View metadata, citation and similar papers at core.ac.uk

brought to you by CORE

provided by University of Dundee Online Publications

University of Dundee

Measurement uncertainty in quantifying delta-9-tetrahydrocannabinol (THC) in blood using SPE and LC/MS/MS

Klu, Joyce K.; Officer, Jane A.; Park, Alexandra ; Mudie, Roy; Nic Daeid, Niamh

Published in:
Forensic Science International

DOI:
10.1016/j.forsciint.2021.110744

Publication date:
2021

Document Version
Publisher's PDF, also known as Version of record

Link to publication in Discovery Research Portal

Citation for published version (APA):
Klu, J. K., Officer, J. A., Park, A., Mudie, R., & Nic Daeid, N. (2021). Measurement uncertainty in quantifying delta-9-tetrahydrocannabinol (THC) in blood using SPE and LC/MS/MS. *Forensic Science International*, *322*, [110744]. https://doi.org/10.1016/j.forsciint.2021.110744

General rights
Copyright and moral rights for the publications made accessible in Discovery Research Portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

• Users may download and print one copy of any publication from Discovery Research Portal for the purpose of private study or research.
• You may not further distribute the material or use it for any profit-making activity or commercial gain.
• You may freely distribute the URL identifying the publication in the public portal.

Take down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Download date: 20. Apr. 2021

Forensic Science International 322 (2021) 110744

Contents lists available at ScienceDirect

Forensic Science International

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

Measurement uncertainty in quantifying delta-9-tetrahydrocannabinol (THC) in blood using SPE and LC/MS/MS

Joyce K. Klu[a,*], Jane A. Officer[b], Alexandra Park[b], Roy Mudie[a], Niamh NicDaeid[a]

[a] Leverhulme Research Centre for Forensic Science LRCFS, University of Dundee, United Kingdom
[b] Forensic Services, Scottish Police Authority SPA, Edinburgh, United Kingdom

ARTICLE INFO

Article history:
Received 20 July 2020
Received in revised form 15 February 2021
Accepted 19 February 2021
Available online 22 February 2021

Keywords:
Measurement uncertainty
Calibration curve
Method validation
Bottom-up approach
THC, LC/MS/MS

ABSTRACT

A method for the quantitative analysis of delta-9-tetrahydrocannabinol (THC, the main active ingredient of cannabis) in whole blood using solid phase extraction and LC/MS/MS has been developed. A bottom-up approach with method validation data was used to evaluate and estimate the measurement uncertainty (MU) of the analytical method. The sources of uncertainty were identified using a cause and effect diagram. The contribution of each uncertainty component was estimated and were combined to derive the overall uncertainty of the analytical method. The combined uncertainty was estimated to be 0.131 μg/L (< 7%). At a 99.7% confidence level, the expanded uncertainty was 0.393 μg/L for a THC concentration of 2 μg/L in a whole blood sample. The calculations not only enable the laboratory to quantify the uncertainty associated with a quantitative result, but can also be used to identify the sources of uncertainty and determine if the analytical method can be improved.

© 2021 The Authors. Published by Elsevier B.V.
CC_BY_4.0

1. Introduction

It is becoming increasingly important and a necessity for forensic toxicologists to quantify and report the measurement uncertainty (MU) associated with their analytical methods [1,2]. For accreditation standards such as ISO/IEC 17025 [3], it is now a fundamental requirement of quality management systems that many forensic toxicology laboratories adhere to, not only to identify the sources of uncertainty that may contribute to an analytical measurement, but also to estimate the size of their contribution.

Every measurement or analytical result will have an associated uncertainty [4,5], partly due to inevitable errors from random effects. In a field where the interpretation of a quantitative drug result is commonplace, it is essential to know the MU associated with a quantified drug concentration to ensure its appropriate interpretation. This is particularly vital when comparing quantitative results to a legal limit, such as for drink-driving or drug-driving cases. The toxicologist must be confident that a drug level exceeds the legal limit before reporting the result for the purpose of the judicial system. In order to do this, they must evaluate and take into account the MU of their analytical result.

Uncertainty as defined in the International Vocabulary of Metrology (VIM) [6] is a “non-negative parameter characterizing the dispersion of the quantity values being attributed to a measurand, based on the information used”. Appropriately quantifying this parameter will yield a range of values that is believed to contain the true unknown value of the measurand, thereby serving as a key indicator of measurement reliability and suitability for purpose [4,7,8].

The most common methods to estimating MU are either the bottom-up or top-down approach. The bottom-up approach requires a systematic evaluation and a clear description of all possible sources of uncertainty contributing to a measured quantity. To achieve this, a formula underlying the relationship between a measurand and its parameters must be clearly specified and all contributing uncertainties quantified using statistical modelling techniques. The top-down approach uses existing data and information from laboratory test performance and does not require one to systematically identify the source of uncertainty in each step of quantifying a measurand. Existing data from laboratory proficiency test, intra- and inter-laboratory studies, method validation and quality control data can be used to estimate MU [9–11].

Studies comparing both approaches have found no significant difference between the MU values obtained from top-down and bottom-up approaches, thereby concluding that both approaches are approximately equivalent and opted for the use of top-down approaches as a simpler method [10,12].

* Correspondence to: Leverhulme Research Centre for Forensic Science LRCFS, Ewing Building, 1st Floor, Small’s Lane, Dundee DD1 4HN, United Kingdom.
E-mail address: jkklu@dundee.ac.uk (J.K. Klu).

https://doi.org/10.1016/j.forsciint.2021.110744
0379-0738/© 2021 The Authors. Published by Elsevier B.V.
CC_BY_4.0

While the bottom-up approach can be very challenging and, in some cases, too complex to implement, it enables practitioners to optimise an analytical method as all possible uncertainty sources at each stage of the process are likely to be detected and included in the estimation process, as well as finding improvements [12,13].

In this paper, a specific method for the quantitative analysis of delta-9-tetrahydrocannabinol (THC, the main active ingredient of cannabis) in whole blood using solid phase extraction and LC/MS/MS is used to illustrate a possible way of evaluating and estimating the MU of a quantitative analytical method using a bottom-up approach. The sources of uncertainty are identified using a cause and effect diagram. Method validation data is used to outline a step-by-step approach to estimating their contribution to, and the overall uncertainty of, the analytical method.

The methodology underpinning the calculations in deriving the overall uncertainty is being used to develop an open access Measurement Uncertainty Calculator (MUCalc) software as part of a statistical tool box project developed by the Leverhulme Research Centre for Forensic Science (LRCFS) for forensic practitioners (see Appendix B).

This study and the ongoing application development provide a more detailed methodical analysis of how each uncertainty component can be calculated, providing full data sets for each computation in order to make it easy for practitioners needing to calculate MU to follow.

2. Experimental

2.1. Chemicals and reagents

Certified reference standard solutions of Δ^9-THC and Δ^9-THC - d_3 (internal standard) were obtained from Cerilliant (Sigma-Aldrich, UK) at concentrations of 1.00 and 0.100 mg/mL in methanol respectively. An independent certified reference standard solution of Δ^9-THC (1.00 mg/mL in methanol) was obtained from Chiron (Chiron UK Ltd, UK) for quality control purposes. All other chemicals and solvents were purchased from Fisher Scientific UK Ltd, UK, Rathburn Chemicals, UK and VWR International, UK.

THC-free human whole blood (blank blood) was obtained from the Scottish National Blood Transfusion Service (SNBTS).

2.2. Preparation of stock and working standard solutions

For the calibration curve, THC stock solution A_{CAL} was prepared by diluting 20 μL of the Cerilliant certified reference standard to 2 mL with methanol. This was subsequently diluted 200- and 100-fold to give working solutions B_{CAL} (50 μg/L) and C_{CAL} (100 μg/L), respectively.

THC-d_3 stock solution A_{IS} was prepared by diluting 12 μL of the Cerilliant certified reference standard to 1.2 mL with methanol. This was then diluted 20-fold to give working solution B_{IS} (50 μg/L).

For the quality control samples, THC working stock A_{QC} was prepared by diluting 20 μL of the Chiron certified reference standard to 2 mL with methanol. This was then diluted 100-fold to give working solution B_{QC} (100 μg/L).

2.3. Calibration and quality control (QC)

Working solutions B_{CAL} and C_{CAL} were used to prepare calibrators (1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 5.0, 6.0, 8.0 and 10 μg/L) in blank whole blood and the calibration curve was constructed using a least-square linear regression. Linear regression analysis was performed on the peak area ratios of the analyte to internal standard versus the analyte concentrations.

Quality control samples were prepared by spiking blank blood with the QC working solution B_{QC} to give Low (2.0 μg/L), Medium (5.0 μg/L), and High (10.0 μg/L) QC samples. Three replicates for each of the three concentration levels were analysed over 11 different days using a freshly prepared calibration line each day.

2.4. Sample preparation

To 0.5 mL blood, 50 μL of methanol and 20 μL internal standard (THC-d_3) were added. The sample was precipitated by adding cold acetonitrile (2 mL) and then vortex mixed and centrifuged. The sample was concentrated under a flow of nitrogen and 3 mL of 0.1 M acetate buffer (pH4) added. Sample extraction was performed using a United Chemical Technologies Inc. (UCT) CleanScreen® SPE column (200 mg/3 mL) (Chromatography Direct, UK) installed on an Extrahera™ automated system (Biotage AB, Sweden). The column was conditioned with methanol, water and 0.1 M acetate buffer (pH 4). The sample was then loaded onto the column and the column washed with water and 0.1 M hydrochloric acid:acetonitrile (80:20). The sample was eluted with hexane and hexane:ethyl acetate (65:35) and the extract dried and reconstituted in acetonitrile (50 μL) for LC/MS/MS analysis.

2.5. Instrumentation

The analysis was conducted using an Agilent 1260 Infinity II Prime LC System coupled with an Agilent Ultivo triple quadrupole LC/MS (VWR International, UK). Chromatographic separation of a 10 μL injection was achieved at 50 °C and a flow rate of 0.5 mL/min using a ZORBAX RRHD Eclipse Plus C18 column (2.1 mm × 50 mm, 1.8 μm, Crawford Scientific, UK) with a complementary guard column. Mobile phase A was 2 mM ammonium formate, 0.4 mM ammonium fluoride, 0.1% formic acid in water and mobile phase B was 2 mM ammonium formate, 0.4 mM ammonium fluoride, 0.1% formic acid in methanol. The gradient started with 70% mobile phase B, which was increased to 95% in 4 min and then held for 0.5 min. The column was re-equilibrated at 70% B for 0.1 min. The AJS ESI was operated in MRM mode. MRM transitions monitored for THC were m/z 315 → 193 with qualifiers 315 → 123 and 315 → 259; and for THC-d_3 m/z 318 → 196 with qualifier 318 → 123. The LC/MS/MS system was controlled using MassHunter Data Acquisition Software and data analysis was performed using MassHunter Quantitative Analysis software (version B.09.00).

2.6. Method validation

The method was validated in line with international guidelines [14–17] establishing selectivity, matrix effects, limit of detection (LOD), lower limit of quantitation (LLOQ), linearity, precision, accuracy, carryover, stability and dilution integrity. QC samples were prepared, extracted and analysed in triplicate on 11 days alongside a freshly prepared calibration curve to determine accuracy and precision. These results were used to estimate the measurement uncertainty of the method.

3. Identifying uncertainty sources

3.1. Specifying the measurand

The measurand is the concentration of the THC analyte (μg/L) in a blood sample expressed using the relationship:

$$x_{THC} = \frac{x_{cs}}{V} \quad (\mu g/L) \tag{1}$$

where x_{cs} is the amount of THC in the case sample, V is the volume of the case sample.

Updating the measurement function with additional influencing factors from method precision [18,19], Eq. (1) becomes

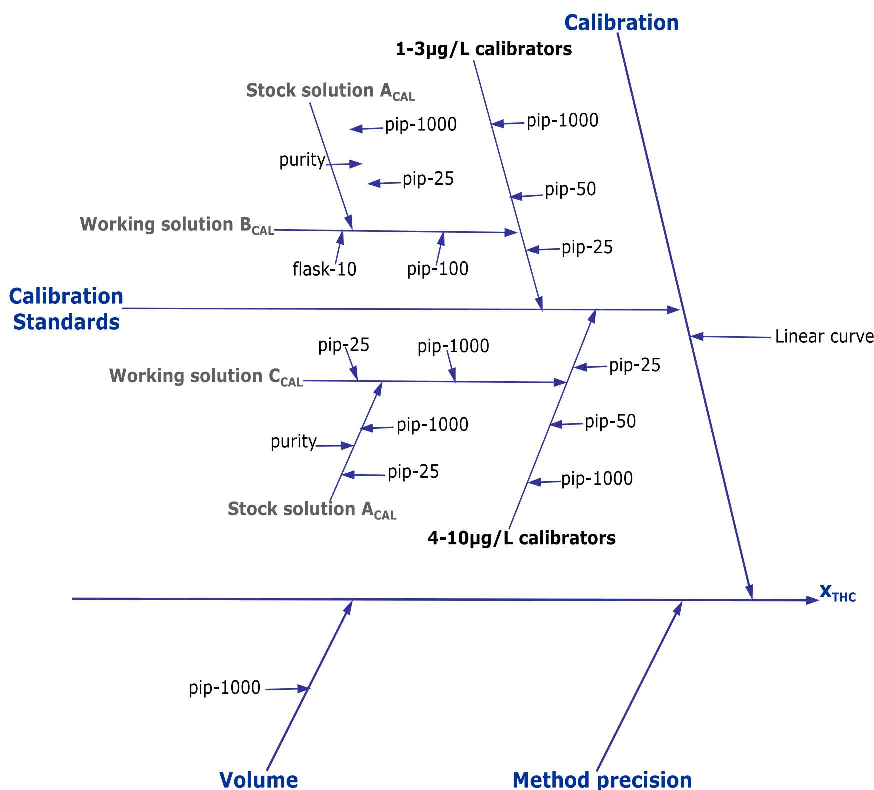

Fig. 1. Cause and effect diagram for identifying the sources of uncertainty in quantifying THC in blood. A 25 μ/L pipette is denoted with pip-25 and a 10 mL volumetric flask is denoted with flask-10.

$$x_{\mathrm{THC}} = \frac{x_{\mathrm{cs}}}{V} \times f_{\mathrm{precision}} \quad (\mu\mathrm{g}/\mathrm{L}) \tag{2}$$

where $f_{\mathrm{precision}}$ is the correction factor for method precision.

3.2. Sources of uncertainty

With reference to Eq. (2), the sources of uncertainties associated with quantifying THC in blood are identified using the cause and effect diagram displayed in Fig. 1. The main uncertainty sources are from method precision, sample volume (in estimating V), calibration curve and the preparation of calibration standards (in estimating x_{cs}).

In the next sections, each of these uncertainty sources are quantified in detail and combined to obtain an overall measure of uncertainty using UKAS guidelines [20], making reference to GUM [21] and EURACHEM [22] guidelines.

4. Quantifying uncertainty sources

The relative standard uncertainty associated with calibration in estimating the amount of THC in case sample x_{cs} is derived by combining uncertainty sources from the preparation of calibration standards and the calibration curve.

For simplicity on how each uncertainty component is calculated, the uncertainty associated with the preparation of calibration standards was calculated separately from that of calibration curve.

4.1. Uncertainty of the calibration standards

The uncertainty associated with the calibration standards sums or combines the uncertainties stated on the Certificates of Analysis of the Certified Reference Materials (CRMs) and the inaccuracies of all measuring equipment (e.g. pipettes and volumetric flasks) used to dilute CRMs and spike blank blood samples when preparing a calibration curve. The structure of how the THC CRM was diluted to make other solutions in preparing the calibration curve is displayed in Fig. 2.

The volume, tolerance and coverage factor (k) of pipettes and volumetric flasks used as given in the manufacturer's reference material, are given in Table 1, along with the number of times each pipette and volumetric flask was used in the preparation process for each standard solution.

The standard uncertainty (u) of THC as well as pipettes and volumetric flasks is given by $u = \frac{\mathrm{Tolerance}}{k}$ and the relative standard uncertainty (RSU) is given by $u_r = \frac{u}{\mathrm{Volume}}$. These are summarised in Table 2 together with the uncertainty associated with the preparation of calibration standards.

The RSU associated with the preparation of calibration standards was obtained by combining the RSU of $u_r(\mathrm{Cal}_{1-3})$ and $u_r(\mathrm{Cal}_{4-10})$ as:

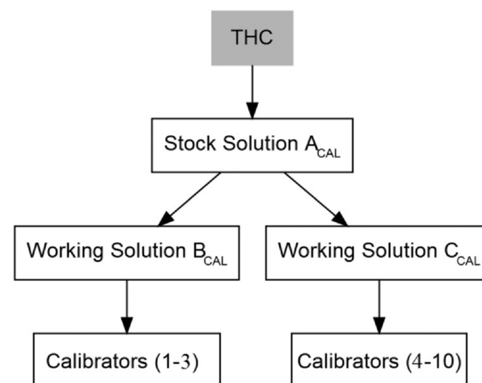

Fig. 2. The structure of THC dilution process for the preparation of the calibration curve.

Table 1
Data on THC CRM purity, pipette and flask used for solutions preparation.

	THC CRM				
	THC	Purity (mg/mL) 1	Tolerance (mg/mL) 0.033	Coverage factor (k) 2	
	Solutions				
	Pipettes/Flask	Volume (μ/L)	Tolerance (μ/L)	Coverage factor(k)	Times used
Stock solution A_{CAL}					
	pip-25	25	0.30	2	1
	pip-1000	1000	5	2	2
Working solution B_{CAL}					
	pip-50	50	0.30	2	1
	pip-1000	1000	5	2	7
Working solution C_{CAL}					
	pip-100	100	0.3	2	1
	flask-10	10,000	25	$\sqrt{3}$	1
	Calibration standards				
1–3 μg/L (Cal_{1-3})					
	pip-25	25	0.30	2	9
	pip-50	50	0.30	2	1
	pip-1000	1000	5	2	5
4–10 μg/L (Cal_{4-10})					
	pip-25	25	0.30	2	7
	pip-50	50	0.30	2	3
	pip-1000	1000	5	2	5

$$\begin{aligned} u_r(\text{CalStd}) &= \sqrt{u_r(\text{Cal}_{1-3})^2 + u_r(\text{Cal}_{4-10})^2} \\ &= \sqrt{0.02716^2 + 0.0252^2} \\ &= 0.0371 \end{aligned} \tag{3}$$

4.2. Uncertainty of the calibration curve

The uncertainty associated with the fitted calibration curve is estimated using the error propagation formula:

$$u(\text{CCur}) = \frac{S_{y/x}}{b_1}\sqrt{\frac{1}{r_{cs}} + \frac{1}{n} + \frac{(x_{cs} - \bar{x})^2}{S_{xx}}}, \tag{4}$$

$$S_{y/x} = \sqrt{\frac{\sum_{i=1}^{n}(y_i - \hat{y}_i)^2}{n-2}} \tag{5}$$

where: $S_{y/x}$ is the residual or standard error of regressing y on x. b_1 is the slope of the regression line. r_{cs} is the number of replicates made on the case sample to determine x_{cs}. n is the number of measurements used to generate the calibration curve. x_{cs} is the mean amount of THC in the case sample. $\bar{x}$ is the mean value of the different calibration standards. x_i is the target calibrator concentration at the i level. S_{xx} is the sum of squared deviation of x given by $\sum_{i=1}^{n}(x_i - \bar{x})^2$.

The relative standard uncertainty is given by:

$$u_r(\text{CCur}) = \frac{u(\text{CCur})}{x_{cs}} \tag{6}$$

Consider the calibration curve data of peak area ratios for 10 concentration levels {1, 1.5, 2, 2.5, 3, 4, 5, 6, 8 and 10} μg/L given in Table 3 along with the coefficients of the linear regression and the sum of squared deviations.

The standard error of regression can be computed using Eq. (5) and values from Table 3 as

$$\begin{aligned} S_{y/x} &= \sqrt{\frac{0.02474}{10-2}} \\ &= 0.05561 \end{aligned} \tag{7}$$

From the calibration curve data in Table 3, at each calibration level, one replicate is analysed for generating the calibration curve according to laboratory protocol. To obtained a more reliable estimate of the standard error, standard errors from previous calibration

Table 2
Calculations of RSU for THC CRM, volumetric flasks, pipettes, stock and working solutions used for calibration standards 1–3 μg/L and 4–10 μg/L.

RSU of THC CRM, volumetric flasks and pipettes

$u(\text{Purity}) = \frac{0.033}{2} = 0.0165$, $u_r(\text{Purity}) = \frac{0.0165}{1} = 0.0165$

$u(\text{pip-25}) = \frac{0.30}{2} = 0.15$, $u_r(\text{pip-25}) = \frac{0.15}{25} = 0.006$

$u(\text{pip-50}) = \frac{0.30}{2} = 0.15$, $u_r(\text{pip-50}) = \frac{0.15}{50} = 0.003$

$u(\text{pip-100}) = \frac{0.30}{2} = 0.15$, $u_r(\text{pip-100}) = \frac{0.15}{100} = 0.0015$

$u(\text{pip-1000}) = \frac{5}{2} = 2.5$, $u_r(\text{pip-1000}) = \frac{2.5}{1000} = 0.0025$

$u(\text{flask-10}) = \frac{25}{\sqrt{3}} = 14.43376$, $u_r(\text{flask-10}) = \frac{14.43376}{10000} = 0.00144$

RSU of working standard solution

$$\begin{aligned} u_r(A_{CAL}) &= \sqrt{u_r(\text{Purity})^2 + u_r(\text{pip-25})^2 + 2 \times u_r(\text{pip-1000})^2} \\ &= \sqrt{0.0165^2 + 0.006^2 + 2 \times 0.0025^2} \\ &= 0.01791 \end{aligned}$$

$$\begin{aligned} u_r(B_{CAL}) &= \sqrt{u_r(A_{CAL})^2 + u_r(\text{pip-50})^2 + 7 \times u_r(\text{pip-1000})^2} \\ &= \sqrt{0.01791^2 + 0.003^2 + 7 \times 0.0025^2} \\ &= 0.019327 \end{aligned}$$

$$\begin{aligned} u_r(C_{CAL}) &= \sqrt{u_r(A_{CAL})^2 + u_r(\text{pip-100})^2 + u_r(\text{flask-10})^2} \\ &= \sqrt{0.01791^2 + 0.0015^2 + 0.00144^2} \\ &= 0.018 \end{aligned}$$

RSU of calibration standards 1–3 μg/L and 4–10 μg/L

$$\begin{aligned} u_r(\text{Cal}_{1-3}) &= \sqrt{u_r(B_{CAL})^2 + 9 \times u_r(\text{pip-25})^2 + u_r(\text{pip-50})^2 + 5 \times u_r(\text{pip-1000})^2} \\ &= \sqrt{0.019327^2 + 9 \times 0.006^2 + 0.003^2 + 5 \times 0.0025^2} \\ &= 0.02716 \end{aligned}$$

$$\begin{aligned} u_r(\text{Cal}_{4-10}) &= \sqrt{u_r(C_{CAL})^2 + 7 \times u_r(\text{pip-25})^2 + 3 \times u_r(\text{pip-50})^2 + 5 \times u_r(\text{pip-1000})^2} \\ &= \sqrt{0.018^2 + 7 \times 0.006^2 + 3 \times 0.003^2 + 5 \times 0.0025^2} \\ &= 0.0252 \end{aligned}$$

Table 3
10 concentration levels versus peak area ratio, linear regression coefficients and the sum of squares of regression for the calibration curve data.

Concentration (x)	Peak area Ratios (y)	$(x-\bar{x})^2$	$\hat{y}=b_0+b_1x$	$(y-\hat{y})^2$
1	0.50936	10.89000	0.46247	0.00220
1.5	0.73972	7.84000	0.72863	0.00012
2	1.00815	5.29000	0.99479	0.00018
2.5	1.24273	3.24000	1.26095	0.00033
3	1.53580	1.69000	1.52711	0.00008
4	2.09479	0.09000	2.05943	0.00125
5	2.50074	0.49000	2.59175	0.00828
6	3.06545	2.89000	3.12407	0.00344
8	4.15375	13.69000	4.18871	0.00122
10	5.34078	32.49000	5.25336	0.00764
$\bar{x}$		$S_{xx}=\sum(x-\bar{x})^2$		$\sum(y-\hat{y})^2$
4.3		78.6		0.02474
		Intercept b_0	−0.06985	
		Slope b_1	0.53232	
		R^2	0.9989	
		n	10	

Table 4
The standard error and sum of squares deviation of 11 different calibration curves.

n	$n-1$	$S_{y/x}$	$(n-1)S^2_{y/x}$
10	9	0.05561	0.02784
10	9	0.05127	0.02366
10	9	0.03796	0.01297
10	9	0.07499	0.05061
10	9	0.04149	0.01549
10	9	0.04626	0.01926
10	9	0.05563	0.02786
10	9	0.04353	0.01705
10	9	0.11674	0.12265
10	9	0.04294	0.01660
9	8	0.08031	0.05160
	$\sum(n-1)$		$\sum(n-1)S^2_{y/x}$
	98		0.38558

curve data can be pooled. Pooling the errors gives a better estimate for the standard error of regression by taking into account different laboratory conditions over different days. The standard errors of a further 10 calibration curve data sets is summarised in Table 4, and the pooled standard error $S_{p_{(y/x)}}$ of regression is calculated using Eq. (8) as:

$$\begin{aligned} S_{p_{(y/x)}} &= \sqrt{\frac{\sum (n-1)S^2_{y/x}}{\sum (n-1)}}, \\ &= \sqrt{\frac{0.38558}{98}} \\ &= 0.06273 \end{aligned} \tag{8}$$

The data for computing the standard errors in Table 4 along with all supplementary data files is given in Appendix A.

Substituting the pooled standard error $S_{p_{(y/x)}}$ as an estimate for $S_{y/x}$, the uncertainty of the calibration curve from Eq. (4) becomes

$$\begin{aligned} u(\text{CCur}) &= \frac{S_{p_{(y/x)}}}{b_1}\sqrt{\frac{1}{r_{cs}}+\frac{1}{n}+\frac{(x_{cs}-\bar{x})^2}{S_{xx}}}, \\ &= \frac{0.06273}{0.53232}\sqrt{\frac{1}{2}+\frac{1}{10}+\frac{(2-4.3)^2}{78.6}} \\ &= 0.09626 \end{aligned}$$

For a given the case sample, two replicates are taken ($r_{cs}=2$) and the average reported. For an average concentration reading of $x_{cs}=2$ µg/L, the relative standard uncertainty of the calibration curve using Eq. (6) is given by

$$\begin{aligned} u_r(\text{CCur}) &= \frac{0.09626}{2} \\ &= 0.04813 \end{aligned} \tag{9}$$

4.3. Uncertainty of the method precision

The quality control (QC) data for evaluating the uncertainty of the method precision is summarised in Table 5. Blank blood samples were spiked with THC at three concentration levels: 2 µg/L (low), 5 µg/L (medium), and 10 µg/L (high). For each concentration level,

Table 5
Quality control data for concentration levels 2 µg/L (Low), 5 µg/L (Medium) and 10 µg/L (High) over 11 different days with three replicates each concentration level.

Concentration	Peak area ratios										
(µg/L)	Run 1	Run 2	Run 3	Run 4	Run 5	Run 6	Run 7	Run 8	Run 9	Run 10	Run 11
Low											
2	2.198	1.825	2.144	2.108	2.065	1.810	1.993	1.829	1.786	2.044	1.851
	1.988	1.920	2.166	2.052	2.002	1.806	1.942	1.768	1.880	1.810	1.822
	2.161	1.851	2.182	1.972	2.152	1.795	1.931	1.826	1.785	1.896	1.701
Mean	2.11567	1.86533	2.16400	2.04400	2.07300	1.80367	1.95533	1.80767	1.81700	1.91667	1.79133
Std. Dev	0.11210	0.04910	0.01908	0.06835	0.07532	0.00777	0.03308	0.03439	0.05456	0.11836	0.07956
Medium											
5	4.885	5.067	4.893	4.986	4.884	4.377	4.969	4.475	4.801	4.731	4.405
	4.869	5.266	5.037	4.906	4.913	4.672	4.641	4.549	4.535	4.718	4.472
	4.806	5.086	5.141	4.867	4.863	4.684	4.737	4.388	4.611	4.709	4.402
Mean	4.85333	5.13967	5.02367	4.91967	4.88667	4.57767	4.78233	4.47067	4.64900	4.71933	4.42633
Std. Dev	0.04177	0.10982	0.12454	0.06067	0.02511	0.17389	0.16863	0.08059	0.13701	0.01106	0.03958
High											
10	9.952	9.945	9.851	10.306	10.054	9.219	9.493	9.732	9.327		8.609
	9.910	10.235	9.940	10.299	9.616	9.249	9.091	9.322	8.988	10.972	
	10.002	9.941	9.740	10.840	10.473	9.275	9.225	9.224	9.255	11.199	8.936
Mean	9.95467	10.04033	9.84367	10.48167	10.04767	9.24767	9.26967	9.42600	9.19000	11.08550	8.77250
Std. Dev	0.04606	0.16860	0.10020	0.31035	0.42854	0.02802	0.20469	0.26950	0.17860	0.16051	0.23122

Table 6
Uncertainty of the method precision calculation for concentration levels 2 μg/L (low), 5 μg/L (medium), and 10 μg/L (high).

Concentration	Nominalvalue (NV) μg/L	Standard.deviation S	Degrees of Freedom ν	$q = S^2 \times \nu$	Pooled S $S_p = \sqrt{\sum q / \sum \nu}$	Case samplereplicate r_{cs}	Standard uncertainty (SU) $u = S_p / \sqrt{r_{cs}}$	RelativeSU $u_r = u/NV$
Low	2	0.11210	2	0.02513	0.06832	2	0.04831	0.02415
		0.04910	2	0.00482				
		0.01908	2	0.00073				
		0.06835	2	0.00934				
		0.07532	2	0.01135				
		0.00777	2	0.00012				
		0.03308	2	0.00219				
		0.03439	2	0.00236				
		0.05456	2	0.00595				
		0.11836	2	0.02802				
		0.07956	2	0.01266				
			$\sum\nu = 22$	$\sum q = 0.10268$				
Medium	5	0.04177	2	0.00349	0.10412	2	0.07362	0.01472
		0.10982	2	0.02412				
		0.12454	2	0.03102				
		0.06067	2	0.00736				
		0.02511	2	0.00126				
		0.17389	2	0.06047				
		0.16863	2	0.05687				
		0.08059	2	0.01299				
		0.13701	2	0.03754				
		0.01106	2	0.00024				
		0.03958	2	0.00313				
			$\sum\nu = 22$	$\sum q = 0.23851$				
High	10	0.04606	2	0.00424	0.22525	2	0.15927	0.01593
		0.16860	2	0.05685				
		0.10020	2	0.02008				
		0.31035	2	0.19263				
		0.42854	2	0.36728				
		0.02802	2	0.00157				
		0.20469	2	0.08379				
		0.26950	2	0.14526				
		0.17860	2	0.06380				
		0.16051	1	0.02576				
		0.23122	1	0.05346				
			$\sum\nu = 20$	$\sum q = 1.01474$				

three replicates were analysed over eleven separate days using a freshly prepared calibration line each day.

The uncertainty associated with the method precision u (Precision) is estimated for each concentration level 2 μg/L (Low), 5 μg/L (Medium) and 10 μg/L (High) using a pooled standard deviation (S_p) approach given by

$$u(\text{Precision}) = \frac{S_p}{\sqrt{r_{cs}}}, \quad (10)$$

where S_p similar to Eq. (8) is given by:

$$S_p = \sqrt{\frac{\sum_i (\nu_i \times S_i^2)}{\sum_i \nu_i}},$$

ν_i is the degrees of freedom of the ith sample, S_i is standard deviation of the ith sample and r_{cs} is the number of case sample replicates. As noted by Kadis [7], r_{cs} is used in the denominator of Eq. (10) to avoid underestimation of the uncertainty associated with the method precision.

The relative standard uncertainty of the method precision is calculated by dividing the standard uncertainty by its nominal value (NV) or by the mean concentration of replicates on NV ($\bar{x}_{NV}$).

$$u_r(\text{Precision}) = \frac{u(\text{Precision})}{NV}$$

The calculations for the uncertainty of method precision are detailed in Table 6 for each concentration level. For a given mean reading from a case sample, the relative standard uncertainty value (Table 6) for which the nominal value (NV) was closest to the mean reading x_{cs} was used. Hence, for $x_{cs} = 2$, the closet nominal value is (NV = 2) and the relative standard uncertainty of the method precision is:

$$u_r(\text{Precision}) = 0.02415$$

4.4. Uncertainty of the sample volume

The RSU of the volume $u(V)$ of case blood sample is equivalent to the uncertainty of the pipette used which is the pipette pip-1000. Where multiple equipment is used in the sample preparation, the uncertainty from each equipment can be combined as shown in Tables 1 and 2. From Tables 1 and 2, the pipette pip-1000 has volume 1000 μ/L with a tolerance of 5 μ/L and a reference certificate coverage factor of 2.

$$u(V) = \frac{5}{2} = 2.5\ \mu/\text{L}$$

$$u_r(V) = \frac{u(V)}{V} = \frac{2.5}{1000} = 0.0025$$

5. Combined and expanded uncertainty

5.1. Calculating the combined uncertainty

The concentration of THC in case sample from Eq. (2) is $x_{\text{THC}} = \frac{2}{1} \times 1 = 2\ \mu\text{g/L}$.

The combined uncertainty u_c is obtained by combing all the individual uncertainty components as follows:

$$\frac{u_c}{x_{\text{THC}}} = \sqrt{u_r(\text{Precision}) + u_r(\text{CalStd}) + u_r(\text{CCur}) + u_r(V)}$$

Hence,

$$\begin{aligned} u_c &= x_{\text{THC}} \times \sqrt{u_r(\text{Precision}) + u_r(\text{CalStd}) + u_r(\text{CCur}) + u_r(V)} \\ &= 2 \times \sqrt{0.02415^2 + 0.0371^2 + 0.04813^2 + 0.0025^2} \\ &= 0.131\ \mu\text{g/L} \end{aligned}$$

5.2. The effective degrees of freedom and coverage factor

The obtain a suitable coverage factor k, the effective degrees of freedom ν_{eff} is calculated using the Welch-Satterthwaite equation generally defined as:

$$\nu_{\text{eff}} = \frac{u_c^4}{\sum_l \frac{u_{(l)}^4}{\nu_l}}, \tag{11}$$

where u_c is the combined uncertainty, $u_{(l)}$ is the individual standard uncertainty component l, combined to obtain u_c, and ν_l is the degrees of freedom for each uncertainty component l. With the use of relative standard uncertainties for the combined uncertainty [21], Eq. (11) becomes.

$$\begin{aligned} \nu_{\text{eff}} &= \frac{[u_c / x_{\text{THC}}]^4}{\sum_l \frac{u_{r(l)}^4}{\nu_l}}, \\ &= \frac{[u_c / x_{\text{THC}}]^4}{\frac{u_r(\text{Precision})^4}{\nu_{(\text{Precision})}} + \frac{u_r(\text{CalStd})^4}{\nu_{(\text{CalStd})}} + \frac{u_r(\text{CCur})^4}{\nu_{(\text{CCur})}} + \frac{u_r(\text{V})^4}{\nu_{(\text{V})}}} \\ &= \frac{(\frac{0.131}{2})^4}{\frac{0.02415^4}{22} + \frac{0.0371^4}{\infty} + \frac{0.04813^4}{8} + \frac{0.0025^4}{\infty}} \\ &= 26.8 \end{aligned}$$

The degrees of freedom for the preparation of calibration standards and sample volume are unknown and therefore $\nu_{(\text{CalStd})} = \nu_{(\text{V})} = \infty$. From the t-distribution table with a 99.7% confidence level [26], a coverage factor of $k_{\nu_{\text{eff}},99.7\%} = 3$ is chosen for calculating the expanded uncertainty.

5.3. Calculating the expanded uncertainty

Finally, the expanded uncertainty is obtained by multiplying the coverage factor by the combined uncertainty:

$$\begin{aligned} U &= k \times u_c \\ &= 3 \times 0.131 \\ &= 0.393\ \mu\text{g/L} \end{aligned}$$

The concentration of THC in the case sample is given by 2 ± 0.393 μg/L. The percentage expanded uncertainty can be calculated as:

$$\begin{aligned} \%U &= \frac{U}{x_{\text{THC}}} \times 100 \\ &= \frac{0.393}{2} \times 100 \\ &= 19.65\% \end{aligned}$$

6. Discussion

Understanding uncertainty sources and appropriately quantifying them can have a potentially significant impact on decision making processes. In the judicial system for example, where THC concentrations in a blood sample can be compared to a legal limit, taking into account a calculated measurement uncertainty associated with an analytical method, MU plays a crucial roll in the decision making process, serving as a measure of confidence in a reported analytical result.

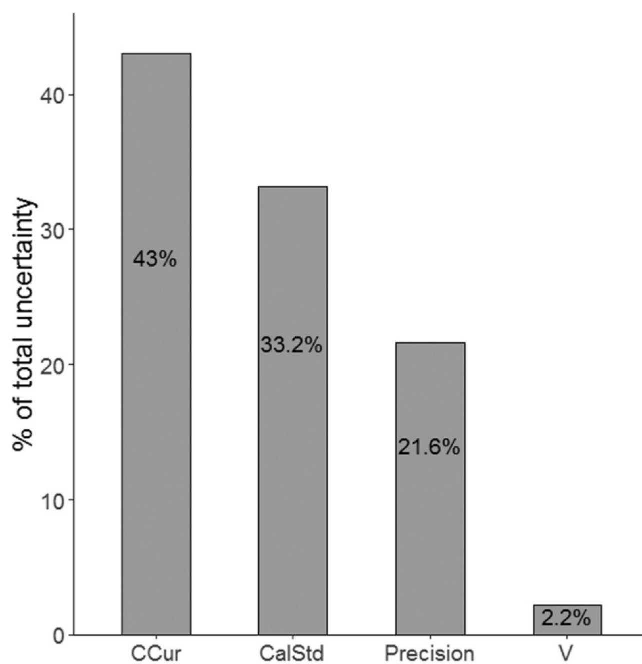

Fig. 3. The contribution of individual uncertainty sources to the total uncertainty.

Using the bottom-up approach with laboratory validation data, the MU for quantifying THC in blood using LC/MS/MS analysis was determined. The combined uncertainty was estimated to be 0.131 μg/L at a concentration of 2 μg/L (<7%). Expanding the combined uncertainty at a 99.7% confidence level gave an expanded uncertainty of 0.393 μg/L.

These calculations not only enable the laboratory to quantify the uncertainty associated with a quantitative result, but can also be used to identify the sources of uncertainty with potential influence and determine if the method can be improved to reduce the uncertainty.

It can be seen from the calculation of the combined uncertainty and Fig. 3 that the largest contribution to the overall method uncertainty for this particular analytical method is from the calibration curve with sample volume contributing the least.

The linear regression of the curve is performed by instrument software, which was optimised during method development and therefore cannot be changed and/or improved. A potential reason for the uncertainty of the calibration curve contributing the most is the problem of double counting, as the uncertainty components are not entirely independent and overlap [7]. Additionally, given that a fresh calibration is prepared on a daily basis according to protocol, the variation in calibrations is partly accounted for in the precision estimate [7].

The risk of double counting is also true for the uncertainty of the method precision and the method recovery (extraction efficiency), which in most cases, it is difficult to separate or avoid [7]. Some authors have minimised the risk of double counting by including the uncertainty of the method recovery and leaving out the uncertainty of the method precision [23,24,5,25,7], with further suggestions on minimising this risk given in [7].

It is worth mentioning two important uncertainty components not calculated for; the uncertainty associated with sample effect and the uncertainty of method recovery. The uncertainty of sample effect which evaluates the effect of different sample matrices was not evaluated due to legal restrictions on the use of case blood samples during validation experiments. According to the guideline [Forensic Service Providers (FSPs) guide for the Analysis of Drugs in support of Section 5a of the Road Traffic Act 1988 (as amended)], an evaluation of analyte recovery was not required if the method was "sufficient to allow detection and quantification at the lowest end of the validated range". Additionally, given the use of deuterated internal standard (THC-d_3) matched to the drug which has similar recovery as the drug of interest (THC), any THC 'lost' during the extraction process is expected to be offset by the loss in THC-d_3. It is, however, acknowledge that recovery is unlikely to be 100% given the analytical procedure and could be accounted for with its associated uncertainty included in the overall uncertainty.

It should be noted that the determination of the method uncertainty outlined above does not take into consideration the bias (accuracy/ trueness) of the method. Whilst the bias of a method should be evaluated (and was in this case −2.938% at 2 μg/L), corrections for bias can be made independently of MU. This is the case for the laboratory casework, in accordance with standard practitioner guidelines [Forensic Science Regulator Codes of Practice and Conduct - FSR -C-133].

In minimising the overall uncertainty, one factor that can possibly be improved is the preparation of the standards and spiking of the calibration curve. Minimising the number of times each pipette is used, or using volumetric flasks instead of pipettes would reduce this uncertainty component. Also, in estimating the uncertainty associated with pipettes and volumetric flasks, additional uncertainty that could possibly influence instrument performance is temperature effect and repeatability. Laboratory temperature was varied during the validation and has therefore been accounted for in the precision experiments.

The experimental design of the validation (3 replicates at 3 concentration levels over 11 days) is one of the more comprehensive designs, nevertheless it is possible that extending the validation to more replicates over more days may provide more accurate or improved precision data. The current design is however, more than adequate for method validation within the forensic toxicological community [Forensic Science Regulator Codes of Practice and Conduct-FSR-C-133].

Using the methodology underpinning these calculations for deriving the overall uncertainty, work is on going to create an application as part of a statistical tool box project that implements these calculations. The statistical tool box will be releasing all applications open source and more information can be found in Appendix B.

7. Conclusion

In summary, a detailed systematic approach has been provided for identifying the sources of measurement uncertainty within a method validated for the purpose of quantifying THC in blood samples and estimating their individual contribution to the overall uncertainty of the analytical method. The data associated with each calculation is given and detailed step-by-step calculations are provided to illustrate a possible way of estimating MU using laboratory validation data.

CRediT authorship contribution statement

Joyce K. Klu: Writing - Original draft, Formal analysis, Visualization, Methodology, Software, Writing - review & editing. **Jane A. Officer:** Conceptualization, Data curation, Investigation, Validation, Writing - review & editing. **Alexandra Park:** Data curation, Validation, Investigation, Writing - review & editing. **Roy Mudie:** Software, Data curation. **Niamh NicDaeid:** Conceptualization, Data curation, Writing - review & editing, Funding acquisition, Supervision.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgements

We would like to appreciate Dr. Sarah Barron and Miss Maria Tamburrini for carrying out all the validation work needed to generate the data.

Funding

This research is funded by the Leverhulme Trust grant RC-1015-011.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.forsciint.2021.110744.

References

[1] S.C.W. de Souza Eller, F. de Oliveira, M. Yonamine, Measurement uncertainty for the determination of amphetamines in urine by liquid-phase microextraction and gas chromatography-mass spectrometry, Forensic Sci. Int. 265 (2016) 81–88.
[2] R.G. Gullberg, Estimating the measurement uncertainty in forensic blood alcohol analysis, J. Anal. Toxicol. 36 (2012) 153–161.
[3] ISO/IEC 17025:2017, General Requirements for the Competence of Testing and Calibration Laboratories, International Organization for Standardization (ISO), 2017.
[4] Y.C. Ma, C.W. Wang, S.H. Hung, Y.Z. Chang, C.R. Liu, G.R. Her, Estimation of the measurement uncertainty in quantitative determination of ketamine and norketamine in urine using a one-point calibration method, J. Anal. Toxicol. 36 (2012) 515–522.
[5] I. Komorowicz, D. Barałkiewicz, Arsenic speciation in water by high-performance liquid chromatography/inductively coupled plasma mass spectrometry–method validation and uncertainty estimation, Rapid Commun. Mass Spectrom. 28 (2014) 159–168.
[6] JCGM 200:2012, International vocabulary of Metrology: Basic and General Concepts and Associated Terms (VIM) 2008 Version with Minor Corrections, third ed., 2012.
[7] R. Kadis, Evaluation of the measurement uncertainty: some common mistakes with a focus on the uncertainty from linear calibration, J. Chromatogr. A 1499 (2017) 226–229.
[8] D. Brockley-Drinkman, H.M. Barkholtz, Estimating uncertainty in Wisconsin's evidential breath alcohol measurements, J. Anal. Toxicol. (2019).
[9] S. Lee, H. Choi, E. Kim, H. Choi, H. Chung, K.H. Chung, Estimation of the measurement uncertainty by the bottom-up approach for the determination of methamphetamine and amphetamine in urine, J. Anal. Toxicol. 34 (2010) 222–228.
[10] J.H. Lee, J.H. Choi, J.S. Youn, Y.J. Cha, W. Song, A.J. Park, Comparison between bottom-up and top-down approaches in the estimation of measurement uncertainty, Clin. Chem. Lab. Med. 53 (2015) 1025–1032.
[11] R. Rigo-Bonnin, A. Blanco-Font, F. Canalias, Different top-down approaches to estimate measurement uncertainty of whole blood tacrolimus mass concentration values, Clin. Biochem. 57 (2018) 56–61.
[12] M. Dabalus Islam, M. Schweikert Turcu, A. Cannavan, Comparison of methods for the estimation of measurement uncertainty for an analytical method for sulphonamides, Food Addit. Contam. 25 (2008) 1439–1450.
[13] F. Martinello, N. Snoj, M. Skitek, A. Jerin, The top-down approach to measurement uncertainty: which formula should we use in laboratory medicine? Biochem. Med. 30 (2020).
[14] S.P. Elliott, D.W. Stephen, S. Paterson, The United Kingdom and Ireland association of forensic toxicologists forensic toxicology laboratory guidelines (2018), Sci. Justice 58 (2018) 335–345.

[15] S.M. Wille, F.T. Peters, V. Di Fazio, N. Samyn, Practical aspects concerning validation and quality control for forensic and clinical bioanalytical quantitative methods, Accrédit. Qual. Assur. 16 (2011) 279.
[16] Scientific Working Group for Forensic Toxicology, Standard practices for method validation in forensic toxicology, J. Anal. Toxicol. 37 (2013) 452–474 (SWGTOX).
[17] F.T. Peters, O.H. Drummer, F. Musshoff, Validation of new methods, Forensic Sci. Int. 165 (2007) 216–224.
[18] A.G. González, M. Á. Herrador, A.G. Asuero, J. Martín, A practical way to ISO/GUM measurement uncertainty for analytical assays including in-house validation data, Quality Control in Laboratory, IntechOpen, 2017.
[19] R.B. da Silva, E. Bulska, B. Godlewska-Żyłkiewicz, M. Hedrich, N. Majcen, S. Marinčić, I. Papadakis, M. Patriarca, E. Vassileva, Analytical Measurement: Measurement Uncertainty and Statistics, Publications Office, 2012.
[20] UKAS M3003. The Expression of Uncertainty and Confidence in Measurement, third ed., United Kingdom Accreditation Service, 2012.
[21] JCGM 100:2008. Evaluation of Measurement Data: Guide to the Expression of Uncertainty in Measurement, GUM 1995 with minor corrections, 2008.
[22] EURACHEM/CITAC Guide CG 4, Quantifying Uncertainty in Analytical Measurement, third ed., 2012.
[23] N. Gros, M. Camoes, R.J. Bettencourt da Silva, Uncertainty budget for simultaneous determination of minor and major ions in seawater with ion chromatography confronted with uncertainties in concentrations of calibration standards, Anal. Lett. 43 (2010) 1317–1329.
[24] H. Tian, Uncertainty analysis of chlormequat residue in fruits by LC–MS–MS, Chromatographia 73 (2011) 457–462.
[25] A. Pavlova, O. Stoyanova, P. Ivanova, T. Dimova, Uncertainty estimation related to analysis of volatile aromatic hydrocarbons by static headspace gas chromatography, J. Chromatogr. Sci. 52 (2014) 567–572.
[26] D.C. Montgomery, E.A. Peck, G.G. Vining, Introduction to linear regression analysis, montgomery 2021 introduction, John Wiley & Sons, 2021.