was 4.452%, which contained one water molecule. The uncertainties contribution of moisture content on the value assignment would be evaluated (Table 3).

### Trace metal residues of reference materials

The content trace metal content for all the illicit drugs was analyzed. The sum of tested 72 trace metal residues content for all the illicit drugs were less 0.1%. The uncertainties contribution of trace metal content on the value assignment would be evaluated when it larger than 0.1%. In this case, its effect on all the nine illicit drug certified value was ignored.

### LC-MS of illicit drugs

The following Table 4 lists the molecular weight and its product ion at optimized LCMS conditions of all the nine illicit drugs, as indicated, the molecular ion peak $(M + H)^+$ of mass charge ratio m/z in the right column, was just the molecular weight (MW) plus the atomic weight of hydrogen (1.0), from which can quantify the nine illicit drugs.

| Illicit drug CRM raw material | Moisture content, % |
|---|---|
| Ketamine hydrochloride | 0.120 |
| Pethidine | 0.089 |
| Cocaine hydrochloride | 0.063 |
| Methandone hydrochloride | 0.068 |
| Diazepam | 0.035 |
| Estazolam | 0.071 |
| Heroin | 4.452 |
| Morphine | 1.176 |
| Methamphetamine hydrochloride | 0.088 |

**Table 3** The moisture content for the illicit drugs.

| Illicit drug CRM | MW | $[M + H]^+$ (m/z) |
|---|---|---|
| Ketamine hydrochloride | 237.72 | 238.20 |
| Pethidine | 247.33 | 248.30 |
| Cocaine hydrochloride | 304.30 | 303.35 |
| Methandone hydrochloride | 310.30 | 309.3 |
| Diazepam | 284.76 | 285.10 |
| Estazolam | 294.74 | 295.10 |
| Heroin | 269.40 | 370.20 |
| Morphine | 285.34 | 286.30 |
| Methamphetamine hydrochloride | 149.20 | 150.20 |

**Table 4:** Molecular weight and its product ion at optimized LC/MS conditions in this study.

## The homogeneity test of samples

After collecting the data under strict repeatability conditions, the homogeneity of the material for the studied compounds was established by comparing the coefficients of variation (CV) obtained for the within-bottle, between-bottle and method uncertainties. The F-test and t-test results of all the studied were listed Table 5. No significant differences were found for the purities of all the studied compounds in homogeneity test as the calculated F-values were lower than the critical F-values. Even a slight difference was noticed, which may be attributed to the analytical method.

## Evaluation of uncertainty of the CRM

### Uncertainty from HPLC-DAD

**A type:** That is the uncertainty from repeatability of HPLC determination, i.e. $u_1$;

**B type:** Uncertainty from difference of response of components at different wavelength: $u_2$. Considering response of main component and impurities, uncertainty from one impurity ($u_{2-i}$) is calculated as followed formula:

$$u_{2-i} = B_{i\max\lambda} - B_{i-assign-\lambda} = \frac{A_{i\max\lambda}}{\Sigma A_{i-assign-\lambda}} - \frac{A_{i-assign-\lambda}}{\Sigma A_{i-assign-\lambda}}$$

in which:

$A_{imax\lambda}$ =Peak area of impurity i at the wavelength, in which it has max absorbance (mAu·s)

$A_{i\text{-}assign\text{-}\lambda}$ =Peak area of impurity i at the wavelength for value assigning (mAu·s)

$B_{i\text{-}assign\text{-}\lambda}$= Peak area percentage of impurity i at the wavelength for value assigning (%)

$B_{imax\lambda}$= Peak area percentage of impurity i at the wavelength, in which it has max absorbance (%)

Uncertainty of all impurities is combined by each uncertainty of impurity:

$$u_2 = \frac{1}{\sum B_i}\left(\frac{\sqrt{\sum u_{2-i}^2}}{\sqrt{3}}\right)$$

Combined standard uncertainty from HPLC-DAD:

$$u_{LC} = \sqrt{u_1^2 + u_2^2}$$

### Uncertainty from GC-FID

**A type:** That is the uncertainty from repeatability of GC determination, i.e. $u_1$;

**B type:** Uncertainty from difference of correction factor of main component and impurities: $u_2$, which was calculated as followed formula:

$$u_2 = \left[\sum_{i=1}^{n}\left(x_i \bullet \frac{\Delta f_i}{f_i}\right)^2\right]^{\frac{1}{2}}$$

in which:

$f_i$- mass response factor of component i

$x_i$- mass fraction (%) of component i

Combined standard uncertainty from GC-FID:

$$u_{GC} = \sqrt{u_1^2 + u_2^2}$$

**Combined uncertainty of the CRM:** The combined uncertainty of the CRM was combined with uncertainties from value assignment ($u_m$), homogeneity ($u_{bb}$) and stability ($u_s$), in which uncertainties of homogeneity and stability were relative standard deviations of corresponding study, in this study, the uncertainties of homogeneity and stability can be neglected as demonstrated in the homogeneity and stability study (Table 6).

The relative expanded uncertainty ($u_c$) of the CRM was calculated by multiplying coverage factor (k) and combined uncertainty.

## The application of CRMs in comparison

Samples from international ring trail specimen (APMP.QM-P20, Government Laboratory, Hong Kong, concentration: MAM 0.5-5 $ng{\cdot}mg^{-1}$, KET 1-10 $ng{\cdot}mg^{-1}$) were measured, of which the CRMs of MAM and KET was used for internal standards. The good results of the comparison confirmed the certified value of the CRMs we developed.

## Conclusion

A series of CRM of illicit drugs were developed with procedures of sample preparation, certification (value assignment), homogeneity study, stability study and evaluation of uncertainty. The certification of the CRMs provides the country with a national reference standard, constituting in one of the main tools for the assurance of the traceability, analytical measurement reliability. The application of purity CRMs with accurate value could eliminated the measurement error in the forensic labs, which could give the accurate and reliable results for the forensic evidence.

### Acknowledgement

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| Homogeneity test | | Ketamine | Pethidine | Cocaine | Methandone | Diazepam | Estazolam | Heroin | Morphine | Methamphetamine |
|---|---|---|---|---|---|---|---|---|---|---|
| $\bar{X}$ | Between-bottle(%, $S_1$) | 99.92 ± 0.012 | 99.42 ± 0.006 | 99.62 ± 0.006 | 99.87 ± 0.008 | 99.94 ± 0.004 | 99.44 ± 0.003 | 99.62 ± 0.006 | 99.61 ± 0.008 | 99.98 ± 0.002 |
| | within-bottle(%, $S_1$) | 99.92 ± 0.012 | 99.42 ± 0.006 | 99.62 ± 0.006 | 99.87 ± 0.007 | 99.94 ± 0.004 | 99.44 ± 0.003 | 99.62 ± 0.006 | 99.61 ± 0.008 | 99.98 ± 0.002 |
| F-test | $F = S_1^2 / S_2^2$ | 1.00 | 1.11 | 1.04 | 1.16 | 1.07 | 1.81 | 1.04 | 1.40 | 1.93 |
| | $F_{0.05}(n,m)$ | 3.38 | 3.96 | 3.96 | 3.96 | 3.96 | 3.96 | 3.96 | 3.96 | 3.96 |
| | $F<F_{0.05}$ | Y | Y | Y | Y | Y | Y | Y | Y | Y |
| t-test | $t = \lvert\overline{x_1} - \overline{x_2}\rvert$ | 0.094 | 0.065 | 0.0267 | 0.03 | 0.107 | 0.051 | 0.253 | 0.27 | 0.004 |
| | $\sqrt{\left(\frac{s_1}{\sqrt{n_1}}\right)^2 + \left(\frac{s_2}{\sqrt{n_2}}\right)^2}$ | 2.025 | 0.269 | 0.262 | 0.34 | 0.193 | 0.129 | 0.261 | 0.34 | 0.074 |
| | $t \le t_\alpha$ | Y | Y | Y | Y | Y | Y | Y | Y | Y |

**Table 5** Results for the contents, the within-bottle, and between-bottle and homogeneity test of 9 illicit drugs.

| Uncertainty components | Ketamine | Pethidine | Cocaine | Methandone | Diazepam | Estazolam | Heroin | Morphine | Methamphetamine |
|---|---|---|---|---|---|---|---|---|---|
| $u_{LC}$(Uncertainty from LC) | 0.017 | 0.09 | 0.005 | 0.01 | 0.007 | 0.02 | 0.01 | 0.01 | 0.03 |
| $u_{GC}$(Uncertainty from GC) | 0.16 | 0.30 | 0.25 | 0.13 | 0.02 | 0.34 | 0.34 | 0.36 | 0.05 |
| $u_{bb}$(Uncertainty of homogeneity) | 0.00054 | 0.0007 | 0.0004 | 0.0011 | 0.0004 | 0.0009 | 0.0004 | 0.002 | 0.0004 |
| $u_s$(Uncertainty of stability) | 0.00004 | 0.002 | 0.0003 | 0.0031 | 0.0007 | 0.0008 | 0.005 | 0.007 | 0.0001 |
| uc(Combined uncertainty) | 0.2 | 0.3 | 0.25 | 0.15 | 0.05 | 0.35 | 0.35 | 0.4 | 0.1 |
| k (Coverage factor) | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 |
| U (expanded uncertainty) | 0.4 | 0.6 | 0.5 | 0.3 | 0.1 | 0.7 | 0.7 | 0.8 | 0.2 |
| Certified value (%) | 99.5 | 99.3 | 99.5 | 99.7 | 99.9 | 99.3 | 95.1 | 98.5 | 99.5 |

**Table 6:** Evaluation of uncertainty for value assignment of 9 illicit drugs CRMs.

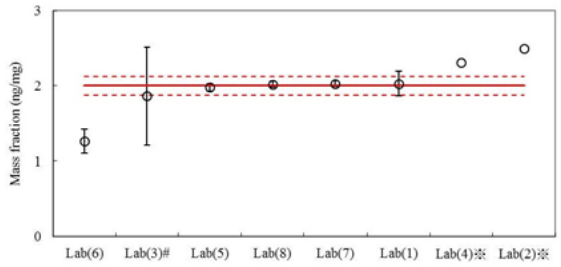


**Figure 2:** Participant's reported results and measurement uncertainties for methamphetamine (unit: ng/mg).

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