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Experimental Comparison of the Different Approaches To Estimate LOD and LOQ of an HPLC Method

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Detection and quantification limits (LOD and LOQ) are two fundamental elements of method validation. Rigorous statistical definitions exist, but in HPLC they could not be implemented. Nevertheless there are several estimation methods for these limits. The most commonly used is the signal-to-noise ratio criterion. Others are based on the dispersion characteristics of the regression line, either simple or weighted. For LOQ, Eurachem proposed an alternate approach based on the use of a target value for the area RSD. Since official guidelines imposed no particular modus operandi, an experimental methodology was set up to investigate the compatibility of the different approaches and their respective reliabilities. Several samples prepared in a concentration range close to the limits were analyzed. It appeared that, both for values and their reliabilities, the different approaches were far from equivalent. In our opinion, the best way to handle the problem of detection and quantification limits was a methodology based on the use of the residual standard deviation of a weighted regression for LOD and on a Eurachem approach for LOQ. Values obtained by these means had the advantage of being reliable, i.e., with a small dispersion, and were still compatible with those obtained with the usual signal-to-noise ratio approach.

Method validation is now a major concern for analysts, and this is particularly true for HPLC methods.[1−9] All the characteristics of the method must be evaluated rigorously through the validation protocol: specificity or selectivity, linearity of calibration, repeatability, accuracy, detection and quantification limits (LOD and LOQ), recovery, and proof of applicability.[10] In the present paper, it was decided to focus on a rather ambiguous point: determination of the detection limit and quantification limit. A precise definition, based on statistical considerations, existed for both limits. However, it was not always convenient to put it directly into practice, especially in HPLC analyses. Consequently, official guidelines[1] present different approaches to estimating these limits but leave the analyst completely free to choose. Since it was not possible to demonstrate theoretically that these approaches could be considered equivalent, experiments were carried out to make comparisons between the different results. Discussion was not restricted solely to the values found, but also took their reliability into consideration.

1. DEFINITIONS

1. LOD. The detection limit (LOD) is the smallest quantity of analyte of which it can be said, with a given level of confidence, that it is present in the sample. As shown in Figure 1, LOD depends on the method dispersion at the blank level σ_B and on two risks values α and β.[11] α corresponds to the risk of detecting the analyte although it is not present. The critical value L_C is determined by three parameters: the blank value (B), which in HPLC analysis is quite often equal to zero, the α value, and the σ_B value. With L_C fixed, LOD depends solely on β, the value of the risk of not detecting the analyte although it is present.

By default α and β are set to 5%. If the distribution of the values is presumed to be Gaussian, and if the dispersion is presumed to be constant in the blank−LOD range, then the LOD value is given by

$$\mathrm{LOD} \cong 3.29\sigma_B \quad (1)$$

The traditional value 3, eq 2 is in fact merely a rounding-off

$$\mathrm{LOD} \cong 3\sigma_B \quad (2)$$

for the purposes of simplification.

2. LOQ. The quantification limit (LOQ) is the smallest quantity of analyte that can be quantified with a given level of confidence. Generally,[4,11] LOQ value is nearly always given by

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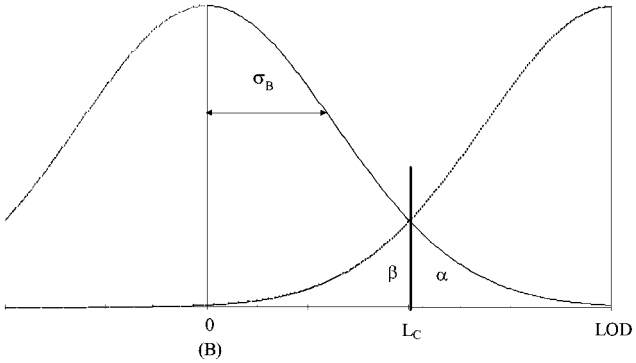

Figure 1. Graphical representation of LOD meaning (adapted from ref 11).

$$\mathrm{LOQ} \cong 10\sigma_B \qquad (3)$$

2. DIFFERENT APPROACHES TO ESTIMATING LOD AND LOQ

In many techniques, especially HPLC, the blank standard deviation is not directly available and can be estimated through different approaches. Below, those described in ICH recommendations[1] or in Eurachem publications[12] are recalled. For the sake of staying close to current practice, the LOD definition used in our expose will be that given in eq 2.

1. From the Noise. This first approach is the most widespread in HPLC methods. The noise magnitude is taken as an estimate of the blank standard deviation. In practice, the noise and signal are measured manually on the chromatogram printout. LOD corresponds to the analyte amount for which the signal-to-noise ratio is equal to 3, and LOQ corresponds to the analyte amount for which the signal-to-noise ratio is equal to 10. This approach has the advantage that it is quite easy to implement, which explains its popularity in most HPLC validations.

2. From Ordinary Least-Squares Regression Data. This approach consists of using the dispersion characteristics of the regression line of the chromatographic peak area against analyte quantity or against concentration if the injection loop volume is determined by the method. Since homoscedasticity, namely, the independence of the area dispersion in relation to analyte quantity, is a necessary condition of ordinary linear regression, it is strongly advised that experimental levels are chosen in a range around LOD and LOQ. When dispersion characteristics have been calculated, there are two options: the standard deviation of the blank is estimated either by the regression residual standard deviation denoted $\hat{\sigma}_{y/x}$, or by the standard deviation of the intercept denoted $\hat{\sigma}_{y_0}$.[13,14] LOD corresponds to the analyte amount for which the area is equal to 3 times the chosen standard deviation, and LOQ corresponds to the analyte amount for which the area is equal to 10 times the standard deviation chosen.

3. From Weighted Least-Squares Regression Data. This approach is quite similar to the former one but weighted regression[13,14] can manage data heteroscedasticity, namely, dependence of the area dispersion on the analyte quantity. In respect to data processing, weights must be normalized so that their sum is equal to the number of observations. There are then two more options, depending on the choice of the standard deviation to use, either $\hat{\sigma}_{y/x}$ or $\hat{\sigma}_{y_0}$. LOD corresponds to the analyte amount for which the area is equal to 3 times the standard deviation chosen, and LOQ corresponds to the analyte amount for which the area is equal to 10 times the standard deviation chosen.

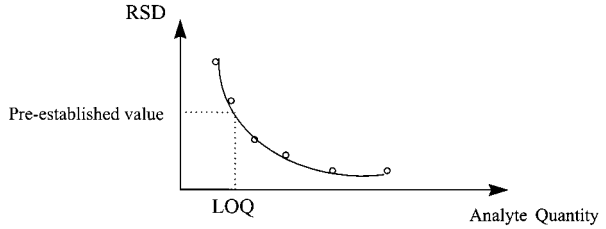

Figure 2. Eurachem approach for LOQ.

4. By the Eurachem Approach. Eurachem proposed an alternate approach to defining the quantification limit.[12] LOQ was the analyte quantity for which the relative standard deviation (RSD) of the analyses reached a preestablished level. This approach required repeated analyses at different levels around the LOQ value and a modeling of the RSD against analyte quantity; see Figure 2.

3. EXPERIMENTAL SECTION

1. Product and HPLC Method. The product chosen for conducting the experiments was Spiramycin.[15] This Rhône-Poulenc Rorer antibiotic may be considered a good example of the kind of products handled in the pharmaceutical industry.

In addition, the HPLC method was fully validated and studied,[16] and its dispersion characteristics were estimated rigorously through a collaborative study.[17−19] Briefly, this method used isocratic elution reversed-phase chromatography and UV detection. Resulting chromatograms were produced and processed by a Shimadzu Class-VP acquisition station. The analysis lasted about 35 min.

The Spiramycin content of the injected samples was expressed as a percentage of the target value of the method, which was 250 mg/L. A typical chromatogram is given in Figure 3.

2. Sample Preparation. A parent solution of Spiramycin at the 10% level was prepared by dissolving 12.5 mg of Spiramycin powder in 500 mL of a mixture of water and acetonitrile at 70:30 v/v. Each sample was prepared by dilution from the parent solution with the help of an automatic diluter (model Hamilton 530B).[20] It was equipped with two syringes: 25 or 250 μL for the parent solution, and 2500 μL for the diluent. Samples were prepared directly in vials used in the automatic injector. Table 1 gives the volumes of parent solution and diluent used for each level of the injection sequence.

3. Injection Sequence. The injection sequence was devised so that data required for implementation of all estimation methods

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(20) Vial, J.; Jardy, A.; Anger, P.; Gorge, G. *Analusis*, in press.

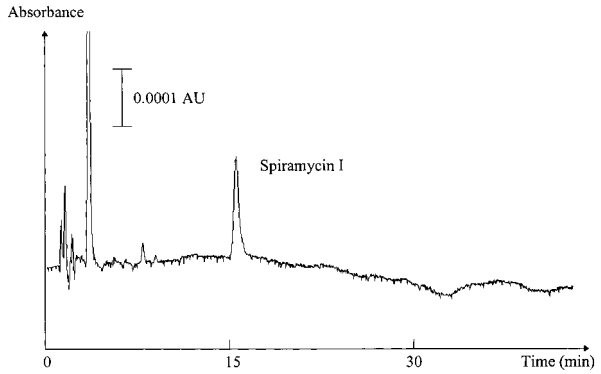

Figure 3. Chromatogram of Spiramicyn at level 0.1%. Column: length, 20 cm; internal diameter, 0,46 cm; temperature, 23 °C. Stationary phase: C8 bonded silica gel, 3 μm (Nucleosil; pore diameter, 120 Å). Mobile phase: acetonitrile−aqueous buffer pH 2.2 ([H_3PO_4] = 6.7 g/L; pH adjusted with sodium hydroxide) 30:70 v/v + 6.5 g/L sodium perchlorate monohydrate. Flow rate, 0.8 mL/min. Pressure drop, 160 bar. Injected volume, 20 μL (sample kept at 4 °C). UV Detection, 232 nm.

Table 1. Volumes Used with the Diluter

level (%)	parent solution (μL)	diluent (μL)	level (%)	parent solution (μL)	diluent (μL)
0.01	2.5	2497.5	0.25	60	2340
0.02	5	2495	0.50	125	2375
0.05	12.5	2487.5	1.00	240	2160
0.10	25	2475			

of the limits described in section 2 were applicable. We chose a seven-level sequence in the range 0−1%. The seven samples were injected in increasing order of Spiramycin concentration. This basic pattern was repeated six times with a blank between replicates. The whole sequence took 42 preparations, 48 injections, and about 28 h of analyses.

4. DATA PROCESSING

Data processing was rather complex because many responses had to be collected or measured. Since, in addition, much modeling had to be undertaken on the various responses, use of a spreadsheet was unavoidable. Consequently Microsoft Excel was chosen to store and display data. Statistical calculations were carried out with the help of the JMP application.[21] The chromatogram was displayed for each injection. The noise and signal (height of the Spiramycin I peak) were measured manually on all the chromatograms by the same operator. After a visual check of integration, the area of the Spiramycin I peak was also reported on the spreadsheet.

1. Estimation and Modeling of the Area RSD. For each level, the six replicates were used to estimate the RSD of the areas. Experimental values are given Table 2.

Then a modeling of the RSD variation was processed by numerical fit using JMP. The function used for the fit eq 4 was

$$\mathrm{RSD} = \mathrm{level} \times p_1^{(1-p_2 \log(\mathrm{level}))} \qquad (4)$$

inspired by the Horwitz relation for RSD of interlaboratory studies,[22] where p_1 and p_2 are two parameters determined by the fitting process. Results are shown in Figure 4, together with experimental estimates.

Table 2. RSD Estimates by Level

level (%)	RSD (%)	level (%)	RSD (%)
0.01	39.7	0.25	5.3
0.02	23.4	0.50	2.6
0.05	20.7	1.00	1.6
0.10	12.3		

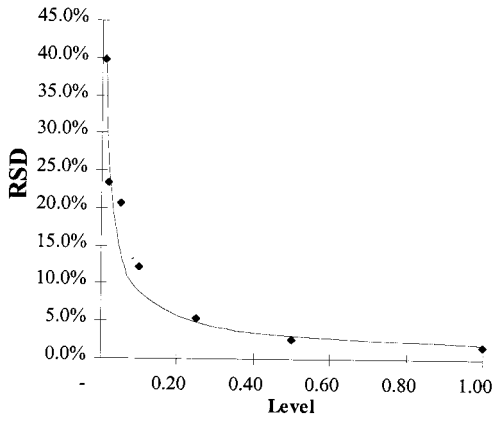

Figure 4. Modeling of the Spiramycin I peak area RSD change as a function of level.

Table 3. Table of Signal-to-Noise Ratio Experimental Values

level (%)	S/N ratio	S/N RSD (%)	level (%)	S/N ratio	S/N RSD (%)
0.01	1.2	20	0.25	41.3	12
0.02	2.7	26	0.50	78.5	9
0.05	7.9	13	1.0	162.5	14
0.10	16.9	12			

2. Measure and Modeling of the Signal-to-Noise Ratio. For each chromatogram the signal-to-noise ratio was calculated from values measured manually. Table 3 shows mean values for each level and their RSDs obtained from the six replicates. The best modeling of the signal-to-noise ratio was obtained with a weighted linear least-squares regression which gave a nonzero intercept. As described in the literature,[13,14] weights used were the inverses of the signal-to-noise ratio variance estimates.

3. Ordinary Linear Regression. From the 42 area values obtained from the 6 replicates of the seven levels, the linear ordinary least-squares (OLS) regression parameters were calculated. They are shown in Table 4.

The *F*-test showed that the slope was significantly different from zero, which meant regression was relevant while the lack of fit tests confirmed the suitability of the linear model. Figure 5 gives a visual plot of the experimental data and regression line.

4. Weighted Linear Regression. From the same 42 area values, and using the inverses of the area variance estimates as weights (normalized to a sum of 42), weighted least-squares (WLS) regression parameters were calculated. They are shown in Table 5.

(21) SAS Institute Inc. *JMP Statistic and Graphics Guide and User's Guide*; Cary, NC, 1995.
(22) Boyer, K. W.; Horwitz, W.; Albert, R. *Anal. Chem.* **1985**, *57*, 454−459.

Table 4. Parameters of Linear Ordinary Least-Squares Regression

parameter	notation	estimate
observations	N	42
correlation coefficient	R	0.999
residual standard deviation	$\hat{\sigma}_{y/x}$	521
slope	b	46364
intercept	a	−364
standard deviation of intercept	$\hat{\sigma}_{y_0}$	104
F-test (regression significance)	F_R	37636 (prob >F) < 0.0001
lack of fit test	F_l	1.92 (prob >F) = 0.116

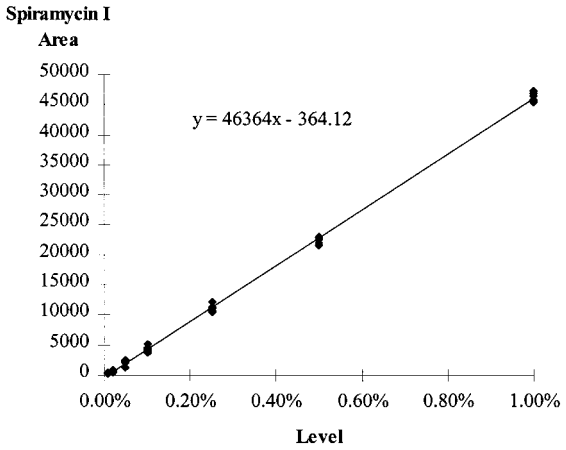

Figure 5. Experimental data and regression line for Spiramycin I area.

Table 5. Weighted Least-Squares Regression Parameters

parameter	notation	estimate
sum of weights	N	42
correlation coefficient	R	0.999
residual standard deviation	$\hat{\sigma}_{y/x}$	239
slope	b	46008
intercept	a	−218
standard deviation of the intercept	$\hat{\sigma}_{y_0}$	39
F-test (regression significance)	F_R	29584 (prob >F) < 0.0001
lack of fit test	F_l	1.79 (prob >F) = 0.140

The F-test showed that the slope was significantly different from zero, which meant regression was relevant, while the lack of fit tests confirmed the suitability of the linear model. It may be noted at this stage that if the slope and the intercept estimates are slightly affected in practice by the use of the weighted regression, standard deviations are significantly modified. This result had already been observed and described.[23,24]

5. RESULTS AND DISCUSSION

Using experimental results and models previously set out, a comparison of the limit estimates obtained with the different approaches was made. Perhaps even more critical than the values themselves is the dispersion that affected these values. This was a reflection of the degree to which the analyst was able to trust the value he had determined.

Table 6. LOD and LOQ Obtained with the Different Approaches

approach		criteria	area	level (%)	S/N
signal-to-noise ratio	LOD	S/N = 3	743	0.021	3.0
	LOQ	S/N = 10	2710	0.064	10.0
	LOD	area = $3\hat{\sigma}_{y_0}$	312	0.015	2.0
ordinary least-squares regression (OLS)	LOQ	area = $10\hat{\sigma}_{y_0}$	1041	0.030	4.5
	LOD	area = $3\hat{\sigma}_{y/x}$	1566	0.042	6.4
	LOQ	area = $10\hat{\sigma}_{y/x}$	5219	0.120	19.3
weighted least-squares regression (WLS)	LOD	area = $3\hat{\sigma}_{y_0}$	117	0.007	0.8
	LOQ	area = $10\hat{\sigma}_{y_0}$	389	0.013	1.7
	LOD	area = $3\hat{\sigma}_{y/x}$	716	0.020	2.9
	LOQ	area = $10\hat{\sigma}_{y/x}$	2387	0.057	8.9

1. Comparison of the Values. For each approach described in section 2 the response values equal to 3 or 10 times the blank standard deviation estimate considered were calculated. These values were converted into analyte quantity or level using modeling described in section 4. Results are shown in Table 6.

These values are shown on the same graph, together with the modeling of the area RSD and of the S/B, on Figure 6 for LOD and on Figure 7 for LOQ.

It clearly appeared that not all the approaches considered in this study were equivalent. Extreme estimates could vary by a factor 6 for LOD and by a factor 10 for LOQ. Consequently, the approach chosen to estimate these limits in a method validation must be rigorously stipulated; if not, this could be a source of serious discrepancies. In respect of the regression approach, as expected, ordinary and weighted gave quite different values, underlining the effect of weighting on dispersion characteristics. Limits obtained from the intercept standard deviation were in all cases weaker than those obtained from residual standard deviation. Differences observed on the example ranged from a factor of 3 to a factor of 4, but they could be even more marked since they depended on many parameters such as the number of levels, their position, the number of replicates per level, and data heteroscedasticity. For both limits, the estimates that were most consistent with those obtained from the usual signal-to-noise ratio were those obtained from the residual variance $\hat{\sigma}_{y/x}$ of the weighted regression. It was also possible to determine area RSD values corresponding to LOD and LOQ obtained with the usual signal-to-noise ratio. For S/B = 3, the corresponding area RSD was 25%, while for S/B = 10, the corresponding area RSD was 12%. It meant using the Eurachem approach with an RSD value of 12% would have given the same LOQ as was obtained from use of the signal-to-noise ratio.

2. Dispersion Comparison. To compare the degree of reliability of the LOD and LOQ estimates, the standard deviation for a simulated implementation of each approach was evaluated. Method characteristics were presumed to be identical to those observed in the present study. For the signal-to-noise ratio approach, it was presumed that the operator carried out successive trials until he obtained the right value. This is the most common procedure in industrial method validation. The regression and Eurachem approaches were presumed to be based on a set of 42

(23) Doyle, E.. *Dev. Drugs Mod. Med.* **1986**, 534−540.
(24) Aarons L. *J. Pharm. Biomed. Anal.* **1994**, *2*, 395−402.

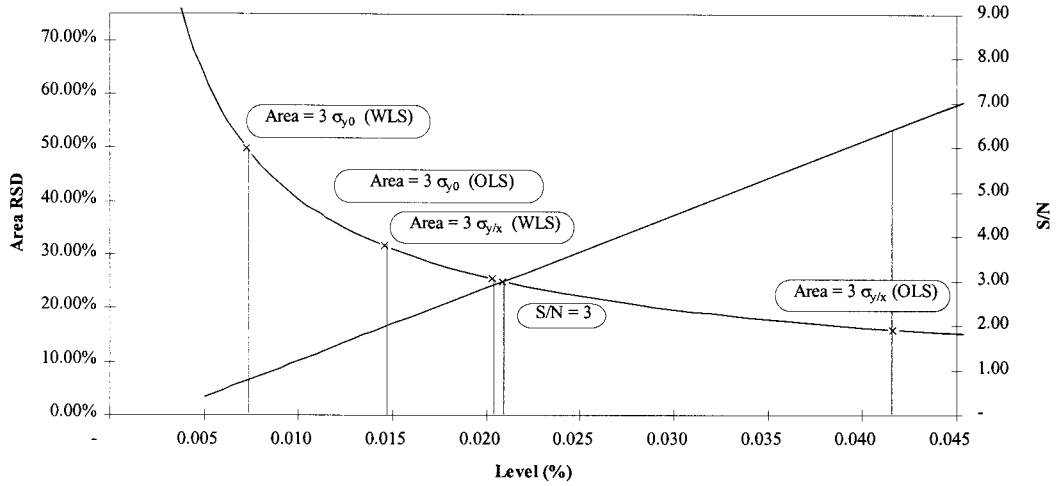

Figure 6. Comparison of LOD estimates obtained with the different approaches.

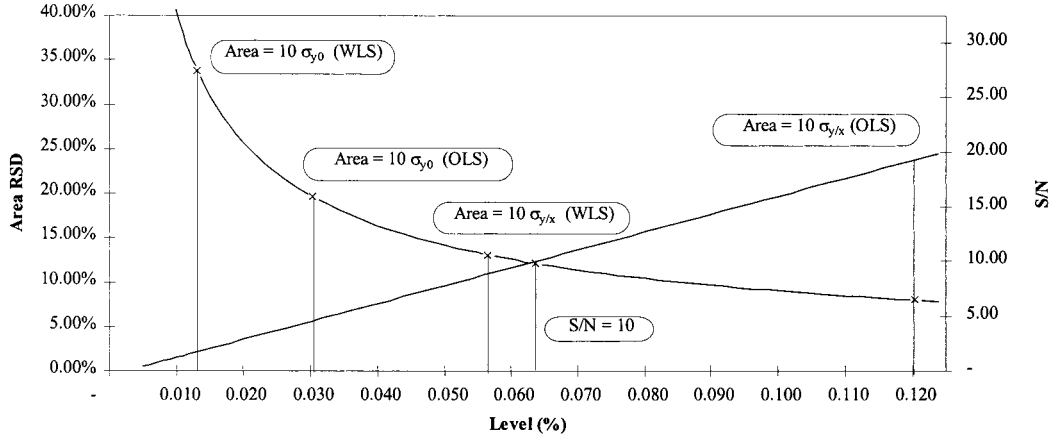

Figure 7. Comparison of LOQ estimates obtained with the different approaches.

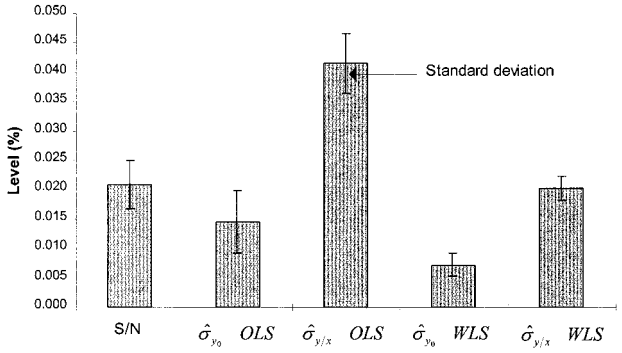

Figure 8. LOD values and their dispersion characteristics.

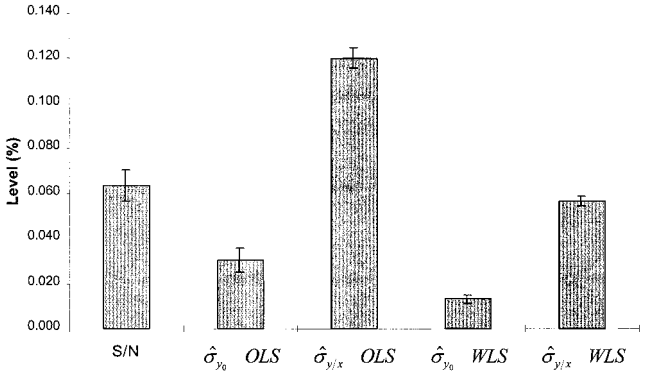

Figure 9. LOQ values and their dispersion characteristics.

injections. LOD results are reported in Figure 8 and LOQ results in Figure 9.

The general behavior is similar for both LOD and LOQ. Values obtained from ordinary least-squares regression presented a large, even the largest, standard deviation. Another drawback of this approach was the dependence of the results on data heteroscedasticity. This could easily be shown by reprocessing the data without the last level (that with the highest absolute dispersion). In this case, limits estimates and their dispersion were radically altered. Consequently, this approach is not advised because of inferior robustness. Weighted least-squares regression produced limits estimates with the weakest standard deviation. In addition, this approach was weakly affected by changes in the levels' design. In this case, reprocessing the data after suppression of the last level did not change the results significantly. The approach using the signal-to-noise ratio seemed, according to present results, to be reasonably dispersed and had the advantage that it was quite simple to implement. Nevertheless, the results of the present paper could in no way reveal the major drawback of this approach. Since manual measuring was involved, the operator's skills and habits were of paramount importance. Another study, carried out for other purposes, underlined the great importance of the human factor. The same analyses were given to five operators who were

given the task of determining the signal-to-noise ratio. Experimental results ranged from 7 to 95! Consequently the signal-to-noise approach had to be proscribed if the method was to be used in collaborative or comparative studies.

3. Recommended Approach. Given the conclusions of the experiments, we thought it would be interesting to propose a global approach for estimating LOD and LOQ. Experimentally, the sequence with 7 levels in the range 0−1% of the method target value appeared quite well-adapted. Six replicates were a good compromise between sequence time length and reliability, i.e., the number of degrees of freedom of the dispersion estimates. If the sequence had absolutely to be shortened, for example in the event of insufficient product stability, it was possible to reduce the number of levels (a minimum of 5 was however required) and to decrease the number of replicates. It must nevertheless be borne in mind that decreasing the sequence would decrease the quality of information. Once experiments were carried out, the area dispersion for each level was evaluated. The LOD estimate was 3 times or 3.3 times $\hat{\sigma}_{y/x}$, the residual standard deviation of the weighted regression. LOQ was estimated using the Eurachem approach with a preestablished value of area RSD of 12%. This methodology has the advantage of compatibility with the results of the traditional signal-to-noise approach without the drawbacks associated with subjective assessment by the operator. The work load is greater, but this should be considered an investment that would enable future discrepancies and additional work to be avoided.

6. CONCLUSION

As explained, detection and quantification limits are two fundamental elements of method validation. There are several estimation methods for these limits: signal-to-noise ratio, dispersion characteristics of the regression line (weighted or not), or area RSD values. According to ICH, all these approaches are possible but none of them is imposed or even advised. The problem is that the different approaches are not equivalent. Whether in terms of the values themselves or their degree of reliability. Consequently, it is necessary to specify in a validation note exactly how the limits have been determined. Otherwise, further comparisons would be meaningless. The methodology we gave at the end of the paper represents, in our opinion, the best compromise for evaluating these limits. It should be regarded as a proposal that we submit to regulatory offices in order to develop more definite guidelines for LOD and LOQ determinations. Such an approach should be useful not only in the case of LC analyses but also in many other analytical methods.

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