The uncertainty and limit of detection in biosensors from immunoassays

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Abstract

This work describes a procedure to obtain the limit of detection (LoD) of a biosensor device from a set of measurements that we call immunoassays, which consists of testing the device's response to different concentrations of the molecule, which has to be detected throughout its working range. The procedure, based on the recommendations of international organizations, provides general expressions to estimate the uncertainty throughout its working range and the LoD. In the metrological model, the contributions to the uncertainty, due to the determination of the parameters of the calibration curve, the resolution, the lack of repeatability and others are taken into account. The model is applied to experimental data from the calibration of a biosensor. Using iterative weighted least squares techniques, a general logistic function (6-parameters) has been fitted and used as a calibration curve. The example studied shows that the lack of repeatability is not always the most important contribution to the LoD. The final expression of the LoD is equivalent to that of the expanded uncertainty (for a coverage probability of 99.9%) assigned to the concentration when $c \cong 0$. This result permits seeing the LoD as the smallest concentration c measured with the device, whereby uncertainty interval $[c - U_{99.9\%}(c), c + U_{99.9\%}(c)]$ does not include negative values.

Keywords: uncertainty, limit of detection, biosensors, calibration functions, immunoassays

(Some figures may appear in colour only in the online journal)

1. Introduction

The development of new biosensing devices and their application to the detection of different types of biomolecules has been a remarkably dynamic field in recent years (Fan *et al* 2008, Sang *et al* 2015, Zancheta *et al* 2017). The biosensors selectively recognize that a certain molecule causes a measurable response. This response has a mathematical relationship with the concentration of the biomolecule that we call the

calibration curve or calibration function (see the International Vocabulary of Metrology (VIM) JCGM/BIPM 2012, definition 4.13 and Barwick and Prichard 2011, section 2.2). The calibration curve relates the target molecule concentration c to some measurable physical property y (see figure 1). Most of the works in this field published in scientific journals or conferences have in common the realization of an immunoassay, which consists of testing the response of the device to different concentrations of the molecule that has to be detected throughout its working range. With these experimental measurements, a calibration curve can be inferred by adjusting

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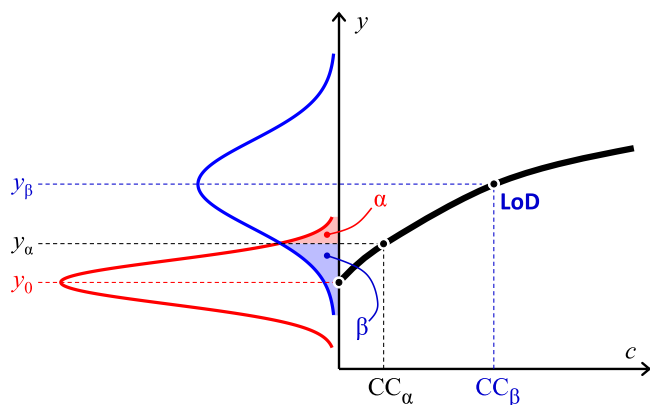
Figure 1. Relationships between measured responses y_0, y_α, y_β, probabilities of false positive (α), false negative (β), critical value (CC_α) and LoD (CC_β).

different mathematical functions to the data set. Once the calibration curve is known, it can be used to relate the signal to the concentration, but not only that; the experimental data used to obtain it determines the uncertainty that we will attribute to the measured concentrations, the limits of detection (LoD) and quantification (LoQ). This method of determining the LoD is called the *Method Detection Limit*, as opposed to the *Instrument Detection Limit* that is based on the repetition of null or very low concentration measurements (Magnusson *et al* 2014 section 6.2.1).

The LoD is one of the parameters used to define the quality of the sensor device and, in this way, to be able to compare different techniques. By analyzing the scientific literature in this field, we have observed that the way to calculate the LoD is not uniform. We believe that the cause may be that, although different international organizations (IUPAC, Eurachem, ISO, etc.) perfectly define the procedures to be used in the analysis of all types of experiments (ISO 1997, IUPAC 1997, Barwick *et al* 2011, Magnusson *et al* 2014), this information is spread and sometimes not accessible or difficult to understand for non-specialists. Our group has recently published a paper where the objective was to help standardize the determination of uncertainty and the limit of detection (LoD) in label-free biosensors (Lavín *et al* 2018). This work established and clarified a simple procedure, based on the recommendations of international organizations, in which, from the data of an immunoassay, a linear calibration curve for the device was determined, as well as an estimation of the expected uncertainty at every point of the calibration line. The value of the LoD derived naturally in this model as the limit at which the uncertainty tends when the concentration tends to zero. This work adds to valuable previous attempts to clarify the procedures for obtaining the LoD and point out some common mistakes (Look *et al* 2012, Evard *et al* 2016).

In the current work, a more general metrological model is presented. The model starts from a general calibration curve that fits the data. The calibration curve can be polynomial of degree one or greater; or can be sigmoidal or logistic. Even other types of calibration curves can be considered: rational functions, logarithmic, exponential, etc (Sit *et al* 1994). Starting from the parameters that determine the calibration

curves and their uncertainty, the concentration uncertainty throughout its working range is obtained, as well as the critical value (CC_α) and the LoD (CC_β, LoD).

Figure 1 shows the relationships between the responses or signals (y_α, y_β) measured in a particular biosensor and the concentration values mentioned above: CC_α and CC_β. The critical value is an essential concept for defining the detection limit of the method. If we look at the ordinate axis of figure 1 we can define the response for the critical concentration as the response y_α, the exceeding of which leads, for a given error probability α, to the decision that the concentration is not zero when measuring a measurand without an analyte (false positive). The probability density function (PDF) corresponding to the sensor response for a measurand without an analyte ($c = 0$) is the red curve. The mean value of this red PDF is y_0. Similarly, the LoD is the concentration whose response produces a PDF (plotted with a blue line), with mean value equal to y_β, in which the probability of obtaining a value below the critical one y_α is β (false negative). The values of the responses associated with the critical value, y_α, and the LoD, y_β, correspond to the critical concentration, CC_α, and the LoD, CC_β through the calibration curve (ISO 1997, Currie 1999, Barwick *et al* 2011). We are following definition 4.18 included in the VIM (JCGM/BIPM 2012).

Following the IUPAC definition, the LoD, expressed as concentration CC_β, is derived from the smallest measure y_β, which can be detected with reasonable certainty for a given analytical procedure (IUPAC 1997, Magnusson 2014). If we assume Gaussian probability distributions of standard deviation σ for all our measurements in the vicinity of $c = 0$, and 5% as a *reasonable* probability of false positive α and false negative β, as IUPAC recommend, then $y_\alpha - y_0 = k_\alpha \sigma \cong 1.65\sigma$ and $y_\beta - y_\alpha = k_\beta \sigma \cong 1.65\sigma$, which leads us to $y_\beta - y_0 \cong 3.3\sigma$. For a linear calibration curve with sensitivity, a, in which the uncertainty of its parameters is negligible (Long 1983, Currie 1995), we could use the following expression for the calculation of CC_β:

$$CC_\beta = 3.3\frac{\sigma}{a} \tag{1}$$

The above formula or similar expressions are widely used in determining the LoD and, in most cases, would provide a fair value. However, we consider that it is important to reflect critically on the situations in which we apply it. The following section presents a more general metrological model that takes into account some sources of uncertainty that affect the determination of the LoD.

2. From calibration curve to uncertainty and limit of detection (LoD)

Let us consider, as the starting point, that a calibration curve has been previously fitted. The process of determining this calibration curve is out of the scope of this work, but it will be admitted that the parameters a_i defining that the curves are known, as well as their uncertainties $u(a_i)$ and correlation coefficients $r(a_i, a_j)$. It should be emphasized that these correlation coefficients are very important because the correlation

between a_i parameters is not negligible; in contrast, this correlation is usually very strong.

The most common calibration curves in the field of biosensors for adjusting immunoassays are shown below: y represents the output signal provided by the sensor and lower case c is the concentration of the analyte that is being measured:

Linear:

$$y(c) = a \cdot c + b = a_1 + a_2 \cdot c \tag{2}$$

Polynomial:

$$y(c) = a_1 + a_2 \cdot c + \cdots + a_n \cdot c^{n-1} \tag{3}$$

Sigmoidal (4-parameter or 5-parameter logistic function) (Findlay and Dillard 2007, Xiang *et al* 2018). For example, the 5PL would be:

$$y = D + \frac{A - D}{\left[1 + \left(\frac{c}{C}\right)^B\right]^G} = a_4 + \frac{a_1 - a_4}{\left[1 + \left(\frac{c}{a_3}\right)^{a_2}\right]^{a_5}} \tag{4}$$

Generalized logistic function (6-parameters) (Richards 1959):

$$y = A + \frac{K - A}{\left[C + Qe^{-Bc}\right]^{\frac{1}{\nu}}} = a_4 + \frac{a_1 - a_4}{\left[a_5 + a_3 e^{-a_2 c}\right]^{1/a_6}} \tag{5}$$

No matter the type of calibration curve chosen, this could be expressed as follows:

$$y = f(c, a_1, a_2, \cdots, a_n) \tag{6}$$

In our metrological model, the estimated value for y, supposing we have an estimation for the concentration c as it happens during the calibration process, is obtained adding the following corrections to the previous estimation of the calibration curve:

- δy_R due to sensor/instrument resolution.
- δy_r due to sensor/instrument repeatability s_r. If heteroscedasticity is significant, a function $s_r = s_r(c)$ is assumed to be known.
- δy_{res} due to other uncertainty sources (environment, reproducibility, drift, bias, stability, etc...) with uncertainty $u(\delta y_{\text{res}}) = u_{\text{res}}$.

It would be supposed that all these corrections have null mean but, probably, not null uncertainties $u(\delta y_i)$.

Thus, the model function used to estimate uncertainty during the calibration according to ISO-GUM (JCGM 2008a) is the following:

$$y = f(c, a_1, a_2, \cdots, a_n) + \delta y_R + \delta y_r + \delta y_{\text{res}} \tag{8}$$

Propagating uncertainties using the mainstream GUM procedure (JCGM 2008a, Ellison *et al* 2012) in this model function gives the following result (expression 9):

$$u_y^2 = u^2(y) = u_f^2(y) + \frac{R^2}{12} + \frac{s_r^2}{N} + u_{\text{res}}^2 \tag{9}$$

where $u_f^2(y) = \sum_{i=1}^n f_i^2 u^2(a_i) + 2\sum_{i=1}^{n-1}\sum_{j=i+1}^n f_i f_j \cdot r(a_i, a_j) \cdot u(a_i) u(a_j)$

where we have used the standard deviation of the mean $u(\delta y_r) = s_r/\sqrt{N}$ for the estimation of the uncertainty associated with the lack of repeatability where N is the number of repeated measures; the expression $u(\delta y_R) = u_R = R/\sqrt{12}$ for the uncertainty component associated with the resolution R of our measuring system assuming a uniform distribution along the interval $[-R/2, +R/2]$ (JCGM 2008a, Ellison *et al* 2012); parameters f_i are the sensitivity coefficients $f_i = \partial f / \partial a_i$ and $u_f(y)$ represent the uncertainty component due to the calibration. Please note that correlation coefficients $r(a_i, a_j)$ have to be taken into account because they are generally not null as we will show during the numerical example.

In the linear case (expression 2) we obtain:

$$u_y^2 = u^2(y) = u^2(b) + c^2 \cdot u^2(a)$$
$$+ 2c \cdot r(a, b) \cdot u(a) u(b) + \frac{R^2}{12} + \frac{s_r^2}{N} + u_{\text{res}}^2 \tag{10}$$

At $c = 0$, $y_0 = f(c = 0)$ and the uncertainty of the signal output is:

$$u^2(y_0) = u^2(b) + \frac{R^2}{12} + \frac{s_r^2}{N} + u_{\text{res}}^2 \tag{11}$$

The critical value CC_α can be calculated as follows:

$$y_\alpha = y_0 + k_\alpha \cdot u(y_0) \text{ and } CC_\alpha = f^{-1}(y_\alpha)$$

In a similar way, the LoD concentration ($c_{\text{LoD}} = CC_\beta$) can be estimated using the following formula:

$$y_\alpha = y_\beta - k_\beta \cdot u(y_\beta) \text{ and } c_{\text{LoD}} = CC_\beta = f^{-1}(y_\beta)$$

In the general case, evaluating $CC_\beta = f^{-1}(y_\beta)$ could be cumbersome. But the distance between y_0 and y_β is usually small enough to permit the linearization of the calibration curve in this interval. So, it would be possible to replace the original non-linear calibration curve with the linearized version, $y(c) = a \cdot c + b$ being $a = \partial f / \partial c$ at $c = 0$ and $b = f(c = 0) = y_0$, where a is the slope of the calibration curve (the sensitivity) in the vicinity of the zero concentration and b the value of the calibration curve at zero concentration. Using this approximated linear calibration curve we obtain:

$$y_\alpha = b + k_\alpha \cdot u(y_0)$$

$$y_\beta = y_\alpha + k_\beta \cdot u(y_\beta) = b + k_\alpha \cdot u(y_0) + k_\beta \cdot u(y_\beta)$$

$$CC_\beta = f^{-1}(y_\beta) = \frac{y_\beta - b}{a} = \frac{k_\alpha \cdot u(y_0) + k_\beta \cdot u(y_\beta)}{a}$$

As y_β is assumed to be close to y_0, then it is possible to approximate $u(y_\beta) \cong u(y_0)$ and taking into account that $k_\alpha = k_\beta \cong 1.65$, we obtain the following expression (12):

$$c_{\text{LoD}} = CC_\beta = 3.30\frac{u(y_0)}{a} = 3.30\frac{\sqrt{u^2(b) + \frac{R^2}{12} + \frac{s_r^2}{N} + u_{\text{res}}^2}}{a} \tag{12}$$

where $u(b)$ is the only uncertainty component coming from calibration. Special care must be taken to avoid forgetting other uncertainty sources: resolution, repeatability and others included in u_{res} (environment, reproducibility, stability, drift, etc…). If we do not consider u_{res} and the resolution uncertainty component and we assume that parameter b of the calibration curve is determined without uncertainty, expression 12 becomes expression (1), which ended the first section of this article. Recently, our group pointed out the importance of the term of uncertainty associated with the resolution $R^2/12$ to guarantee that the uncertainty of the signal is at least of the order of magnitude of the resolution and, therefore, the LoD is consistent with this fact (Lavín *et al* 2018).

3. Uncertainty throughout the working range

The model function for the measured concentration c, when using a calibrated device, is now the following:

$$
\begin{aligned}
c &= f^{-1}(\bar{y} - \delta y_R - \delta y_{res}, a_1, a_2, \cdots, a_n) \\
&= g(\bar{y} - \delta y_R - \delta y_{res}, a_1, a_2, \cdots, a_n)
\end{aligned}
\quad (13)
$$

where $g = f^{-1}$ is the inverse calibration curve and $\bar{y}$ is the average of N repeated measurements y_i over the measurand which concentration c we want to know. Please note that now the input value is the measured signal y provided by the biosensor and our goal is to estimate concentration c and its uncertainty. By propagating uncertainties using the mainstream GUM method (JCGM 2008a), but retaining terms associated with usually non-null correlation coefficients $r(a_i, a_j)$, we obtain the following combined standard uncertainty for c:

$$
u^2(c) = g_y^2 \cdot \left(\frac{s_r^2}{N} + \frac{R^2}{12} + u_{res}^2 \right) + \sum_{i=1}^{n} g_i^2 u^2(a_i) \\
+ 2 \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} g_i g_j \cdot r(a_i, a_j) u(a_i) u(a_j) \quad (14)
$$

where $g_i = \partial g / \partial a_i$ and $g_y = \partial g / \partial y$.

As we are only interested in the interval between $c = 0$ and the LoD, we can use instead, the linearized version of the calibration curve. Then, in the linear case, the new model function is:

$$
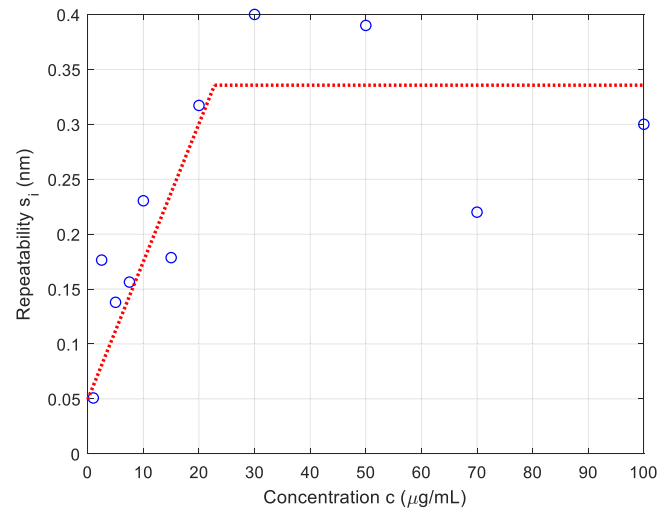c = \frac{(\bar{y} - \delta y_R - \delta y_{res}) - b}{a} \quad (15)
$$

And the estimation $u(c)$ of the uncertainty of the measured concentration would be:

$$
u^2(c) = \frac{1}{a^2} \cdot \left(\frac{s_r^2}{N} + \frac{R^2}{12} + u_{res}^2 \right) + \frac{u^2(b)}{a^2} \\
+ \left(\frac{b - \bar{y}}{a^2} \right)^2 u^2(a) + 2 \left(\frac{b - \bar{y}}{a^2} \right) \left(-\frac{1}{a} \right) r(a, b) u(a) u(b)
$$

$$(16)$$

When working near the origin ($c \cong 0$, $\bar{y} \cong b$), previous expression can be simplified:

Table 1. Calibration data.

Concentration c_i (μg ml^{-1})	Output Signal y_i (nm)	Repeatability s_i (nm)	min(y_{ij}) (nm)	max(y_{ij}) (nm)
1	0.09	0.05	0.00	0.15
2.5	0.33	0.18	0.03	0.52
5	0.53	0.14	0.35	0.72
7.5	0.75	0.16	0.51	0.90
10	1.22	0.23	0.85	1.43
15	2.01	0.18	1.71	2.22
20	3.16	0.32	2.60	3.40
30	3.73	0.40	3.14	4.18
50	4.39	0.39	3.73	4.82
70	5.05	0.22	4.77	5.38
100	5.61	0.30	5.05	6.14

Figure 2. Observed repeatability s_i versus concentration c.

$$
u^2(c) \cong \frac{1}{a^2} \cdot \left(\frac{s_r^2}{N} + \frac{R^2}{12} + u_{res}^2 \right) + \frac{u^2(b)}{a^2} \quad (17)
$$

$$
u(c) = \frac{\sqrt{\frac{s_r^2}{N} + \frac{R^2}{12} + u_{res}^2 + u^2(b)}}{a} \quad (18)
$$

If we choose a coverage probability of 99.9% the coverage factor (assuming normality) is $k_{99.9\%} = 3.30$. So, the expanded uncertainty near the origin ($c = 0$) would be:

$$
U_{99.9\%}(c) = k_{99.9\%} \cdot u(c) = 3.30 \frac{\sqrt{\frac{s_r^2}{N} + \frac{R^2}{12} + u_{res}^2 + u^2(b)}}{a}
$$

Therefore, $U_{99.9\%}(c)$ near the origin is equal to the LoD (when choosing $\alpha = \beta = 5\%$):

Table 2. Calibration curve: fitting results.

Parameter		Value a_i	Uncertainty $u(a_i)$	Matrix of correlation coefficients $r(a_i, a_j)$					
a_1	K	0.0064	0.0046	1	+0.20	+0.11	+0.32	−0.18	−0.27
a_2	B	0.079	0.011	+0.20	1	+0.55	+0.73	−0.52	−0.58
a_3	Q	0.034 19	0.003 71	+0.11	+0.55	1	+0.52	−0.80	+0.11
a_4	A	−0.33	0.16	+0.32	+0.73	+0.52	1	−0.76	−0.70
a_5	C	0.965 53	0.004 29	−0.18	−0.52	−0.80	−0.76	1	+0.15
a_6	ν	0.012 613	0.001 410	−0.27	−0.58	+0.11	−0.70	+0.15	1
b	f_0	0.014	0.048	1	−0.32				
a	f_c	0.075	0.016	−0.32	1				

$$U_{99.9\%}(c \approx 0) = c_{\text{LoD}} \tag{19}$$

4. Experimental example

Table 1 shows experimental results from a biochip calibration (Hernández *et al* 2016). This biochip, formed by six BICELLs (Biophotonic Sensing Cells), (Holgado *et al* 2010) is going to be used as an antibody-based (anti-IgG) label-free biosensor. The calibration has been performed over $m = 11$ calibration points from $c_1 = 1\,\mu\text{g ml}^{-1}$ to $c_{11} = 100\,\mu\text{g ml}^{-1}$ where uncertainties are negligible against other uncertainty sources. The output signal y_i is the resonant dip shift, expressed in nm, observed when the sensor is exposed to antibody concentrations c_i. The readout instrument resolution is $R = 0.12$ nm. Values $y_i = \sum_j y_{ij}/6$ represent the average output over six BICELL readouts, and y_{ij} and s_i represent the observed repeatability at this calibration point.

If we plot observed repeatabilities s_i versus concentration c (see figure 2) we can observe that, for lower concentrations, repeatability increases with concentration. But for $c \geqslant 30\,\mu\text{g ml}^{-1}$, observed repeatabilities s_i pass the Hartley's test (Hartley 1950) indicating that repeatability remains constant over $30\,\mu\text{g ml}^{-1}$. In this range (30–$100\,\mu\text{g ml}^{-1}$) we have estimated the repeatability using the root mean square of s_i:

$$s_r(c \geqslant 30\,\mu\text{g ml}^{-1}) = \sqrt{\sum_{c_i \geqslant 30\,\mu\text{g ml}^{-1}} s_i^2} = 0.34\text{ nm}$$

For concentrations lower than $30\,\mu\text{g ml}^{-1}$ we have supposed that repeatability increases linearly with concentration:

$$s_r(c) \cong a_s \cdot c + b_s$$

In order to estimate parameters a_s, b_s we have used a weighted least squares procedure with weights inversely proportional to observed repeatabilities s_i as described in section 5.3.2 of ISO 11843-2 (ISO 2000). The final result is:

$$s_r(c) \cong (0.049 + 0.0126 \cdot c\,[\mu\text{g ml}^{-1}])\text{ nm}$$

Now, the normalized repeatibilities $s_i = s_r(c_i)/s_i$ pass the Hartley's test, indicating that the estimated function $s_r(c)$ explain satisfactorily the variation of the repeatability along the range 0–$30\,\mu\text{g ml}^{-1}$.

The point where straight lines $s_r(c) = 0.34$ nm and $s_r(c) \cong (0.049 + 0.0126 \cdot c\,[\mu\text{g ml}^{-1}])$ nm intersect is $c' = 23\,\mu\text{g ml}^{-1}$ (see figure 2, dotted red lines). Therefore, the final result for the whole range 0–$100\,\mu\text{g ml}^{-1}$ would be:

$$s_r(c) = \begin{cases} c < 23\,\mu\text{g ml}^{-1} & (0.049 + 0.0126 \cdot c\,[\mu\text{g ml}^{-1}])\text{ nm} \\ c \geqslant 23\,\mu\text{g ml}^{-1} & 0.34\text{ nm} \end{cases}$$

Back to the estimation of the calibration curve, we have used an iterative weighted least squares procedure to fit a general logistic function (6-parameters) to the points (c_i, y_i). Weights have been chosen inversely proportional to observed repeatabilities s_i. The uncertainty propagation has been performed using Monte-Carlo simulation (JCGM 2008b). The fitting results are presented in table 2, where f_0 and f_c represent the parameters $b = f(c = 0)$ and $a = \partial f / \partial c$ at $c = 0$ of the linearized calibration curve $y = b + a \cdot c$. Figure 3 represents the fitted calibration curve (red line) with uncertainty bands $\pm k_{99.9\%} \cdot u_f$ (green lines) and calibration data (blue circles and error bars). Remember that u_f represents the uncertainty contribution coming from calibration, see expression (9).

Introducing results from table 2 in the expression of the LoD (expression 12) we obtain:

$$c_{\text{LoD}} = 3.30 \frac{\sqrt{u^2(b) + \frac{R^2}{12} + \frac{s_r^2}{N} + u_{\text{res}}^2}}{a} = 3.30$$

$$\frac{\sqrt{(0.048\text{ nm})^2 + \frac{(0.12\text{ nm})^2}{12} + \frac{(0.049\text{ nm})^2}{3} + 0}}{0.075\,\mu\text{g ml}^{-1} \times \text{nm}^{-1}} = 2.9\,\mu\text{g ml}^{-1}$$

where we have supposed that:

- we repeat the measurement three times ($N = 3$);
- the repeatability s_r is close to $s_r(c = 0) = 0.049$nm;
- the uncertainty component u_{res} is negligible in comparison with the previous ones.

But in $c = 2.9\,\mu\text{g ml}^{-1}$ the repeatability is $s_r(c) \cong (0.049 + 0.0126 \cdot 2.9) = 0.085$ nm, which is a value significantly higher than $s_r(c = 0) = 0.049$ nm. Therefore we have to reevaluate c_{LoD} using, in this case, the value of $s_r(c)$ at the center of the interval 0–$2.9\ \mu\text{g mL}^{-1}$:

$$s_r\left(c = \frac{1}{2} \cdot 2.9\,\mu\frac{\text{g}}{\text{ml}}\right) \cong (0.049 + 0.0126 \cdot 1.45) = 0.067\text{ nm}$$
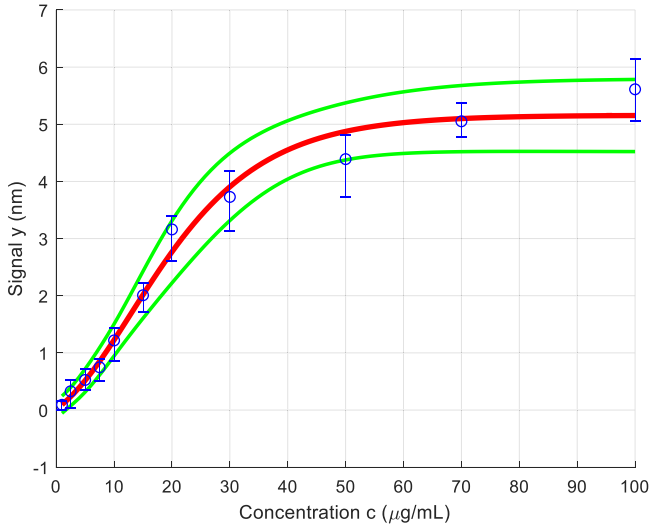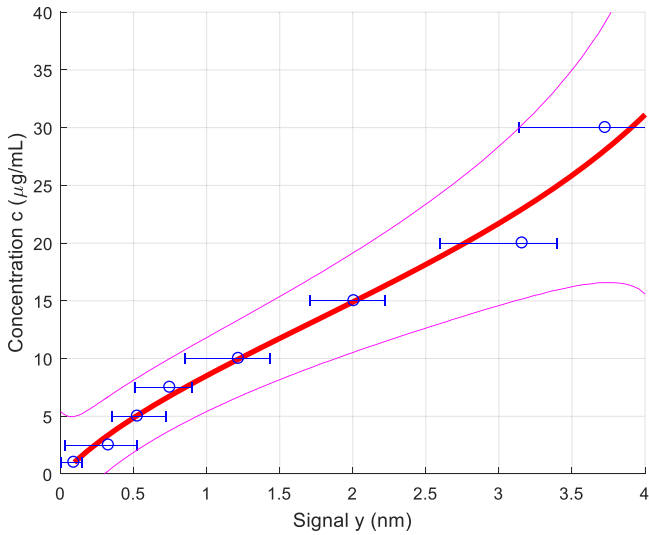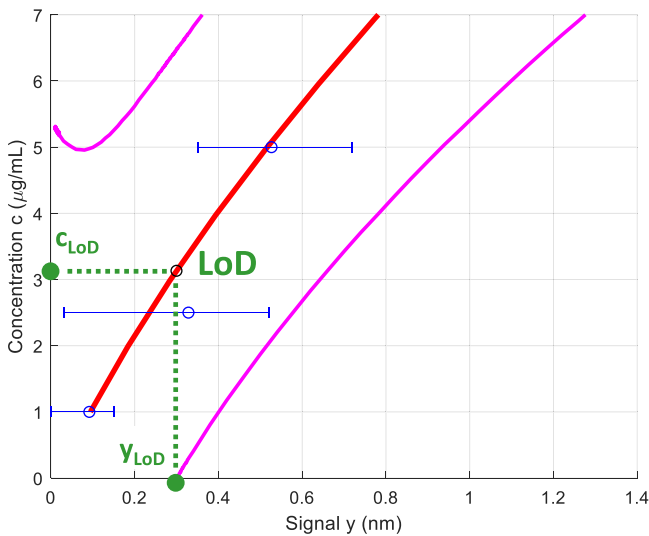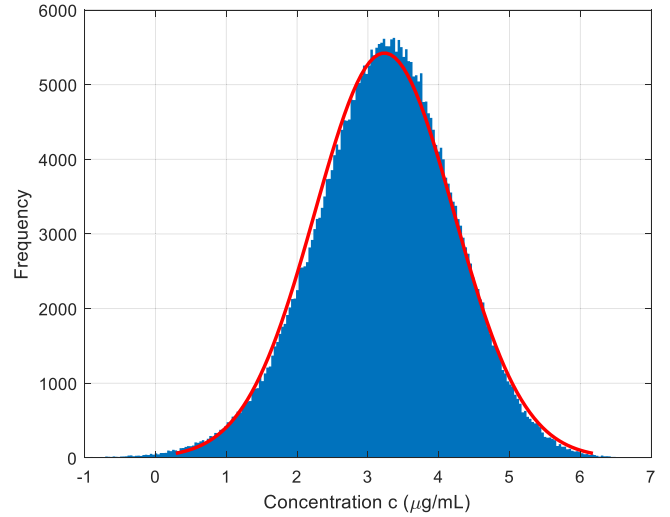
Figure 3. Calibration curve.

Figure 4. Inverse calibration curve: full range.

Figure 5. Inverse calibration curve: near $c = 0$.

Figure 6. Distribution of measured concentration c at LoD $(3.1\,\mu\mathrm{g\,ml^{-1}})$.

The final estimation of c_{LoD} would be:

$$c_{\mathrm{LoD}} = 3.30 \frac{\sqrt{(0.048\,\mathrm{nm})^2 + \frac{(0.12\,\mathrm{nm})^2}{12} + \frac{(0.067\,\mathrm{nm})^2}{3} + 0}}{0.075\,\mu\mathrm{g\,ml^{-1}} \times \mathrm{nm^{-1}}} = 3.1\ \mu\mathrm{g\,ml^{-1}}$$

In this particular case, the most important contribution to the LoD is the uncertainty $u(b)$ of parameter $b = f_0 = f(c = 0)$ (intersection of the calibration curve with the y-axis). Contributions of repeatability s_r and resolution R are very similar.

Using Monte-Carlo simulation techniques again, it is possible to estimate the uncertainty $u(c)$ of the measured concentration c using model function (13). This is the uncertainty estimation needed when we use the biochip after calibration. Figure 4 shows the inverse calibration curve $c = g(y)$ (red line) with uncertainty bands ($U_{99.9\%}(c) = k_{99.9\%} \cdot u(c)$, magenta lines) assuming that the measurement of the output signal y has been repeated $N = 3$ times. It has been assumed that all the random variables involved are normally distributed. This inverse calibration curve serves to obtain the estimate of the measured concentration c from the output signal y provided by the sensor.

For example, let us suppose that the observed output signal is $y = 2.00$ nm (y is the average of $N = 3$ measurements). Then, the estimated concentration would be $c = 14.9\,\mu\mathrm{g\,ml^{-1}}$ with an expanded uncertainty $U_{99.9\%}(c) = 4.3\,\mu\mathrm{g\,ml^{-1}}$.

Figure 5 shows the inverse calibration curve near the origin ($c = 0$). In this figure we have represented the LoD estimated previously. Please note that the lower end of the uncertainty interval $[c - U_{99.9\%}(c), c + U_{99.9\%}(c)]$ is zero at $c = c_{\mathrm{LoD}}$ according to expression (18).

Figure 6 shows the histogram (blue vertical bars, estimation of the PDF) of measured concentration c at LoD for $N = 3$. The distribution is slightly asymmetric but differences from a normal distribution (red line) are small.

Figure 7 shows the histograms of the estimated signal y at $c = 0$ (red line) and at $c_{\mathrm{LoD}} = 3.1\,\mu\mathrm{g\,ml^{-1}}$ (solid blue
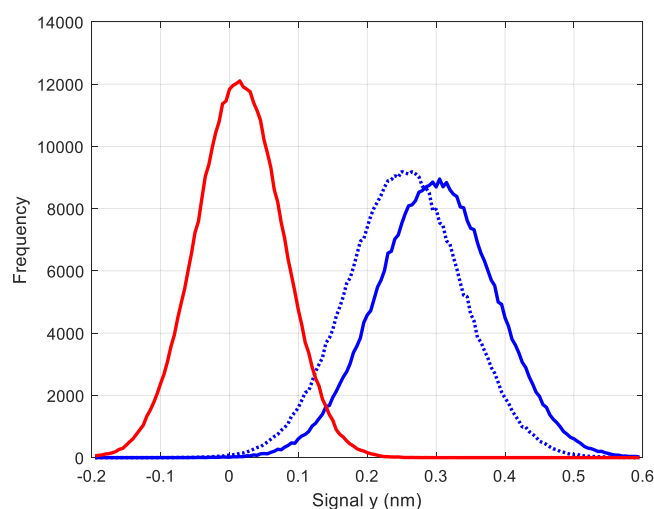
Figure 7. Distribution of estimated signal y at $c = 0$ (red line), at $c = 2.9\,\mu\text{g}\,\text{ml}^{-1}$ (first estimation of LoD, blue line) and at $c = 3.1\,\mu\text{g}\,\text{ml}^{-1}$ (final estimation of LoD, dotted blue line).

line) according to the model described by expression (8). This is figure 1 particularized for the data of the numerical example. Using the red histogram, we can estimate CC_α. Choosing $\alpha = 5\%$ (probability of a false positive) we obtain $y_\alpha = CC_\alpha = 0.117\text{nm}$.

Using the solid blue histogram we can now estimate $\beta = \text{Pr}\left\{y \leqslant y_\alpha | c = c_{\text{LoD}} = 3.1 \ \mu\text{g}\,\text{ml}^{-1}\right\} = 1.6\%$ (probability of a false negative). This value is smaller than the objective $\beta = 5\%$. The reasons are that we have estimated c_{LoD} assuming:

- a linear calibration curve near the origin $c = 0$, but figure 4 shows a slight nonlinearity;
- all random variables involved are normally distributed, but figure 6 shows a slight difference from a normal distribution, mainly in their tails;
- repeatability is constant in the interval $0 < c < c_{\text{LoD}}$, but figure 2 shows that there is a significant variation in repeatability along this interval;
- $u(y_0) \cong u(y_\beta)$ but, in the example, $u(y_\beta) = 0.086$ nm is clearly higher than $u(y_0) = 0.063$ nm.

In order to get a better estimation of c_{LoD} we can follow a trial and error procedure. For example, with $c_{\text{LoD}} = 2.7\,\mu\text{g}\,\text{ml}^{-1}$ (see figure 7, dotted blue line, final estimation of LoD) we get $\beta = \text{Pr}\left\{y \leqslant y_\alpha | c = c_{\text{LoD}} = 2.7 \ \mu\text{g}\,\text{ml}^{-1}\right\} = 4.9\%$, which is very close to the objective $\beta = 5\%$. Therefore, a better estimation of the LoD would be $c_{\text{LoD}} = 2.7\,\mu\text{g}\,\text{ml}^{-1}$.

5. Conclusions

We have presented a procedure to estimate the uncertainty of the concentration measured with a biosensor that has been calibrated fitting a general calibration curve.

We have also shown a procedure to estimate the LoD, based on the definition of the VIM that is a somewhat more rigorous version than that of the IUPAC, which permits taking into account uncertainty components such as resolution and calibration that sometimes can be more important than repeatability, as we have shown in an example.

The final expression of the LoD is equivalent to that of the expanded uncertainty (for a coverage probability of 99.9%) assigned to the concentration when $c \cong 0$. This result permits us to see the LoD as the smallest concentration c where uncertainty interval $[c - U_{99.9\%}(c), c + U_{99.9\%}(c)]$ does not include negative values.

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