Taking Into Account Both Preparation and Injection in High-Performance Liquid Chromatography Linearity Studies

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Abstract

As mentioned in International Conference on Harmonisation publications, linearity is a principal parameter in method validation. The most popular statistical tool used is linear least-squares regression. Contrary to what is still very often practiced, the correlation coefficient can in no way be considered as an indicator of the fit quality. There is in fact a test called the "lack-of-fit test" that enables one to answer the question, "Is the linear model adapted to the calibration curve?". However, this test can give erroneous conclusions when, at each level, several sources of variation for the response are influent. It often occurs in high-performance liquid chromatography, as shown in a following example, where the calibration curve is obtained from repeated injections of repeated dilutions of a parent solution. The lack-of-fit test rejected linearity, although it was at least questionable. In fact, the reason for a discrepancy of this kind lies in the presence of a double source of variation: injection and dilution. It is possible to overcome the problem by mixing a nested ANOVA with the standard least-square linear regression. As shown in an example, implementing this methodology for data processing allows one not only to carry out an unbiased lack-of-fit test but also give estimates of the dispersion introduced respectively by the preparation and the injection.

Introduction

Today, each analytical method must be fully validated before being used in a routine. Several publications deal with the subject (1–5), especially in high-performance liquid chromatography (HPLC) (6–10). Linearity of response against analyte quantity is one of the main concerns of validation. In the field of HPLC with ultraviolet (UV) detection, the analyst must ensure that the area (or height) of the peaks is a linear function of the concentration in the range defined for the analysis. Generally, the experiment consists of analyzing samples of different concentration levels obtained by dilution from a parent solution. A common approach, as mentioned in the latest International Conference on Harmonisation (ICH) recommendations (1), is to process data by least-square regression. Then, it allows one to carry out statistical tests, generally on the intercept and slope. The correlation coefficient measures the proportion of the variation around the mean explained by the model. Therefore, it is not convenient to test the adequacy of the latter (i.e., linearity), and its use is thus rightfully considered inadvisable or at least not sufficient (1,3,5,11). Nevertheless, it is still current practice to rely solely upon the correlation coefficient (8–9,12–17). However, a lack-of-fit test has been proposed (16) and is now integrated in several statistical applications (19–20), but it is not commonly used for at least two reasons. First, it is not very popular, and few analysts are even aware of its existence. Second, as will be shown by our example, it can lead to rejection of the linear model, although it is considerably less obvious when considering the residuals. The reason lies in the fact that there is more than one source of variation for the response at each level (generally two: the preparation of the calibration solutions and the injection) that affects the results. After a brief review of the theoretical basis of the lack-of-fit test, it will be shown how to combine linear regression with a nested analysis of variance (ANOVA) to solve the problem. This approach enables not only the lack-of-fit test to be carried out rigorously, bearing in mind the current experimental design, but also to give reliable estimates of the dispersion introduced respectively by the preparation and the injection. The example will illustrate practically how to proceed.

Experimental

Definition of the lack-of-fit test

Linear regression

Linear regression is used to estimate the parameters of a linear model linking a dependent variable Y and an independent variable X. The model is given by Equation 1.

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$$Y = \beta_0 + \beta_1 X + \varepsilon \qquad \text{Eq. 1}$$

where ε is the Gaussian variable for the effect of random error, and β_0 and β_1 are unknown parameters estimated using the least-squares method from experimental data (x_i, y_i) (21). Least-squares estimates are denoted b_0 and b_1 and are given in Equations 2 and 3.

$$b_1 = \frac{\Sigma_i (x_i - \bar{x})(y_i - \bar{y})}{\Sigma_i (x_i - \bar{x})^2} \qquad \text{Eq. 2}$$

$$b_0 = \bar{y} - b_1 \bar{x} \qquad \text{Eq. 3}$$

where $\bar{x}$ and $\bar{y}$ stand for the x_i mean and y_i mean, respectively.

From these estimates and for each value x_i of x, it is possible to calculate $\hat{y}_i$, the y value predicted by the model (Figure 1).

$$\hat{y}_i = b_0 + b_1 x_i \qquad \text{Eq. 4}$$

ANOVA of least-squares regression

The traditional ANOVA table of least-squares regression is given in Table I. The *F* test, which consists of comparing the regression and the error mean squares, is not in any sense a test of linearity. It is merely a test that allows one to detect whether there is a significant relation between the y and x values. Mathematically, it is strictly equivalent to the t-test on the slope of the regression line.

The lack-of-fit test

The lack-of-fit test is the one that enables one to check whether the linear model is suitable for the set of experimental data. For each level x_i, it requires several (at least two) experimental y values denoted $y_{i\alpha}$. The aim is to determine whether the dispersion of the level means $\bar{y}_i$ around the regression line can be explained by the dispersion inside the levels. If this is the case, then linearity cannot be rejected. The situation is illustrated by Figure 2.

Numerically, it is merely a decomposition of the error sum of squares of the regression into two terms. The first corresponds to the lack of fit, whereas the second is a pure error term, as shown in Equation 5.

$$\underbrace{\sum_{i\alpha}(y_{i\alpha} - \hat{y}_i)^2}_{Q_r} = \underbrace{\sum_{i}(\bar{y}_i - \hat{y}_i)^2}_{Q_1} + \underbrace{\sum_{i\alpha}(y_{i\alpha} - \bar{y}_i)^2}_{Q_{\text{intra}}} \qquad \text{Eq. 5}$$

The corresponding ANOVA table is given in Table II. The statistical test to be used is an *F* test. If the linear model is appropriate, then q_1 and q_{intra} are two independent estimates of the same variance. The ratio q_1/q_{intra} is therefore a Fischer Snedecor variable with $(p-2)$ and $(n-p)$ degrees of freedom.

Validity

The conditions of validity of the linear regression (and therefore of the lack-of-fit test) are that (*a*) each x_i is known exactly (i.e., without error) and (*b*) the data are homoscedastic (i.e., dispersion on y values does not depend on x values) and there is no preponderant source of variation for the response except the value of x. A tolerance of a factor 5 in the extreme standard deviations is, however, mentioned as acceptable in the literature (22).

Neglecting these hypotheses is, unfortunately, current practice and often leads to ambiguous situations or erroneous conclusions.

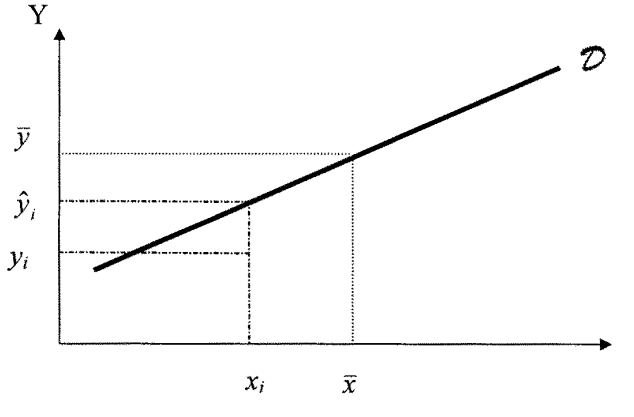

Figure 1. Predicted and experimental data.

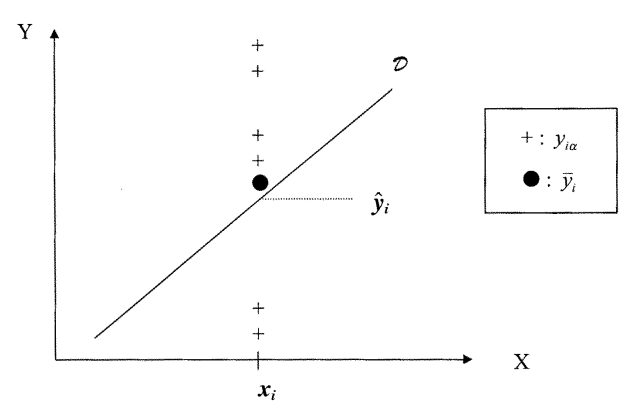

Figure 2. Principle of lack of fit test.

Table I. ANOVA Least-Squares Regression Table*

Source of variation	Sum of squares	Degrees of freedom	Mean square
Regression	$Q_l = b_l^2 \Sigma_i (x_i - \bar{x})^2$	1	$q_l = b_l^2 \Sigma_i (x_i - \bar{x})^2$
Error	$Q_r = \Sigma_i (y_i - \hat{y}_i)^2$	$n-2$	$q_r = Q_r / n-2$
Total	$Q_T = \Sigma_i (y_i - \bar{y})^2$	$n-1$	

* n = number of experimental data used for the regression.

Table II. Lack-of-Fit ANOVA Table*

Source of variation	Sum of squares	Degrees of freedom	Mean square
Lack of fit	$\Sigma_i (\bar{y}_i - \hat{y}_i)^2$	$p-2$	$q_l = Q_l / p-2$
Pure error	$\Sigma_i (y_{i\alpha} - \bar{y}_i)^2$	$n-p$	$q_{\text{int ra}} = Q_{\text{int ra}} / n-p$
Total error	$\Sigma_{i\alpha} (y_{i\alpha} - \hat{y}_i)^2$	$n-2$	

* p = number of levels.

Limitation of the lack-of-fit test

In practice, the hypothesis of linearity can be rejected by the lack-of-fit test, whereas an examination of the residuals indicates clearly that the relation is probably linear. It is quite frequent in HPLC with UV detection. Paradoxically, it is more frequent the better the repeatability of the HPLC device. The following example is a typical illustration of what can happen.

Experimental

The linearity study chosen was carried out on the analysis of Spiramycin, an antibiotic developed by Rhône-Poulenc Rorer (Centre de Recherche de Vitry Alfortville, France). This product has already been the subject of other studies (23–25).

The method used isocratic elution reversed-phase chromatography with a Nucleosil C_8 120Å 3-µm (200 × 4.6 mm) column. The mobile phase was a mixture of acetonitrile (HPLC grade, J.T. Baker, Phillipsburg, NJ) and phosphate buffer pH 2.2 (30:70, v/v). The pump was a Varian (Les Ulis, France) 9012, the flow rate of which was set at 0.8 mL/min. The injector was a Waters (St. Quentin, France) 715 Ultra Wisp with a cooling system set at 4°C. The injection volume was 20 µL. The detector was an adjustable wavelength Varian 2050 set at 232 nm. The column temperature was maintained at 23°C using an Alltech (Deerfield, IL) Water Jacket and a Bioblock Polystat 5. Acquisition and integration were performed on a personal computer with the Shimadzu (Courtaboeuf, France) Chromatography Data System Class-Vp. The mobile phase was recycled with an Ecosaver (Touzart & Matignon, Courtaboeuf, France).

Each sample was prepared by dilution from a parent solution: 125 mg of Spiramycin in 100 mL of a mixture of acetonitrile (HPLC grade, J.T. Baker) and Milli-Q (Millipore, Milford, MA) water (30:70, v/v). Rather than using flasks and pipettes, we chose to use a Hamilton (Reno, NV) Microlab 530B diluter. The exact protocol is not included, because this is not the purpose of the present paper. Nevertheless, preliminary experiments showed that diluter accuracy and repeatability were at least as good as, or better than, those of an experienced handler using standard flasks and pipettes (26).

Six levels of concentration were chosen, arbitrarily denoted 25, 50, 75, 100, 125, and 150. For each level, 3 independent samples were prepared, and each sample was injected twice. The 100 level corresponds to a chromatographic peak with a maximum absorbance of approximately 0.4 AU.

If we call L_i the ith preparation of the L level and B a blank, the sequence can be written as follows: $B/25_1/50_1/75_1/100_1/125_1/150_1/B/25_2/50_2/75_2/100_2/125_2/150_2/B/25_3/50_3/75_3/100_3/125_3/150_3/B/25_1/50_1/75_1/100_1/125_1/150_1/B/25_2/50_2/75_2/100_2/125_2/150_2/B/25_3/50_3/75_3/100_3/125_3/150_3$.

Results

The response chosen was the area of the peak of Spiramycin I, the main component. Numerical data obtained (in arbitrary integration units) are shown in Table III.

Data processing

First, we chose to process the data in a traditional way (i.e., without taking into account the preparation factor). Calculations were achieved using the least-square method on a personal computer with the JMP software (SAS Institute, Cary, NC).

A plot of experimental data, together with the regression line, is given in Figure 3. Estimated parameters of the regression line are

Table III. Area of the Spiramycin 1 Peak

Level	Preparation	First injection area	Second injection area
25	1	2457524	2391693
25	2	2450828	2391252
25	3	2444638	2360293
50	1	4693194	4844527
50	2	4835596	4878092
50	3	4809226	4722253
75	1	7142763	7182769
75	2	7135550	7173920
75	3	7216871	7076359
100	1	9496553	9537788
100	2	9405825	9439201
100	3	9609870	9707734
125	1	12031958	12027037
125	2	11935594	11930086
125	3	12154132	12096462
150	1	14298064	14396607
150	2	13964716	14221039
150	3	14283992	14042220

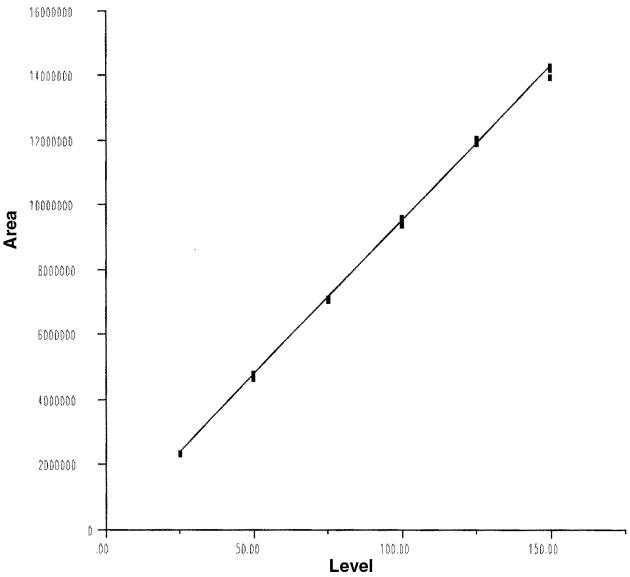

Figure 3. Plot of experimental results together with regression line.

Table IV. ANOVA Table for Regression on Experimental Data

Source of variation	Sum of squares	Degrees of freedom	Mean square	*F* ratio
Regression	$Q_l = 5.90 \times 10^{14}$	1	$q_l = 5.90 \times 10^{14}$	49037
Error	$Q_r = 4.09 \times 10^{11}$	34	$q_r = 1.20 \times 10^{10}$	Prob>*F*
Total	$Q_T = 5.91 \times 10^{14}$	35		< 0.0001

b_1 = 94857 and b_0 = 55207. The ANOVA table of the regression is given in Table IV.

The high F ratio value and the low probability that it occurred only by chance indicates that there is in fact a relation between y and x. This test is not able to state if this relation is linear or not. Only the lack-of-fit test, the table for which is given in Table V, can answer this point.

With an α level of 5%, the hypothesis of linearity must be rejected. Effectively, the value of the F ratio is too high (i.e., the probability that such a value occurred by chance is too low to consider the hypothesis of linearity as acceptable). Nevertheless, an examination of the residuals of the regression ($y_i - \hat{y}_i$) (Figure 4) does not confirm the conclusions of the lack-of-fit test. No deviation from linearity seems obvious, because residuals are rather randomly distributed around zero.

Apparently, the lack-of-fit test is in contradiction with a visual examination of the residuals. If data homoscedasticity seems to be not strictly respected, that cannot explain alone such an observation. Effectively, standard deviations vary in a factor of 5 or less, which can be considered as tolerable (22). On the other hand, it must be remembered that in the former approach, the preparation factor had not been taken into account. Does this factor introduce a dispersion that cannot be neglected in comparison with those of injection (intrinsic dispersion inherent to the HPLC device), and how would the conclusions be modified if this new source of variation was introduced in the model? In other words, how is it possible to take into account the presence of both genuine replicates and repeated injections (27) in the data processing of the experimental design of an HPLC linearity study?

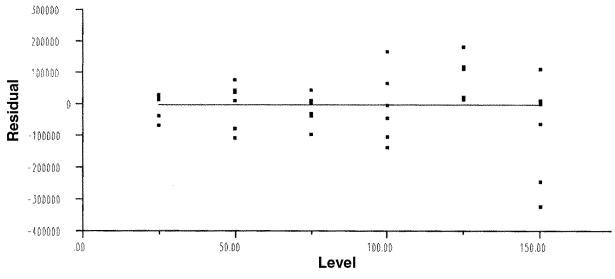

Figure 4. Plot of the regression residuals.

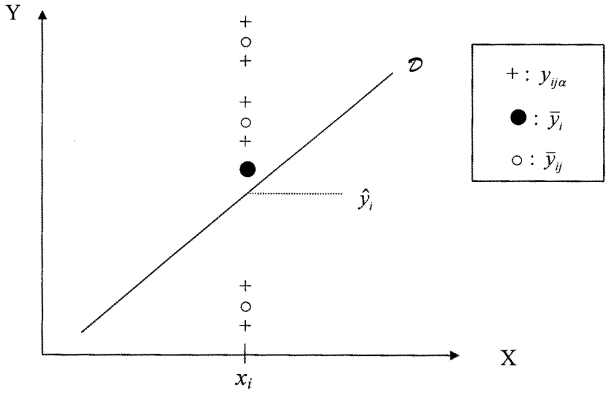

Figure 5. Preparation taken into consideration.

Table V. Lack-of-Fit Table for Experimental Data

Source of variation	Sum of squares	Degrees of freedom	Mean square	F ratio
Lack of fit	1.25×10^{11}	4	$q_1 = 3.13 \times 10^{10}$	3.31
Pure error	2.84×10^{11}	30	$q_{intra} = 9.47 \times 10^{9}$	Prob > F
Total error	4.09×10^{11}	34		0.023

Separation between preparation and injection effects: another approach to test linearity

It had been previously shown in an example that the traditional lack-of-fit test could not be well adapted to assert the linearity of UV detection of an HPLC method because, in fact, its validity conditions were not respected within the actual experimental design. If it is possible to bypass the difficulty with a single injection per preparation, this solution appears not fully satisfactory, because in this case, it is no longer possible to make a difference between preparation and injection dispersion. We will henceforth show theoretically and practically how to proceed to overcome the problem without any loss of information.

Theoretical approach

Any variation in x due to the preparation step induced a corresponding variation in y. Because a random effect on x values occurring during the preparation of calibration solutions could be neither measured nor quantified, it would therefore be treated as an additional source of variation on y. So, the aim of the proposed modification is to compare the dispersion of the level means with the dispersion between preparations and no longer with an overall dispersion including both injection and preparation. The lack-of-fit test conducted with this approach will be called the "modified lack-of-fit test". Figure 5 corresponds to Figure 2 when differences between preparations are taken into account. $\bar{y}_{ij}$ is the mean for the jth preparation of level i.

Mathematically, the pure error sum of squares of the traditional lack-of-fit test (Table V) is separated into two terms. The first corresponds to the dispersion introduced by the preparation of the samples, and the second stands only for the injection repeatability. Consequently, the entire decomposition of the sum of squares is given in Equation 6.

$$\underbrace{\sum_{ija}(y_{ija} - \hat{y}_i)^2}_{Q_r} = \underbrace{\sum_{i}(\bar{y}_i - \hat{y}_i)^2}_{Q_1} + \underbrace{\sum_{ij}(\bar{y}_{ij} - \bar{y}_i)^2}_{Q_{prep}} + \underbrace{\sum_{ija}(y_{ija} - \bar{y}_{ij})^2}_{Q_{intra}} \qquad \text{Eq. 6}$$

The corresponding table is given in Table VI.

First, the influence of the preparation factor is to be tested. The statistical test used is an F test. If the hypothesis of noninfluence of the preparation factor is not to be rejected, then q_{prep} and q_{intra} are two independent estimates of the same variance. The ratio q_{prep}/q_{intra} is therefore a Fischer Snedecor's variable with ($p[r-1]$) and ($n-pr$) degrees of freedom. The way to test the appropriateness of the linear model is then different, whether the preparation factor is found to be influential or not. If the preparation factor is not influential, the lack-of-fit F test uses the ratio q_1/q_{intra}, which is a Fischer Snedecor's variable with ($p-2$) and ($n-pr$) degrees of freedom in the case of appropriateness of the linear model. Otherwise, the lack-of-fit F test uses the ratio q_1/q_{prep},

which is a Fischer Snedecor's variable with $(p - 2)$ and $(p[r - 1])$ degrees of freedom in the case of appropriateness of the linear model.

With such changes, the difficulty inherent to the multiplicity of sources of variation for the response is bypassed. The lack-of-fit test actually tests the lack of fit. If linearity is rejected, either the response is nonlinear (saturation phenomena), or the accuracy of the x value must be questioned.

Experimental

The same set of data as in the Limitation of the lack-of-fit test section is used, but data processing is carried out using the modified lack-of-fit test methodology. The ANOVA table is given in Table VII.

The influence of the preparation factor must be tested first. The observed value for the F ratio is $1.45 \cdot 10^{10}/6.10 \cdot 10^{9} = 2.34$, and the probability that a Fischer Snedecor's variable with 12 and 18 degrees of freedom takes a value greater than 2.34 is 4.71%. With an α level of 5%, the preparation factor can be considered significant. Consequently, to test the lack of fit, the F ratio q_1/q_{prep} must be used. The observed value is $3.13 \cdot 10^{11}/1.45 \cdot 10^{11} = 2.16$, and the probability that a Fischer Snedecor's variable with 4 and 12 degrees of freedom takes a value greater than 2.16 is 13.55%. With an α level of 5%, the hypothesis of appropriateness of the linear model cannot be rejected; in other words, there is no problem with the linearity.

To determine the dispersion introduced respectively by the preparation and the injection, it is necessary to calculate estimates of their respective standard deviation (SD), or better, of their relative standard deviation (RSD, normalization by the value of the response at the 100 level). The residual SD, corresponding to the injection, is given by the square root of the pure error mean square. Numerically, residual SD = 78124 and residual RSD = 0.82%. The preparation SD is given by Equation 7.

$$q_{prep} = SD_r^2 + 2SD_{prep}^2 \qquad \text{Eq. 7}$$

Numerically, preparation SD = 64864 and preparation RSD = 0.68%.

As shown in this example, by taking into account the possible influence of the preparation on the results' dispersion, the modified lack-of-fit test prevents erroneous conclusions. So, validation notes can be less ambiguous, because approximate explanations are no longer necessary to justify that response is linear despite the rejection of the linear hypothesis by the lack-of-fit test. Moreover, the analyst can, in the same time, give estimates of the dispersion introduced respectively by the preparation and the injection. Such information is of great interest, because it enables an optimization of the future routine injection sequence.

Table VI. Lack-of-Fit Table for the Modified Lack of Fit Test*

Source of variation	Sum of squares	Degrees of freedom	Mean square
Lack of fit	$\sum_{i}(\bar{y}_i - \hat{y}_i)^2$	$p - 2$	$q_1 = Q_1/(p-2)$
Preparation	$\sum_{ij}(\bar{y}_{ij} - \bar{y}_i)^2$	$p(r-1)$	$q_{prep} = Q_{prep}/p(r-1)$
Pure error	$\sum_{ij\alpha}(y_{ij\alpha} - \bar{y}_{ij})^2$	$n - pr$	$q_{intra} = Q_{intra}/(n-pr)$
Total error	$\sum_{ij\alpha}(y_{ij\alpha} - \hat{y}_i)^2$	$n - 2$	

* r = number of preparations per level.

Table VII. Experimental ANOVA Table for the Modified Lack-of-Fit Test

Source of variation	Sum of squares	Degrees of freedom	Mean square
Lack of fit	1.25×10^{11}	4	3.13×10^{10}
Preparation	1.74×10^{11}	12	1.45×10^{10}
Pure error	1.10×10^{11}	18	6.10×10^{9}
Total error	4.09×10^{11}	34	

Conclusion

It was shown that linearity of a response could be confirmed rigorously using a test described in-depth in statistical treatises and called the "lack-of-fit test". However, problems could arise when, as shown in the example, an additional source of variation was involved but not taken into consideration. It could, for example, be the dispersion resulting from the dilution of a parent solution. To overcome this difficulty, a modified lack-of-fit test was developed by mixing a nested ANOVA with traditional regression. The efficiency of this methodology was demonstrated, because it not only avoided erroneous rejection of the linearity hypothesis but also provided estimates of the dispersion of each source of variation involved. At first sight, our approach could seem fastidious, because it involves complex calculations. However, the use of computers and statistical software substantially reduces the validity of this observation. The present study also clearly underlines the need to proceed with repeated preparations, because a single preparation per level and repeated injections were highly likely to produce discrepancies. One could still object that repeated injections are not necessary, but it is bad policy not to extract the maximum of information from an analysis method, and full knowledge of each dispersion characteristic of the method is an uncontestable asset for any further development. In any event, the methodology we have proposed here is a new tool that can be used by analysts for method validation.

Acknowledgments

The authors would particularly like to thank Rhône-Poulenc Rorer (Centre de Recherche de Vitry Alfortville, France) for supporting the research and experiments.

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Manuscript accepted March 3, 2000.