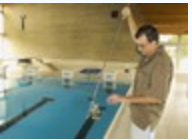




Validation of multiresidue methods

Beyond the validation CD 2002/657/EC



The “dynamic” range in multi-residue analysis



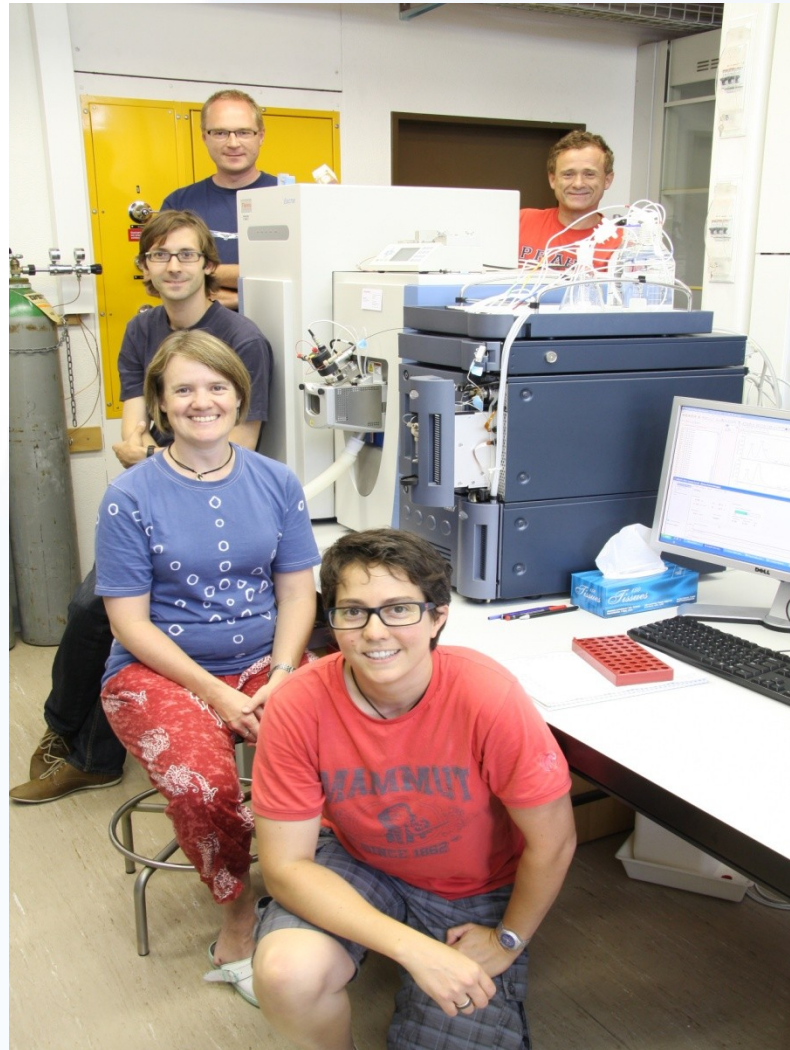
Activity of our laboratory



- Reference laboratory for antibiotic residues
- Measurements for various monitoring programs (some 2800 sample per year)
- Border inspection point analysis (24 hour analysis time)
- Some method development and applied research.
- 4 Laboratory assistants in my lab



Our group



Our view on method validation

- A successful validation should be the proof that a method produces accurate and precise results.
- We develop and validate methods which are afterwards heavily used.
- Validation should resemble more a driving licence than a religious pilgrim journey.
- Validation concepts have to be cost-efficient and suited for modern multi-residue methods.





Does the CD: CE/657/2002 meet these expectations ?



- The CD unified older validation strategies
- The CD eliminated troublesome measurement uncertainty calculation methods
- The CD permitted single laboratory validation
- The CD defined clear confirmation criteria

Does the CD: CE/657/2002 meet these expectations ?

- The CD was not written for multi-residue methods.
- The CD ruggedness test procedure is not feasible for most involved laboratories.
- The CD confirmation criteria are outdated by technological developments (UPLC & HRMS).
- The CD CC α and CC β concept is too complex.





Validation of multiresidue methods



1. Design of validation strategy
2. Calculation of performance parameters
3. Complexity of concepts
4. Economical aspects of validation

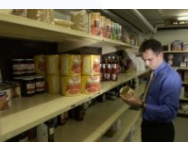


1. Design of validation strategy

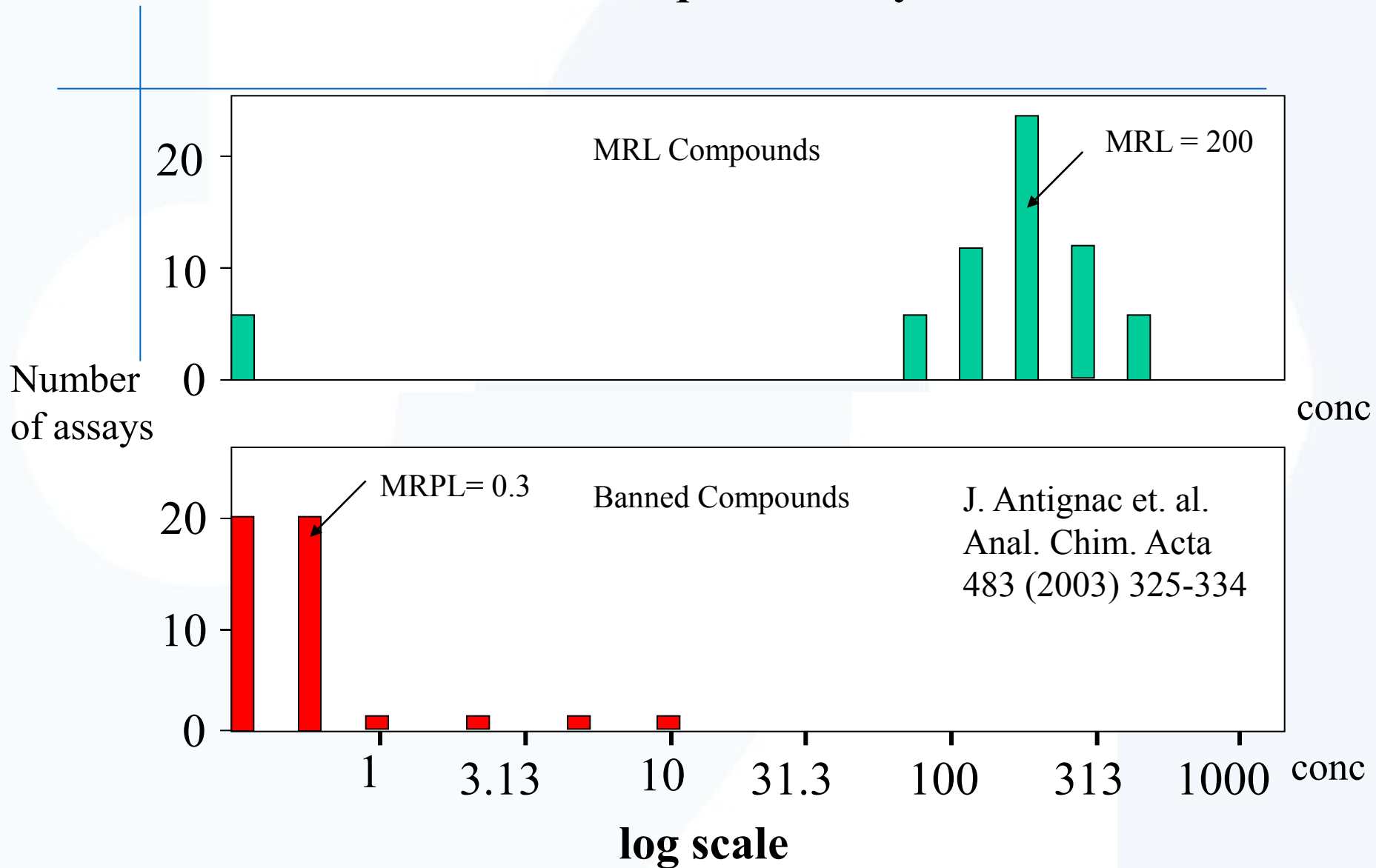


What makes multiresidue methods unique and validation difficult?

- Large number of compounds from different classes
- Many „good“ and a few „bad“ compounds
- Each analyte in each matrice has a different MRL
- Regulated and banned compounds should be covered by the same analytical method



Number of required assays

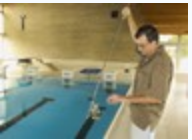




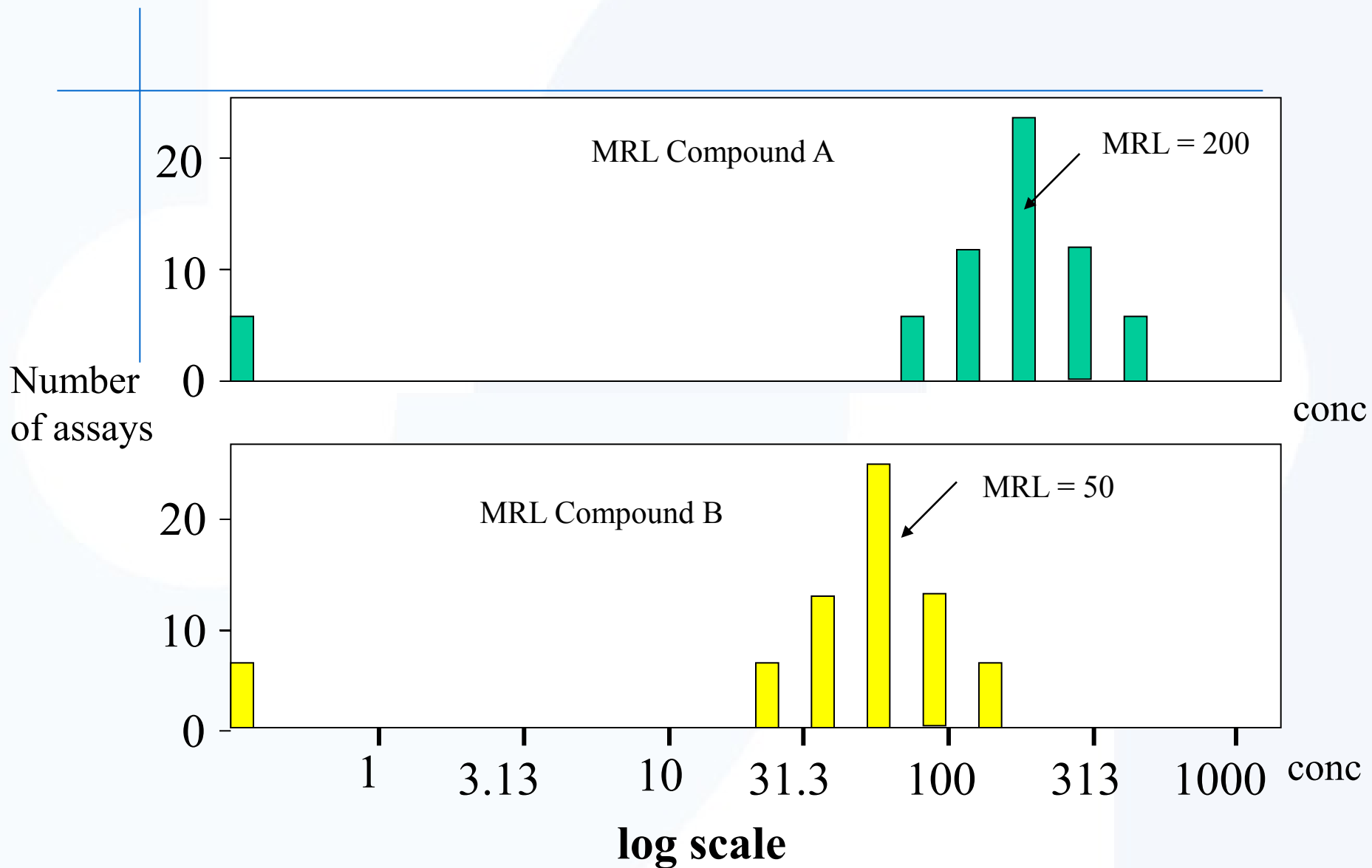
How do we produce individual fortifications ?

- Normally one standard solution containing all analytes
- various added volumes produce the different spike levels

Stop this will not work !



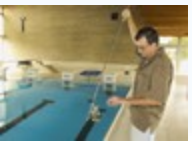
Different MRL's



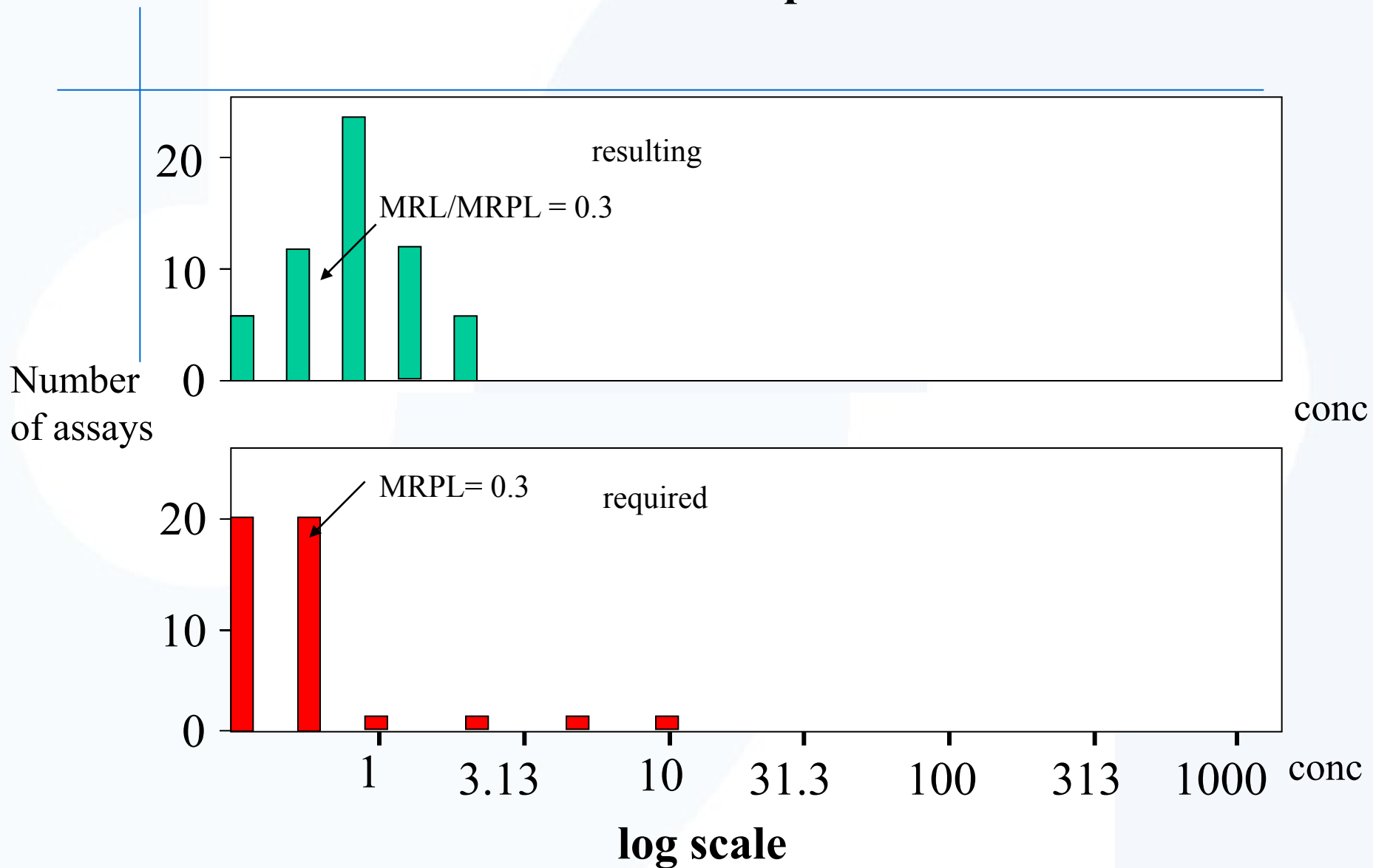


Possible but !

- Producing appropriate concentrations for 100 analytes in a single solution is not simple !
- Different concentrations will be required for spikes into other matrices !
- Practical problems:
Some analytes are not soluble, stable in certain solvents
(Sulfadiazin in proficiency test)

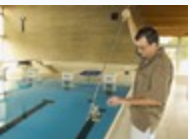


For banned compounds



Still possible ?

- Producing appropriate concentrations is virtually impossible
- Even if attempted, large number of different spiking solutions and various volumes would make validation procedures very complex and error prone
- What happens when the MRL of one compound is modified ?





Change of an MRL

- Phenoxymethylpenicillin in eggs:

2011: not permitted

2012: 25 µg/kg

- Altrenogest in pork:

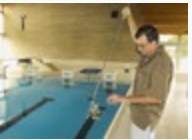
2011: 1 µg/kg

2012: 4 µg/kg

- Ivermectin in beef

■ 2010: 100 µg/kg

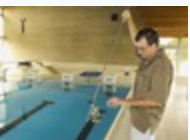
2011: 20 µg/kg





Change of an MRL

- Following the CD the change of an MRL requires a revalidation of the method.
- Revalidation of a multimethod because of a single compound?
- In the real world: The method is still being used to test if meat or meat products are non-compliant.



Any clever lawyer will be able to contest such analytical findings!

Alternative approaches

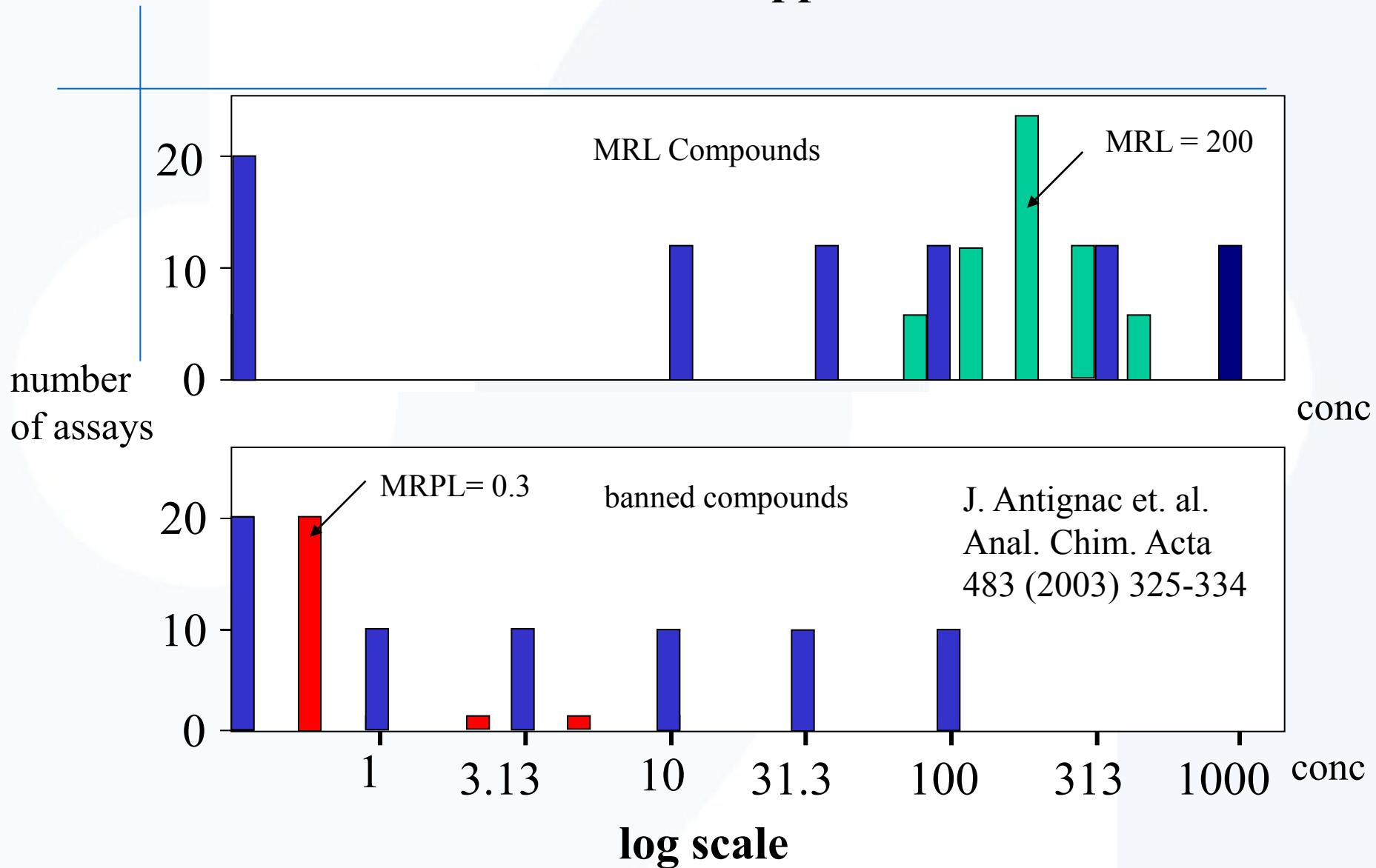
The CD permits alternative approaches:

3.1.3. Validation according to alternative models

The underlying model and strategy with the respective prerequisites, assumptions and formulas shall be laid down, or at least references shall be given on their availability.



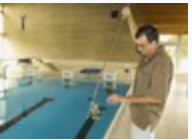
An alternative approach





The advantage ?

- MRL and banned compounds are combined
- Sum MRL compounds like sulfonamides are properly addressed
- A modified MRL does not require a revalidation
- Time and expenses significantly reduced



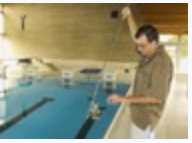


2. Calculation of performance parameters



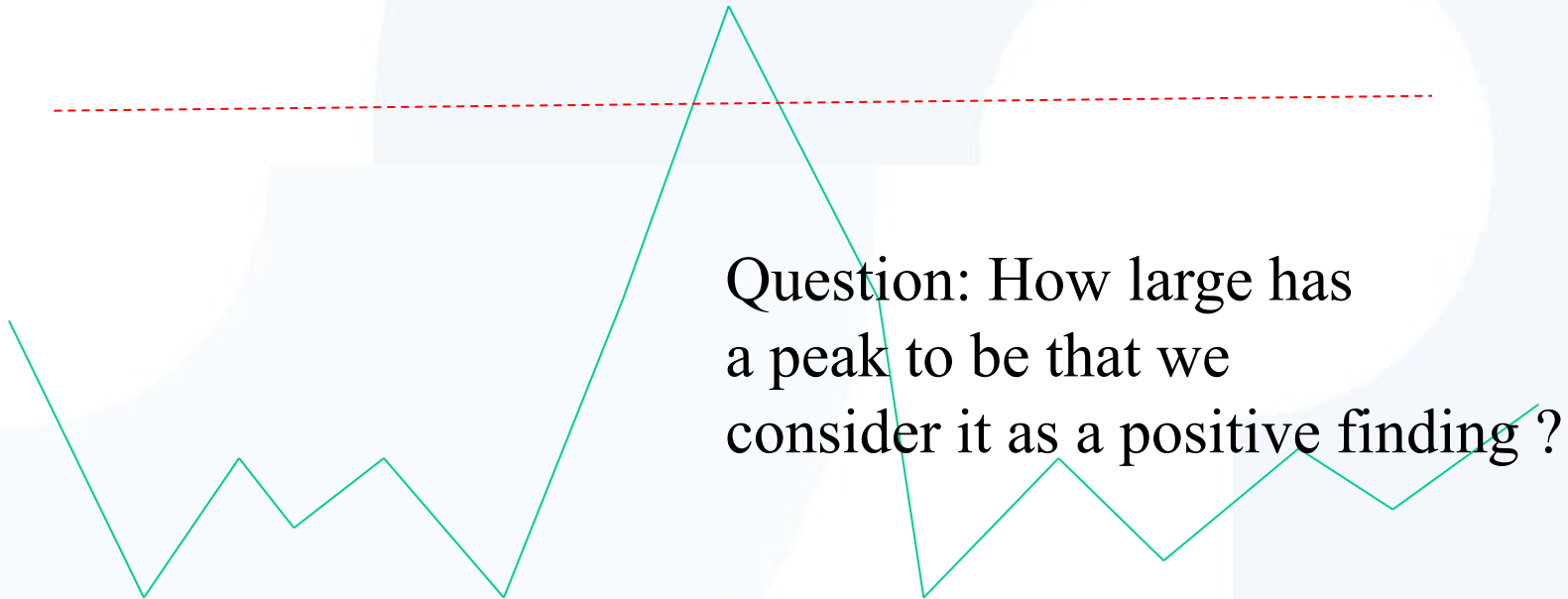


False Positive Findings $CC\alpha$



Am I a peak?
Am I a Fata Morgana?

Question: How large has
a peak to be that we
consider it as a positive finding ?





False negative Findings CC β



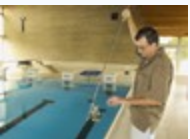
Question: How large has
a moose to be so that
I do not overlook it ?



How to determine CC α for banned compounds?

Two different approaches

- Comparing the signal of a spiked sample to the noise of a blind sample
- Determine the intercept of a linear regression according to ISO 11843



How to determine $CC\alpha$?

Banned compound (20 assays of a blank)

Signal

20 spikes
for $CC\beta$

20 blanks
for $CC\alpha$

$$CC\beta = \frac{2.33\sigma + .64\mu (R.S.D.)_s}{a[1 - .64(R.S.D.)_s]}$$

Conc.

s/n 6:1

approximation for $CC\beta$

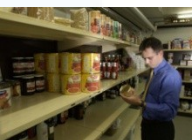
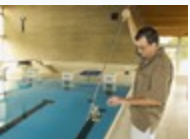




How to determine $CC\alpha$?

Comparing the signal of a spiked sample to the noise of a blind sample

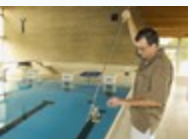
- This requires the detection of noise
- High resolution instruments like TOF, Orbitrap, but also some LC-MS-MS instruments show no classical noise





Noise of a blank

Dicloxacilline in muscle by LC-ESI-TOF



33 $\mu\text{g/kg}$

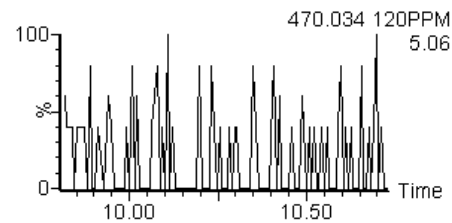
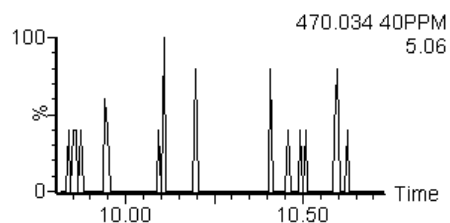
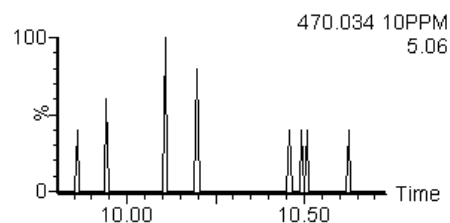
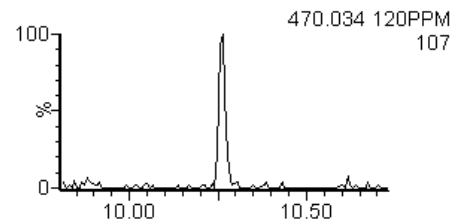
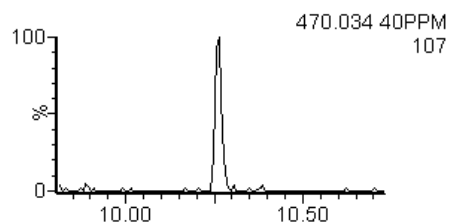
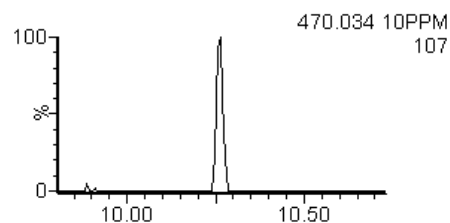
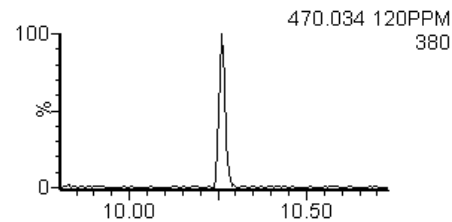
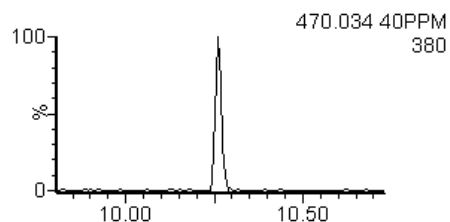
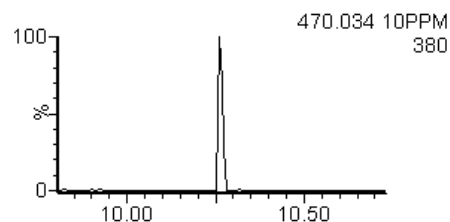
10 $\mu\text{g/kg}$

blind

10 ppm

40 ppm

120 ppm

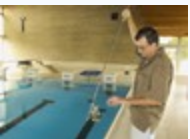




How to determine CC_{α} ?

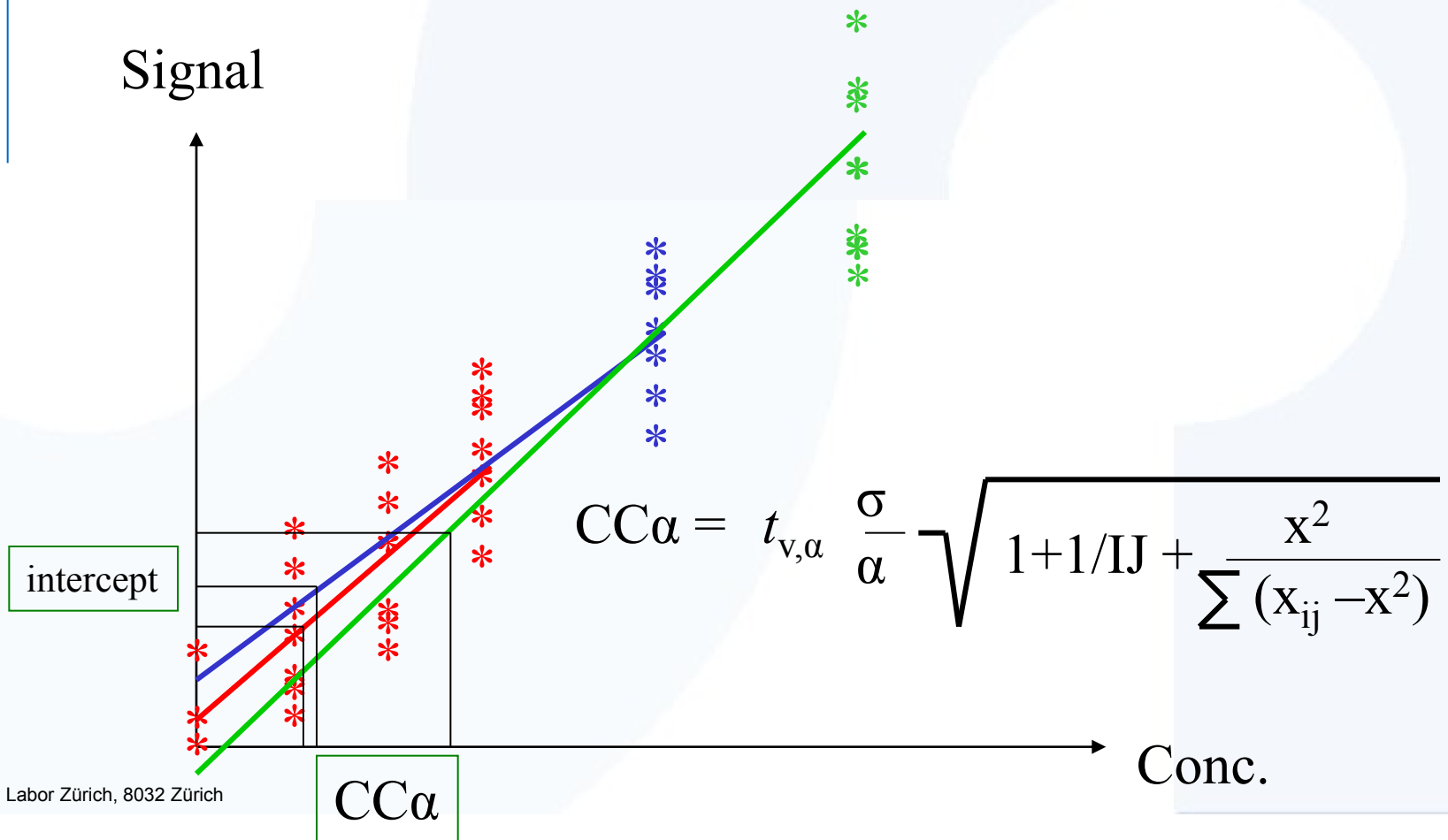
**Determine the intercept of a linear regression
according to ISO 11843**

- This works with multiresidue data but:



banned compound according to ISO 11843

Signal





The multiresidue method validation problem

- Validation has to cover large dynamic ranges
- The ISO 11843 approach is very questionable if the calibration curve covers a large dynamic concentration range
- Problems are aggravated if data shows non-linearity or heteroscedasticity
- Non-linearity of the detector, adsorption of analytes at low concentration will be observed for some analytes

We need a calculation which does not requires that underlying data follow strict statistical requirements (e.g. linearity, Gaussian distribution, homoscedascitiy)



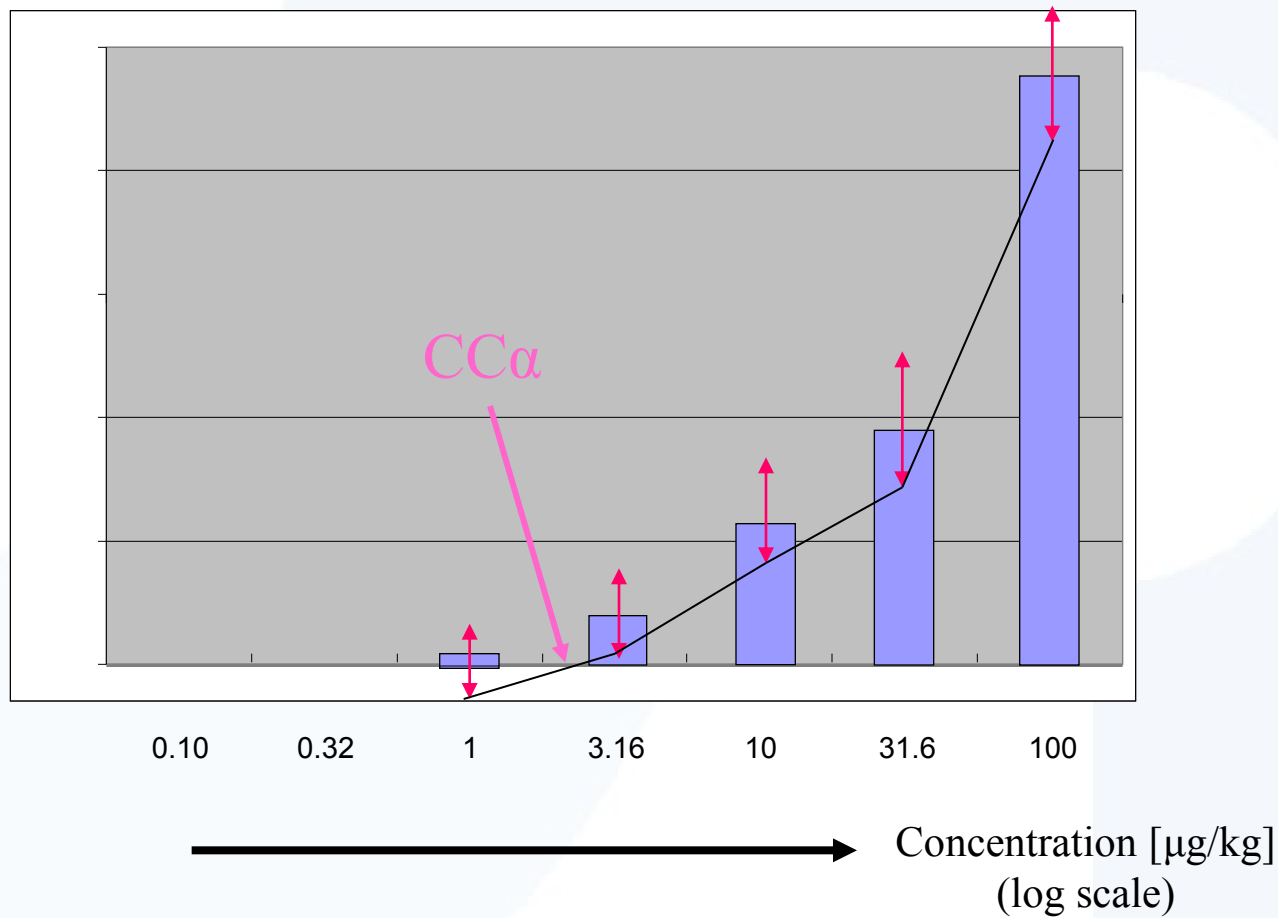


Robust $CC\alpha$

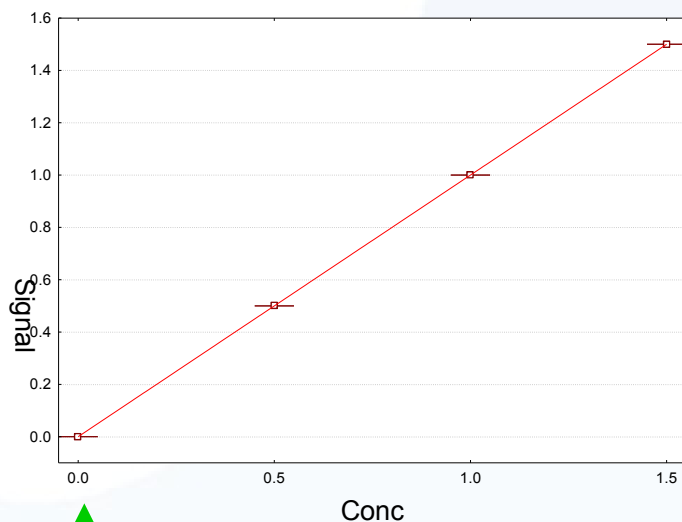
Average Peak area – $3.143 \text{ RSD} * (\text{average peak area})$ – noise



peak
area



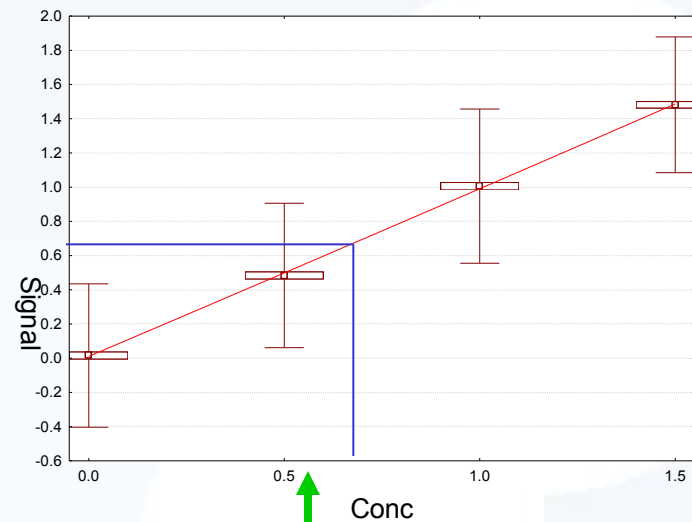
Poor repeatability gives high CC's



ISO CC_{α} = 0

Noise based = 0

Robust CC_{α} = 0



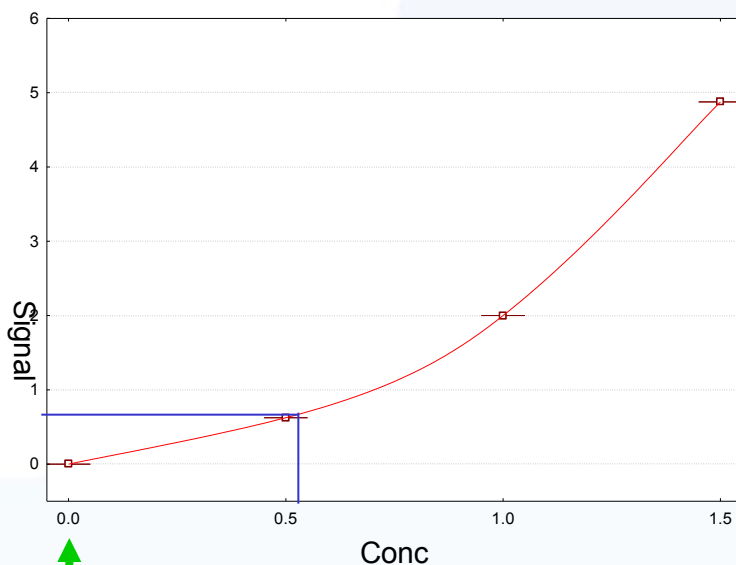
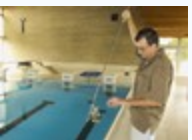
ISO CC_{α} = 0.677

Noise based = 0.496

Robust CC_{α} = 0.524



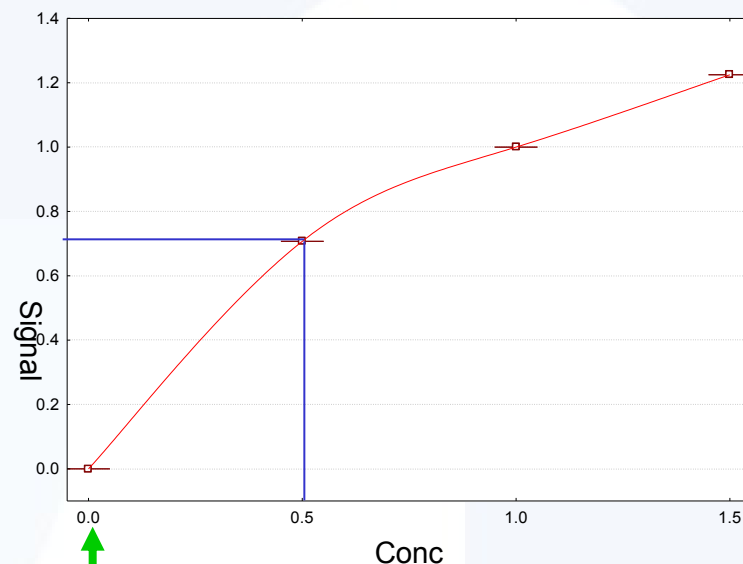
Non-linear calibration curves produce high CC's, even if there is a perfect reproducibility



ISO CC_{α} = 0.526

Noise based = 0

Robust CC_{α} = 0



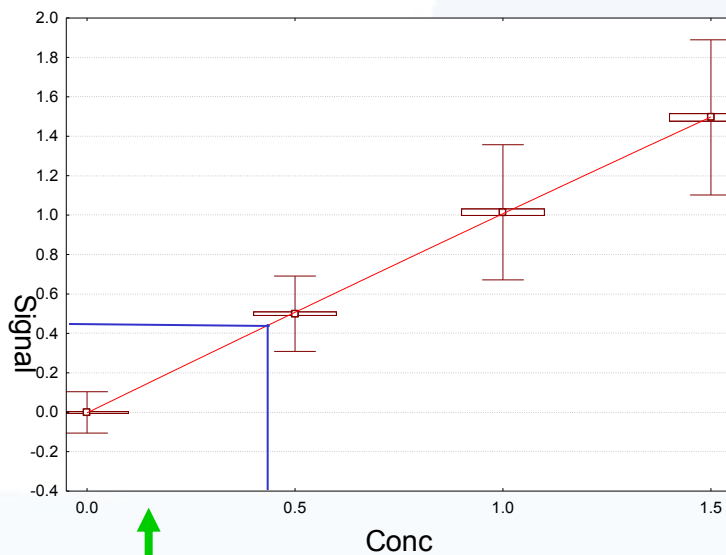
ISO CC_{α} = 0.505

Noise based = 0

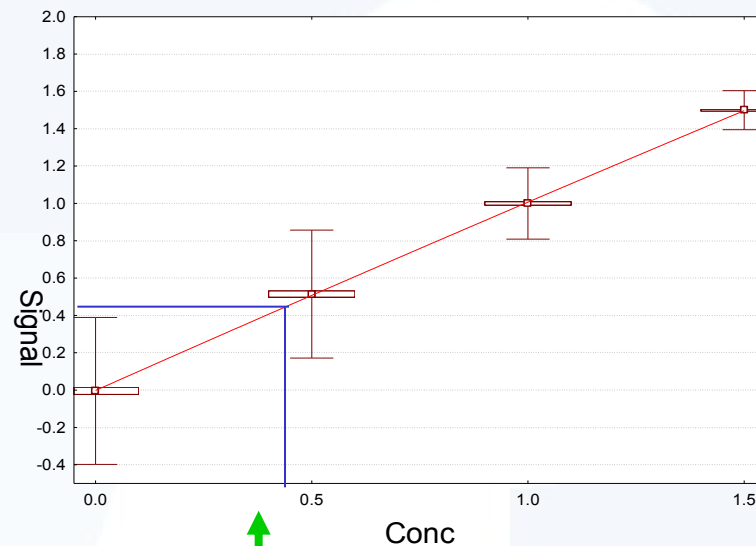
Robust CC_{α} = 0



Violation of homoscedasticity



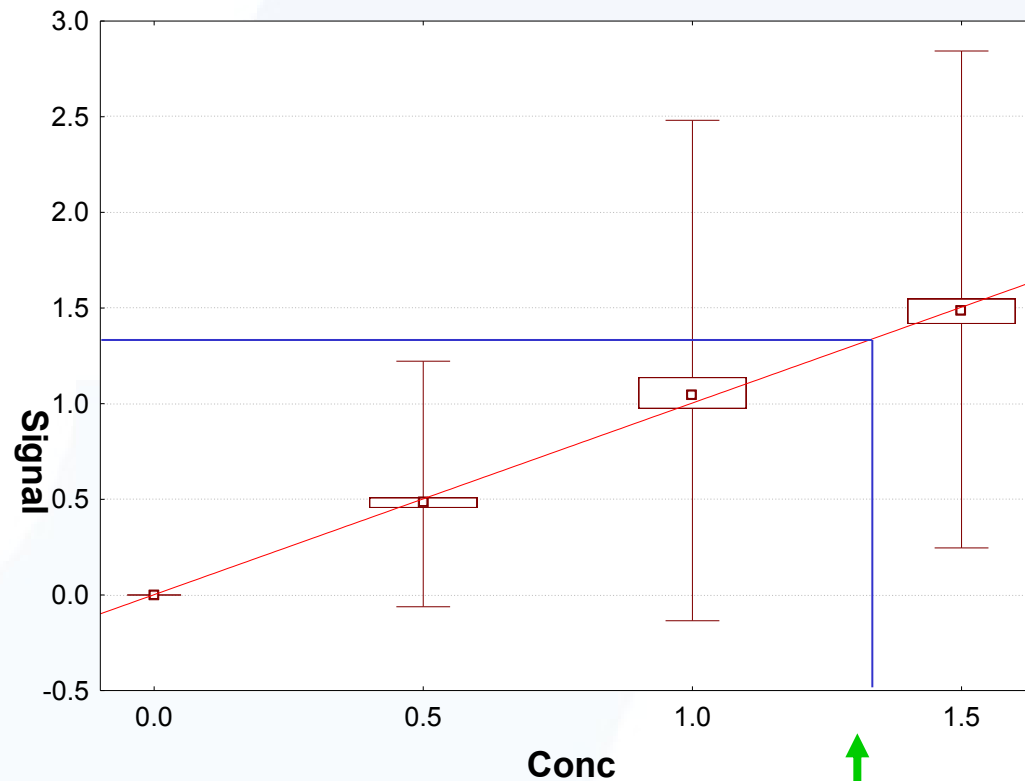
ISO CC_{α} = 0.445
Noise based = 0.122
Robust CC_{α} = 0.153



ISO CC_{α} = 0.445
Noise based = 0.459
Robust CC_{α} = 0.397



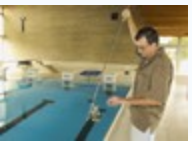
No noise and lack of homoscedasticity



ISO CC_α = 1.311

Noise based = 0

Robust CC_α = 1.290

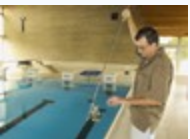




Robust CC α

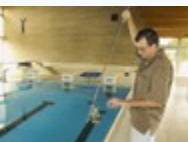
- Does not require noise for blanks
- Does not require linearity
- Does not require homoscedasticity
- Easy to comprehend
- Value has to be interpolated

Statistically not highly sophisticated
Designed for real life data
Very intuitive

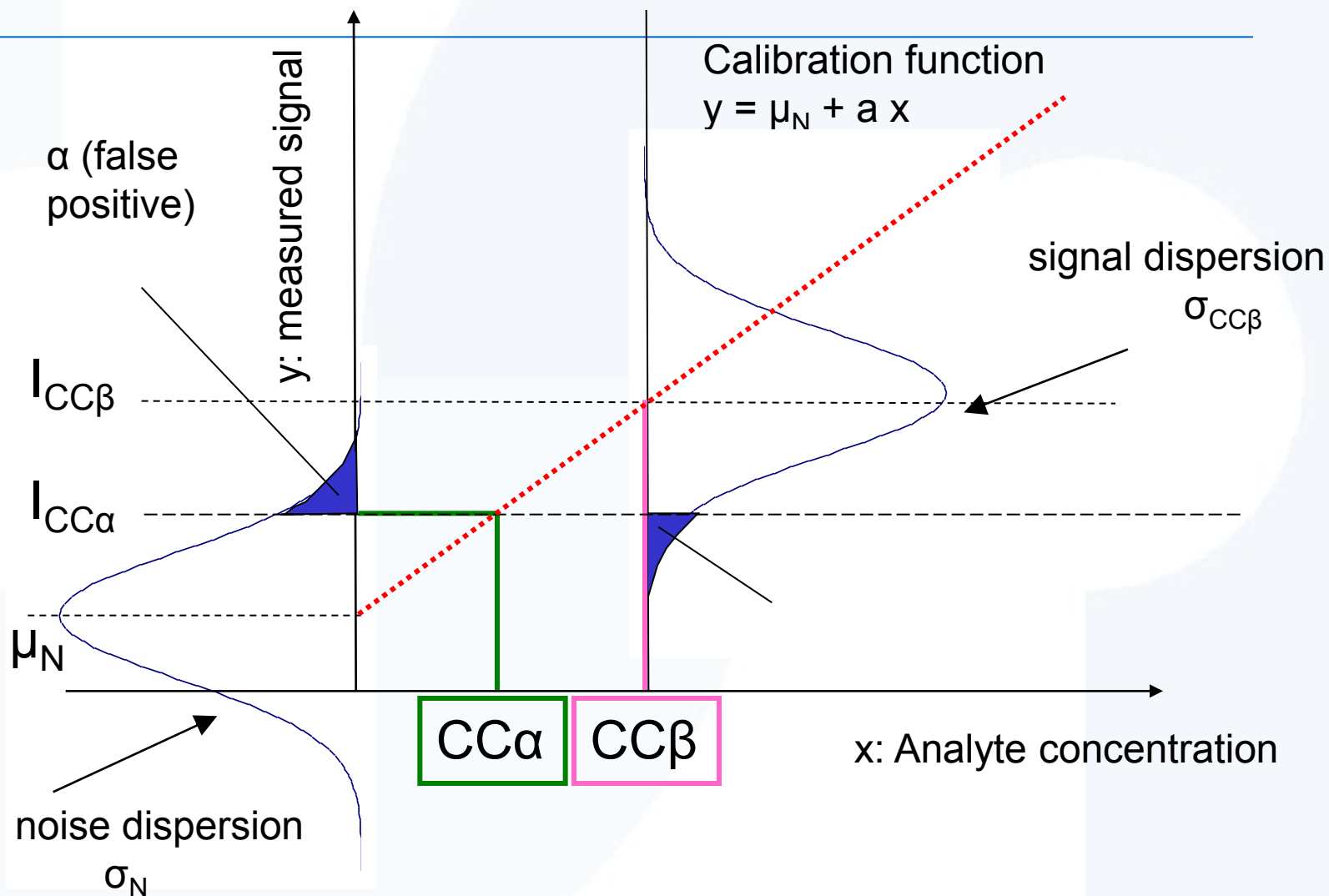




3. Complexity of concepts



3. Complexity of concepts





The minefield between $CC\alpha$ and $CC\beta$

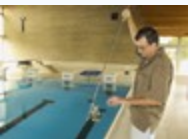
- $CC\alpha$ makes sense for regulated compounds, but what means $CC\beta$ in this context ?

100 ppb : MRL

120 ppb : ($CC\alpha$) The concentration where I know that a sample is bad.

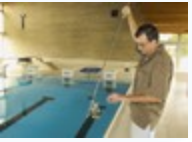
140 ppb : ($CC\beta$) The concentration where a bad sample will not anymore be considered to be a good sample

130 ppb: The sample is bad, but to be honest, the sample would not have been bad, if I had analyzed it on some other day



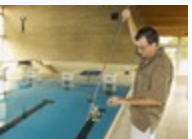
Complexity of concepts

- $CC\alpha$ and $CC\beta$ are complex statistical concepts.
- How many validating analysts fully comprehend the concept ?
- What does the information, that a sample shows a concentration between $CC\alpha$ and $CC\beta$ mean to a owner or producer of that piece of meat?
- Determining $CC\beta$ requires the prior knowledge of that very value
- What is the difference between the minimum required performance level and minimum required performance limit ?





4. Economical aspects of validation

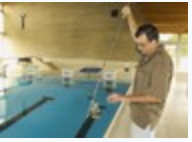




Experimental design

Systematical test of Factors

- Sex of animal: Male / Female A/a
- Feeding regime: Low-land / Alps B/b
- Batch of solid phase cartridge: Batch 1/ Batch 2 C/c
- Analyst performing the assay: John/Brigitte D/d





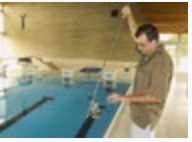
Experimental design



Sample Nr.	Factor A	Factor B	Factor C	Factor D	Factor E
1	+	+	+	+	-
2	+	+	-	-	+
3	+	-	+	-	-
4	+	-	-	+	+
5	-	+	+	-	+
6	-	+	-	+	-
7	-	-	+	+	+
8	-	-	-	-	-



Experimental design



- Where do I get my male pig fed on a alpine pasture in the month of February?
- How do I know which variables I have to test for?
- How about the involved work?



Will experimental design detect error sources ?

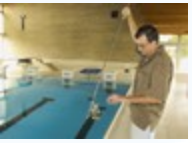
If we know the error generating variables,
experimental design will determine their relative importance

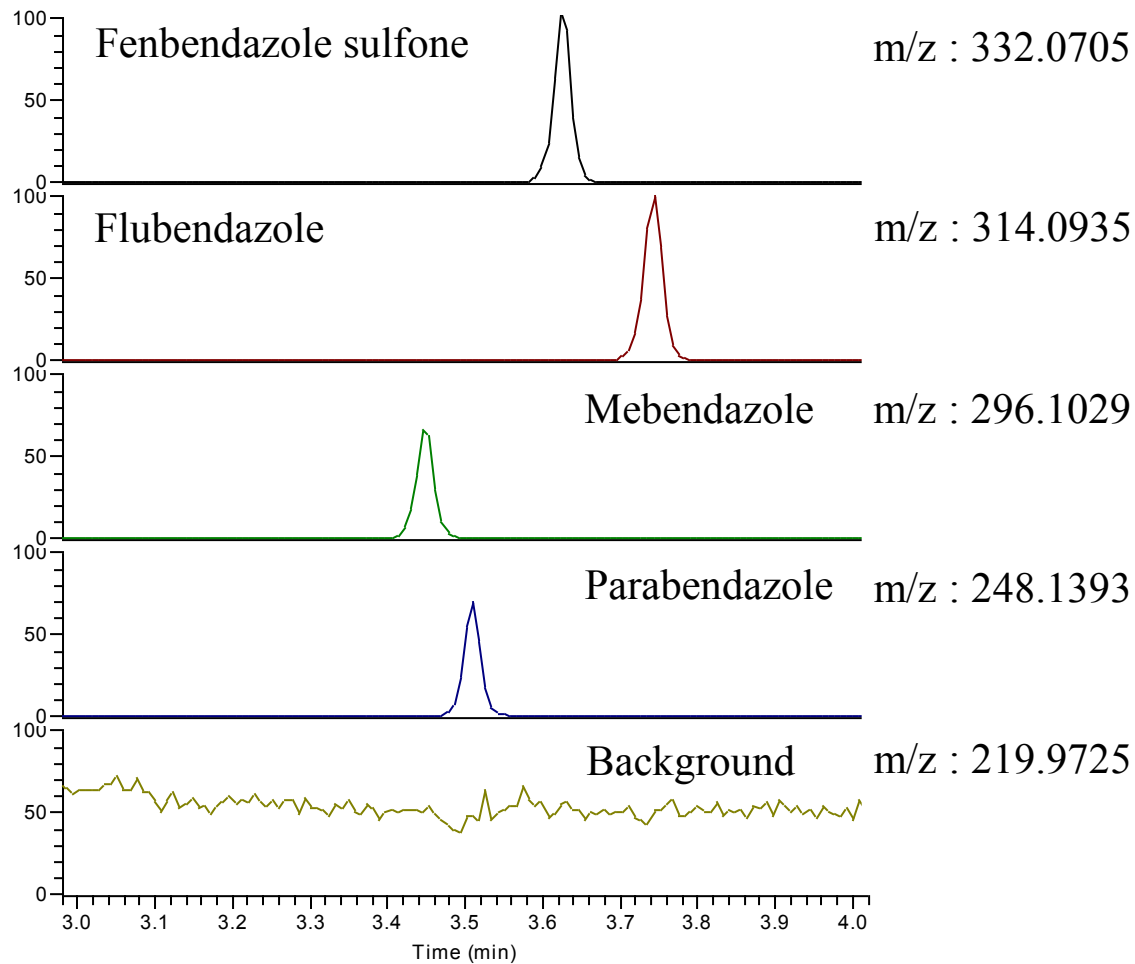
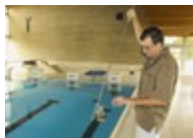
However: The dangerous errors are the unexpected, the unknowns

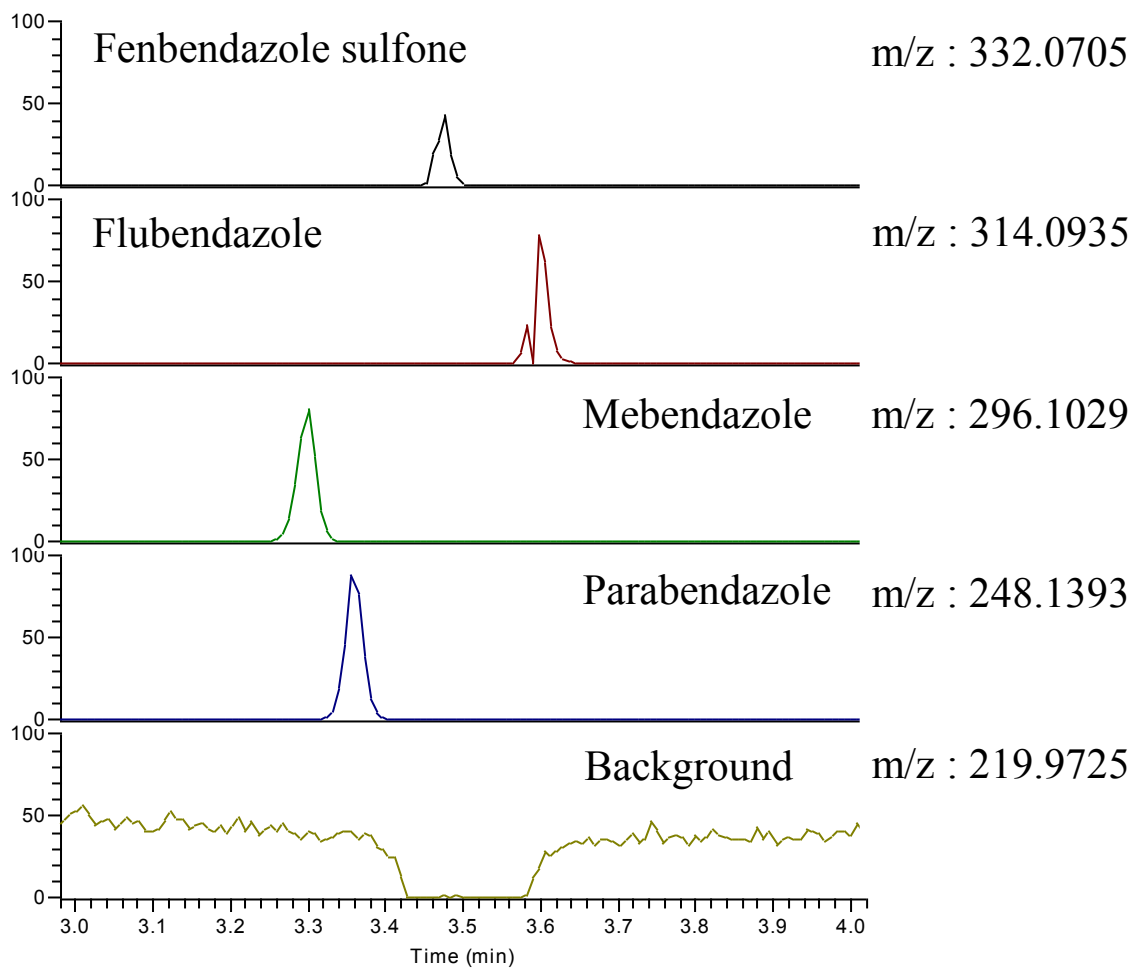
(Example: Neutralization with ammonia hydroxide)

(Example: Phase separation in autosample vial)

(Example: Signal suppression)





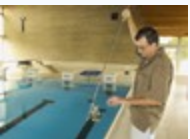




Testing 20 blank samples

Organizing 20 samples,
reflecting the variety within a given matrix,
is very difficult for many laboratories,
analyzing such negatives can be very boring

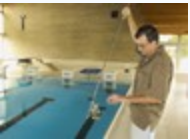
- Reliable CC's can be obtained by using less blanks
- False positives are mostly related to carryover, or a new matrix





Do we need 20 blank samples?

Interferences within a given matrix are either the rule or the very exception



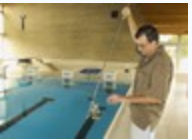
Confirming of rule requires only a few samples, proofing the exception needs much more than 20 samples!

Always or once in a blue moon

If an interfering substance is a endogenous (e.g produced by the metabolism or is an integral compound of a given organ), than it will be present in every sample (Gaussian distribution).

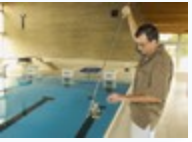
If an interfering substance is exogenous (e.g. originating from environmental contamination or a particular fodder) than it will be very rarely encountered (Poisson distribution)

But: A new matrix can cause a blue moon !!



Robustness of a method

- History tells us that humans are probably not very good in imaging of what can go wrong
- Method robustness tests are as good as bank stress tests
- Banks passed a stress test, just to collapse the next day
- My nicely working method collapses as soon as somebody else tries to reproduce it!





Robustness of a method

Don't ask yourself
Ask your method!

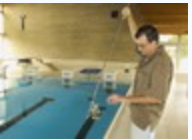
- Test your method by using a matrix for which it was not developed
- Let other people in other laboratories with other samples test your method





Validated method, correct results?

- Nitrofurantoin methods were properly validated
e.g. R. Draisci; J. Chrom. A 777 (1997) 201-211
- Residues were never detected
- Newer methods focused on covalently bound metabolites
- Suddenly a high percentage of samples were positive !
- Do our current validation concepts prevent such things?

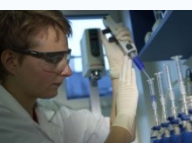
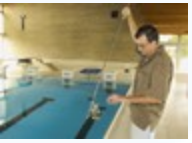




How much validation ?

- Many laboratories have limited or even shrinking resources
- More time spent for validation means less sample controlled

How to simplify validation protocols
without sacrificing quality?





Suggestions of how to reduce assays

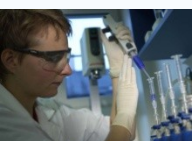
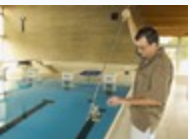
- Considering one organ as one matrix (e.g. pork and bovine liver)
- Testing between day reproducibility ($n=3$) only for the main matrix
- Reducing the testing of blanks samples





Why are there different validation procedures?

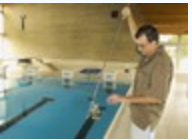
- The CD describes the very details of validations of methods used for residue detection in meat and meat products.
- Pesticides in fruit or marine toxins in shells or mycotoxins in animal feed use different validations techniques.
- How will we validate future methods which quantify mycotoxins, marine toxins and vet drugs in one run?
- There is no analytical reason for validating vet drugs and pesticides in a different way !!!!





Conclusion

- **Multimethods, based on high resolution MS instrumentation require new validation approaches**
- **The concept of $CC\alpha$ and $CC\beta$ is complex and not fully understood by many analysts**
- **The two ways to calculate $CC\alpha$ and $CC\beta$ leads to significantly different results**
- **Validation has also an economical dimension:
What is nice and what is essential to have ?**
- **Validation concepts should be designed that they are not perceived as an ugly mechanical requirement but as an intellectual task to more fully comprehend and master an analytical method**





Calculating $CC\alpha$, $CC\beta$, accuracy, reproducibility and filling folders & DVD's does not improve a method

