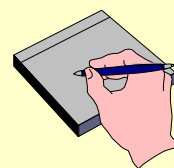
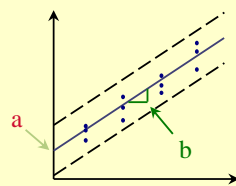
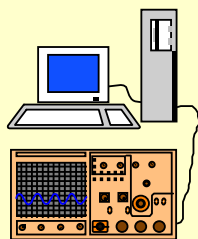


# Validation of Methods

## Fundamentals



Ralf Lippold

## Fundamentals (1)

- **Subject matter and scope (article 1)**
  - testing of official samples taken pursuant to article 15 (1) sentence 2 of DC 96/23/EC
  - This Decision shall not apply to substances for which more specific rules have been laid down in other Community legislation
- **Analytical methods (article 3)**
  - are documented in test instructions, preferably according to ISO 78-2
  - comply with Part 2 of the Annex to this Decision
  - validated according to the procedures described in Part 3 of the Annex
  - comply with the relevant minimum required performance limits (MRPL)
- **MRPL-values (article 4)**
  - establishing of **m**inimum **r**equired **p**erformance **l**imits (MRPL) of analytical methods to be used for substances for which no permitted limit has been established

## Fundamentals (2)

- **Quality control (article 5)**
  - The Member States shall ensure the quality of the results of the analysis of samples taken pursuant to Directive 96/23/EC, in particular by monitoring tests and/or calibration results according to chapter 5.9 of ISO 17025 (1)
- **Interpretation of results (article 6)**
  - The result of an analysis shall be considered non-compliant if the **decision limit** ( $CC\alpha$ ) of the confirmatory method for the analyte is exceeded
  - If a MRL has been established for a substance, the **decision limit** is the concentration **above** which it can be decided with a statistical certainty of  $1 - \alpha$  that the permitted limit has been truly exceeded ( $\alpha = 5\%$ )
  - If no permitted limit has been established for a substance, the **decision limit** is the lowest concentration level at which a method can discriminate with a statistical certainty of  $1 - \alpha$  that the particular analyte is present. ( $\alpha = 1\%$ )

## Fundamentals (3)

- **Detection capability ( $CC\beta - 1.12$ )**
  - Detection capability ( $CC\beta$ ) means the smallest content of the substance that may be detected, identified and/or quantified in a sample with an error probability of  $\beta$
  - MRL-substances: the detection capability is the concentration at which the method is able to detect MRL-concentrations with a statistical certainty of  $1 - \beta$  ( $\beta = 5\%$ )
  - Substances with no permitted limit: the detection capability is the lowest concentration at which a method is able to detect truly contaminated samples with a statistical certainty of  $1 - \beta$  ( $\beta = 5\%$ )
- **Alpha ( $\alpha - 1.2$ )**
  - error means the probability that the tested sample is compliant, even though a non-compliant measurement has been obtained ("**false non-compliant decision**")
- **Beta ( $\beta - 1.4$ )**
  - error means the probability that the tested sample is truly non-compliant, even though a compliant measurement has been obtained ("**false compliant decision**")
- **Handling of samples (2.1.1)**
  - Samples shall be obtained, handled and processed in such a way that there is a maximum chance of detecting the substance
  - Sample handling procedures shall prevent the possibility of accidental contamination or loss of analytes

## Fundamentals (4)

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- **Recovery (2.1.2.1)**
  - recovery shall be determined in each batch of samples, if a **fixed recovery correction factor** is used
  - If the recovery is within limits, the **fixed correction factor** may then be used
  - Otherwise the **recovery factor** obtained for that specific batch shall be used
  - unless the specific recovery factor of the analyte in the sample is to be applied in which case the standard addition procedure (see 3.5) or an internal standard shall be used for the quantitative determination of an analyte in a sample

## Fundamentals (4)

---

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  - unless the specific recovery factor of the analyte in the sample is to be applied in which case the standard addition procedure (see 3.5) or an internal standard shall be used for the quantitative determination of an analyte in a sample.
- **Consequence: all results must be corrected using the recovery rate!**

**Trueness of quantitative results (2.3.2.1)**

Minimum trueness of quantitative methods

Mass fraction	Range
$\leq 1 \mu\text{g/kg}$	-50% to +20%
$> 1 \mu\text{g/kg}$ to $10 \mu\text{g/kg}$	-30% to +10%
$\geq 10 \mu\text{g/kg}$	-20% to +10%

With certified reference materials (CRM)

If no CRM is available: recovery of additions of known amounts of the analyte(s) to a blank matrix

**Mass spectrometric detection (2.3.3.2)**

Maximum permitted tolerances for relative ion intensities using a range of mass spectrometric techniques

Relative intensity (% of base peak)	EI-GC-MS (relative)	CI-GC-MS, GC-MS <sup>n</sup> LC-MS, LC-MS <sup>n</sup> (relative)
>50%	$\pm 10\%$	$\pm 20\%$
> 20% - 50%	$\pm 15\%$	$\pm 25\%$
> 10% - 20%	$\pm 20\%$	$\pm 30\%$
$\leq 10\%$	$\pm 50\%$	$\pm 50\%$

## Performance Characteristics

- Validation shall demonstrate that the analytical method complies with the criteria applicable for the relevant performance characteristics

- Table 9 -

		Detection capability CC $\beta$	Decision Limit CC $\alpha$	Trueness/ Recovery	Precision	Selectivity/ Specificity	Applicability/ Ruggedness/ Stability
Qualitative methods	S	+	-	-	-	+	+
	C	+	+	-	-	+	+
Quantitative methods	S	+	-	-	+	+	+
	C	+	+	+	+	+	+

S = screening methods; C = confirmatory methods; + = determination is mandatory

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	C	+	+	-	-	+	+
Quantitative methods	s	+	-	-	+	+	+
	<b>C</b>	<b>+</b>	<b>+</b>	<b>+</b>	<b>+</b>	<b>+</b>	<b>+</b>

S = screening methods; C = confirmatory methods; + = determination is mandatory

## VALIDATION PROCEDURES

### ▪ Preamble to annex 1, chapter 3.1

This chapter provides examples and/or references for validation procedures of analytical methods. Other approaches to demonstrate that the analytical method complies with performance criteria for the performance characteristics may be used, provided that they achieve the same level and quality of information.

Validation can also be performed by conducting an interlaboratory study such as established by Codex Alimentarius, ISO or the IUPAC (12), or according to alternative methods such as single laboratory studies or in-house validation (13)(14). This part focuses on single laboratory studies (on in-house validation) using a *modular* approach. This approach consists of:

1. a set of common performance characteristics independent of the validation model used and
2. more specific model-dependent procedures as described in Table 10.

## VALIDATION PROCEDURES

Table 10  
Model-independent and model-dependent performance parameters

Validation		
Model-independent performance parameters	Model-dependent performance parameters	
Common performance characteristics (3.1.1.)	Conventional validation approach (3.1.2.)	In-house validation approach (3.1.3.)
Specificity	Recovery	Recovery
Trueness	Repeatability	Repeatability
Ruggedness: minor	Within-laboratory	Within-laboratory
Stability	Reproducibility	Reproducibility
	Decision limit (CC <sub>α</sub> )	Decision limit (CC <sub>α</sub> )
	Detection capability (CC <sub>β</sub> )	Detection capability (CC <sub>β</sub> )
	Calibration curves	Calibration curve
	Ruggedness: major changes	Ruggedness

## Model-independent performance characteristics (1)

---

- **Specificity (3.1.1.1)**
  - Analyse an appropriate number of representative blank samples ( $n \geq 20$ ) and check for any interferences (signals, peaks, ion traces) in the region of interest where the target analyte is expected to elute
  - Select a range of chemically related compounds (metabolites, derivatives, etc.) or other substances likely to be encountered with the compound of interest that may be present in the samples
- **Trueness (3.1.1.2 and 3.1.2.1)**
  - Analyse 6 replicates of a certified reference material (CRM)
  - If no CRM is available, instead of trueness, the recovery can be determined

## Model-independent performance characteristics (2)

---

- **Applicability/ruggedness (minor changes) (3.1.1.3)**
  - **Checks for ruggedness (major changes) should be carried out during the development of a method!**
  - Such studies use the deliberate introduction of minor reasonable variations by the laboratory and the observation of their consequences
  - Select factors of the sample pre-treatment, clean up and analysis, which may influence the measurement results. Such factors may include the analyst, the source and the age of reagents, solvents, standards and sample extracts, the rate of heating, the temperature, the pH-value as well as many other factors that may occur in the laboratory.

## Model-independent performance characteristics (3)

- **Applicability/ruggedness (minor changes) (3.1.1.3)**
  - Identify possible factors that could influence the results
  - Vary each factor slightly (in an order of magnitude that matches the deviations usually encountered among laboratories)
  - Conduct a ruggedness test using the approach of Youden
    - The Youden approach is a fractional factorial design - interactions between the different factors cannot be detected
  - Factors that significantly influence the results should be identified clearly in the method protocol
- After describing clearly the method protocol start performing a validation study

## Model-independent performance characteristics (4)

Table 11  
Experiment design for ruggedness studies (minor changes)

	Combination of Determinations No.							
Factor value F	1	2	3	4	5	6	7	8
A/a	A	A	A	A	a	a	a	a
B/b	B	B	b	b	B	B	b	b
C/c	C	c	C	c	C	c	C	c
D/d	D	D	d	d	d	d	D	D
E/e	E	e	E	e	e	E	e	E
F/f	F	f	f	F	F	f	f	F
G/g	G	g	g	G	g	G	G	g
Observed Result R	<b>S</b>	<b>T</b>	<b>U</b>	<b>V</b>	<b>W</b>	<b>X</b>	<b>Y</b>	<b>Z</b>

## Model-independent performance characteristics (4)

- **Stability (3.1.1.4) – Analyte in solution**

- Prepare fresh stock solutions, dilute and make aliquots
- Measure the analyte content in the freshly prepared solution according to the test instructions

**Scheme for the determination of analyte stability in solution**

	- 20°C	+ 4°C	+ 20°C
Dark	10 aliquots	10 aliquots	10 aliquots
Light			10 aliquots

- The storing time could be selected as 1, 2, 3, and 4 weeks or longer if necessary, e.g. until the first degradation phenomena are observable during identification and/or quantification
- -> The maximum storing time and the optimum storing conditions have to be recorded

## Model-independent performance characteristics (4)

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- -> The maximum storing time and the optimum storing conditions have to be recorded

## Model-independent performance characteristics (5)

---

- **Stability (3.1.1.4) – Analyte in solution**
  - Do it in routine: when ever preparing fresh stock solutions – compare it with your old solution
  
- **Stability (3.1.1.4) – Analyte in matrix**
  - Should be done with incured material
  - Impossible with spiked material
  - Should be a duty during registration



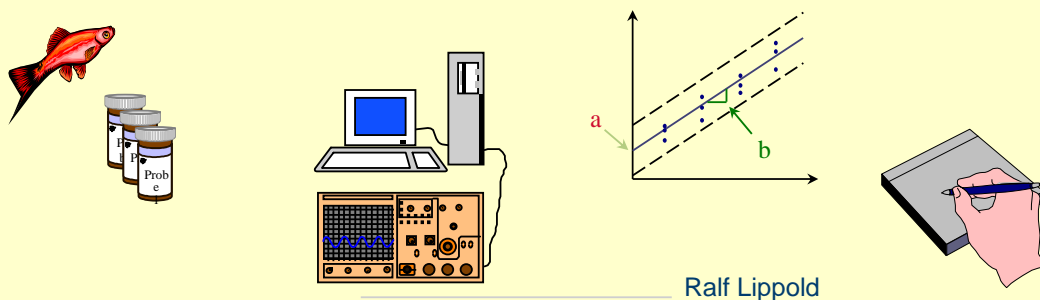
## Model-independent performance characteristics (6)

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- **Calibration curves (3.1.1.5)**
  - At least five levels (including zero) should be used
  - The working range of the curve should be described
  - The mathematical formula of the curve and the goodness-of-fit of the data to the curve should be described
  - Acceptability ranges for the parameters of the curve should be described

# Validation of Methods

## Classical Approach and Alternative Model



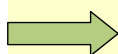
Ralf Lippold

## Conventional validation

Validation approaches, e.g. according to

- AOAC
- Codex alimentarius
- ISO Standards
  - ISO Guide 25
  - ISO 5725-2
  - ISO 11843

are based on repeatability and reproducibility standard deviation. They consider differences between identical samples only. All differences due to major changes (species, matrix) are considered systematic effects.



Consequence: Each matrix and each species has to be validated separately!

## Conventional validation procedures (1)

- **Recovery (3.1.2.1)**
  - Analyse 6 replicates of a certified reference material (CRM)
  - Select 18 aliquots of a blank material and fortify 6 aliquots at each
    - of 1, 1.5 and 2 times the minimum required performance limit
    - of 0.5, 1 and 1.5 times the permitted limit
  - Calculate recovery and cv
- **Recovery (standard addition method)**
  - The complete procedure for determination of the recovery by mean of the standard addition method is described in 3.5

## Conventional validation procedures (2)

- **Repeatability (3.1.2.2)**
  - Repeat the procedure for the recovery on at least two other occasions
  - Calculate the overall mean concentrations and CVs for the fortified samples
- **Within-laboratory reproducibility (3.1.2.3)**
  - Select 18 aliquots of a blank material and fortify 6 aliquots at each
    - of 1, 1.5 and 2 times the minimum required performance limit
    - of 0.5, 1 and 1.5 times the permitted limit
  - Repeat the procedure for the recovery on at least two other occasions (with different operators, equipment, samples...)
  - Calculate the mean concentration, standard deviation and the coefficient of variation (%) of the fortified samples

## Conventional validation procedures (3)

- **Reproducibility (3.1.2.4)**
  - participate in collaborative studies according to ISO 5725-2
- **Decision Limit ( $CC\alpha$ ) (3.1.2.5) – MRL components**
  - By the calibration curve procedure according to ISO 11843
    - blank material shall be used, which is fortified around the permitted limit in equidistant steps. Analyse the samples.
    - Plot the signal against the added concentration.
    - The corresponding concentration at the permitted limit plus 1.64 times the standard deviation of the within-laboratory reproducibility equals the decision limit ( $\alpha = 5\%$ )
  - Analyse at least 20 blank materials per matrix fortified with the analyte(s) at the permitted limit. The concentration at the permitted limit plus 1.64 times the corresponding standard deviation equal the decision limit ( $\alpha = 5\%$ )

## Conventional validation procedures (4)

- **Detection capability  $CC\beta$  (3.1.2.6) – MRL-Stoffe**
  - By the calibration curve procedure according to ISO 11843
    - blank material shall be used, which is fortified around the permitted limit in equidistant steps. Analyse the samples.
    - Plot the signal against the added concentration.
    - The corresponding concentration at the decision limit plus 1.64 times the standard deviation of the within-laboratory reproducibility equals the detection capability ( $\beta = 5\%$ )
  - Analyse at least 20 blank materials per matrix fortified with the analyte(s) at the decision limit. The concentration at the decision limit plus 1.64 times the corresponding standard deviation equal the detection capability ( $\beta = 5\%$ )



# Error probabilities

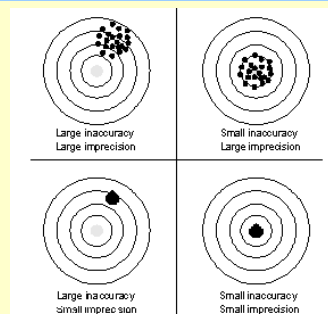
	decision: compliant (meas. value < $CC\alpha$ )	decis.: not compliant (meas. value > $CC\alpha$ )
true content: uncontaminated or below MRL/MPL, resp.	ok	error probability = $\alpha$ (false non-compliant decision)
true content: contaminated or above MRL/MPL, resp.	error probability = $\beta$ (depends on true content; false compliant decision)	ok

# Error probabilities depend on the calculation of accuracy and precision

Underlying model:

Measurement value  
= true value  
+ systematic error (Bias)      **⇒ Accuracy**  
+ random error                      **⇒ Precision**

Trueness = Accuracy+Precision



## Typical factors in food analyses

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- Species (e.g. fish, cattle, pig)
- Compartment (e.g. plasma, muscle, shrimps, mussels, liver, eggs, milk, honey)
- Condition of sample (fresh ... Not fresh)
- Homogenisation (lyophilised yes/no)
- Storage conditions (duration of storage, temperatures, frozen yes/no etc.)
- Storage of extract
- Operator
- Instruments
- Condition of columns
- ...

## The idea: systematic and simultaneous assessment of noise factors

---

In order to assess the impact of the noise factors to the precision of test results, a large number of measurements is required if the samples are selected randomly.



More cost-effective: systematic assessment of error (see EURACHEM Guide)

## Conventional versus alternative approach

### Conventional:

Random selection of samples

⇒ random variation of noise factors

⇒ Precision = random variation of measurement result

⇒ Many samples required

### Alternative:

Selection of samples by factorial design

⇒ systematic variation of noise factors

⇒ Precision = factorial effects + remaining random variation

⇒ Reduced number of samples

## Orthogonal designs

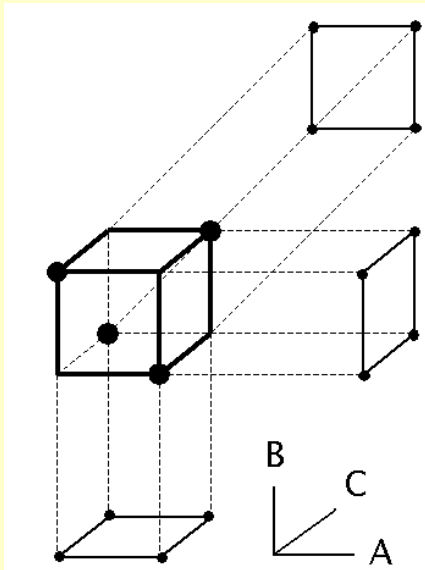
Simultaneous variation of several factors reduces experimental effort considerably.

But: with 7 factors each with 2 factor levels there are 128 different factor settings - far to much

Therefore a special selection of settings is required, according to the principle of orthogonality.

## Orthogonal designs

3 Factors A, B and C, each with 2 levels + und -



A	B	C
+	+	+
+	+	-
+	-	+
+	-	-
-	+	+
-	+	-
-	-	+
-	-	-

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## Orthogonal designs

7 Factors A, B, C, E, F, G each with 2 levels + and -

A	+	+	+	+	-	-	-	-
B	+	+	-	-	+	+	-	-
C	+	-	+	-	+	-	+	-
D	+	+	-	-	-	-	+	+
E	+	-	+	-	-	+	-	+
F	+	-	-	+	+	-	-	+
G	+	-	-	+	-	+	+	-

Each combination ++, +-, -+ and - of each factor pair appears twice.

This design is performed with 4 concentration levels, i.e.: **32 measurements in total** (plus 8 blanks)

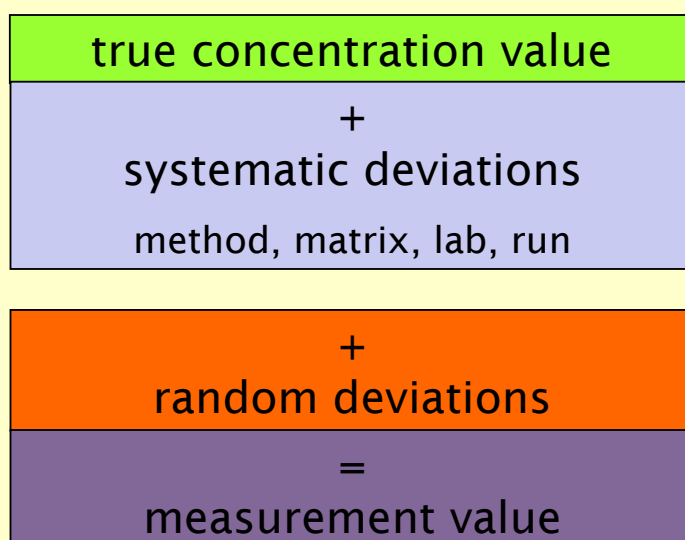
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# Principles of in-house validation experiments

- Factor levels shall be determined so that the full bandwidth of realistic conditions is covered, e.g.
  - operator: experienced / unexperienced
  - Storage of extracts: 0d / 3d
  - fat content: low / high
  - etc.
- Apart from one factor with up to 4 levels only 2 level per factor shall be used (proper definition of factors required)
- Randomisation of order of experiments to avoid effects of temporal trends.
- Not all experiments within one week

# Model components



## Example: Avermectines in Milk

- **Analytes**
  - Abamectine, Ivermectine, Doramectine, Moxidectine
  - Internal Standard: Nemadectine
- **Factors**
  - Staff (2 different persons)
  - Storage of samples in the laboratory, frozen (< 3 and > 5 weeks)
  - Measurement of the final extract (at the same day and after 2 days)
- **Concentration levels [ $\mu\text{g}/\text{kg}$ ]**
  - Abamectine, Ivermectine, Doramectine: 0, 1, 2, 3, 4
  - Moxidectine (MRL=40): 0, 20, 40, 60, 80
  - Internal Standard Nemadectine: 3

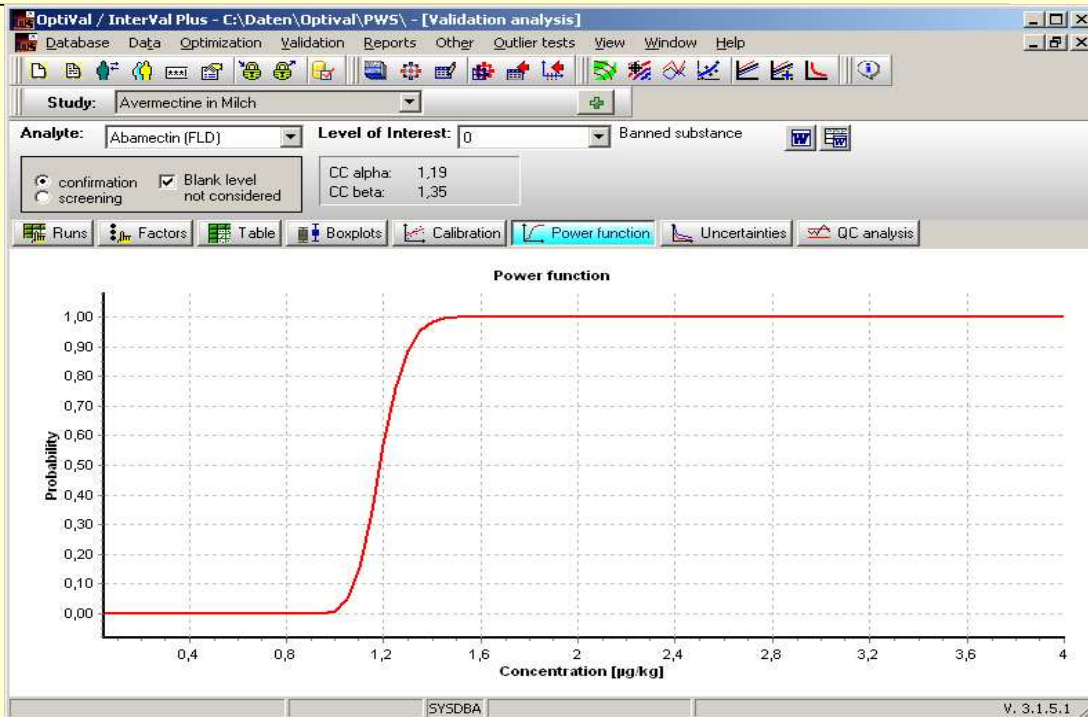
## Example: Avermectines in Milk

	A	B	C	D
2	RUN	Name	storage samples	measurement
3	1	staff 1	> 5 weeks	2 days
4	2	staff 1	> 5 weeks	at once
5	3	staff 1	< 3 Wochen	2 days
6	4	staff 1	< 3 Wochen	at once
7	5	staff 2	> 5 weeks	2 days
8	6	staff 2	> 5 weeks	at once
9	7	staff 2	< 3 Wochen	2 days
10	8	staff 2	< 3 Wochen	at once

	A	B	C	D
2	RUN	Name	storage samples	measurement
3	1	'+	'+	'+
4	2	'+	'+	'-
5	3	'+	'-	'+
6	4	'+	'-	'-
7	5	'-	'+	'+
8	6	'-	'+	'-
9	7	'-	'-	'+
10	8	'-	'-	'-



# Example: Avermectines in Milk



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## classic <-> inhouse

Overview	classical approach	inhouse
No. Samples	about 100	32
Matrices Liver / kidney	1	2
Species Cattle / swine	1	2
effective samples	about 100	8

High number of analyses for classical validation for 2002/657 can be reduced by inhouse-validation concept giving same results

(8 beef livers + 8 pork livers + 8 beef kidneys + 8 pork kidneys =  
32 samples / 8 per matrix – species)

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