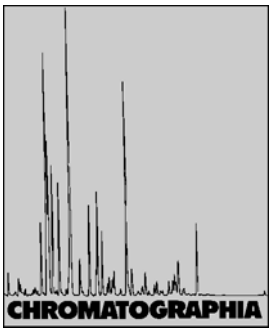
What is the Best Means of Estimating the Detection and Quantification Limits of a Chromatographic Method?



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Key Words

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Statistical data processing
Method validation
Limits of detection and quantification

Summary

Limits of detection and quantification (*LOD* and *LOQ*) are two fundamental elements of method validation. Although strict statistical definitions exist, a variety of procedures based on different means of estimating the standard deviation of the blank can be used to evaluate these limits. An experiment set up to investigate their compatibility for an LC-UV method has proved they are not equivalent. We therefore recommend an alternative and absolute methodology: *LOQ* is obtained from the target *RSD* chosen for the assay and *LOD* is taken as three tenths of the *LOQ*. To demonstrate the method is not restricted to LC-UV it has been applied to LC-MS and GC-FID.

Introduction

The lower limits of the analytical range, i.e. the limits of detection and quantification (*LOD* and *LOQ*) are two fundamental aspects of method validation [1–7]. Although rigorous statistical definitions exist [8, 9], e.g. "the lowest quantity of analyte that can be either detected or quantified with a given confidence level", practical determination of *LOD* and *LOQ* is complex. According to the definitions *LOD* and *LOQ* correspond to the quantities injected which produce signals equal to a given multiple of the standard deviation of the blank, σ_B. In chromatography σ_B cannot be measured and must be estimated. Several means of obtaining this estimate have been proposed in official guidelines, e.g. those of the FDA [10], but are they equivalent? It will be shown for liquid chromatographic analysis with ultraviolet detection (LC-UV) that they are not. An alternative method, reported by Eurachem [11] and based on the target value of the relative standard deviation (*RSD*), will be considered. The applicability of this method will be demonstrated not only for LC-UV but also for two other chromatographic techniques – LC coupled with a mass spectrometer (MS) as detector and gas chromatography with flame ionization detection (GC-FID).

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Experimental

LC-UV

Experiments were performed on spiramycin. This Aventis antibiotic can be regarded as a good example of the kind of products handled by the pharmaceutical industry. The LC-UV method was fully validated and studied and its dispersion characteristics were estimated rigorously by means of a collaborative study [12–15]. Briefly, the method uses reversed-phase chromatography under isocratic conditions. The resulting chromatograms were produced and processed by means of a Shimadzu Class-VP acquisition station. A typical chromatogram is shown in Figure 1.

For sample preparation a parent solution of spiramycin was obtained by dissolving spiramycin powder (12.5 mg) in 70:30 (*v/v*) water-acetonitrile (500 mL). Solutions affording injected quantities ranging from 0.5 ng to 50 ng were obtained by dilution of the parent solution. For each of the seven levels chosen six independent solutions were prepared and each solution was injected once.

LC-MS

The LC-MS method considered was from the field of environmental chemistry – analysis of polar phenolic compounds in

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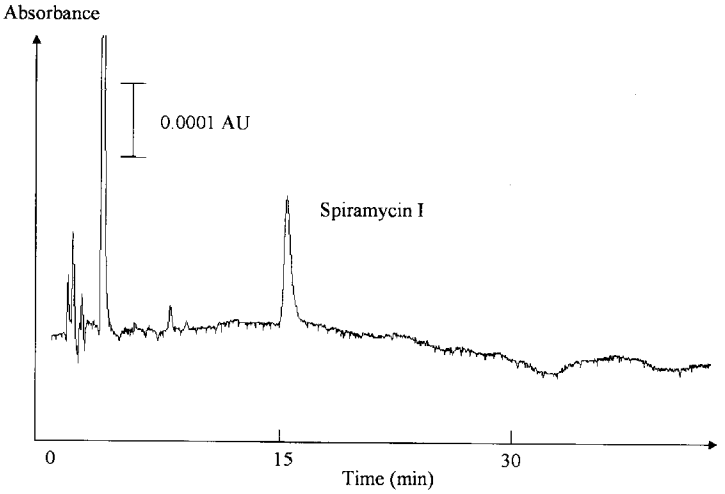

Figure 1. Chromatogram of spiramycin (quantity injected = 5 ng) Column: length, 20 cm; internal diameter, 0.46 cm; stationary phase, C_8-bonded silica gel, 3 µm (Nucleosil); pore diameter, 120 Å; column temperature 23 °C. Mobile phase: acetonitrile-aqueous buffer, pH 2.2 ($[H_3PO_4] = 6.7\ g\ L^{-1}$; pH adjusted with sodium hydroxide) 30:70 (*v/v*) + 6.5 g L^{-1} sodium perchlorate monohydrate; flow rate, 0.8 mL min^{-1}; pressure drop, 160 bar. Volume injected, 20 µL (sample temperature 4 °C). UV detection at 232 nm.

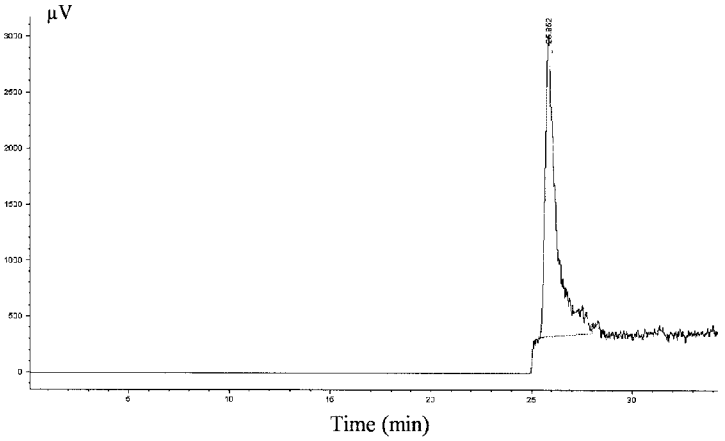

Figure 2. SIM chromatogram (*m/z* = 163) of *p*-coumaric acid (quantity injected 20 ng). APCI, negative-ion mode. Column: length, 10 cm; i.d., 0.46 cm; stationary phase, Hypercarb porous graphitic carbon (Hypersil), 5 µm; column temperature ambient. Mobile phase: acetonitrile-methanol-acidified water ($[formic\ acid] = 0.2\ mol\ L^{-1}$), 40:40:20 (*v/v/v*) and THF gradient; flow-rate, 1 mL min^{-1}; pressure drop, 80 bar. Volume injected, 10 µL. Detection: simple quadrupole.

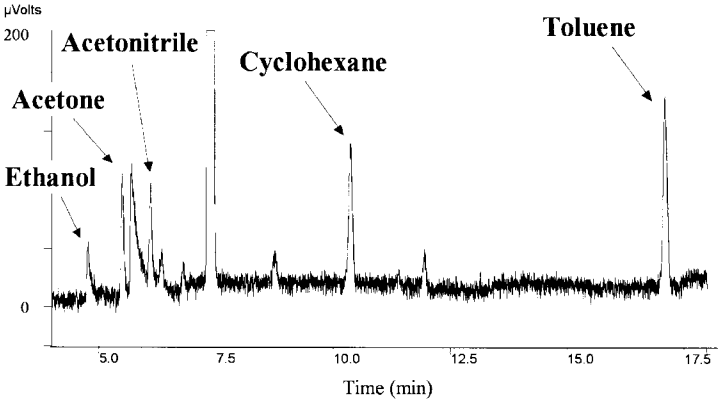

Figure 3. Column: length, 30; internal diameter, 0.32 m; stationary phase, CP-Select 624 CB; film thickness, 1.8 µm. Carrier gas, helium; flow rate, 1.5 mL min^{-1}; split ratio, 40. Detection: FID at 270 °C. Temperature gradient from 40 °C to 250 °C. Volume injected, 1 µL; quantity of each solvent injected, 25 pg.

olive mill wastewater. The analytical technique involved use of a porous graphitic carbon column coupled with atmospheric-pressure chemical ionization and mass detection [16]. *p*-Coumaric acid was chosen as model compound to evaluate the applicability of the target *RSD* approach. A chromatogram is shown in Figure 2. The response considered was the peak area and was obtained by use of the Chemstation (Agilent) integration software. To apply the target *RSD* approach seven levels ranging from 5 to 80 ng were injected and six replicate analyses were performed at each level.

GC-FID

The GC-FID method was the determination of the residual solvent content of pharmaceutical products. Analyses were performed with a Varian 3800 CX chromatograph which was equipped with a 1078 split/splitless injector, the temperature of which was set at 300 °C. The detector temperature was set at 270 °C, the make up flow rate at 25 mL min^{-1}, hydrogen flow at 30 mL min^{-1}, and air flow at 300 mL min^{-1}. Helium was used as carrier gas at a flow of 1.5 mL min^{-1}. Analytes were separated on a 30 m length × 0.25 mm i.d., film thickness 1.8 µm, CP-Select 624 CB column (Chrompack, Les Ulis, France). The column temperature was held at 40 °C for 3 min after injection then increased at 3°C min^{-1} to 100 °C and then at 20°C min^{-1} to 250 °C, which was held for 5 min. A typical chromatogram is shown in Figure 3. Five solvents were analyzed – ethanol, acetone, acetonitrile, cyclohexane, and toluene. Both peak areas and peak heights were recorded by the Star (Varian) integration software.

Comparison of Approaches Based on Estimation of σ_B

When considering existing procedures, i.e. approaches based on estimation of σ_B, the difficulty does not lie in determination of the multiplying coefficient [8] but rather in estimation of σ_B. Five different quantities, measured or calculated from a set of experimental data taken around the expected limits, can be used to estimate σ_B:

- the chromatogram baseline noise;
- the residual standard deviation of an ordinary least squares (OLS) regression line, denoted $\sigma_{y/x}$(OLS);

- the standard deviation of the intercept of an OLS regression line, denoted σ_{y0}(OLS) (here it is assumed the zero intercept hypothesis is verified, otherwise it must be taken into account [8]);
- the residual standard deviation of a weighted least squares (WLS) regression line, denoted $\sigma_{y/x}$(WLS); and
- the standard deviation of the intercept of a WLS regression line, denoted σ_{y0}(WLS) (again it is assumed the zero intercept hypothesis is verified, or must be taken into account [8]).

Because no theoretical background guarantees, a priori, that all the preceding approaches are equivalent, an experimental comparison was performed for the LC-UV method described in the experimental section. The response considered was the peak area of the main compound, spiramycin I. Five estimates of σ_B were calculated from the 42 experimental chromatograms. Details of the data processing are available elsewhere [17]. *LOD* and *LOQ* values (taken as equal to $3\sigma_B$ and $10\sigma_B$, respectively), with their respective reliability, were then compared as shown in Figure 4.

LOQ values were very different; depending on the approach used extreme values could vary by a factor of 10. Reliability also varied much from one approach to another. Similar behavior was observed for *LOD*. This ambiguity made it difficult to compare *LOD* and *LOQ* values given in the literature, because the values are highly dependent on the procedure used for determination. Thus providing an *LOD* or *LOQ* value without specifying how it had been obtained is meaningless [18]. Moreover, previous results did not take into account between-operator variability in the measurement of the noise, a consequence of different aspects of the noise (short-term noise, long-term noise, drift ...) [19]. The versatility of noise measurement can be illustrated by an anecdote. The same chromatogram of spiramycin was provided to five different operators who were asked to measure the noise. Reported values differed by a factor of 5. This discredits the signal-to-noise approach, despite its simplicity and the apparently acceptable results if a single operator is considered. The lack of equivalence between approaches based on estimation of σ_B make it very attractive to devise a unified and unambiguous approach which could deal with the problem of the limits in the lower working range.

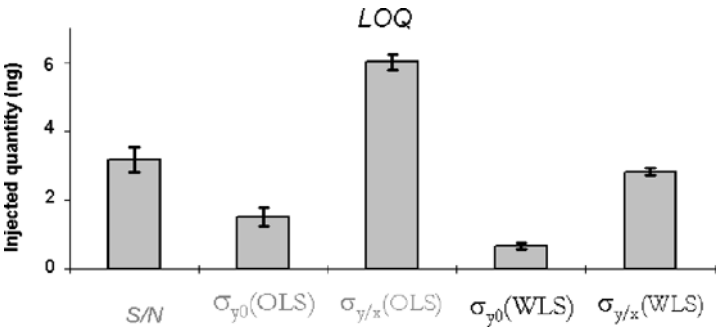

Figure 4. Comparison of *LOQ* values obtained from the five different estimates of σ_B. Error bars indicate the reliability of each value.

The Target *RSD*: An Alternative Approach

Methodology

The target *RSD* approach is different in essence from those based on an estimate of the standard deviation of the blank. It is an approach based on direct application of a definition – *LOQ* is defined as the minimum amount of analyte that can be analyzed with a precision equal to a chosen target *RSD*. So this approach is an absolute, or reference, method. In procedures described previously it is assumed without further verification that when the signal reaches a given multiple of the noise the precision of the measurement can be considered sufficient. In the target *RSD* approach the precision of the measurement, i.e. the defining criterion of the *LOQ*, is directly measured. A typical value for the target *RSD* is 10% but it can, and must, be adapted to the requirements of the method. *LOD* can then be defined by another approach but, for the sake of simplicity and to keep the usual ratio between *LOD* and *LOQ*, it is better to define *LOD* as three tenths of the *LOQ* value. For quantitative analytical methods the *LOD* value is mainly given for information. In practice the target *RSD* approach can be divided into different steps:

1. choice of the target *RSD* value, depending on the requirements of the method;
2. definition of at least six levels, corresponding to given quantities of the analyte, in the range around the expected *LOQ*;
3. experimental realization of independent replicates of the analysis (a minimum of six) at each level;
4. calculation of the observed *RSD* at each level;
5. modeling of variations of the *RSD* as a function of quantity of analyte, for example by use of an "Horwitz-like function" [17, 20] as given in Eq. (1):

$$RSD = level \cdot p_1^{(1-p_2 \cdot \log(level))} \quad (1)$$

where p_1 and p_2 are two terms determined by the fitting process, by means of numerical resolution (a graphical plot is recommended to verify the fit with experimental data); and

6. determination of the *LOQ* by reporting the target *RSD* on the modeled curve.

The target *RSD* approach can seem tedious to implement because of the large number of experiments required. It must, nevertheless, be regarded as an investment justified by the quality of the results provided. The reliability of the limit value is directly dependent on the target *RSD* chosen.

Applications

The target *RSD* approach, with a target value set at 10%, was applied in the spiramycin analysis. The data set was the same as that used previously, because it fulfilled all the requirements – seven levels and six replicates at each level. The observed *RSD* and the modeling are presented in Figure 5. The *LOQ* was found to be 4 ng. It is apparent that this value was of the same order of magnitude as those obtained from approaches based on estimation of the standard deviation of the blank. This first example demonstrated the applicability of the target *RSD* approach to LC-UV analytical methods.

The target *RSD* approach, with a target value set at 10%, was then applied to the LC-MS analysis of phenolic compounds. *LOQ* was found to be 13 ng. This rather high value was not limiting here because the real samples provided for analysis were concentrated and dilution was re-

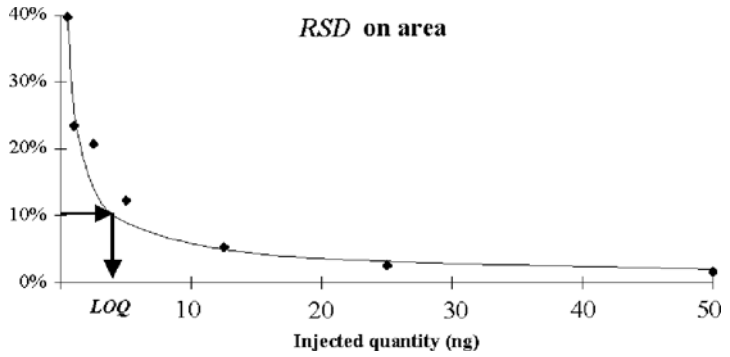

Figure 5. Application of the target *RSD* approach to determination of the *LOQ* of spiramycin. The curve represents modeling of the *RSD* variation as a function of the quantity injected. Arrows indicates how *LOQ* is obtained from the curve.

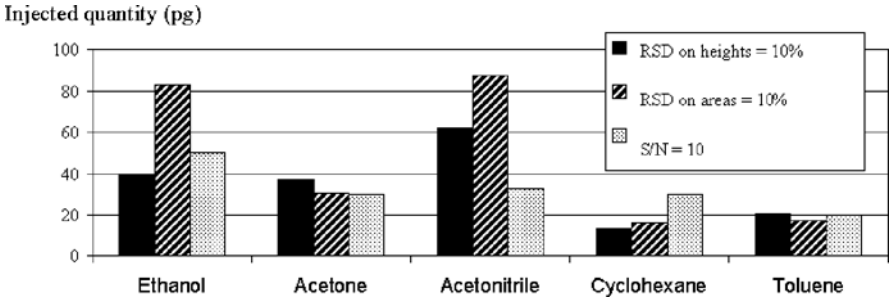

Figure 6. Comparison of *LOQ* obtained from the target *RSD* approach by use of peak areas and peak heights for the five solvents. The *LOQ* obtained from the *S/N* criterion is given for information.

quired before injection. In this analysis MS detection was chosen because of its selectivity rather than its detectability. This example demonstrated, nevertheless, that the target *RSD* approach could also be applied to LC-MS analysis.

Finally, the target *RSD* approach was applied to the GC-FID method for analysis of residual solvents, again with the target *RSD* set at 10%. To enable comparison peak areas and peak heights were both considered as responses. *LOQ* was found to be close to 80 pg if peak areas were considered and 40 pg if peak heights were considered. Figure 6 confirms a difference can be observed between values obtained from peak areas and those obtained from peak heights. As a trend, use of peak heights resulted in a lower (i.e. better) *LOQ* for peaks with severe tailing, e.g. that of ethanol, or those, e.g. that of acetonitrile, on the tail of another peak. Otherwise peak areas gave slightly better or equivalent results. Anyway, values remained always of the same order of magnitude. *LOQ* based on the traditional signal-to-noise criterion were also obtained for information, with the limitations discussed above. This example demonstrated that the target *RSD* approach could also apply to GC-FID analysis, and that working with peak areas or peak heights did not give exactly the same results.

Conclusion

For determination of *LOD* and *LOQ* approaches based on the use of different means of estimating the standard deviation of the blank gave results not compatible with each other. This was true not only for the value itself but also for its reliability. The between-operators variability in the dispersion obtained by use of the traditional *S/N* ratio measurement, the most commonly used procedure, seemed, moreover, to be the very reason that must dissuade any reasonable analyst from relying on this approach only. Indeed, the lack of compatibility of all the approaches implied the *LOD* or *LOQ* values obtained could not be compared or discussed if the method of estimation was not clearly specified. As a consequence, and for the sake of simplicity, we recommend an alternative and absolute method based on the chosen target *RSD* value to define the *LOQ* level. Now the meaning and reliability of *LOQ* value are quite unambiguous – the *LOQ* is the minimum quantity that can be measured with a precision equal to the target *RSD*. Further rigorous comparisons could henceforth be performed without any problem. *LOD* can then be defined as three tenths of the *LOQ*, to preserve the usual ratio of *LOQ* to *LOD*. The applicability of the target *RSD* approach was demonstrated not only in LC-UV but also in LC-MS and GC-FID, which augurs well for the general applicability of this approach to other analytical techniques.

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