Talanta 85 (2011) 1535–1542

Contents lists available at ScienceDirect

Talanta

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

Uncertainty profiles for the validation of analytical methods

T. Saffaj [a,b,*], B. Ihssane [b]

[a] Centre Universitaire Régional d'Interface, Université Sidi Mohamed Ben Abdallah, BP 2626, Route d'Imouzzar-Fès, Morocco
[b] Laboratoire de Chimie Organique Appliquée, Faculté des Sciences et Techniques, BP 2626, Route d'Imouzzar-Fès, Morocco

ARTICLE INFO

Article history:
Received 15 April 2011
Received in revised form 10 June 2011
Accepted 14 June 2011
Available online 21 June 2011

Keywords:
Method validation
Uncertainty profile
β-Content tolerance interval
Measurement uncertainty
Bioanalytical method

ABSTRACT

This article aims to expose a new global strategy for the validation of analytical methods and the estimation of measurement uncertainty. Our purpose is to allow to researchers in the field of analytical chemistry get access to a powerful tool for the evaluation of quantitative analytical procedures. Indeed, the proposed strategy facilitates analytical validation by providing a decision tool based on the uncertainty profile and the β-content tolerance interval. Equally important, this approach allows a good estimate of measurement uncertainty by using data validation and without recourse to other additional experiments.

In the example below, we confirmed the applicability of this new strategy for the validation of a chromatographic bioanalytical method and the good estimate of the measurement uncertainty without referring to any extra effort and additional experiments. A comparative study with the SFSTP approach [1] showed that both strategies have selected the same calibration functions.

The holistic character of the measurement uncertainty compared to the total error was influenced by our choice of profile uncertainty. Nevertheless, we think that the adoption of the uncertainty in the validation stage controls the risk of using the analytical method in routine phase.

1. Introduction

Nowadays, an analytical result is indispensible to take decisions. It has a significant impact on our society. For this reason, several standards and guides have been written and established for the sole purpose of ensuring the quality of results returned. All scientists know that a measure and particularly an analytical result must be necessarily accompanied by a measurement uncertainty. Indeed, the expression of the uncertainty associated with measurement results becomes an important parameter of performance of an analytical method so as to be considered as the analytical validation. However, the question to be asked with intensity now is: "how to quantify the uncertainty with certainty?"

To resolve this problem, standards, guidelines and guidance have been developed to offer practical concepts for estimating uncertainty [2–6]. These documents are mandatory passages for laboratories attempting to be accredited and certified. Despite these efforts, the above approaches are complex, costly and often cumbersome for daily practice laboratories.In fact, the practical implementation of the normative documents cited above is a major problem for industries which are trying to ensure quality results and satisfy their customers. Generally, the problems are technical and economic order. For example, a quality control laboratory of a pharmaceutical industry produces each year more than 10,000 analysis. With such figures, one can imagine an enormous cost, generated by the implementation of guides, as EURACHEM [3] and GUM [4].

To treat these drawbacks, researchers have attempted to offer simpler approaches to estimate the measurement uncertainty from validation data. A recent draft of guide ISO/DTS 21748 [2] suggests using repeatability, reproducibility and trueness to estimate measurement uncertainty. In 2000, Barwick and Ellison have published a guide through the VAM project [6] entitled "Protocol for uncertainty evaluation from validation data". This manual and the ISO Guide have provided an important service for analysts.Recently, SFSTP published an interesting document on the validation of quantitative analytical procedures [1]. It attempted in this manual to harmonize the procedures to follow to validate an analytical method by introducing the concept of accuracy profiles based on the concept of total error and the β-expectation tolerance interval. Feinberg et al. [7] are the first who proposed a relationship between the measurement uncertainty and the β-expectation tolerance interval. In 2006, Gonzalez and Herrador [8], through a very interesting publication have tried to make, a double cost. Indeed, they developed a validation strategy based on the notion of accuracy profiles and measurement uncertainty.

The goal of this publication is to propose a new strategy for the analytical validation using the uncertainty profile as a decision tool as well as to describe an original method to estimate uncertainty of measurement. The formula that we offer to assess the

* Corresponding author.
E-mail address: saffajt@gmail.com (T. Saffaj).

0039-9140/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.talanta.2011.06.042

uncertainty is based on the calculation of the β-content tolerance interval by two different procedures. The power and efficiency of the proposed approach is demonstrated by application to evaluate the performance of a chromatographic bioanalytical method.

2. Theory and computation

2.1. β-Content tolerance intervals

The theory and computation of tolerance intervals has been developed at an exponential rate in recent decades. Nowadays, the statistical tolerance regions are applied in areas including quality control, environment, industry, health and other... Three basic types of tolerance intervals have received considerable attention: (i) β-content tolerance intervals, (ii) β-expectation tolerance intervals and (iii) fixed-in-advance tolerance interval.

In this section, we discuss procedures for computing two sided β-content tolerance intervals for balanced one-way random effects models by using Mee's method [9,10] and Liao's approach [11].

2.1.1. Mee's approach

During the validation of analytical methods, the experimental design involves to make measurements in different days, with replicate determinations within each day. A statistical model to describe the measured values is given by:

$$Y_{ij} = \mu + b_i + e_{ij}; \quad j = 1, 2, \ldots, n; \quad i = 1, 2, \ldots, a \tag{1}$$

where Y_{ij} denotes the jth replicate observation corresponding to ith run, μ is an unknown general mean, b_i's represent random effects and e_{ij}'s represent error terms. It is assumed that b_i's and e_{ij}'s are all independent having the distributions $b_i \sim N(0, \sigma_b^2)$ and $e_{ij} \sim N(0, \sigma_e^2)$. Thus, $Y_{ij} \sim N(0, \sigma_b^2 + \sigma_e^2)$ and σ_b^2 and σ_e^2 represent the two variance components in the model.

We define

$$\bar{Y} = \frac{1}{an}\sum_{i=1}^{a}\sum_{j=1}^{n} Y_{ij}, \qquad \bar{Y}_i = \frac{1}{n}\sum_{j=1}^{n} Y_{ij},$$

$$SS_b = n\sum_{i=1}^{a}(\bar{Y}_i - \bar{Y})^2 \quad \text{and} \quad SS_e = \sum_{i=1}^{a}\sum_{j=1}^{n}(Y_{ij} - \bar{Y}_i)^2, \tag{2}$$

Also we identify the expected mean squares under the model (1):

$$MS_b = \frac{SS_b}{a-1} \quad \text{and} \quad MS_e = \frac{SS_e}{a(n-1)} \tag{3}$$

The ANOVA estimators of σ_b^2 and σ_e^2 are given by

$$\hat{\sigma}_b^2 = \frac{1}{n}(MS_b - MS_e) \quad \text{and} \quad \hat{\sigma}_e^2 = MS_e \tag{4}$$

The two-sided tolerance limits that we shall construct will be functions of $\bar{Y}$, $\hat{\sigma}_b^2$ and σ_e^2. It will be as follows:

$$\bar{Y} \pm k\hat{\sigma}_m \tag{5}$$

where

$$\hat{\sigma}_m^2 = \hat{\sigma}_b^2 + \hat{\sigma}_e^2 \tag{6}$$

and k is the tolerance factor to be determined. Thus, k should satisfy the condition

$$P_{\bar{Y},\hat{\sigma}_b^2,\hat{\sigma}_e^2}\left\{P_Y\left[\bar{Y} - k\hat{\sigma}_m < Y < \bar{Y} + k\hat{\sigma}_m \middle| \bar{Y}, \hat{\sigma}_m\right] \geq \beta\right\} = \gamma \tag{7}$$

The constants β and γ are the interval content and coverage, respectively.

If we use the Satterthwaite approximation to get an approximate Chi-square distribution associated with $\hat{\sigma}_m^2$. We write

$$R = \frac{\sigma_b^2}{\sigma_e^2} \quad \text{and} \quad R_0 = \frac{\sigma_b^2 + \sigma_e^2}{n\sigma_b^2 + \sigma_e^2} = \frac{R+1}{nR+1} \tag{8}$$

We note that

$$\bar{Y} \sim N\left(\mu, \frac{n\sigma_b^2 + \sigma_e^2}{an}\right), \frac{SS_b}{n\sigma_b^2 + \sigma_e^2} \sim \chi_{a-1}^2 \quad \text{and} \quad \frac{SS_e}{\sigma_e^2} \sim \chi_{a(n-1)}^2 \tag{9}$$

where χ_ν^2 denotes the chi-square distribution with ν degrees of freedom.

Then we conclude that

$$\frac{\hat{\sigma}_m^2}{\sigma_m^2} \sim \frac{\chi_f^2}{f} \tag{10}$$

where

$$f = \frac{(R+1)^2}{(R+n^{-1})^2/(a-1) + (1-n^{-1})/(an)} \tag{11}$$

Thus, Eq. (7) can be written as

$$P_{Z,\chi_f^2} = \left[\Phi\left(\frac{Z}{\sqrt{anR_0}} + k\sqrt{\frac{\chi_f^2}{f}}\right) - \Phi\left(\frac{Z}{\sqrt{anR_0}} - k\sqrt{\frac{\chi_f^2}{f}}\right) \geq \beta\right] = \gamma \tag{12}$$

where $\Phi(\,)$ denotes the standard normal distribution function and Z is a standardized normal variable.

Since R is unknown, R^* is used in its place. Where R^* is an upper $100\eta\%$ confidence bound for R; that is

$$R^* = \max\left[0, \frac{F \cdot F_\eta - 1}{n}\right] \tag{13}$$

where F is the MS ratio MS_b/MS_e and F_η is the 100η percentile of an F distribution with $\nu_1 = a(n-1)$ and $\nu_2 = (a-1)$. However, based on numerical results, the recommended values of η are 0.85, 0.905 and 0.975, corresponding to $\gamma = 0.90$, 0.95 and 0.99, respectively. Then k is computed from

$$P_{Z,\chi_f^2} = \left[\Phi\left(\frac{Z}{\sqrt{anR_0^*}} + k\sqrt{\frac{\chi_f^2}{f}}\right) - \Phi\left(\frac{Z}{\sqrt{anR_0^*}} - k\sqrt{\frac{\chi_f^2}{f}}\right) \geq \beta\right] = \gamma \tag{14}$$

The estimation of k requires solving the following integral

$$\sqrt{\frac{2}{\pi h}}\int_0^\infty P_L\left(L \geq \frac{\chi_{1;\beta}^2(x^2)}{k^2}\right) e^{-x^2/2h}dx = \gamma$$

$$\text{with} \quad h = \frac{1}{anR_0^*} \quad \text{and} \quad L = \frac{\chi_f^2}{f} \tag{15}$$

where $\chi_{\nu;\alpha}^2(\tau)$ denotes the α quantile of a noncentral chi-square distribution with dfν and noncentrality parameter τ.

An approximation to k, called k_s, which satisfies the integral above is given by

$$k_s \simeq \sqrt{\frac{f^*\chi_{1;\beta}^2(h)}{\chi_{f^*;1-\gamma}^2}} \tag{16}$$

Note that f^* and R_0^* are estimate of f and R_0 that can be obtained by replacing the R by R^*.

Finally, the two sided β-content tolerance interval provided by

$$\bar{Y} \pm k_s \hat{\sigma}_m \tag{17}$$

2.1.2. Liao's approach

In order to estimate the β-content tolerance intervals, Liao et al. have used the generalized pivotal confidence interval idea. In fact, the generalized p-value approach for hypothesis testing has been introduced by Tsui and Weerahandi [12] and the generalized confidence interval by Weerahandi [13]. Together, these are referred to as the generalized variable approach or generalized inference procedure. The concepts of generalized p-values and generalized confidence intervals have turned out to be extremely fruitful for obtaining tests and confidence intervals for some complex problems where standard procedures are difficult to apply. Thus, Liao et al. have exploited this procedure to compute the two-sided tolerance interval for the normal distribution with several variance components.

Let us now consider the case of one-way random model with balanced data; Note that, in order to derive a two-sided tolerance interval, we have to obtain a margin of error statistic D, a function of σ_b^2 *and* σ_e^2, so that

$$P_{\bar{Y},\hat{\sigma}_b^2,\hat{\sigma}_e^2}\left\{P_Y\left[\bar{Y} - D < Y < \bar{Y} + D \,\middle|\, \bar{Y}, \hat{\sigma}_m\right] \geq \beta\right\} = \gamma \tag{18}$$

where $Y \sim N(\mu, \sigma_b^2 + \sigma_e^2)$. Once D is obtained, the tolerance interval is given by

$$\bar{Y} \pm D \tag{19}$$

We define

$$\sigma_1^2 + \sigma_b^2 + \sigma_e^2 \quad \text{and} \quad \sigma_2^2 = \frac{n\sigma_b^2 + \sigma_e^2}{an} \tag{20}$$

From Eq. (20), we can write

$$\sigma_1^2 + \sigma_2^2 = \left(1 + \frac{1}{a}\right)\frac{n\sigma_b^2 + \sigma_e^2}{n} + \left(1 - \frac{1}{n}\right)\sigma_e^2 \tag{21}$$

To calculate D, we must determine γ upper confidence limit for $\sigma_1^2 + \sigma_2^2$ using the generalized confidence interval idea.

The following algorithm can be used to compute D and, consequently, to estimate the two-sided β-content tolerance interval.

Algorithm.

1. Once the data are obtained, compute the observed values $\bar{Y}$, ss_b and ss_e.
2. Let M denotes the number of simulation runs. For $\boldsymbol{i} = 1,2,\ldots, M$, perform the following steps.
3. Generate independent random variables $T_{b,i}^2 \sim \chi_{a-1}^2$ *and* $T_{e,i}^2 \sim \chi_{a(n-1)}^2$
4. Compute

$$D_{1,i} = \frac{(1/n)(1 + 1/a)ss_b}{T_{b,i}^2} + \frac{(1 - 1/n)ss_e}{T_{e,i}^2}$$

The γ quantile of the $\boldsymbol{D_{1,i}}$ values gives an estimate of the γ upper confidence limit for $\sigma_1^2 + \sigma_2^2$

The square root of this upper confidence limit, multiplied by $Z_{(1+\beta)/2}$ provides a margin of error statistic $\boldsymbol{D}$ needed for computing the two-sided β-content tolerance interval.

In the above, $Z_{(1+\beta)/2}$, denotes the $(1+\beta)/2$ quantile of a standard normal distribution.

3. Uncertainty profile

Several approaches are proposed for estimating measurement uncertainty. They are normative documents, guides and guidances or articles published by researchers in the field of Analytical Chemistry [19–25]. The VAM (Valid Analytical Measurement) [6] is the first protocol used to evaluate the measurement uncertainty from validation data. As well, after that, SFSTP had adopted the concept of profile accuracy to validate analytical methods; Feinberg [7] has exploited this concept to estimate the measurement uncertainty. Although his method is original, but it presents an underestimation of uncertainty by the negligence of terms that can be sources of errors for the measurand. Gonzalez [8] has proposed a new approach for estimating uncertainty and validation of analytical procedures based on the work of Feinberg. Indeed, he proposes to estimate the measurement uncertainty from validation data and robustness study. Even though, his approach gives a more or less correct estimate of the uncertainty, it has disadvantages of a technical and economic nature in the practical implementation.

In this section, we present an original method so as to validate first the analytical procedures and second, to estimate correctly the measurement uncertainty from validation data without having recourse to other experiments.

3.1. Uncertainty profile: principle

According to the LGC/VAM protocol [6] and the recommendations of the ISO/DTS 21748 guide [2], a basic model for the uncertainty of the measurand Z, can be expressed by Eq. (22):

$$u^2(Z) = S_R^2 + u^2(\hat{\delta}) + \sum c_i^2 u^2(x_i) \tag{22}$$

where S_R is the reproducibility standard deviation, $u(\hat{\delta})$ is the uncertainty associated with the bias of the method, and $\Sigma c_i^2 u^2(x_i)$ is the sum of all of the effects due to other deviations.

When Feinberg has used the concept of accuracy profile and validation data to estimate the measurement uncertainty, he has ignored the third term of Eq. (22). Well, uncertainty can be expressed by the following equation:

$$u^2(Z) = S_R^2 + u^2(\delta) \tag{23}$$

The accuracy profile can be built using the β-expectation tolerance interval, according to Mee's method. This interval is equal to:

$$\bar{Y} \pm t(\nu) k \hat{\sigma}_M \tag{24}$$

Thus, one can verify that:

$$u^2(Z) = k^2 \hat{\sigma}_M^2 \tag{25}$$

And the mathematical model that brings the uncertainty and the β-expectation tolerance interval is given by:

$$\bar{Y} \pm t(\nu) u(Z) \tag{26}$$

Note that: $\hat{\sigma}_M = S_R$

Gonzalez [8] has proposed a formula of three terms to estimate the measurement uncertainty, it can be expressed by:

$$u^2(Z) = S_R^2 + u^2(\hat{\delta}) + u_{\text{rob}}^2(Z) \tag{27}$$

where u_{rob} is the uncertainty coming from the robustness study.

To estimate u_{rob}, the authors have employed the landmark procedure based on the experimental design of Plackett–Burmann. Thus, they have suggested to validate analytical methods by the construction of accuracy profile across the uncertainty using the following equation:

$$\bar{Y} \pm k u(Z) \tag{28}$$

With regard to the strategy that we propose for the analytical validation and estimation of measurement uncertainty, is a holistic approach that takes the analytical procedure as a whole. Indeed, it is not necessary to pass by the bias, reproducibility, and robustness study to validate first the methods and second, to estimate uncertainty. Simply, to calculate the tolerance limits by using the β-content tolerance intervals.

The basic idea is simple, according to Eq. (26), we can write that

$$U = \bar{Y} + t(\nu)u(Z) \tag{29}$$

And

$$L = \bar{Y} - t(\nu)u(Z) \tag{30}$$

where U is upper tolerance interval; L is lower tolerance interval; Finally, the Uncertainty can be expressed as:

$$u(Z) = \frac{U - L}{2t(\nu)} \tag{31}$$

It should be noted that there are two scenarios to calculate the uncertainty according to Eq. (31):

1. If one considers that the uncertainty can be expressed by Eq. (23), and then the tolerance interval [L, U] is calculated using the β-expectation tolerance intervals. Where $t(\nu)$ is the $(1+\beta)/2$ quantile of the student t distribution with ν degrees of freedom.
2. If one considers that the uncertainty is expressed by Eq. (22), that is to say, we take into account other terms that affect the measurand errors such as robustness. In this case, we use the β-content tolerance intervals to calculate U and L.

   Where:

   - $t(\nu)$ is the $(1+\gamma)/2$ quantile of the student t distribution with ν degrees of freedom.

$$\upsilon = \frac{(\hat{R}+1)^2}{(\hat{R}+n^{-1})^2/(a-1) + (1-n^{-1})/(an)} \tag{32}$$

$$\hat{R} = \max\left[0, \left(\frac{MS_b}{MS_e} - 1\right)\frac{1}{n}\right] \tag{33}$$

Therefore, we propose the following equation to build the uncertainty profile and as a decision tool for the validation of analytical methods.

$$\left|\bar{Y} \pm ku(Z)\right| < \lambda \tag{34}$$

where k is a Coverage factor. The choice of the factor k is based on the level of desired confidence. For an approximate level of confidence of 95%, $k = 2$ [3].

It should be noted that the calculation of $u(z)$ by the first case of Eq. (31) is equivalent to the uncertainty calculated by Feinberg's method [7], according to Eq. (23). However, Marini et al. [14] have been able to demonstrate through a comparative study that:

- The uncertainties estimated by the inter-laboratory approach, or the robustness study are statistically different from uncertainties calculated by Feinberg's method.
- The inter-laboratory and robustness uncertainties are always higher than calculated in the Feinberg's method.

For this reason, we adopted the second case of Eq. (31), to assess correctly the uncertainty and then to use it in Eq. (34), so as to adjudicate on the decision of validating or not analytical methods.

In order to demonstrate the power and efficiency of our approach in estimating measurement uncertainty and analytical validation, the following paragraph, will be dedicated to implement our strategy to a chromatographic bioanalytical method.

Table 1
Experimental data used to illustrate the calculation of the uncertainty profile.

Concentration (ng mL^{-1})	Signal (ratio)		
	Series 1	Series 2	Series 3
Calibration standards			
25.3533	0.0485	0.0358	0.0449
25.3533	0.0448	0.0402	0.0415
48.2417	0.0959	0.1025	0.0987
48.2417	0.087	0.0993	0.0892
96.4833	0.1974	0.2046	0.2036
96.4833	0.2057	0.1996	0.2082
223.8496	0.5589	0.5371	0.5095
223.8496	0.5667	0.5066	0.5756
437.8235	1.1041	0.9963	1.1726
437.8235	1.0961	1.0568	1.1772
964.8233	2.396	2.2877	2.4528
964.8233	2.3861	2.25	2.3147
Validation standards			
25.3533	0.0439	0.0371	0.0444
25.3533	0.0488	0.0422	0.0457
25.3533	0.048	0.0461	0.0502
25.3533	0.0484	0.0448	0.0475
48.2417	0.0949	0.0922	0.0956
48.2417	0.0927	0.0916	0.1023
48.2417	0.0887	0.0854	0.1007
48.2417	0.1015	0.0918	0.1092
437.8235	0.9873	0.9718	1.0392
437.8235	1.0136	1.0322	1.1132
437.8235	1.0288	1.0342	1.1419
437.8235	1.0173	1.0319	1.0751
838.6479	2.022	1.9252	2.1272
838.6479	1.9901	2.0284	2.2127
838.6479	2.0937	2.0127	2.2699
838.6479	2.0189	2.0273	2.2546

4. Illustrative example

To illustrate our strategy, the statistical methodology which allows us to conclude correctly about the validity of a procedure is proposed in this section. In fact, all the steps to attain the decision tool, namely the uncertainty profile, are described and illustrated step by step by a numerical example.

The example, which has been the object of this illustration, concerns the validation of a method for the determination of sotalol in plasma by HPLC using atenolol as an internal standard. The data of this method is taken from SFSTP guide [15].

Table 1 summarizes all results obtained in the validation phase. The concentrations are expressed as ng mL^{-1}, and the instrumental signal is a ratio between the peak areas of sotalol and atenolol.

In the purpose of testing our approach to select the best regression model and at the same time to make a comparison with the strategy based on the profile accuracy, we have generated all responses functions adopted by SFSTP. For details see article 15. In fact, eight regression models have been fitted in order to analyze the relationship between concentrations an analytical response.

4.1. Uncertainty profile: computation and building procedure

Uncertainty profile is a graphical decision tool to help the analyst in deciding whether an analytical procedure is valid. It is based on the combination in the same graphic of the uncertainty interval and the acceptability limit. Whereas, validation must cover up the complete application domain of the method, the uncertainty profile combines several tolerance intervals computed at several levels of concentration.

Therefore, the building of the uncertainty profile can be achieved through the following steps:

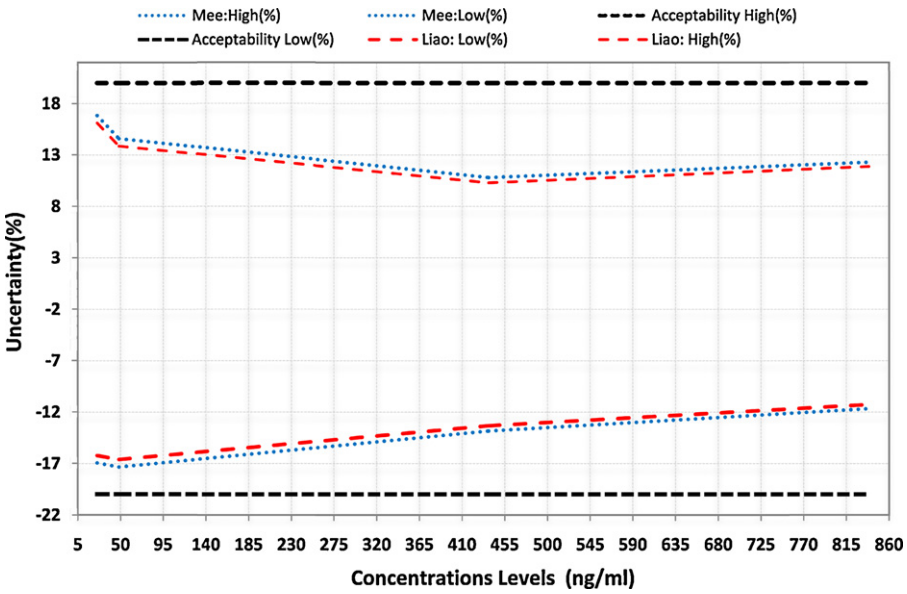

Fig. 1. Uncertainty profile of the analytical procedure results when the linear regression after square root transformation model is chosen as response function. Acceptance limits are set at ±20%.

Table 2
Point estimates of the relative expanded uncertainty and uncertainty limits obtained with each response function using the β-content tolerance interval computed by Mee's method.

Model	Concentration level ($ng\,mL^{-1}$)	Uncertainty ($ng\,mL^{-1}$)	Expanded uncertainty ($ng\,mL^{-1}$)	Relative expanded uncertainty (%)	Relative uncertainty limits (%)	
					Lower	Upper
Linear 0–max	25.3533	1.31	2.62	10.34	−36.49	−15.8
	48.2417	3.45	6.91	14.32	−32.95	−4.32
	437.8235	30.19	60.39	13.79	−16.11	11.48
	838.6479	59.8	119.6	14.26	−12.26	16.27
Linear 0–223	25.3533	1.33	2.67	10.53	−36.34	−15.29
	48.2417	4.18	8.37	17.34	−35.57	−0.89
	437.8235	37.85	75.7	17.29	−19.11	15.47
	838.6479	74.61	149.23	17.79	−15.27	20.32
Linear	25.3533	2.3	4.61	18.17	−18.24	18.09
	48.2417	1.54	3.08	6.39	−11.92	0.86
	437.8235	27.86	55.72	12.73	−14.83	10.63
	838.6479	54.47	108.94	12.99	−11.59	14.39
Weighted linear, $1/X$	25.3533	2.06	4.13	16.29	−7.85	24.72
	48.2417	3.63	7.27	15.07	−16.47	13.67
	437.8235	27.21	54.42	12.43	−14.77	10.09
	838.6479	51.11	102.22	12.19	−11.32	13.06
Linear regression after logarithm transformation	25.3533	1.77	3.53	13.93	−13.83	14.02
	48.2417	4.38	8.76	18.16	−15.4	20.91
	437.8235	23.66	47.31	10.81	−12.63	8.98
	838.6479	39.97	79.93	9.53	−13.6	5.46
Linear regression after square root transformation	25.3533	2.14	4.28	16.88	−16.94	16.83
	48.2417	3.85	7.7	15.97	−17.34	14.59
	437.8235	26.99	53.97	12.33	−13.84	10.81
	838.6479	50.22	100.45	11.98	−11.67	12.29
Quadratic	25.3533	5.05	10.11	39.86	−19.24	60.49
	48.2417	5.37	10.73	22.25	−19.66	24.84
	437.8235	27.55	55.11	12.59	−17.18	8
	838.6479	57.99	115.99	13.83	−12.49	15.17
Weighted quadratic, $1/X$	25.3533	2.49	4.99	19.68	−8.91	30.44
	48.2417	3.59	7.18	14.87	−16.17	13.58
	437.8235	25.89	51.77	11.83	−15.17	8.48
	838.6479	55.98	111.97	13.35	−11.98	14.72

Table 3
Point estimates of the relative expanded uncertainty and uncertainty limits obtained with each response function using the β-content tolerance interval computed by Liao's method.

Model	Concentration level ($ng\,mL^{-1}$)	Uncertainty ($ng\,mL^{-1}$)	Expanded uncertainty ($ng\,mL^{-1}$)	Relative expanded uncertainty (%)	Relative uncertainty limits (%)	
					Lower	Upper
Linear 0–max	25.3533	1.29	2.59	10.2	−36.34	−15.94
	48.2417	3.29	6.57	13.62	−32.26	−5.01
	437.8235	28.96	57.92	13.23	−15.54	10.91
	838.6479	58.05	116.11	13.84	−11.84	15.85
Linear 0–223	25.3533	1.32	2.64	10.4	−36.21	−15.42
	48.2417	4.01	8.01	16.61	−34.84	−1.62
	437.8235	36.84	73.68	16.83	−18.65	15.01
	838.6479	71.93	143.86	17.15	−14.63	19.68
Linear	25.3533	2.2	4.39	17.32	−17.4	17.24
	48.2417	1.48	2.95	6.12	−11.65	0.58
	437.8235	26.65	53.3	12.17	−14.27	10.07
	838.6479	52.85	105.69	12.6	−11.2	14
Weighted linear, 1/*X*	25.3533	1.97	3.94	15.55	−7.11	23.98
	48.2417	3.46	6.92	14.34	−15.74	12.94
	437.8235	26.03	52.05	11.89	−14.23	9.55
	838.6479	49.11	98.22	11.71	−10.84	12.58
Linear regression after logarithm transformation	25.3533	1.75	3.49	13.79	−13.69	13.88
	48.2417	4.17	8.35	17.3	−14.55	20.06
	437.8235	22.59	45.18	10.32	−12.14	8.5
	838.6479	38.27	76.54	9.13	−13.19	5.06
Linear regression after square root transformation	25.3533	2.05	4.1	16.16	−16.22	16.11
	48.2417	3.67	7.35	15.24	−16.61	13.86
	437.8235	25.87	51.74	11.82	−13.33	10.3
	838.6479	48.51	97.01	11.57	−11.26	11.88
Quadratic	25.3533	4.94	9.87	38.93	−18.31	59.56
	48.2417	5.22	10.45	21.65	−19.07	24.24
	437.8235	26.19	52.38	11.96	−16.55	7.37
	838.6479	55.57	111.14	13.25	−11.91	14.6
Weighted quadratic, 1/*X*	25.3533	2.39	4.78	18.86	−8.09	29.62
	48.2417	3.43	6.86	14.21	−15.51	12.92
	437.8235	24.78	49.56	11.32	−14.66	7.97
	838.6479	53.96	107.91	12.87	−11.5	14.24

- Define the limits of acceptance of the method. For the chosen example, the acceptance limit was set at ±20%. This value was used because the method is a bioanalytical technique [15].
- Generate all possible calibration models, using the calibration data. Then, calculate the concentrations of validation standards using the inverse prediction equation.
- Compute the β-content tolerance intervals for each level, according to Eq. (17) or (19).
- Compute the uncertainty for each level using Eq. (31).
- Compute the uncertainty profile according to Eq. (34) and make 2D-graphical representation results for the acceptability limit and uncertainty;

All the steps listed below have been applied successfully to evaluate the performance of a chromatographic bioanalytical method. As decision rule, we have selected 4-6-20 rule (i.e. $\beta = 66.7\%$ and $\gamma = 90\%$) [16–18].

The whole uncertainties estimated and intervals calculated by generating different calibration model are reported in Tables 2 and 3. As well, an example of a plot of uncertainty profile is illustrated in Fig. 1.

The analysis of all results in Tables 2 and 3 have shown that:

- Some response functions are better than others (i.e., calibration models most appropriate to quantify, with uncertainties not exceeding the limits of acceptability are: the linear regression model and the linear regression after square root transformation).
- A comparison of our approach and that developed by SFSTP has shown that both approaches select the same functions of calibrations (see Table 4).
- It should be noted that the calculation of tolerance intervals presented by the commission SFSTP [15] corresponds to $\beta = 90\%$. Whereas, tolerance intervals reported in Table 4 corresponds to $\beta = 95\%$.
- Uncertainty as estimated by our approach is always greater than that estimated by the Feinberg's method. (see Table 5).

5. Discussion

Before building the uncertainty profile, it is necessary, firstly, to generate all possible response functions. Indeed, this step is of great importance since the reliability of the validation results depend on the selected regression model.

In this direction, the FDA guidance on Bioanalytical Method Validation [18] requires that "The simplest model that adequately describes the concentration–response relationship should be used." For that, we have introduced the use of the uncertainty profile based on the statistical tolerance intervals to decide if a calibration model will give sufficient quality results. The models based on the accuracy of the back-calculated results regardless of the statistical properties should be retained or rejected.

Concerning our example for the determination of sotalol in human plasma, the response function was evaluated from three

Table 4
Estimates of error limits and uncertainty limits obtained with each response function.

Model	Concentrationlevel ($ng\,mL^{-1}$)	Relative error limits (%) (95-expectation tolerance interval)[a]		Relative uncertainty limits (%) (66.7, 90) tolerance interval)[b]		Relative uncertainty limits (%)(66.7, 90) tolerance interval)[c]	
		Lower	Upper	Lower	Upper	Lower	Upper
Linear 0–max	25.3533	−37.54	−14.75	−36.34	−15.94	−36.49	−15.8
	48.2417	−31.87	−5.4	−32.26	−5.01	−32.95	−4.32
	437.8235	−17.09	12.47	−15.54	10.91	−16.11	11.48
	838.6479	−16.48	20.49	−11.84	15.85	−12.26	16.27
Linear 0–223	25.3533	−37.29	−14.34	−36.21	−15.42	−36.34	−15.29
	48.2417	−35.91	−0.55	−34.84	−1.62	−35.57	−0.89
	437.8235	−24.44	20.8	−18.65	15.01	−19.11	15.47
	838.6479	−24.65	29.7	−14.63	19.68	−15.27	20.32
Linear	25.3533	−17.23	17.08	−17.4	17.24	−18.24	18.09
	48.2417	−14.82	3.75	−11.65	0.58	−11.92	0.86
	437.8235	−15.04	10.83	−14.27	10.07	−14.83	10.63
	838.6479	−14.24	17.04	−11.2	14	−11.59	14.39
Weighted linear, $1/X$	25.3533	−6.5	23.37	−7.11	23.98	−7.85	24.72
	48.2417	−15.72	12.92	−15.74	12.94	−16.47	13.67
	437.8235	−14.85	10.17	−14.23	9.55	−14.77	10.09
	838.6479	−13.06	14.81	−10.84	12.58	−11.32	13.06
Linear regression after logarithm transformation	25.3533	−13.99	14.19	−13.69	13.88	−13.83	14.02
	48.2417	−14.77	20.28	−14.55	20.06	−15.4	20.91
	437.8235	−12.27	8.63	−12.14	8.5	−12.63	8.98
	838.6479	−14	5.86	−13.19	5.06	−13.6	5.46
Linear regression after square root transformation	25.3533	−15.52	15.41	−16.22	16.11	−16.94	16.83
	48.2417	−16.39	13.64	−16.61	13.86	−17.34	14.59
	437.8235	−13.96	10.93	−13.33	10.3	−13.84	10.81
	838.6479	−13.48	14.1	−11.26	11.88	−11.67	12.29
Quadratic	25.3533	−47.87	89.12	−18.31	59.56	−19.24	60.49
	48.2417	−28.35	33.52	−19.07	24.24	−19.66	24.84
	437.8235	−17.42	8.24	−16.55	7.37	−17.18	8
	838.6479	−15.11	17.8	−11.91	14.6	−12.49	15.17
Weighted quadratic, $1/X$	25.3533	−9	30.54	−8.09	29.62	−8.91	30.44
	48.2417	−15.54	12.95	−15.51	12.92	−16.17	13.58
	437.8235	−14.83	8.14	−14.66	7.97	−15.17	8.48
	838.6479	−14.6	17.34	−11.5	14.24	−11.98	14.72

[a] Approach adopted by SFSTP.
[b] β-Content tolerance interval computed by Liao's method.
[c] β-Content tolerance interval computed by Mee's method.

calibration curves constructed from the calibration standards by using six concentration levels (Table 1). Then, several regression models were fitted in order to analyze the relationship between concentration and analytical response.

From every obtained response function, the concentrations of the validation standards were back-calculated in order to determine, by concentration level, the upper and the lower β content tolerance limits, the expanded uncertainty and the relative uncertainty limits.

From these data, different uncertainty profiles were plotted to select the most suitable regression model for the intended use of the analytical method. The acceptance limits were set at ±20%. Although we have adopted two methods to calculate the tolerance interval we have found that only two response

Table 5
Point estimates of the different uncertainties obtained by Eq. (31) using the β-content tolerance interval and β-expectation tolerance interval by considering the linear regression after square root transformation.

Statistical model	Concentration level ($ng\,mL^{-1}$))	Uncertainty ($ng\,mL^{-1}$)	Expanded uncertainty ($ng\,mL^{-1}$)	Relative expanded uncertainty (%)
β-Expectation tolerance intervals	25.3533	1.7	3.4	13.43
	48.2417	2.93	5.87	12.16
	437.8235	20.61	41.21	9.41
	838.6479	39.54	79.08	9.43
β-Content tolerance intervals (Mee's method)	25.3533	2.14	4.28	16.88
	48.2417	3.85	7.7	15.97
	437.8235	26.99	53.97	12.33
	838.6479	50.22	100.45	11.98
β-Content tolerance intervals (Liao's method)	25.3533	2.05	4.1	16.16
	48.2417	3.67	7.35	15.24
	437.8235	25.87	51.74	11.82
	838.6479	48.51	97.01	11.57

functions allow demonstrating the capability of the method to quantify sotalol over the whole concentration range considered, since the uncertainty limits were totally included inside the acceptance limits. These functions are the linear regression model and the linear regression after square root transformation.

In addition, we have used the SFSTP strategy to assess the same response functions. In fact, this strategy is founded on the concept of accuracy profiles based on the notion of total error and the β-expectation tolerance interval. As illustrated in Table 4, the upper and the lower β expectation tolerance limits would not exceed the acceptance limits settled at ±20% only if we selected two response functions, namely the linear regression model and the linear regression after square root transformation. Consequently, if we adopt the selected calibration curves, the method can be considered as accurate over the concentration range investigated.

On the other hand, the proposed strategy allows a good estimate of measurement uncertainty by using data validation and without having recourse to other experiments (Tables 2 and 3). We have proposed an original formula for estimating uncertainty based on the β-content tolerance interval and computed by two different methods. As shown in Table 5, whatever the method adopted to calculate the tolerance interval, the uncertainty as estimated by our approach is always greater than that estimated by the Feinberg's method. This can be explained by the following reasons: Feinberg method's is based on the β-expectation tolerance interval while our approach relies on the β-content tolerance. Indeed, the β-expectation tolerance interval is an interval covers on average 100β% of the distribution given the estimated parameters. This interval is referred to by Mee [26] as a "mean-coverage" tolerance interval. On the contrary, the β-content tolerance interval is an interval that contains at least 100β% of the population with given confidence level γ. This interval is referred to by Mee [26] as a "guaranteed-coverage" tolerance interval. As a result, β-content tolerance intervals are much wider than β-expectation tolerance intervals [27]. Accordingly, the proposed method resulted in the greater uncertainty compared with the previous method. These outcomes, prompt us to say that Feinberg's method underestimates the measurement uncertainty. Moreover, this finding has been confirmed by Marini et al. in a collaborative study [14].

Finally, it should be noted that the proposed strategy is principally destined for chromatographic methods, but it could also be applied for other analytical methods based on techniques such as spectrofluorimetry, polarography, spectrophotometry, capillary electrophoresis, etc.

6. Conclusion

In this article we have tried to propose a global strategy for the validation of analytical methods and estimation of measurement uncertainty. Indeed, the proposed strategy facilitates analytical validation by providing a decision tool based on both the uncertainty profile and the β-content tolerance interval. Equally important, this approach has allowed a good estimate of measurement uncertainty by using data validation and without having recourse to other experiments in addition.

The holistic character of the measurement uncertainty compared to the total error was influenced by our choice of profile uncertainty. Nevertheless, we think that the adoption of the uncertainty in the validation stage controls the risk of using the analytical method in routine phase.

Acknowledgements

The authors thank Y. Ben Abderrazik of the University of Sidi Mohamed Ben Abdallah for reviewing the manuscript. Many thanks are also due to the anonymous reviewers for providing important comments that led to clarify a number of points in this article.

References

[1] Ph. Hubert, J.J. Nguyen-Huu, B. Boulanger, E. Chapuzet, P. Chiap, N. Cohen, P.A. Compagnon, W. Dewe, M. Feinberg, M. Lallier, M. Laurentie, N. Mercier, G. Muzard, C. Nivet, L. Valat, STP Pharm. Pratiques 13 (2003) 101.
[2] ISO/DTS 21748 Guide to the Use of Repeatability, Reproducibility and Trueness Estimates in Measurement Uncertainty Estimation, ISO, Geneva, 2003.
[3] EURACHEM/CITAC Guide: Quantifying Uncertainty in Analytical Measurement, 2nd ed., EURACHEM/CITAC, Budapest, 2000, http://www.eurachem.org/.
[4] Guide to the Expression of Uncertainty in Measurement, ISO, Geneva, Switzerland, 1995.
[5] EA-4/16, EA Guidelines on the Expression of Uncertainty in Quantitative Testing, 2004, http://www.european-accreditation.org/.
[6] V.J. Barwick, L.R. Ellison, VAM Project 3.2.1, Development and Harmonization of Measurement Uncertainty Principles. Part D: Protocol Uncertainty for Evaluation from Validation Data, January 2000. Report No.:LGC/VAM/1998/088.
[7] M. Feinberg, B. Boulanger, W. Dewé, Ph. Hubert, Anal. Bioanal. Chem. 380 (2004) 502.
[8] A.G. Gonzalez, M.A. Herrador, Talanta 70 (2006) 896.
[9] R. Mee, Technometrics 26 (1984) 251.
[10] R. Mee, D.B. Owen, J. Am. Stat. Assoc. 78 (1983) 901.
[11] C.T. Liao, H.K. Iyer, Stat. Sinica 14 (2004) 217.
[12] K.W. Tsui, S. Weerahandi, J. Am. Stat. Assoc. 84 (1989) 602.
[13] S. Weerahandi, J. Am. Stat. Assoc. 88 (1993) 899.
[14] R.D. Marini, P. Chiap, B. Boulanger, S. Rudaz, E. Rozet, J. Crommen, Ph. Hubert, Talanta 68 (2006) 1166.
[15] Ph. Hubert, J.J. Nguyen-Huu, B. Boulanger, E. Chapuzet, N. Cohen, P.A. Compagnon, W. Dewé, M. Feinberg, M. Laurentie, N. Mercier, G. Muzard, L. Valat, E. Rozet, J. Pharm. Biomed. Anal. 45 (2007) 82.
[16] D. Hoffman, R. Kringle, Pharm. Res. 24 (2007) 1157.
[17] D. Hoffman, AAPS J. 11 (2009) 570.
[18] Food and Drug Administration, Guidance for Industry, Bioanalytical Methods Validation, 2001.
[19] E. Hund, D.L. Massart, J. Smeyers-Verbeke, Anal. Chim. Acta 480 (2003) 39.
[20] J.O. De Beer, P. Baten, C. Nsengyumva, J. Smeyers-Verbeke, J. Pharm. Biomed. Anal. 32 (2003) 767.
[21] P. Dehouck, Y. Vander Heyden, J. Smeyers-Verbeke, D.L. Massart, J. Crommen, Ph. Hubert, R.D. Marini, O.S.N.M. Smeets, G. Decristoforo, W. Van de Wauw, J. De Beer, M.G. Quaglia, C. Stella, J.L. Veuthey, O. Estevenon, A. Van Schepdael, E. Roets, J. Hoogmartens, Anal. Chim. Acta 481 (2003) 261.
[22] A.G. Gonzalez, M.A. Herrador, A.G. Asuero, Talanta 65 (2005) 1022.
[23] A. Dıaz, L. Vazquez, F. Ventura, M.T. Galceran, Anal. Chim. Acta 506 (2004) 71.
[24] A. Maroto, J. Riu, R. Boque, F.X. Rius, Anal. Chim. Acta 391 (1999) 173.
[25] A.G. Gonzalez, M.A. Herrador, TrAC Trends Anal. Chem. 26 (2007) 227.
[26] R. Mee, Technometrics 32 (1990) 83.
[27] R.D. Wolfinger, J. Qual. Technol. 30 (1998) 18.