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Spotlights

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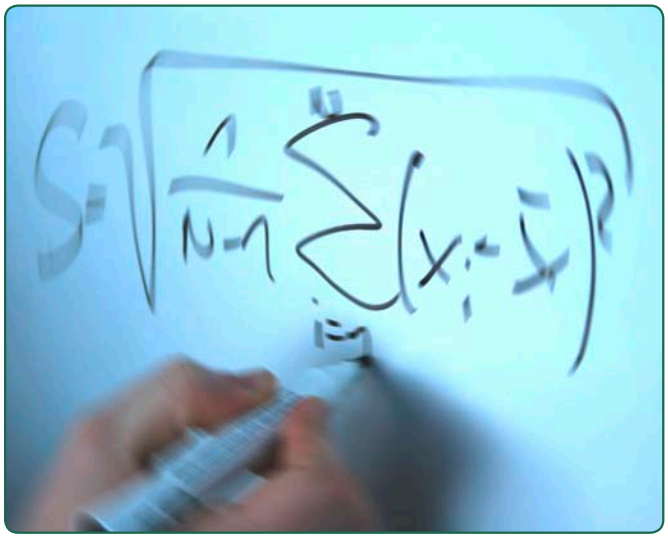
> EDITORIAL

Laboratories provide analytical results to their customers. These results often have to meet international regulations. A method validation is an internationally recognized tool, applied by laboratories, to assure the quality of the analysis' results. It is often part of the laboratory's quality management system.

Eurachem states that "the laboratory and its staff have clear responsibility to justify the customer's trust by providing the right answer to the analytical part of the problem."[1] This means it is the obligation of the laboratory to ensure that the provided results are accurate and reliable. To fulfill these requirements, the laboratory uses quality assurance, quality control and analytical method validation systems.

Romer Labs®, the supplier for food safety testing solutions with worldwide expertise, aims to support its customers with this guideline to analytical method validation. This guide will show the customer an in-house validation process and helps to assure accurate and reliable results when Romer Labs® products are being used.

Alois Schiessl



This Romer Validation Guide is a helpful tool when implementing an HPLC method validation with IAC or SPE cleanup. The information provided in this document can be considered as a working protocol including the most important steps for a validation. The procedures are kept in general, therefore a few modifications may be necessary when applying this guide to different mycotoxins (e.g. adaptation of the spiking levels for different toxins – depending on regulatory limits).

Romer Validation Guide

When validating an IAC or an SPE cleanup column in combination with an HPLC analysis the following parameters have to be obtained and checked:

- LOD and LOQ
- Linearity and Range
- Specificity
- Precision and Robustness
- Accuracy and Trueness

Definitions

LOD and LOQ:

The limit of detection (LOD) is defined as "the smallest concentration or amount of an analyte that can be reliably shown to be present or measured under defined conditions; the smallest amount that is clearly distinguishable from background or 'blank". Definition for

[1] *EURACHEM Working Group: The Fitness for Purpose of Analytical Methods. A Laboratory Guide to Method Validation and Related Topics. First Internet Version, December 1998, http://www.eurachem.org/guides/pdf/valid.pdf, last accessed 24.01.2011*

the limit of quantification (LOQ) "... defines the point at which the analysis becomes just feasible"[2].

Linearity and Range:
Linearity describes the correlation to a regression line that has been fitted to the acquired data. The range is any value that lies between the lowest concentration that can be accurately measured and the highest concentration that can be accurately measured.

Specificity:
Specificity means the ability of a method to distinguish between the analyte being measured and other substances. This characteristic is predominantly a function of the measuring technique described, but can vary according to class of compound or matrix[3].

Precision and Robustness:
Precision means the closeness of agreement between independent test results obtained under stipulated (predetermined) conditions. The measure of precision usually is expressed in terms of imprecision and computed as standard deviation of the test result. Less precision is determined by a larger standard deviation[4].

Accuracy and Trueness:
Trueness is described as closeness of agreement between the average of an infinite number of replicate measured quantity values and a reference quantity value[5]. Also ISO 5725[6] describes how to get these values.

Working Procedures

Calibration without Matrix:
The calibration which is used for result interpretation needs to be up-to-date (a new calibration is ideal for a new validation). To attain the calibration curve a set of five different standard levels (e.g. using Biopure™ standards) is measured in triplicates. These five levels should be evenly distributed over the targeted quantitation range.

Specificity:
Before working with matrix, the specificity of the HPLC method needs to be checked. An HPLC method is specific if the chromatogram is free of any interfering substances. This can be checked visually by comparing a blank chromatogram and a low level spike chromatogram. The target analyte must be a clear peak without any interference.

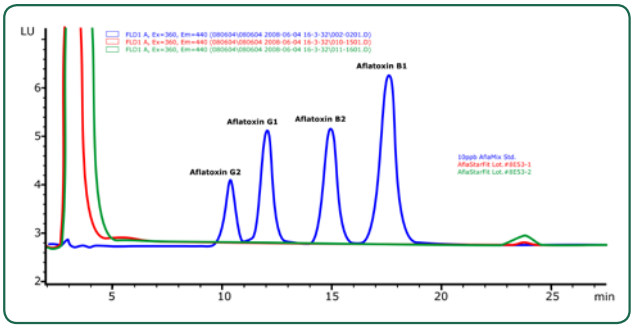


Figure 1: *HPLC chromatogram using an aflatoxin mix showing good specificity*

Calibration with Matrix:
For a matrix validation three spiking levels (low, medium and high level – absolute values depend on regulation requirements) should be performed. For a statistical interpretation a minimum of 6 replicates per level is a must (ideally 6 separate matrix spikes per level). To test the robustness of a method these 6 replicates should be analyzed by 2 different analysts over 2 separate days. For example both analysts A and B analyze 6 spikes per level on day 1 and 6 spikes per level on day 2.

Ring trials:
To determine the trueness of a method the participation in a ring trial is essential. If there is no available ring trial at the time of validation, the characterization of a matrix reference material (e.g. Biopure™ MRMs) can be performed too. Again, a minimum of 6 replicates is necessary for interpretation.

Calculations

Before starting the calculation, all data points (per spiking level) need to be checked for normal distribution and outliers. The quickest way of doing this would be by applying a validation software, such as Validat 2007 by ICD (http://www.icd.eu/seiten/english/produkte_validat_validat_idx.html) or SQS 2010 by Joachim Kleiner (http://www.kleiner-j.de/Download/SQS2010_Flyer.pdf).
Alternatively the normal distribution of the data points can be checked with a "David-Test". The test value G is calculated with R/s, where R is the difference between the highest and the lowest data point and s the standard deviation calculated according to the formulas found in chapter "LOD and LOQ".

$$G = \frac{R}{s} \qquad Z_{min} < G < Z_{max}$$

The test value should then fit into the upper and lower border Z_{min} and Z_{max} of the David Test (can be found in the "David-table"); typically at a 95 % confidence level.
Outliers can for example be checked with the "Grubbs Outlier Test". Typically the two data points (suspicious values) which are the farthest off the mean value are checked for being an outlier. The test value G is calculated according to the following formula.

$$G = \frac{\left| \bar{x} - x_{susp} \right|}{s}$$

$$Z_A = \frac{N-1}{\sqrt{N}} \sqrt{\frac{\frac{t^2\alpha}{2N}\,N-2}{N-2+r^2\alpha_{\frac{}{2N}N-2}}} \qquad G < Z_A$$

If the calculated test value G is lower than the Grubbs test value z_A (calculated with t-values from the t-table) the analyzed data point is not an outlier. When analyzing more than 6 data points, an outlier can be eliminated.

LOD and LOQ:
Mean value and standard deviation of the 6 data points from the low level matrix spike are calculated according to the formulas found below. The low level spike should be as close as possible to the expected LOD of the method. If it is much higher an additional (low level) matrix spike can be performed.

Mean Value: $\bar{x} = \frac{1}{N} \sum_{i=1}^{N} x_i$ *N=6 for 6 replicates*

Standard Deviation: $s = \sqrt{\frac{1}{N-1} \sum_{i=1}^{N} (x_i - \bar{x})^2}$

The LOD and the LOQ are then calculated according to these formulas:

Limit of detection: $LOD = 3.3 * s$

Limit of quantification: $LOQ = 10 * s$

Linearity and Range:

First the calibration data without matrix is checked for linearity. This can be done with a validation software (e.g. Validat 2007, SQS 2010, etc.) or by visual interpretation. The target concentrations vs. the HPLC signals (area) are entered into a coordinate system. The calibration is linear if a straight line can be drawn through the data points.

Then the linearity of the matrix calibration needs to be checked. This is also done visually with a coordinate system. The spiking concentrations vs. the actual concentrations are entered into the coordinate system and a straight line is drawn through the data points. The range of quantitation is set to be within the linear section of the matrix calibration curve.

Precision and Robustness:

The precision and robustness can be measured in two levels, repeatable or reproducible conditions.

First, the relative standard deviation under repeatable conditions RSDr is calculated for each spiking level from standard deviations and mean values (one operator, same day) according to the following formula.

$$RSD_r = \frac{S_r}{X_r} * 100$$

Then the relative standard deviation under reproducible conditions RSDR is calculated using data points from different analysts, different days and different laboratories (e.g. ring trial).

$$RSD_R = \frac{S_R}{X_R} * 100$$

The RSD_r and RSD_R values are then compared to the requirements found in the European Commission Regulation (EC) 401/2006[7].

To ensure the robustness of the method all possible variations should be checked. Possible variations are

- Different lots of HPLC columns and cleanup columns
- Different instruments (e.g. 2 HPLC systems)
- Different lots of Reference Materials, solvents, consumables
- Different room temperatures

Accuracy and Trueness:

The accuracy is determined by calculating the spiking recovery (in %). For that the target concentrations versus actual concentrations are entered into a coordinate system. Then a straight line (the recovery function) is drawn through the data points. The slope of the function is determined which equals the overall method recovery for the matrix spike. This recovery is used to correct the results of all samples which are analyzed with this validated method.

To determine the trueness of the measurement results the method is checked against a ring trial material or a certified matrix reference material (CRM). The results are corrected with the recovery established in the previous step. An independent one-sample t-Test is used to compare the measured results to the assigned value of the CRM. The test value t is calculated according to the following formula.

$$t = \frac{\bar{x} - \mu_0}{\frac{s}{\sqrt{N}}}$$

μ_0... *CRM assigned value*

$$t < t(1 - \frac{\alpha}{2}, n-1)$$

1-a/2... two-tailed confidence level;
n-1... degrees of freedom

This calculated test value is compared to the t-test value (found in a t-table). The calculated test value must be lower than the t-test value to show no significant difference between the measured concentration and the assigned concentration of the CRM.

Quality Control Chart:

For long term performance testing it is mandatory to set up a control chart during or shortly after the validation process. The control chart should be based on naturally contaminated material with assured long term supply (e.g. Biopure™ Check Samples).

In a pre-period 20 replicates of the material are analyzed by different operators, on different instruments and different days. The 20 values are used to set up a control chart (mean ±2s and ±3s). These data points are then entered into the chart.

Whenever the method is performed one replicate of the naturally contaminated material is analyzed and entered into the chart. If it fits within the ±2s range the result is ok. If the result lies beyond the ±2s range (Warning limit) further investigation should be taken. If the result lies beyond the ±3s range (Action limit) immediate action is required. Every laboratory is required to define its own "out of control" situations and the corresponding consequences. The following rules are just an example.

Warning rule
(if occurring, then data requires further investigation):

- One control result beyond Warning Limit.

Rejection rules (if occurring, then data are rejected):

- One control result beyond Action Limit.
- Two successive control results beyond same Warning Limit.
- Ten successive control results are on the same side of the mean.
- Whenever results seem unlikely (plausibility check).

Measurement Uncertainty and Confidence Level:

Additionally, the measurement uncertainty (bias) can be determined using the quality control chart. The ±2s data is extracted from the chart and can be used as the measurement uncertainty.

Uncertainty: $U = 2 * s_{rec}$

Alternatively the measurement uncertainty can be calculated according to EUROCHEM / CITAC Guide "Quantifying Uncertainty in Analytical Measurement (QUAM)"[8].

The method confidence level is then defined as the mean value plus/ minus the measurement uncertainty.

Confidence Level = $\bar{x} \pm U$

Conclusion:

After fulfilling these validation criteria the new HPLC method with IAC or SPE cleanup is considered as an in-house validated method according to Romer Labs®.

[7] European Commission. Commission Regulation (EC) No 401/2006 of 23 February 2006 laying down the methods of sampling and analysis for the official control of the levels of mycotoxins in foodstuffs. Official Journal of the European Union, L70, 12-34, 2006.

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Disclaimer:

Many guides are available for validations. Some use different numbers of replicates, some use different formulas to calculate certain parameters like e.g. LOD and LOQ. Romer Labs® validation guide gives recommendations based on the experiences of our own in-house service laboratories.
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David Table						
N	Lower border			Upper border		
	5 %	1 %	0.1 %	5 %	1 %	0.1 %
5	2.15	2.02	1.83	2.83	2.80	2.80
6	2.28	2.15	1.83	3.16	3.16	3.10
7	2.40	2.26	1.87	3.46	3.46	3.34
8	2.50	2.35	1.87	3.74	3.74	3.54
9	2.59	2.44	1.90	4.00	4.00	3.72
10	2.67	2.51	1.90	4.24	4.24	3.88
11	2.74	2.58	1.92	4.47	4.01	3.80
12	2.80	2.64	1.92	4.69	4.13	3.91
13	2.86	2.70	1.93	4.90	4.24	4.00
14	2.92	2.75	1.93	5.10	4.34	4.09
15	2.97	2.80	1.94	5.29	4.44	4.17
16	3.01	2.84	1.94	5.48	4.52	4.24
17	3.06	2.88	1.94	5.66	4.60	4.31
18	3.10	2.92	1.94	5.83	4.67	4.37
19	3.14	2.96	1.95	6.00	4.74	4.43
20	3.18	2.99	1.95	6.16	4.80	4.49
25	3.34	3.15	1.96	6.93	5.06	4.71
30	3.47	3.27	1.97	7.62	5.26	4.89
35	3.58	3.38	1.97	8.25	5.42	5.04
40	3.67	3.47	1.98	8.83	5.56	5.16
45	3.75	3.55	1.98	9.38	5.67	5.26
50	3.83	3.62	1.98	9.90	5.77	5.35
55	3.90	3.69	1.98	10.39	5.86	5.43
60	3.96	3.75	1.98	10.86	5.94	5.51
65	4.01	3.80	1.98	11.31	6.01	5.57
70	4.06	3.85	1.99	11.75	6.07	5.63
75	4.11	3.90	1.99	12.17	6.13	5.68
80	4.16	3.94	1.99	12.57	6.18	5.73
85	4.20	3.99	1.99	12.96	6.23	5.78
90	4.24	4.02	1.99	13.34	6.27	5.82
95	4.27	4.06	1.99	13.71	6.32	5.86
100	4.31	4.10	1.99	14.07	6.36	5.90
150	4.59	4.38	1.99	17.26	6.64	6.18
200	4.78	4.59	2.00	19.95	6.84	6.39
500	5.37	5.13	2.00	31.59	7.42	6.94

t-Table			
f	P=95 %	P=99 %	P=99,9 %
1	12.706	63.657	636.619
2	4.303	9.925	31.598
3	3.182	5.841	12.924
4	2.776	4.604	8.610
5	2.571	4.032	6.869
6	2.447	3.707	5.959
7	2.365	3.499	5.408
8	2.306	3.355	5.041
9	2.262	3.250	4.781
10	2.228	3.169	4.587
11	2.201	3.106	4.437
12	2.179	3.055	4.318
13	2.160	3.016	4.221
14	2.145	2.977	4.140
15	2.131	2.947	4.073
16	2.120	2.921	4.015
17	2.110	2.898	3.965
18	2.101	2.878	3.922
19	2.093	2.861	3.883
20	2.086	2.845	3.850
21	2.080	2.831	3.819
22	2.074	2.819	3.792
23	2.069	2.807	3.767
24	2.064	2.797	3.745
25	2.060	2.787	3.725
26	2.056	2.779	3.707
27	2.052	2.771	3.690
28	2.048	2.763	3.674
29	2.045	2.756	3.659
30	2.042	2.750	3.646
∞	1.960	2.576	3.291

[8] *Ellison S.L.R., M. Rosslein, A. Williams. EURACHEM/CITAC Guide - Quantifying Uncertainty in Analytical Measurement (EURACHEM / CITAC Guide CG 4), 2nd Edition, 2000*

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