Bundesamt für
Verbraucherschutz und
Lebensmittelsicherheit

EURL Guidance on confirmatory method validation

The “alternative” validation approach

Contents

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Characteristics of the alternative approach

Experimental design based validation

- Factorial approach: controlled variation of selected factors
  - Statistically sound precision data (combination of factorial and random effects and estimation of its size)
  - Integrated ruggedness investigation
  - Stability investigations not included
- Validation of **concentration ranges** instead of distinct levels
- Efficient use of experiments / sample numbers for maximum information output
  - Precision, recovery, sensitivity, measurement uncertainty and critical concentrations determined simultaneously; (method optimisation potential)
- Applicable to semi-quantitative (screening) methods

4.2.1. Selection of analytes and concentration range

- **Selection of analytes**
  - Consideration of EURL recomendations (minimum required …)

- **Selection of concentration ranges**
  - Lowest fortification level should yield reliable signal
    - Requirements for confirmation do not need to be fulfilled at this level in all cases
  - Minimum of 5 different fortification levels is recommended

Residue	Concentration range
RPA	0.5 - 1.5 RPA
Unauthorised	1.0 - 3.0 LCL
Authorised	0.1 - 1.5 MRL/ML

4.2.2. Design of Experiments

- **Brainstorming (based on a method description)**
  - Which factors might have an influence on the result ?
  - Which factors might be controlled / set ?
  - Which factors are random ?

- **Types of Factors**
  - “design factors” (mainly method-specific)
  - “noise factors” (mainly sample-specific)

In general:

- design factors are parameters which can be defined in the method
- Noise factors may vary from analytical series to analytical series.

4.2.2. Design of Experiments

- **Examples of Factors and Factor Levels**
  - **Matrix**
    - Species, matrix (muscle, liver, plasma, …), fat content, …
  - **Measurement**
    - Instrument, injection volume, dilution, …
  - **Operator**
    - Familiar/unfamiliar with the method, A-team/B-team, …
  - **Sample preparation**
    - Lot/supplier of chemicals/cartridges, sample size, filtration, …
  - **Sample storage**
    - Storage conditions / storage duration of samples/extracts…
  - **Technical factors**
    - HPLC column (different manufactures, lot, age), evaporation devices, …

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Basis : Orthogonal Experimental Design

European Commission

Variation of 7 factors (A-F) at 2 levels (A/a, B/b, C/c, …)

		1	2	3	4	5	6	7	8
Factors	A	+	+	+	+	-	-	-	-
	B	+	+	-	-	+	+	-	-
	C	+	-	+	-	+	-	+	-
	D	+	+	-	-	-	-	+	+
	E	+	-	+	-	+	-	+	-
	F	+	-	-	+	+	-	-	+
	G	+	-	-	+	-	+	+	-
Effects		s	t	u	v	w	x	y	z

Runs (factor/level combinations)

Experimental Design

		Runs							
		1	2	3	4	5	6	7	8
Factors	A	+	+	+	+	-	-	-	-
	B	+	+	-	-	+	+	-	-
	C	+	-	+	-	+	-	+	-
	D	+	+	-	-	-	-	+	+
	E	+	-	+	-	+	-	+	-
	F	+	-	-	+	+	-	-	+
	G	+	-	-	+	-	+	+	-
Effects		s	t	u	v	w	x	y	z

Example:

$t = A \circ B \circ c \circ D \circ e \circ f \circ g$

To assess effect of a ⟶ A:

$(s+t+u+v) / 4 - (w+x+y+z) / 4$

Limited number of **experiments** but **maximised** number of investigated **effects!**

Study design : Example

Selected factors and factor levels

Factor		Level "+"	Level "-"
A	matrix	plasma	serum
B	species	pig	turkey
C	operator	unfamiliar	familiar
D	amount of matrix	2 g	1 g
E	storage of final extract	2-3 days of storage at +4 °C	immediate analysis
F	filtration	none	100 kDa
G	final volume	250 µL	150 µL

…. + analyte list and concentration range

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4.2.3 Validation Experiments

8 „runs“ (8 different factor level combinations)

- Random order to minimise influence of systematic effects
- recommendation: max. 2 runs per week

Validation series	Run	Matrix	Species	Operator	Amount of matrix	Storage of extract	Filtration	Final volume
1	run 04	plasma	turkey	familiar	1 g	immediate analysis	no	250 µL
2	run 08	serum	turkey	familiar	2 g	2-3 days of storage at +4 °C	no	150 µL
3	run 01	plasma	pig	unfamiliar	2 g	2-3 days of storage at +4 °C	no	250 µL
4	run 07	serum	turkey	unfamiliar	2 g	immediate analysis	yes	250 µL
5	run 02	plasma	pig	familiar	2 g	immediate analysis	yes	150 µL
6	run 06	serum	pig	familiar	1 g	2-3 days of storage at +4 °C	yes	250 µL
7	run 03	plasma	turkey	unfamiliar	1 g	2-3 days of storage at +4 °C	yes	150 µL
8	run 05	serum	pig	unfamiliar	1 g	immediate analysis	no	150 µL

4.2.4 Validation study and samples

Practical implementation : 8 validation „runs“

– Each run consists of : - Spiked matrix samples
- Calibration curve
- „QA samples“

Minimum required samples for one run (one validation series)

	# Samples	Performance characteristic
5 aliquots from 1 batch, fortified prior to extraction at 5 different levels#	**5**	within-lab reproducibility, repeatability, trueness, CCα, (CCβ‡), absolute recovery*, ruggedness
5 aliquots from 1 batch, fortified prior to extraction at 5 different levels#	**5**	matrix calibration curve
5 standard solutions#	**(5)**	standard calibration curve
1 matrix blank sample**	**1**	specificity / selectivity
1 matrix blank sample fortified with internal standard(s)	**1**	specificity / selectivity
1 matrix blank sample fortified with analyte(s) and internal standard(s) at a relevant level	**1**	relative matrix effect***
Total	**13 (18)**	For 8 runs : 104 (144) samples

4.2.4 Validation study and samples

Required samples / analysis

- Minimum of 104 (144) sample preparations for a full validation
- 9 -16 different blank matrix samples („batches“) required
- Minimum time of 4 weeks

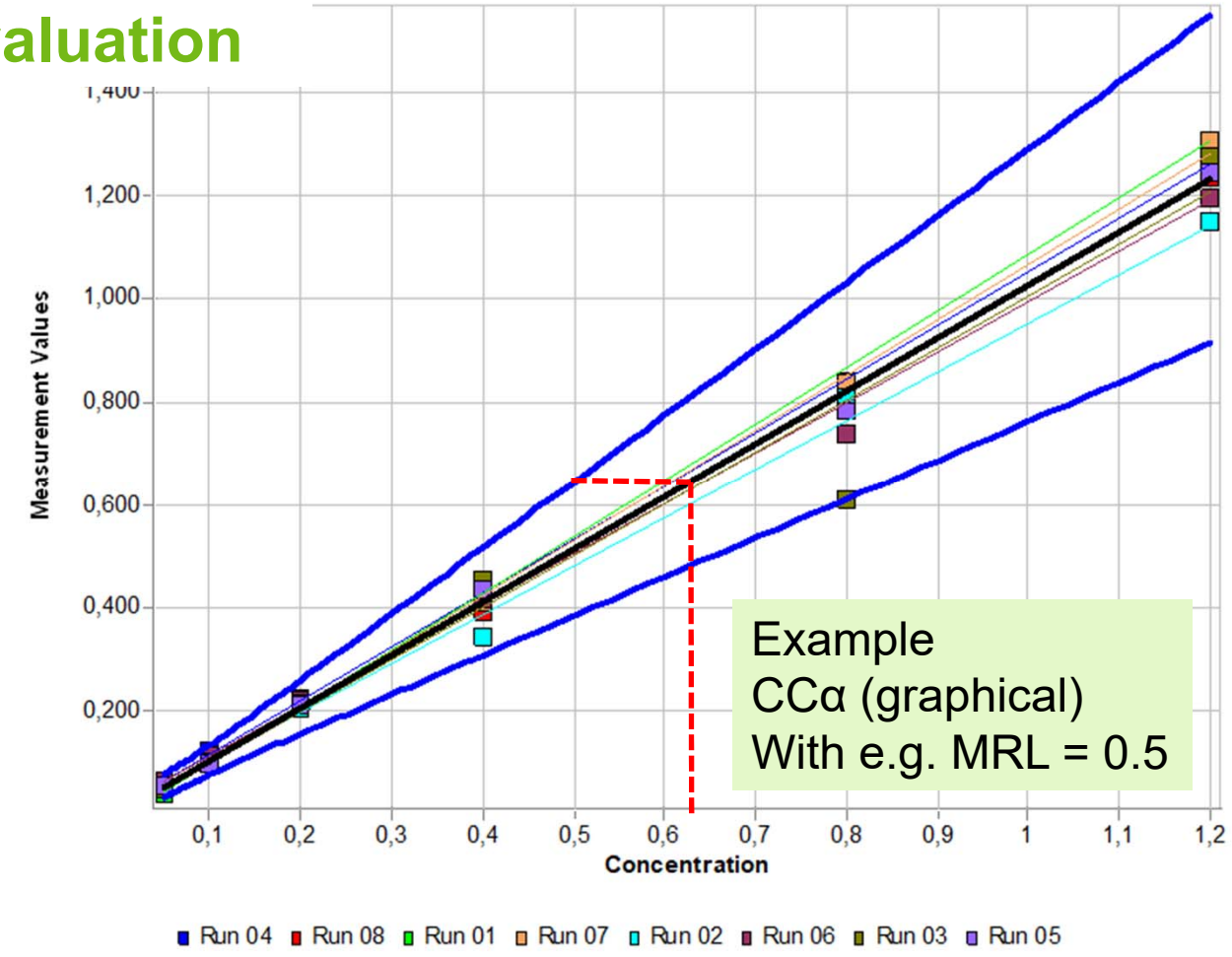
Data preparation

- Quantification of the samples against matrix/standard calibration
- Check of Fulfilment of confirmation critieria (RT, ion ratios) for each sample

4.2.5 Validation parameters

Example : Data evaluation

- „Calibration curves“ for each of the 8 runs
- Overall calibration curve
- Confidence interval
- Decision limit



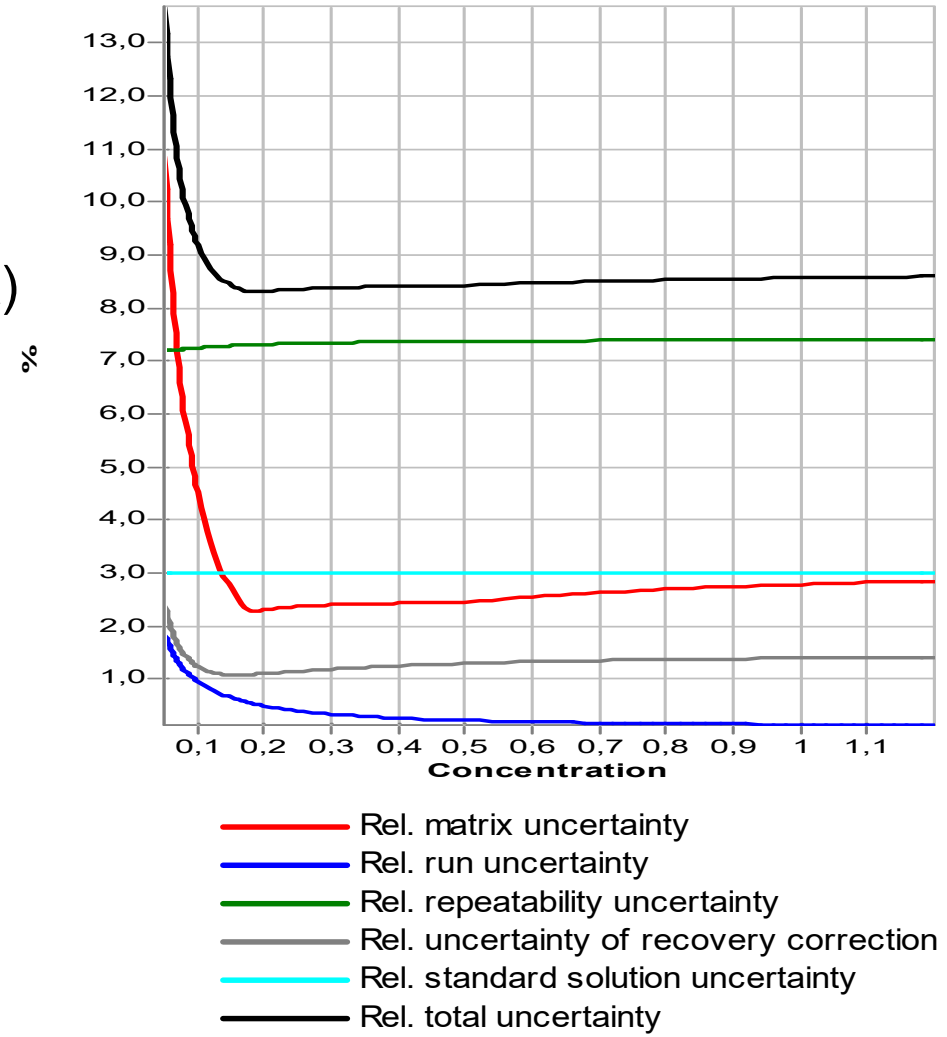
Calculation details : Gowik, P., Jülicher, B.,Uhlig, S. (1999) Analyst 124, 537 - Commercial software „Interval“

4.2.6 Interpretation of results

Further Data evaluation

Uncertainty contributions (concentration dependent)

- rel. total uncertainty u („within laboratory reproducibility“)
- Repeatability
- Additional uncertainty from standard solution uncertainty
- Matrix uncertainty
- (…)



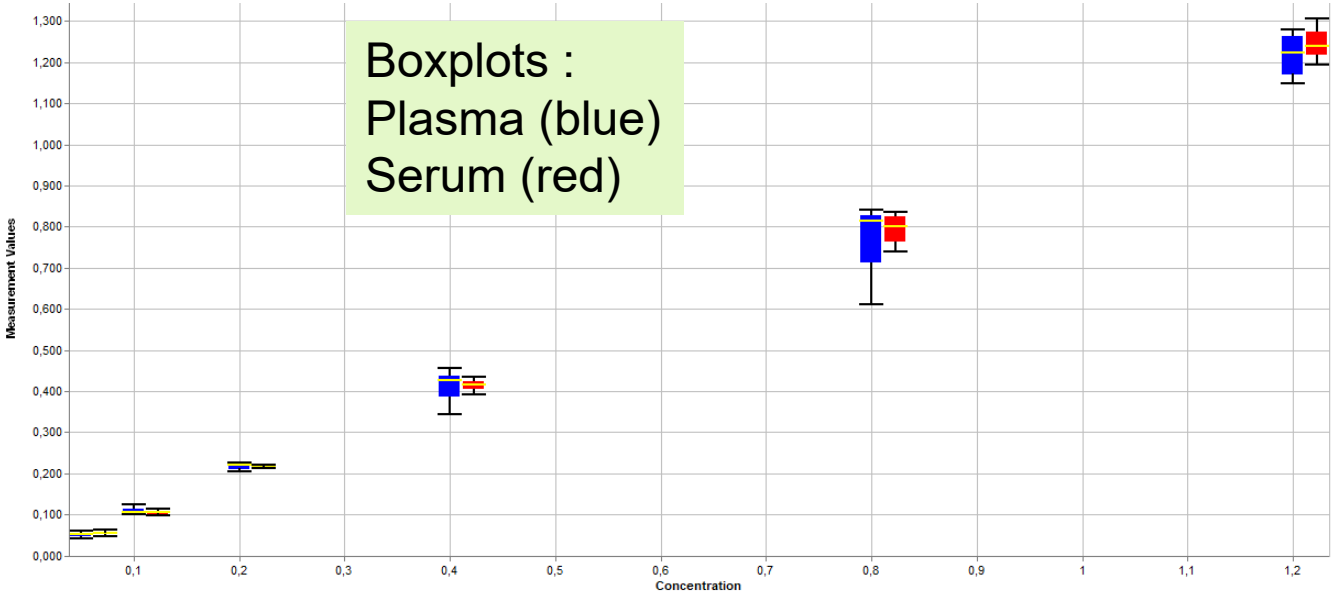
$$u(total)=\sqrt{\{(u_{matrix})^2+(u_{run})^2+(u_{repeatability})^2+(u_{recovery})^2+(u_{standard})^2\}}$$

4.2.6 Interpretation of results

„Bonus“ Data Evaluation

factorial effects

- Relative factor influences
- Graphical evaluation of each factor
- Overall factor evaluations



- Useful for method optimisations (extensions)

Factor	Level	slope deviation	Constant deviation
matrix	plasma (+); serum(-)	0.13	0.28
species	turkey(+); pig(-)	1.13	0.98
operator	unfamiliar (+); familiar(-)	-2.56	-1.18
amount of matrix	2 g(+); 1 g(-)	0.98	0.13
storage of extract	direct analysis(+); 2-3 days storage(-)	-0.23	-0.01
filtration	yes (+); no(-)	-2.25	-2.04
volume	200 uL (+); 120 uL final volume(-)	2.33	2.24

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Validation report and fitness for purpose

Decision limit, recovery, repeatability and in-house reproducibility

- evaluation of acceptance criteria for every analyte.
- Example : method performance data for the determination of metronidazole (MNZ) in plasma and serum

Analyte	Calibration interval	Number of values	CCα	Recovery [%] at CCα	Rel sR [%] at CCα
MNZ	0.050 - 1.200	48	0.072	107.0	10.7

= > requirements regarding the performance parameters are fulfilled)

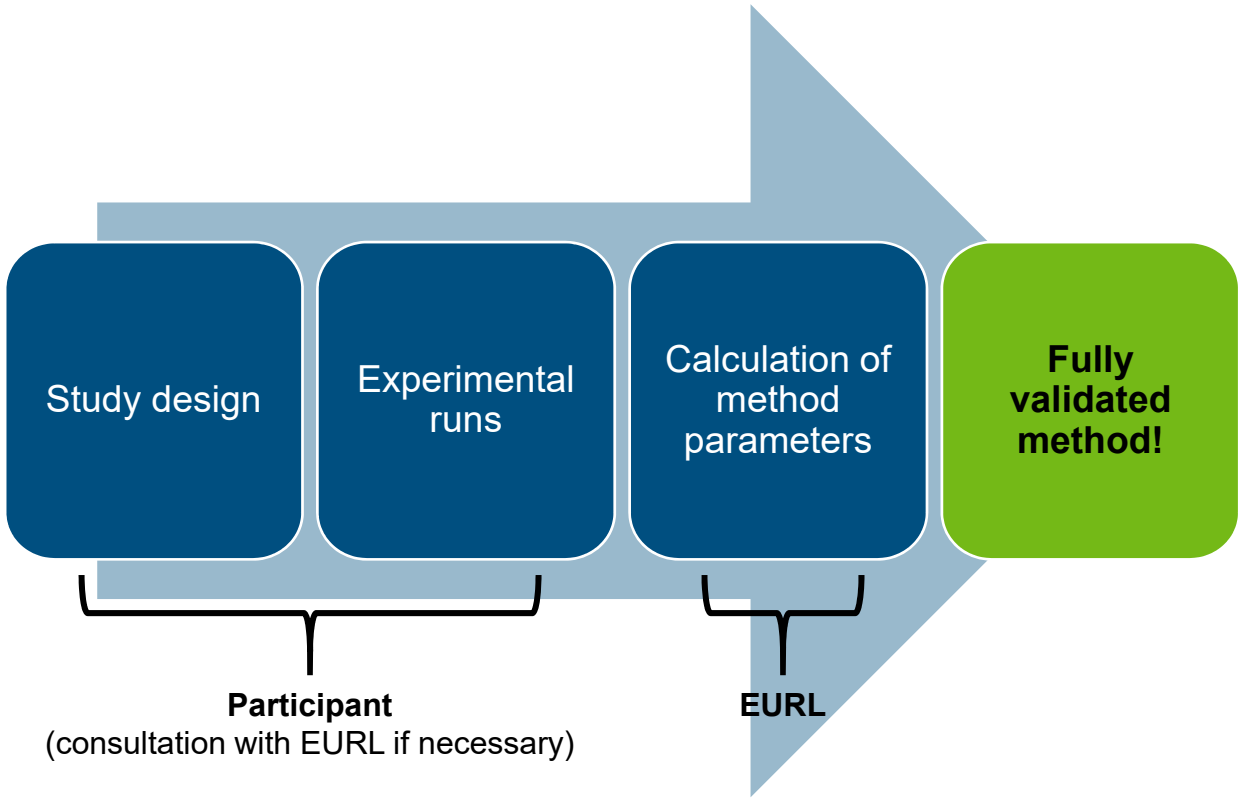
- **If acceptance criteria are not met**
  - Redefine **method applicability**
    (e.g. confirmation → screening, exclusion of certain analytes)
  - Continue method development

Fit for purpose ??

Performance characteristic	Acceptance criteria
Identification	Sufficient amount of identification points as derived from the measurement technique, see 1.2.3.3, Annex of Commission Implementing Regulation (EU) No 11188/2018
CCα	No numerical criteria -authorised substances: higher than but as close to the MRL / ML as analytically achievable -prohibited / unauthorised substances with RPA: lower than or equal to the RPA -prohibited / unauthorised substances without RPA: as low as analytically achievable
CCβ	No numerical criteria -authorised substances: lower than or equal to the MRL / ML -prohibited / unauthorised substances with RPA: lower than or equal to the RPA -prohibited / unauthorised substances without RPA: as low as analytically achievable
Precision	Concentration dependant, see 1.2.2.2, Annex of Commission Implementing Regulation (EU) No 11188/2018
Trueness	Concentration dependant, see 1.2.2.1, Annex of Commission Implementing Regulation (EU) No 11188/2018
Stability	See 2.5
Relative matrix effect	See 2.10
Absolute recovery	No fixed criteria for absolute recovery, specificity/selectivity and ruggedness. The results for these parameters shall be evaluated using expert knowledge. The responsible scientist shall identify critical aspects which may require method improvements.
Specificity / selectivity	
Ruggedness	

4.2.7 EURL Service

- Design of a study using EURL template
- Prepared templates for methods validated in collaborative trials are available on the EURL website



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Thank you for your attention!

Thanks to the EURL team in Berlin

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