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EURL Guidance Document on standard addition in the field of the analysis of residues of pharmaco-logically active substances

Contents

This document serves as a guidance to support official control laboratories in the harmonised application of standard addition approaches. Laboratories operating under Commission Implementing Regulation (EU) 2021/808 are **not obliged** to follow this guidance minutely; different approaches are acceptable, if they provide the same level and quality of information.

Abbreviations

CCα	Decision limit
CIR	Commission Implementing Regulation
CV	Coefficient of variation
LCL	Lowest calibrated level
ME	Matrix effect
ML	Maximum level

MMPR	Minimum method performance requirements
MRL	Maximum residue level
MSA	Multiple standard addition
RF	Response factor
RL	Relevant level
RM	Reference material
RPA	Reference point for action
SA	Standard addition
SAM	Standard addition method
SSA	Single standard addition

1. Introduction

Matrix effects arising from the presence of components other than the analyte of interest may in some cases severely hinder the quantification in a specific analyte-matrix combination. One approach, allowing for the correction for some of these effects, is the application of standard addition (SA). In addition, SA is useful for the quantification of endogenous analytes like testosterone or thiouracil. In SA, the sample is analysed as is, as well as fortified with the analyte(s) of interest at multiple concentration levels (henceforth called SA samples). The quantification is then possible through construction of a calibration curve using the results of all of these samples. Since the SA samples are prepared under exactly the same conditions as the actual sample with regards to the matrix, some of the matrix effects (MEs) of this specific analyte-matrix combination are automatically corrected for.

This guidance describes in which cases SA can be applied (non-exhaustive list), how the SA-approach is applied, which quality control measures should be considered, as well as how to estimate an adapted CCα (if relevant).

2. Scope of standard addition

In order for SA to yield reliable results, a basic understanding of the analyte behaviour is necessary. Therefore, a validated physico-chemical analytical method (qualitative or quantitative, screening or confirmatory) covering the analyte(s) of interest should be applied. If no validated method for the analyte(s) in the same matrix as the sample to be investigated is available, the use of methods validated for similar matrices is possible. The decision for any analytical method should be guided by expert opinion and extra care in the evaluation of the quality control samples should be taken in these cases.

It is worth noting that SA can only correct for rotational ME[1-2]. With this type of ME, the size of the analyte signal is influenced by the general presence of compounds in the sample. The effect is usually dependent on the analyte concentration and therefore influences the slope of the overall calibration curve. In contrast, translational MEs, caused by interfering signals co-eluting with the analyte(s) of interest, cannot be corrected for by the application of SA. The influence of these MEs can be understood as an offset in the calibration curve and hence changes the intercept of the overall calibration curve. In

order for SA to work properly, any significant translational ME needs to be eliminated, for example by modification of the sample preparation procedure or the chromatographic separation (within the scope of the underlying validated analytical method). More details and figures can be found in the literature[1-2].

If the aforementioned prerequisites are fulfilled, SA can be used for screening, as well as confirmation purposes, including quantitation. SA's fields of application pertain to specialties of the matrix, to the analytical method to be applied and to the concentration of the analyte(s) of interest. Applications include (but are not limited to) the following cases:

- unique or exotic matrix (e. g. crocodile muscle)
- lack of blank matrix for the preparation of a matrix calibration (e. g. with endogenous hormones)
- Not fully identified matrix (e. g. fish species)
- degraded matrix (e. g. muscle deteriorated due to delay in shipment; in agreement with laboratory policy)
- matrix with significant ME (e. g. feed, processed foods)
- analytical method is in principle suitable for the relevant analyte(s), but the quantitative method validation parameters do not comply with all of the requirements prescribed by CIR 2021/808
- lack of a suitable internal standard
- analyte concentration(s) significantly above the validated concentration range are expected

3. Single standard addition for semi-quantitative screening purposes

Single standard addition (SSA) can be applied when a sample is to be screened for the presence and/or the semi-quantitative determination of an analyte/analytes of interest (the use of SSA is not limited to these two cases). For SSA, weigh a minimum of two aliquots of the sample to be investigated. At least one aliquot is analysed as is and at least one other aliquot is fortified with the relevant analyte(s) at a relevant level (RL). It is recommended to fortify the samples prior to the sample preparation procedure. A suitable internal standard may also be added, in order to account for any analyte loss during the sample preparation procedure. When the amount of analyte(s) in the sample is unknown, it can be difficult to decide on an adequate fortification level. In this case, it can be an option to fortify with an amount representing the legal limit (if applicable) or corresponding to the middle of the validated linear range.

For the evaluation of the SSA, record the signal areas attributed to the analyte(s) of interest, or the ratio of the analyte area and the area of the internal standard (if using) in the unfortified and fortified samples. The concentration of the analyte(s) in the investigated sample for such a single point calibration can be calculated as:

$$c_0 = \frac{Area_0}{Area_f - Area_0} \cdot c \qquad (1)$$

With

c_0 analyte concentration in sample of interest

$Area_0$	signal area of analyte in sample of interest without fortification
$Area_f$	signal area of analyte in fortified sample
c	fortified concentration of analyte

If an internal standard is used, $Area_0$ and $Area_f$ are to be substituted by the ratio of the signal of the analyte of interest and the signal of the corresponding internal standard in the fortified and unfortified sample (response factor RF).

4. Multiple standard addition for quantitative, confirmatory purposes

If an approximate concentration of the analyte(s) of interest is already known and a more accurate quantitative result is needed, it is advised to perform SA on multiple levels (multiple standard addition, MSA). This approach also allows for an estimation of an adapted CCα. For MSA weigh the sample in several aliquots (at least 5 aliquots including one unfortified and 4 fortified are recommended). At least one aliquot is analysed as is and the other aliquots are fortified with increasing amounts of the relevant analyte(s). It is recommended to fortify the samples prior to the sample preparation procedure. The amount of analyte added to the aliquots should be adapted to the estimated concentration in the sample. If available, a suitable internal standard should be added to each aliquot, so as to account for any loss of analyte(s) during the sample preparation procedure. For the evaluation of the MSA, record the signal areas attributed to the analyte(s) of interest, or the ratio of the analyte area and the area of the internal standard (if using) in the unfortified and fortified samples. Perform a linear regression on the signal areas (or ratios of analyte signal area and internal standard signal area) and the fortified concentrations. The concentration in the unfortified sample is then identified as:

$$c_0 = \left|-\frac{b}{a}\right| \quad (2)$$

With

c_0	analyte concentration in sample of interest
b	intercept of the MSA calibration curve
a	slope of the MSA calibration curve

5. Quality control

A usual set of quality control samples (at least a blank and fortified sample) as described in the respective EURL Guidance Document[3] should be analysed together with the SA samples (see Annex 1).

For all analytes of interest, the requirements of CIR 2021/808 with regard to chromatographic separation, mass spectrometry performance and identification (confirmatory methods only) need to be respected for all relevant samples included in an analytical series.

The quality control parameters of highest importance for SA are the criteria mentioned in CIR 2021/808 in addition to the sufficient linearity and/or repeatability (Annex 2) of the MSA calibration curve (confirmatory methods) according to specific laboratory requirements.

Furthermore, the following quality control samples with relation to trueness/recovery are recommended:

5.1. SA on a sample of known concentration

Choose a sample with a matrix as similar as possible to the sample the SA is performed for, incurred with residues of the analyte(s) of interest, for which the concentration has already been determined (ideally a (inhouse) reference material); analyse one aliquot of this sample as is and at least one more aliquot fortified with the analyte(s) of interest (see option 1 in Annex 1). If the result calculated from the SA is in agreement with the previously determined analyte content, this supports the claim, that the incurred residues of the analyte of interest behave sufficiently similar to the fortified analyte portion.

5.2. Fortification with surrogate compound(s)

In addition to the fortification of the aliquots with the analyte(s) of interest, fortify each of the samples with increasing concentrations of an analyte exhibiting a similar physico-chemical behaviour. Usually, this would be a compound structurally-related to the analyte(s) of interest (e. g. sample is suspect for tylosin, add tilmicosin as a proxy substance, see option 2 in Annex 1). Make sure that the structurally-related analyte does not interfere with the analysis. Whether or not a compound might be a useful surrogate can be assessed by a comparison of the validation parameters for the analyte(s) of interest and the candidate substance.

Example:

A sample is suspect for the presence of tylosin at around 1·MRL. Therefore, one aliquot of the sample would be analysed as is and four other aliquots would be fortified with tylosin at different levels (denoted aliquots A-E in the table). As a proxy for tylosin, all aliquots, including the original sample not fortified with tylosin, are also fortified with the structurally-related substance tilmicosin as given in the table below:

Aliquot	Tylosin	Tilmicosin	Internal standard (if applicable)
A	-	1.0·MRL	yes
B	0.5·MRL	1.0·MRL + 0.5·MRL	yes
C	1.0·MRL	1.0·MRL + 1.0·MRL	yes
D	1.5·MRL	1.0·MRL + 1.5·MRL	yes
E	2.0·MRL	1.0·MRL + 2.0·MRL	yes

The proof of the validity of the experiment can be assessed by calculating the concentration of tilmicosin in sample A. If the concentration for tilmicosin calculated from the linear regression overall results as given in (2), corresponds to the known actual concentration (1·MRL) in sample A within the accuracy limits according to laboratory policy, it can be inferred that the calculated result for tylosin is also credible. This quality control measure is especially useful for sample matrices not previously included within the validated scope.

6. Calculation of CCα

There are different possibilities for attributing a decision limit for confirmation (CCα) to a quantification by MSA. The straightforward approach would be to refer to the CCα determined for the underlying

validated method. This is easily acceptable, if the matrix of the sample of interest falls within the scope of the validated method.

If the MSA experiments are based on a validated analytical method, which does not cover the matrix of the sample of interest, it can be difficult to justify the transfer of the initially determined CCα to the MSA at hand – Even more so, if the validation of the analytical method covers only a single matrix/species. This is due to the lack of experimental data demonstrating a similar behaviour of the analyte(s) in the matrix of the sample of interest. An alternative for the derivation of a valid CCα is based on Method 2 (authorised substances, considering the MRL/ML) and Method 3 (unauthorised and prohibited substances, considering the LCL) given in paragraph 2.6 of CIR 2021/808, respectively. In these approaches, the calculation of CCα starts by checking whether the repeatability of the calculated concentration is within the repeatability criteria of CIR 2021/808 (Table 2, ⅔ of the given CVs). If the repeatability is within limits, CCα can be estimated by Equations 3 or 4, where the maximum with-in-laboratory reproducibility s_{RL_MAX} is deducted from the maximum CV (Table 2, CIR 2021/808):

$$CC\alpha = MRL\ (or\ ML) + k(one - sided,\ 95\ \%) \cdot s_{RL\_MAX} \quad (3)$$

$$CC\alpha = LCL + k(one - sided,\ 99\ \%) \cdot s_{RL\_MAX} \quad (4)$$

With

MRL	maximum residue level
ML	maximum level
LCL	lowest calibrated level
k	coverage factor
s_{RL_MAX}	maximum standard deviation at RL according to Table 2 of CIR 2021/808

An example of this approach is presented in Annex 2. A CCα is usually calculated such that it considers the within-laboratory reproducibility, thereby representing a range of influences on the measurement results for example caused by intra-species and also inter-species variances (the latter only, if the method scope covers several species). In consequence, the derivation of a CCα using either of the two approaches presented above, would usually overestimate the actual CCα, because MSA negates rotational matrix effects and is only performed on a single sample within a single analytical series. However, it is guaranteed, that the α-error requirement is fulfilled and these approaches are therefore acceptable. Additionally, both approaches presented in this section offer the advantage that the CCα remains constant and hence the decision regarding the presence of an analyte above or below a concentration threshold is coherent within different analytical series and not dependant of the instrument, the operator or the day of analysis.

References

[1] M. Thompson, S.L.R. Ellison, *Accreditation and Quality Assurance* 2005, 10, 82-97.

[2] S.L.R. Ellison, M. Thompson, *Analyst* 2008, 133, 992-997.

[3] EURL Guidance Document on the quality control during routine analysis (ongoing method performance verification), Version 1.1, October 2020.

[4] G. Bagur, M. Sanchez-Vinas, D. Gazquex, M. Ortega, R. Romero, *Talanta* 2005, 66, 1168-1174.

Annex 1 Day-to-day approach for standard addition

For all cases, a quality check of the detection system should be prepared and evaluated.

SA for semi-quantitative screening purposes

	Fortification level
Sample as is	-
Sample + fortification at relevant level	Relevant level (RL)**
Negative control/blank sample*	-
Positive control/fortified sample*	RL

*matrix closely related to the sample to be analysed, preferably a reference material RM

**RL can be MRL, ML, RPA, MMPR or other

SA for quantitative, confirmatory purposes

Option 1: Reference material (RM) of closely related matrix is available

	Fortification level
Sample as is	-
Sample + fortification nr 1	0.5·screening concentration
Sample + fortification nr 2	1.0·screening concentration
Sample + fortification nr 3	1.5·screening concentration
Sample + fortification nr 4	2.0·screening concentration
RM as is	-
RM + fortification nr 1	0.5·previously determined concentration
RM + fortification nr 2*	1.0·previously determined concentration
RM + fortification nr 3	1.5·previously determined concentration
RM + fortification nr 4	2.0·previously determined concentration

* at least one fortification level is needed, the other three are optional

Option 2: No reference material is available, sample to be analysed also fortified with related analyte (example of tylosine and tilmicosin in chapter 'Quality Control')

	Fortification level of suspect analyte	Fortification level of related analyte
Sample as is	-	RL
Sample + fortification nr 1	0.5·screening concentration	RL + 0.5·RL
Sample + fortification nr 2	1.0·screening concentration	RL + 1.0·RL
Sample + fortification nr 3	1.5·screening concentration	RL + 1.5·RL
Sample + fortification nr 4	2.0·screening concentration	RL + 2.0·RL

Annex 2 Calculation of CCα after verification of repeatability

Before calculating the CCα of an analyte in a non-validated matrix, the standard deviation (s_s) of the determined concentration in the MSA-experiment can be calculated as follows[4]:

$$s_s = \frac{s_{res}}{a}\sqrt{\frac{1}{n} + \frac{\bar{y}^2}{a^2 \sum(x_i - \bar{x})^2}} \quad (5)$$

$$s_{res} = \sqrt{\frac{\sum\left(y_i - \mathrm{y_{est}}\right)^2}{n-2}} \quad (6)$$

With

s_s	standard deviation of the calculated concentration in the actual sample
s_{res}	standard deviation of the residuals in the regression analysis
a	slope of the MSA calibration curve
n	number of data points included in the MSA calibration curve
$\bar{y}$	mean of the analyte areas or response factors (if using internal standard) of the sample aliquots included in the MSA calibration curve (including the sample as is)
y_i	analyte areas or response factors (if using internal standard)
$\mathrm{y_{est}}$	analyte areas or response factors (if using internal standard) estimated from the calibration function
x_i	fortified concentrations of the individual sample aliquots included in the MSA calibration curve (including the sample as is)
$\bar{x}$	average of the concentrations of the individual sample aliquots included in the MSA calibration curve (including the sample as is).

With this s_s the repeatability of the calculated result can be determined by:

$$Repeatability\ of\ result = \frac{standard\ deviation\ of\ concentration\ (s_s)}{calculated\ concentration} \quad (7)$$

s_s should be ≤ ⅔·CV of the maximum acceptable reproducibility given in Table 2 of CIR 2021/808.

Example:

A porcine muscle sample is suspected to contain tulathromycine. The concentration shall be elucidated by MSA. Raw data are given in blue, parameters from Equations 5 and 6 are given in orange. Units have been omitted for clarity of presentation.

Spiked concentration tulathromycine ppb	Area analyte	Area internal standard	Response factor RF (area analyte/ area IS)	Spiked concentration – average concentration, squared
0	55230000	3344000	16.5	$=(0-1080)^2$ =1166400
300	68290000	3007000	22.7	$=(300-1080)^2$ =608400
600	83870000	3058000	27.4	230400
1500	112000000	2677000	41.8	176400
3000	158500000	2619000	60.5	3686400

$n = 5, \bar{x} = 1080$	$\bar{y}^2 = 1143$ $\sum(x_i - \bar{x})^2 = 5868000$

Slope a	0.0145
Intercept b	18.1
correlation	0.997

$$c_0 = \left|-\frac{b}{a}\right| = \frac{18.1}{0.0145} = 1252\ \mu g/kg$$

From equation (2)

Spiked concentration tulathromycine ppb	Actual response factor RF	Calculated response factor RF	(RF–calculated RF)²
0	16.5	=0.014*0 +18.1=18.1	2.66
300	22.7	=0.014*300 +18.1=22.5	0.05
600	27.4	26.8	0.34
1500	41.8	39.9	3.80
3000	60.5	61.6	1.24

sum	8.08
s_{res}	$=\sqrt{\left(\frac{8.08}{5-2}\right)} = 1.64$

$$s_s = \frac{s_{res}}{a}\sqrt{\frac{1}{n} + \frac{\bar{y}^2}{a^2 \sum(x_i - \bar{x})^2}} = \frac{1.64}{0.0145}\sqrt{\frac{1}{5} + \frac{1143}{0.0145^2 \cdot 5868000}} = 120\ \frac{\mu g}{kg}$$

From equation (5)

Repeatability of result	$= \frac{120\ \mu g/kg}{1252\ \mu g/kg} \sim 9.6\ \%$
Acceptable $CV_{repeatability}$:	$= \frac{2}{3} \cdot 16\ \% = 10.7\ \%$
	$9.6\ \% < 10.7\ \%$

From equation (7)

Repeatability is within limits

$$CC\alpha = MRL + 1.64 \cdot s_{RL\_MAX} = 800\ \frac{\mu g}{kg} + 1.64 \cdot 0.22 \cdot 800\ \frac{\mu g}{kg} = 1089\ \frac{\mu g}{kg}$$

Calculation of CCα using equation (3)

$$1248 \frac{\mu g}{kg} > 1089\ \frac{\mu g}{kg}$$

Sample is non-compliant