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Limit of detection estimator for second-order bilinear calibration

Ricard Boqué [a,*], Joan Ferré [a], Nicolaas (Klaas) M. Faber [b], F. Xavier Rius [a]

[a] *Department of Analytical and Organic Chemistry, Institute of Advanced Studies, Rovira i Virgili University,*
Plaça Imperial Tàrraco 1, E-43005 Tarragona, Catalunya, Spain
[b] *Department Production and Control Systems, ATO, P.O. Box 17, 6700-AA Wageningen, The Netherlands*

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Abstract

A new approach is developed for estimating the limit of detection in second-order bilinear calibration with the generalized rank annihilation method (GRAM). The proposed estimator is based on recently derived expressions for prediction variance and bias. It follows the latest IUPAC recommendations in the sense that it concisely accounts for the probabilities of committing both types I and II errors, i.e. false positive and false negative declarations, respectively. The estimator has been extensively validated with simulated data, yielding promising results. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Limit of detection; Second-order bilinear calibration; GRAM; Prediction variance; Prediction bias

1. Introduction

The limit of detection (LOD) is an important figure of merit in method validation when the determination is performed at low levels of analyte concentration. The subject has been extensively studied and several methodologies have been proposed for its calculation in both zero-order (univariate) [1–3] and first-order (multivariate) [4–7] calibration. As any other analytical determination, second-order calibration methods must be validated at trace levels. This validation, however, presents a major problem, because LOD estimation for second-order data has not been systematically addressed. Different attempts have been made, but they all suffer from limitations. Some deal with the problem by collapsing the second-order data matrix to a scalar or vector, and then applying zero- or first-order strategies for the LOD calculation [8,9]. Other approaches simply calculate LOD as three times the standard deviation of the blanks [10,11]. It is stressed that the previously proposed LOD estimators do not comply with the latest IUPAC recommendation [12], formulated for zero-order data. This recommendation demands the incorporation of the probabilities of committing both types I and II errors in the LOD calculation. Finally, Faber et al. [13] propose an LOD estimator that is consistent with the IUPAC criteria. However, the practical utility of this estimator is limited, because it is formulated in terms of signal, rather than analyte concentration.

The aim of this paper is to extend the latest IUPAC recommendations to the realm of multi-way analysis. To this end, we propose an LOD estimator for second-order bilinear calibration with the generalized rank annihilation method (GRAM). GRAM is a method for curve resolution and calibration that requires only a single calibration sample [14]. Unlike conventional first-order methods such as principal component regression (PCR) or partial least squares (PLS), GRAM does not construct a model from data

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[*] Corresponding author. Tel.: +34-977-559564;
fax: +34-977-559563.
E-mail address: boque@quimica.urv.es (R. Boqué).

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obtained for a calibration set. Instead, it works by constructing a *joint* model for the calibration sample and prediction sample data matrices. An important consequence of the construction of a joint model is that correct prediction of the analyte of interest is feasible without modelling the interferences. This property is known as the second-order advantage. The practical value of the second-order advantage is likely to increase with the complexity of the prediction samples. It is noted that several alternatives have been proposed to solve this particular calibration problem. They comprise the relatively old method of alternating least squares [15], as well as a suite of new methods, i.e. alternating trilinear decomposition [16], alternating coupled vector resolution [17], alternating slice-wise diagonalisation [18], alternating coupled matrices resolution [19], and self-weighted alternating trilinear decomposition [20]. The reason for focusing on GRAM is that LOD estimation requires that expressions be available for the expected prediction error. Unfortunately, the necessary error analysis has not yet been developed for any of these methods. Restricting ourselves to GRAM is further motivated by the observation that GRAM compares well with the alternatives under standard assumptions for noise [21]. The proposed estimator is built using the expressions for prediction variance recently developed for GRAM [22], which also take into account the uncertainty due to correcting for prediction bias [23]. These expressions are approximate, because they are obtained by truncating a Taylor series expansion. It is important to note that truncated Taylor series expansions yield good approximations only if the noise in the data is relatively small (say, less than 10%). Otherwise, higher-order terms must be included. However, working out the resulting problem may well be mathematically untractable for complicated methods such as GRAM. The demand of sufficiently low noise level, which is often met by modern analytical instruments, could certainly preclude the use of these approximations in fields where low noise levels are the exception, e.g. environmental chemistry and the social sciences. It is important to note that the currently proposed approach [24] has worked well for LOD estimation in first-order calibration of near-infrared (NIR) data using PCR [6].

The proposed LOD estimator is extensively validated with simulated excitation-emission fluorescence spectroscopy (EEM-FL) data. The reason for performing simulations is that a thorough validation amounts to investigating the distribution of predictions. Such an investigation typically requires a very large number of samples, which is not practical in the real world. While, according to us, validation of an approach is best done using simulated data, the practical utility is only demonstrated using real examples. We are currently investigating the practical utility of the proposed LOD estimator in our laboratory.

2. Theory

2.1. Notation

Boldface uppercase letters represent matrices, e.g. $\boldsymbol{A}$, while scalars are indicated by italic uppercase or lowercase letters, e.g. A and a. Measured quantities are distinguished from their errorless counterparts (true values) by adding a 'tilde', e.g. $\tilde{\boldsymbol{A}}$. Likewise, estimated or predicted quantities carry a 'hat', e.g. $\hat{\boldsymbol{A}}$.

2.2. Second-order bilinear calibration using GRAM

In the general set-up of GRAM the calibration sample may contain analytes that are not present in the prediction sample, and vice versa. In this scenario, the two data matrices can be expressed as

$$\tilde{\boldsymbol{R}}_0 = \boldsymbol{X}\boldsymbol{C}_0\boldsymbol{Y}^{\mathrm{T}} + \boldsymbol{E}_0, \qquad \tilde{\boldsymbol{R}}_{\mathrm{u}} = \boldsymbol{X}\boldsymbol{C}_{\mathrm{u}}\boldsymbol{Y}^{\mathrm{T}} + \boldsymbol{E}_{\mathrm{u}} \tag{1}$$

where $\tilde{\boldsymbol{R}}_0$ and $\tilde{\boldsymbol{R}}_{\mathrm{u}}$ are measured for the calibration and prediction sample, $\boldsymbol{X}$ $(J_1 \times K)$ and $\boldsymbol{Y}$ $(J_2 \times K)$ contain the column and row profiles (K is the number of constituents present in both samples), $\boldsymbol{C}_0$ and $\boldsymbol{C}_{\mathrm{u}}$ are $K \times K$ diagonal concentration matrices (with zero diagonal elements for the analytes that are absent), and $\boldsymbol{E}_0$ and $\boldsymbol{E}_{\mathrm{u}}$ are $J_1 \times J_2$ error matrices.

Calibration and prediction with GRAM is a three-step process.

1. Singular value decomposition (SVD) of the sum matrix $\tilde{\boldsymbol{Q}} = \tilde{\boldsymbol{R}}_0 + \tilde{\boldsymbol{R}}_{\mathrm{u}}$ as $\tilde{\boldsymbol{Q}} = \hat{\boldsymbol{U}}\hat{\boldsymbol{S}}\hat{\boldsymbol{V}}^{\mathrm{T}}$ to get truncated orthogonal factor matrices $\hat{\boldsymbol{U}}(J_1 \times \boldsymbol{A})$ and $\tilde{\boldsymbol{V}}(J_2 \times \boldsymbol{A})$. The number of factors A is an estimate of K.
2. Solution of the eigenvalue problem $(\hat{\boldsymbol{S}}^{-1}\hat{\boldsymbol{U}}^{\mathrm{T}}\tilde{\boldsymbol{R}}_{\mathrm{u}}\hat{\boldsymbol{V}})\hat{\boldsymbol{T}} = \hat{\boldsymbol{T}}\hat{\boldsymbol{\Pi}}$. In this step, $\tilde{\boldsymbol{R}}_{\mathrm{u}}$ is projected onto the

factor space defined by $\tilde{\boldsymbol{Q}}$ to obtain the eigenvector matrix $\hat{\boldsymbol{T}}$ and the matrix of eigenvalues $\hat{\boldsymbol{\Pi}}$.

3. Estimation of the column and row profiles ($\boldsymbol{X}$ and $\boldsymbol{Y}$) by applying the transformation matrix $\hat{\boldsymbol{T}}$ on $\hat{\boldsymbol{U}}$ and $\hat{\boldsymbol{V}}$, respectively. The prediction for the analyte of interest is obtained by combining the measured concentration in the calibration sample, $\tilde{c}_0$, and the associated eigenvalue, $\hat{\pi}$, as

$$\hat{c}_\mathrm{u} = \frac{\tilde{c}_0 \hat{\pi}}{1 - \hat{\pi}} \tag{2}$$

This calculation requires $\tilde{c}_0$ and $\hat{\pi}$ to be matched, which in practice is achieved by comparing the estimated profiles (appropriate column of $\hat{\boldsymbol{X}}$ and $\hat{\boldsymbol{Y}}$) with reference profiles. Throughout this paper the number of the analyte is not made explicit to simplify the notation.

2.3. Standard error of prediction and prediction bias when using GRAM

Accuracy is defined as the closeness of agreement between a test result and the accepted reference value and, when applied to a set of test results, involves a combination of random components and a common systematic error or bias component [25]. In other words, accuracy is the sum of two components: so-called trueness and precision (Fig. 1). Whereas trueness indicates how close the mean of a set of results is to the true value, precision is a measure of how close results are to one another. Trueness and precision are usually expressed in terms of bias and standard error (square root of variance), respectively.

Standard error of prediction can be estimated using [22]

$$\hat{\sigma}(\hat{c}_\mathrm{u}) = [\hat{s}^{-2}(1 + \hat{h}_\mathrm{u})\hat{V}(\tilde{\boldsymbol{R}}) + \hat{h}_\mathrm{u}\hat{V}(\tilde{c}_0)]^{1/2} \tag{3}$$

where $\hat{s}$ is the sensitivity, $\hat{h}_\mathrm{u}$ the prediction sample leverage, $\hat{V}(\tilde{\boldsymbol{R}})$ the variance of the elements of $\boldsymbol{E}_0$ and $\boldsymbol{E}_\mathrm{u}$ (estimated in reproducibility conditions) and $\hat{V}(\tilde{c}_0)$ is the variance of the measurement error in $\tilde{c}_0$, respectively (all quantities are estimated). The sensitivity in Eq. (3) is the generalisation of Lorber's multivariate sensitivity [26] to bilinear second-order data [13]. It is obtained as the ratio of an appropriately defined net analyte signal (NAS) and the analyte concentration. The prediction sample leverage is the same as for a classical zero-order model with a single calibration sample [13], i.e. $h_\mathrm{u} = (c_\mathrm{u}/c_0)^2$. Thus, from the perspective of standard error of prediction, GRAM behaves as zero-order ordinary least squares (OLS). It is noted that a GRAM model does not have an intercept, since the second-order advantage implies that interferences are handled "mathematically". Consequently, a GRAM model can be (exactly) presented as a zero-intercept zero-order calibration graph constructed with a single calibration sample. This observation makes the development of an LOD estimator intuitive, because it allows one to visualise prediction bands in the usual way (see Fig. 2).

Previous studies have demonstrated that random measurement errors cause the GRAM eigenvalues to

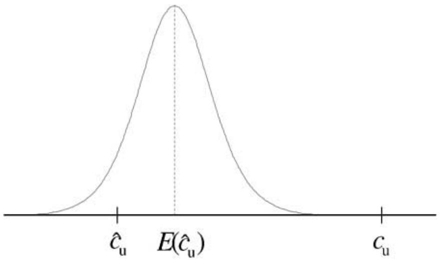

Fig. 1. Illustration of the concept of prediction bias. The distribution of predicted values, $\hat{c}_\mathrm{u}$, around the mean or expected value, $E(\hat{c}_\mathrm{u})$, deviates from the true value c_u.

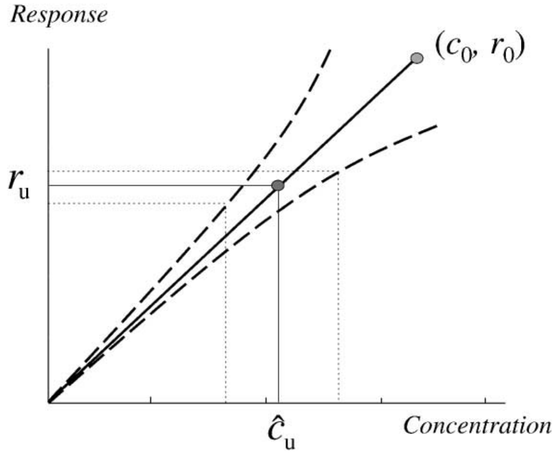

Fig. 2. Prediction bands of a zero-intercept zero-order calibration graph constructed with a single calibration sample. The symbols are explained in the text.

be biased [27,28]. This bias directly translates into prediction bias, which can be estimated using [23]

$$\hat{B}(\hat{c}_{\mathrm{u}}) = \frac{\tilde{c}_0 \hat{B}(\hat{\pi})}{(1 - \hat{\pi} + \hat{B}(\hat{\pi}))(1 - \hat{\pi})} \quad (4)$$

where $\hat{B}(\hat{\pi})$ is the bias in the associated eigenvalue, see Eq. (17) in [23]. It is worth mentioning that this bias arises from the non-linear character of the estimation method. GRAM shares this non-linear character with the alternatives listed in Section 1. Eq. (4) enables removing most of the prediction bias [23]. Clearly, one must remove the prediction bias; otherwise a decision cannot be taken with respect to the presence or absence of the analyte in the sample. Consequently, a bias-corrected prediction has to be calculated:

$$\hat{c}_{\mathrm{u}}^{\mathrm{bc}} = \hat{c}_{\mathrm{u}} - \hat{B}(\hat{c}_{\mathrm{u}}) = \frac{\tilde{c}_0(\hat{\pi} - \hat{B}(\hat{\pi}))}{1 - \hat{\pi} + \hat{B}(\hat{\pi})} \quad (5)$$

However, when a result is corrected for bias, this must be included in the uncertainty budget. The reason for this is that correction for bias amounts to subtracting a number that carries an uncertainty itself. Consequently, the standard error of prediction is increased. The standard error in the bias-corrected prediction can be estimated using (Eq. (22) in [23] and neglecting the contribution of $\tilde{c}_0$)

$$\hat{\sigma}(\hat{c}_{\mathrm{u}}^{\mathrm{bc}}) = \left[\left(\frac{\tilde{c}_0 + \hat{c}_{\mathrm{u}}^{\mathrm{bc}}}{1 - \hat{\pi} + \hat{B}(\hat{\pi})} \right)^2 \hat{V}(\hat{\pi}) + \left(\frac{-(\tilde{c}_0 + \hat{c}_{\mathrm{u}}^{\mathrm{bc}})}{1 - \hat{\pi} + \hat{B}(\hat{\pi})} \right)^2 \hat{V}(\hat{B}(\hat{\pi})) \right]^{1/2} \quad (6)$$

where $\hat{V}(\hat{\pi})$and $\hat{V}(\hat{B}(\hat{\pi}))$ follow from inserting estimates in Eqs. (14) and (64) in [23].

2.4. LOD estimator

The LOD is commonly defined as the minimum amount or concentration of substance that can be *reliably* detected by a given analytical method. In the last IUPAC document [12], LOD is considered as a fundamental performance characteristic of a method of analysis. Thus, LOD is an a priori defined value, because it is fixed *before* the measurements are made. The LOD is essentially different from a detection decision, because the latter is taken once the result of the measurement is known. In other words: a posteriori. The decision of whether a given analyte is present or not in a sample is based on a comparison with the critical level, L_{C}, which is defined as

$$L_{\mathrm{C}} \equiv z_{1-\alpha}\sigma_0 \quad (7)$$

where $z_{1-\alpha}$ is the upper-α percentage point of a normal distribution and σ_0 is the standard error of prediction when the analyte is not present in the sample (zero concentration level). The value of α is the risk one accepts of committing a false positive decision, i.e. stating that the analyte is present when in fact it is not. For predictions below L_{C} there is no statistical evidence that the analyte is present. This is illustrated in Fig. 3 that shows the distribution of results obtained for a sample where the analyte of interest is not present. Half of the predictions are above zero and half of them below. The L_{C} is defined in order to mark a minimum value for which a prediction is considered as being caused by the analyte. By doing so, there exists a risk α of committing a type I error, i.e. a false positive decision. Then the natural question arises: why not take L_{C} as the LOD? This would imply that, with the proposed method, the presence of analyte could be detected at concentration levels as low as L_{C}. This reasoning is, however, naive. Consider, for example, a collection of samples, all of them containing the analyte at a concentration level equal to L_{C}. Approximately half of the samples would give rise to a prediction below L_{C} and the analyte would not be detected. Consequently, we would run a risk of about 50% of committing a false negative decision, i.e. stating that the method is not able to detect the presence of analyte at that level

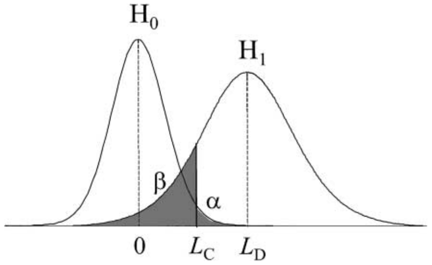

Fig. 3. Normal distributions at zero concentration level (null hypothesis H_0) and at the level of the LOD (alternative hypothesis H_1). The α and β represent the probabilities of types I error (false positive) and II error (false negative), respectively (see text for more details).

of concentration, while in fact, it was claimed that the substance could be *reliably* detected. To keep the risk of a false negative decision (called β) low, the LOD of the method, L_D, must be higher (Fig. 3) by taking into account both probabilities of error:

$$L_D \equiv z_{1-\alpha}\sigma_0 + z_{1-\beta}\sigma_D \tag{8}$$

where $z_{1-\beta}$ is the upper-β percentage point of a normal distribution and σ_D is the standard error of prediction when the analyte is present in the sample at the level of the LOD.

In the remainder of this section we will first develop a simplified approach to LOD estimation by neglecting prediction bias. Next, we will present the proposed LOD estimator as a straightforward adaptation of the simplified approach.

2.4.1. LOD estimator that does not take account of prediction bias

The two standard deviations, σ_0 and σ_D, can be estimated from Eq. (3). Formally, their calculation requires a sample without analyte (zero concentration level) and a sample with a concentration equal to the LOD, respectively. However, these samples may not be available. Fortunately, the problem can be solved by performing a GRAM calibration and setting the prediction to zero ($\hat{c}_u = 0$) and to the LOD ($\hat{c}_u = \hat{L}_D$), respectively, in the prediction sample leverage in Eq. (3). It is important to note that the resulting LOD estimate is specific for a certain combination of analytes. For each sample a different LOD is calculated, which may be similar for groups of samples with similar composition. Lorber [26] has already emphasised the sample-specific character of LOD when going beyond zero order data. The reason for this is the incorporation of the interferents in the model.

Assuming that the variance of the measurement error in the calibration sample concentration, $\hat{V}(\tilde{c}_0)$, is negligible, the critical level and the LOD are estimated by

$$\hat{L}_C = z_{1-\alpha}\hat{\sigma}(\hat{c}_u)_0 = z_{1-\alpha}\hat{\sigma}(\tilde{\boldsymbol{R}})\hat{s}^{-1} \tag{9}$$

$$\begin{aligned}\hat{L}_D &= z_{1-\alpha}\hat{\sigma}(\hat{c}_u)_0 + z_{1-\beta}\hat{\sigma}(\hat{c}_u)_D \\ &= z_{1-\alpha}\hat{\sigma}(\tilde{\boldsymbol{R}})\hat{s}^{-1} + z_{1-\beta}\hat{\sigma}(\tilde{\boldsymbol{R}})\hat{s}^{-1}\sqrt{1+\left(\frac{\hat{L}_D}{\tilde{c}_0}\right)^2}\end{aligned} \tag{10}$$

where $\hat{\sigma}(\tilde{\boldsymbol{R}}) = \hat{V}(\tilde{\boldsymbol{R}})^{1/2}$ and the two standard deviations, $\hat{\sigma}(\hat{c}_u)_0$ and $\hat{\sigma}(\hat{c}_u)_D$ are the analogues of σ_0 and σ_D.

Eq. (10) is a quadratic equation in $\hat{L}_D$. It has an exact solution, which further simplifies when making the common choice $z_{1-\alpha} = z_{1-\beta} = z$ to

$$\hat{L}_D = \frac{2\hat{L}_C}{1-(\hat{L}_C/\tilde{c}_0)^2} \tag{11}$$

Eqs. (9)–(11) illustrate that for second-order bilinear calibration using GRAM, the LOD depends on:

- the probabilities α and β of committing false positive and false negative decisions;
- the noise level in the response data;
- the sensitivity of the method;
- the analyte concentration in the calibration sample. For example, for high values of $\tilde{c}_0$, Eq. (11) simplifies to $\hat{L}_D = 2\hat{L}_C$.

2.4.2. LOD estimator that takes account of prediction bias

As we have mentioned above, both standard error and bias have an influence on detection limits. When checking the presence or absence of the analyte in a given sample, a bias-corrected prediction has to be compared to $\hat{L}_C$. This correction introduces an uncertainty, which must be included in $\hat{L}_C$ and subsequently in $\hat{L}_D$. The resulting modifications of Eqs. (9) and (10) are

$$\hat{L}_C = z_{1-\alpha}\hat{\sigma}(\hat{c}_u^{bc})_0 \tag{12}$$

$$\hat{L}_D = z_{1-\alpha}\hat{\sigma}(\hat{c}_u^{bc})_0 + z_{1-\beta}\hat{\sigma}(\hat{c}_u^{bc})_D \tag{13}$$

where the two standard deviations, $\sigma(\hat{c}_u^{bc})_0$ and $\sigma(\hat{c}_u^{bc})_D$, are the analogues of σ_0 and σ_D. They can be estimated by performing a GRAM calibration and setting the *bias-corrected* prediction to zero or LOD in Eq. (6). Since the LOD is unknown, an iterative procedure must be carried out in the latter case. We have found it convenient to use twice the critical value as an initial guess of LOD. This leads to an initial value of $\sigma(\hat{c}_u^{bc})_D$, which can be subsequently inserted in Eq. (13) to obtain an improved estimate of LOD, etc.

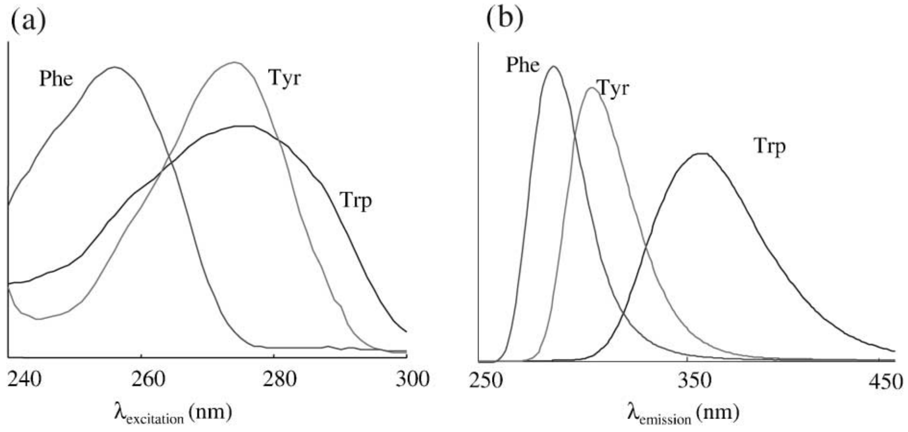

Fig. 4. Normalised (a) excitation and (b) emission spectra of tryptophan (Trp), tyrosine (Tyr) and phenylalanine (Phe).

3. Experimental

3.1. Data and simulations

Simulated data were used to demonstrate the validity of the approximate expression for the LOD. Fig. 4 shows the excitation (***X***) and emission (***Y***) fluorescence spectra of the amino acids tryptophan, tyrosine and phenylalanine that were used to simulate the data. These spectra were obtained from a three-factor PARAFAC model applied to the five samples contained in the dataset *claus.mat*, which is available in the N-way toolbox for Matlab [29]. Noise-free calibration and prediction sample response matrices, $\boldsymbol{R}_0$ and $\boldsymbol{R}_{\mathrm{u}}$, were simulated according to Eq. (1) with the concentrations indicated in Table 1. Next, the following three steps were repeated 200 times.

1. Measured calibration and prediction sample response matrices, $\tilde{\boldsymbol{R}}_0$ and $\tilde{\boldsymbol{R}}_{\mathrm{u}}$, were simulated by adding noise sampled from a normal distribution with mean zero and standard deviation $\sigma(\tilde{\boldsymbol{R}}) = 3$ to $\boldsymbol{R}_0$ and $\boldsymbol{R}_{\mathrm{u}}$. This noise level (approximately 0.6% in terms of relative standard deviation at the maximum of $\boldsymbol{R}_{\mathrm{u}}$) is realistic for EEM-FL data.
2. GRAM was performed to obtain the estimated profiles and the predictions. The sensitivity was calculated as explained in [13].
3. The critical level and LOD were calculated using Eqs. (9), (10), (12) and (13) with $\alpha = \beta = 0.10$.

Table 1
Analyte concentrations

Analyte	Calibration (mol/l)	Prediction (mol/l)
Tryptophan	8.79×10^{-7}	1.58×10^{-6}
Tyrosine	4.40×10^{-6}	5.44×10^{-6}
Phenylalanin	2.97×10^{-4}	3.55×10^{-4}

Once the critical level and LOD for each of the analytes had been calculated, the following three steps were repeated 200 times for each amino acid.

1. Three noise-free prediction data matrices were simulated. Each prediction sample contained the amino acid in study at three different concentrations: (1) almost no analyte present (10^{-15} M); (2) analyte with a concentration equal to the critical level (from step 3); and (3) analyte with a concentration equal to the LOD (from step 3). The concentrations of the other two analytes in the samples were kept fixed. For each situation, measured calibration and prediction sample data matrices $\tilde{\boldsymbol{R}}_0$ and $\tilde{\boldsymbol{R}}_{\mathrm{u}}$ were simulated by adding noise sampled from a normal distribution with mean zero and standard deviation $\sigma(\tilde{\boldsymbol{R}}) = 3$ to $\boldsymbol{R}_0$ and $\boldsymbol{R}_{\mathrm{u}}$.
2. For each pair $\tilde{\boldsymbol{R}}_0$ and $\tilde{\boldsymbol{R}}_{\mathrm{u}}$, predictions were calculated in three ways: (1) bias-corrected using Eq. (5); (2) bias-corrected using the mean value from all simulations (correction for 'true bias'); and (3) without bias correction. The purpose of the latter two calculations is to enable a better assessment of the merit of the proposed bias correction using Eq. (5).

3. The percentage predictions below the critical level and LOD (from step 3) were calculated.

It is essential to construct a large number of independent models and associated estimates for critical level and LOD by repeating steps 1–3. This can be understood as follows. Repeating steps 1–3 many times enables one to calculate an accurate *average value* of the percentage predictions below the critical level and compare it to the nominal values: 90% when $c_\mathrm{u} = 10^{-15}$, 50% when $c_\mathrm{u} = L_\mathrm{C}$ and 10% when $c_\mathrm{u} = L_\mathrm{D}$. It is critical to calculate such an average value, because some models are better than others are, simply by chance alone. A 'relatively good' model would systematically result in 'better' predictions than a 'relatively bad' model. Consequently, both models would not yield the target values mentioned above, but the average of many randomly generated (hence independent) models would approach the target values if the approximate expressions were adequate.

Finally, it is noted that the procedure described in steps 1–3 to calculate the LOD is the one to be used in a real application, the only difference being the estimation of $\sigma(\boldsymbol{R})$. A good way to estimate the noise level on real data is from replicated analysis or from the residuals of SVD [30].

3.2. Software

Calculations were performed using in-house Matlab 6.0 (Mathworks Inc., Natick, MA) functions on a 500 MHz Pentium computer.

4. Results and discussion

4.1. Analytical figures of merit

Before turning attention to the quantitative results, the analytical figures of merit are summarised (Table 2). These numbers have been calculated for the errorless calibration sample. Eq. (3) shows how standard error of prediction depends on the sensitivity (SEN) and the level of noise present in the data. As expected, $\hat{L}_\mathrm{C}$ and $\hat{L}_\mathrm{D}$ are (approximately) inversely proportional to sensitivity. The selectivity (SEL) quantifies the amount of analyte signal that is overlapped with the interferences [26]. It is calculated as the ratio of the NAS, which is constructed to be free of overlap, and the total signal. Consequently, SEL ranges between zero (complete overlap) and unity (no overlap). Dividing the SEN values by the associated SEL values yields the maximum sensitivity that would be obtained in absence of interferents. Such a calculation also yields the ideal values for critical level and LOD achievable when using this specific instrumentation.

4.2. Distribution of GRAM predictions

LOD estimation based on Fig. 3 assumes that the distributions are sufficiently normal. Fig. 5 shows a normal probability plot of the predictions for tryptophan at the level of the LOD. The predictions are bias-corrected using Eq. (5). It is observed that the distribution deviates only slightly from normality when $\alpha < 0.02$. Consequently, for the common values of α chosen here, the results are considered to be excellent. Similar results were obtained for the other analytes at the concentration levels studied (not shown).

4.3. Validation of the LOD estimator

The LOD estimator is validated by counting the occurrences where the predicted concentration is smaller than the critical level and LOD, respectively (Table 3). The numbers listed in columns three and six are obtained for the predictions that are bias-corrected using Eq. (5). The adequacy of this bias correction follows from the comparison with the results obtained

Table 2
Analytical figures of merit for the errorless calibration sample[a]

Analyte	NAS ($\times 10^3$ FI)	SEN ($\times 10^9$ FI l/mol)	SEL	$\hat{L}_\mathrm{C}$ ($\times 10^{-7}$ mol/l)	$\hat{L}_\mathrm{D}$ ($\times 10^{-7}$ mol/l)
Tryptophan	2.29	2.61	0.25	0.015	0.029
Tyrosine	1.49	0.34	0.22	0.11	0.23
Phenylalanin	3.51	0.012	0.58	3.25	6.50

[a] The symbols are explained in the text.

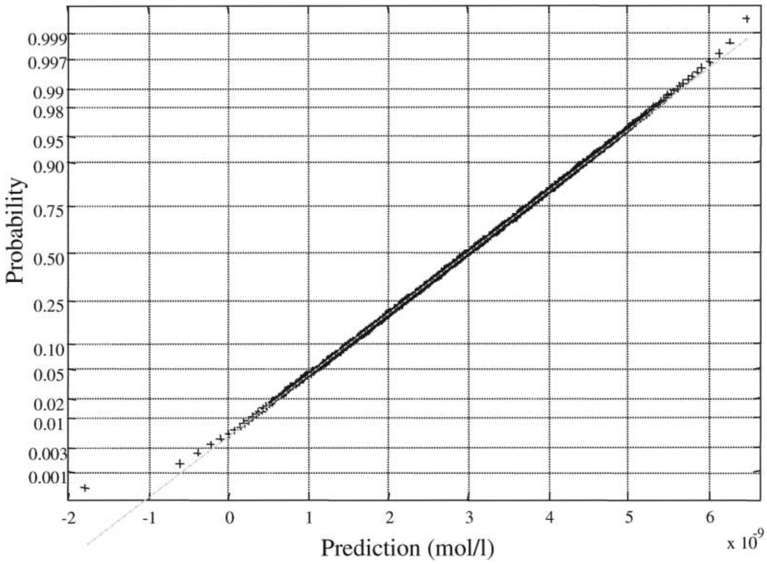

Fig. 5. Normal probability plot of bias-corrected GRAM predictions for tryptophan at the level of the LOD.

by correcting for the 'true' bias, which is calculated as the difference between the true concentration and the mean value (see columns four and seven). Owing to the relatively large number of predictions (200) that determine the mean value, the estimate of 'true' bias is very precise. It is seen that, in average (steps 1–3 are repeated 200 times and numbers in Table 3 are *average values*), this 'ideal' procedure yields slightly better results than Eq. (5). However, it is clear that in a real case, in which the true concentration of the analyte is unknown, bias correction using Eq. (5) is feasible. In general, the results agree well with what can be expected for the α and β values used in the simulations (10%), namely:

Table 3
Percentage of predictions below $\hat{L}_C$ and $\hat{L}_D$

Analyte	Level	Below $\hat{L}_C$			Below $\hat{L}_D$		
		I[b]	II[c]	III[d]	I[a]	II[b]	III[c]
Tryptophan	0	89.71	89.91	81.47	99.44	99.47	98.55
	L_C	49.07	49.88	34.47	89.90	90.36	81.10
	L_D	9.55	9.83	4.71	49.27	50.03	34.73
Tyrosine	0	89.70	90.05	77.74	99.50	99.53	98.08
	L_C	49.53	49.94	30.15	90.06	90.36	77.74
	L_D	9.85	10.13	3.52	49.21	50.07	30.13
Phenylalanin	0	89.77	90.03	87.12	99.47	99.51	99.14
	L_C	49.59	49.93	43.26	89.96	90.08	86.97
	L_D	9.77	9.84	7.28	50.07	50.13	43.37

[a] The symbols are explained in the text.
[b] Corrected for bias using Eq. (5).
[c] Corrected for bias using true bias.
[d] Not corrected for bias.

- when the analyte is absent, 90% of the predictions should be below $\hat{L}_{\mathrm{C}}$;
- when the analyte is present at a concentration equal to $\hat{L}_{\mathrm{C}}$, 50% of the predictions should be below $\hat{L}_{\mathrm{C}}$ and 90% below $\hat{L}_{\mathrm{D}}$;
- when the analyte is present at a concentration equal to $\hat{L}_{\mathrm{D}}$, 90% of the predictions should be below $\hat{L}_{\mathrm{C}}$ and 50% below $\hat{L}_{\mathrm{D}}$.

It is important to note that part of the deviations from the nominal probabilities can be explained from the fact that $\hat{L}_{\mathrm{C}}$ and $\hat{L}_{\mathrm{D}}$ are estimated. Clearly, the true values are unknown so that the analytes are not present at the true postulated levels. It is expected that the results would have been better if the analytes were present at the true postulated levels.

Finally, Table 3 also shows the results that are obtained without correcting for bias (see columns five and eight). The large deviation from the target values mentioned above illustrates the necessity of the proposed bias-correction procedure.

5. Conclusions and future research

We have presented an LOD estimator for second-order bilinear data when GRAM is used as a calibration method. To obtain consistent results, predictions must be bias-corrected. Simulations have confirmed the approximate normality of the distribution of the bias-corrected predictions, which is essential for the derivation of the LOD expressions. Since the bilinear model is very rigorous, data that really follow this model are not particularly abundant. EEM-FL has been shown to be a technique that provides second-order data that do not deviate seriously from bilinearity. The current work is part of ongoing research with respect to LOD estimation in our laboratory. The validation and application of the developed estimator to real fluorescence data is currently in progress.

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