农药分析的方法验证与实验室质量控制

产品分析的重要要素

- 可靠的分析方法： CIPAC、AOAC、 国标、行业标准、经过验证的企业标准、权威文献报道的方法
- 可靠的分析实验室质量控制手段： 内部质量控制IQC； 外部质量控制
- 公认的原则： 采用标准分析方法；开发并验证用于质量控制的分析方法、使用实验室熟练掌握的分析方法

外部质量控制手段

- 初步实验室间研究： 由两个或多个实验室参加， 评价一种方法， 确定其是否具备条件作为协作研究的对象。

- 实验室间检测能力测试 Performance Test：

  分析经仔细制备的均匀样本， 以证实和考核实验室或分析人员的试验水平。

Q1: 测量值的组成

- **Observed value**:

  xi = m + B + e

- where
- ***M:*** *平均值*
- ***B*** is bias ;
- ***e*** is 随机误差;
- μ is 真值; often not known

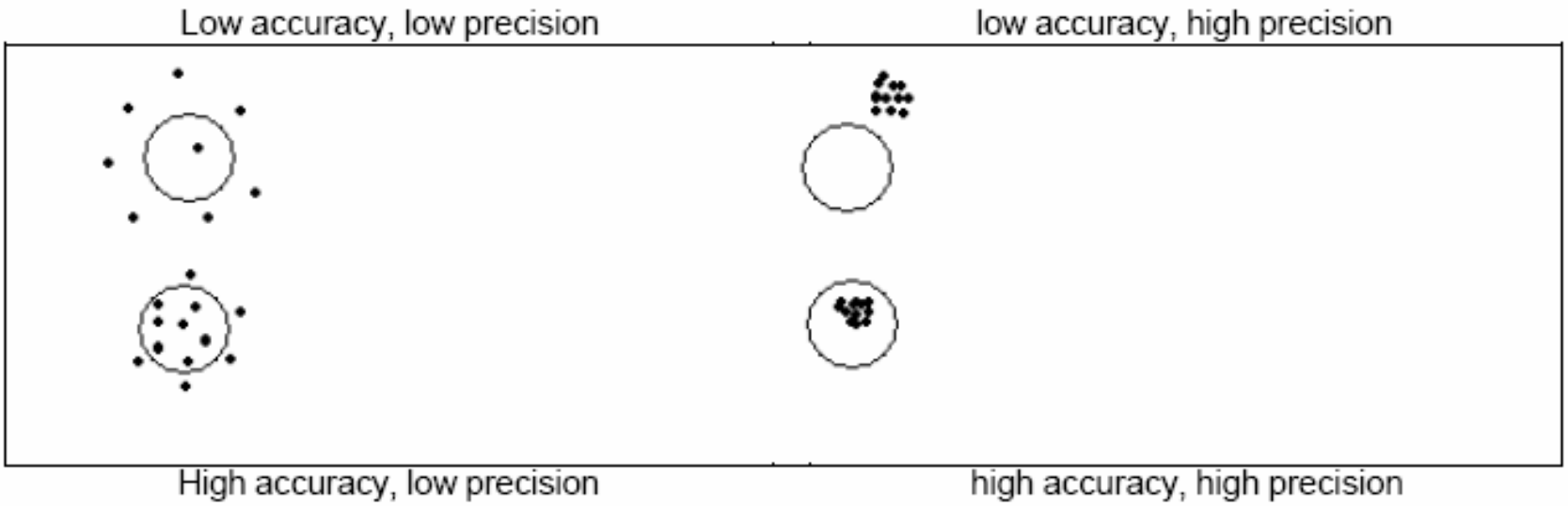
单个实验室内部的误差来源

1- 每个分析测试员之间的差异,

2- 仪器之间的差异,

3- 实验试剂与消耗品之间的差异,

4- 一定时间内以上1、2、3的差异,



结果的表述

- X = Average(Xi) +(-) t * Sigma * / Squareroot(n)
- n＝测定次数； Sigma：方法的重复性； t：一定容错概率下包含因子； Xi：平均值

方法验证的需要

农药生产企业的质量分析部门应该建立产品的分析方法验证程序。

It is recognized that most Agrochemical manufacturers will have internal procedures for analytical method validation.

- 农药产品登记要求： 如EU

为什么进行方法验证？— 重要性

- 确定方法的应用范围与局限性
- 由质量控制样本确定结果的可接受范围
- 验证分析测定是否真实可靠

何时进行方法验证

新方法开发	$F^{1,2,3}$
现有方法的适应（新的基质等）	$F^{1,2,}$F or P^{3}
标准方法的改良	P or F^{1}
质量控制显示分析方法有偏离	P or F^{1}
不同实验室之间方法交换	P^{1} or E^{2}
仪器、操作人员改变	P^{1}
新的试剂与配件	P^{1}
已验证方法长时间未采用	P^{2}
实验室管理或相关的改变	P^{2}
新的协作研究方法	P^{2}
经过验证但未经协作研究验证	P + E^{2}
文献报道、有方法的特征参数	P + E^{2}
文献报道、无方法的特征参数	$F^{1,2,3}$

F: full validation; E: extensive validation; P: partial validation;

农药分析方法验证的内容（1）

准确性 Accuracy：与真值的偏离程度

线性范围 Linearity: 分析的可靠范围（定量分析的基础）

精确性 Precision: 结果之间的接近程度

农药分析方法验证的内容（2）

灵敏度 Sensitivity: 不同浓度样本的响应大小

特异性 Specificity: 分析物定性的考察

添加回收率Recovery: 测定样本中分析物全部的能力 (usually within an acceptable range, e.g., 70-120%)

重现性 Reproducibility

稳定性 Stability 分析方法各步骤中分析物稳定性

抗干扰能力

分析范围

SST:

5.1 Default Values from Regulatory Guidelines

There are numerous guidelines which detail the expected limits for typical chromatographic methods. In the current FDA guidelines on "Validation of Chromatographic Methods" , the following acceptance limits are proposed as initial criteria:

Parameter	**Limit**
Capacity factor	k' > 2
Injection precision	RSD < 1% for n ≥ 5
Resolution	Rs >2
Tailing factor	T ≤ 2
Theoretical plate	N > 2000

These suggested limits may be used as a reference to set up the initial system suitability criteria in the early method development process.

方法验证手段

不同分析人员在不同天对同一样本或方法进行至少4次测定

添加可能干扰样本分离的类似物以确立色谱分离方法并保证分析的特异性

通过添加标准品确定回收率水平

添加稳定性分析物测试分析流程重样本制备与储存的稳定性

标准校正曲线　（1*5 或2*3）

标准曲线

斜率 Slope (可置信范围？)

- 截距 Intercept (可置信范围？
- 相关系数 Correlation Coefficient (R^2>0.997)
- 方差 Variance
- 相对残差的标准偏差 Standard deviation of relative residual (SS< 0.01 or 0.02)

残差在结果评价中的应用

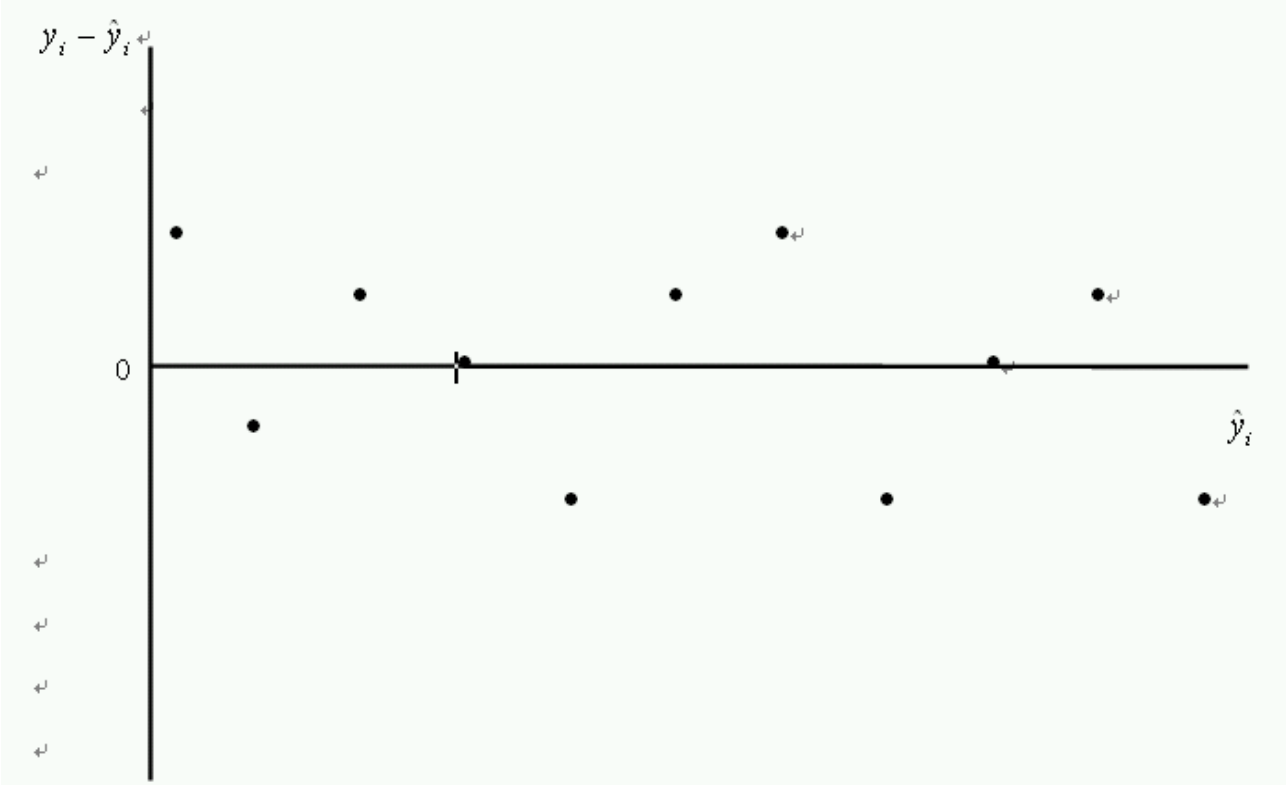


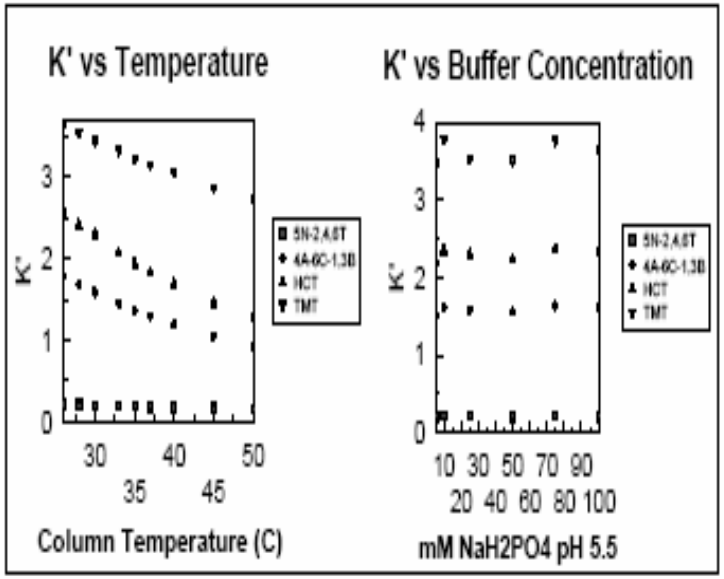
Fig. 9.2 Residual Plot

LOQ

- 1. Analysis repeatability and injection repeatability data at the quantitation limit.
- 2. Use of an additional reference standard at the quantitation limit level in the test method.

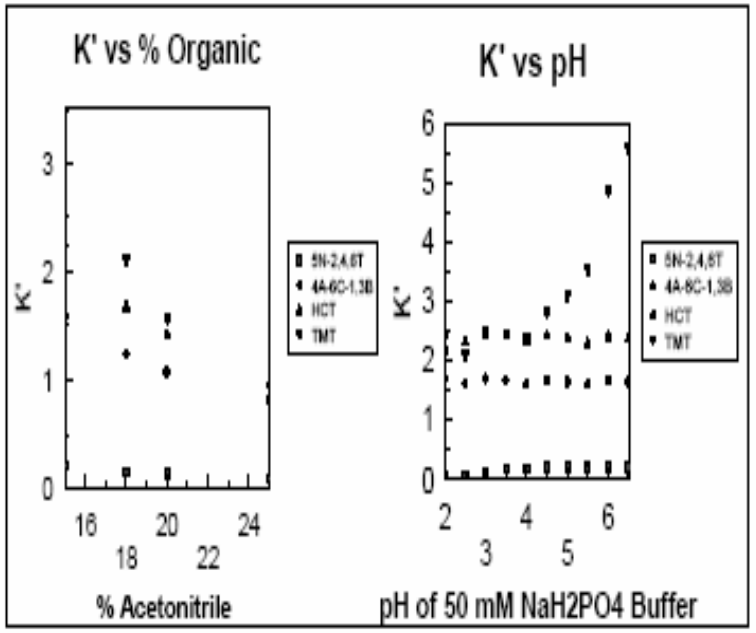
Robustness Study

k' Versus Temperature and Buffer Concentration



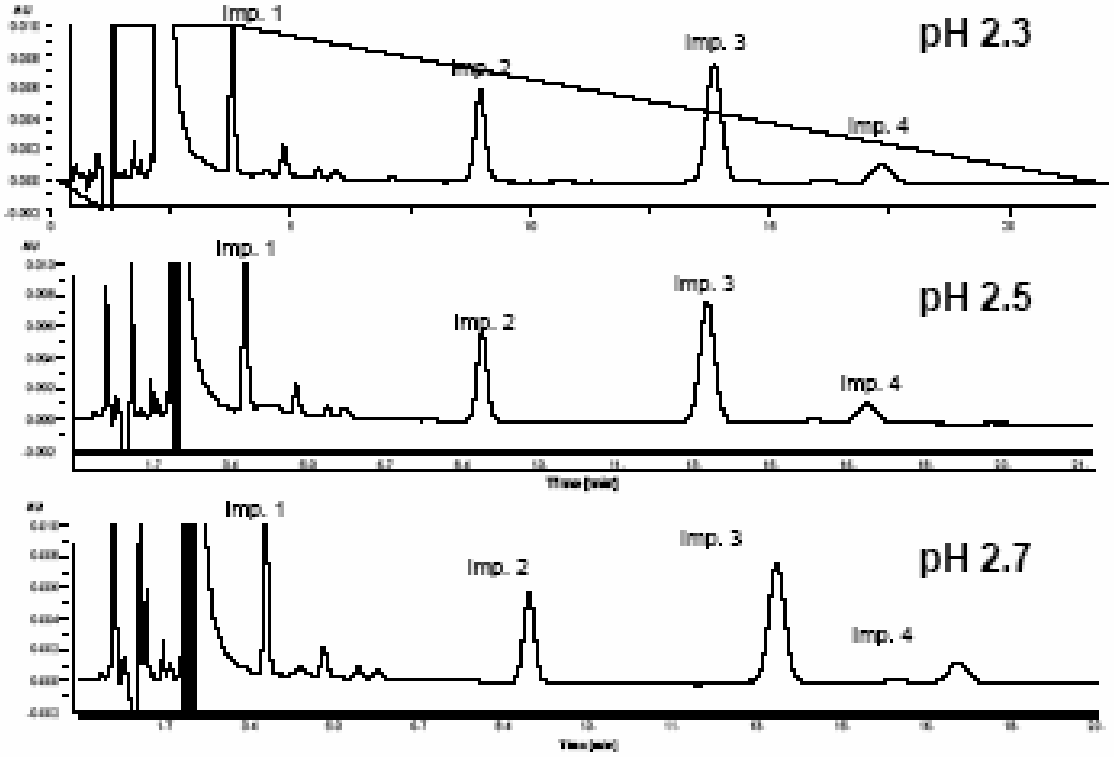
HPLC Method Robustness Study

k' Versus % Organic and pH



AZT: Robustness Testing

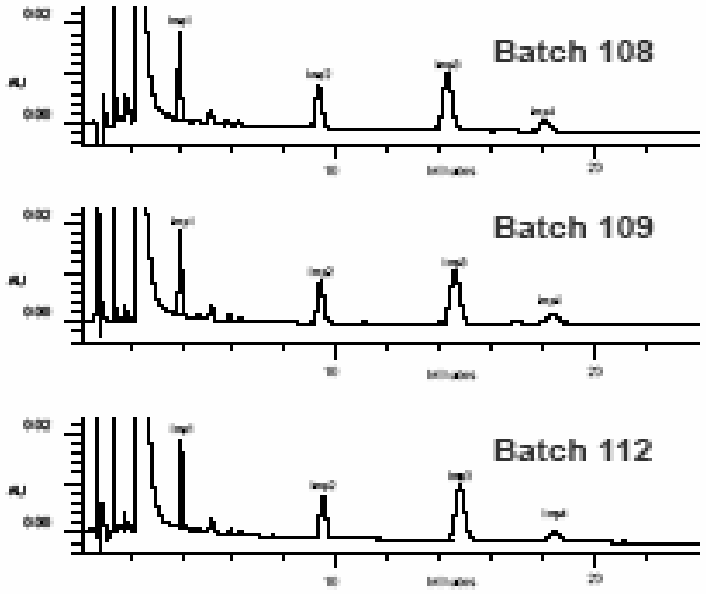
6% Methanol, 6% THF



Method Ruggedness

- Analyst to analyst
- Instrument to instrument
- Lab to lab
- Column to column
- Batch to batch

Column Batch-to-Batch Reproducibility



Sample: AZT

Injection: 150 µL of 0.6 mg/mL solution

Column: Symmetry C18, 3.9 mm x 150 mm

Temperature: 45 °C

Mobile Phase: 6% MeOH/ 6% THF/ 88%

10 mM potassium phosphate buffer, pH 2.6

Flow rate: 1.7 mL/min

Detector: UV at 288 nm

	Quantification and analysis of a.i. in technical material	Quantification and analysis of significant impurities (>0.08 % and substances of toxicological concern below this level) in technical material	Qualitative analysis of low level impurities (<0.08 %) in technical material	Quantification and analysis of a.i. in a matrix (formulation)		Quantification and analysis of a.i. in drinking water (0.1 $\mu g\ l^{-1}$)
				High Concentration (≥1 %w/w)	Low Concentration (≤1 %w/w)	
Accuracy	✓	✓	x	✓	✓	✓
Repeatability	✓	✓	x	✓	✓	✓
Reproducibility	Where the method is to be used in other laboratories reproducibility should be addressed					
Specificity	✓	✓	✓	✓	✓	✓
LOD	x	x	✓	x	✓	✓
LOQ	x	x	x	x	✓	✓
Linearity	✓	✓	x	✓	x	✓
Range	x	✓	x	✓	✓	✓
Robustness	Robustness should be addressed as part of the method development					

FAO 规格

〔II〕固、液体原药，易挥发液体（Bp < 50 ℃）及粘稠液体（粘度 1×10^{-3} m^2/s，20 ± 2 ℃）均用 g/kg 表示含量．其它所有液体的有效成分为 g/kg 或 g/L（20 ± 2 ℃）表示．下表为原药和制剂的有效成分允许范围．

标明值 g/kg 或 g/L（20 ± 2 ℃）	允许
⩽25	±15 %为均匀制剂（EC、SC、SL 等）的标明值含量或 ±25 %为不均匀制剂（GR、WG 等）
25 ~ 100	±10 %标明值含量
100 ~ 250	±6 %标明值含量
250 ~ 500	±5 %标明值含量
> 500	±25g/kg，或 g/L

注：每个范围是包括最大范围

500g/kg product, +_ 25g/kg

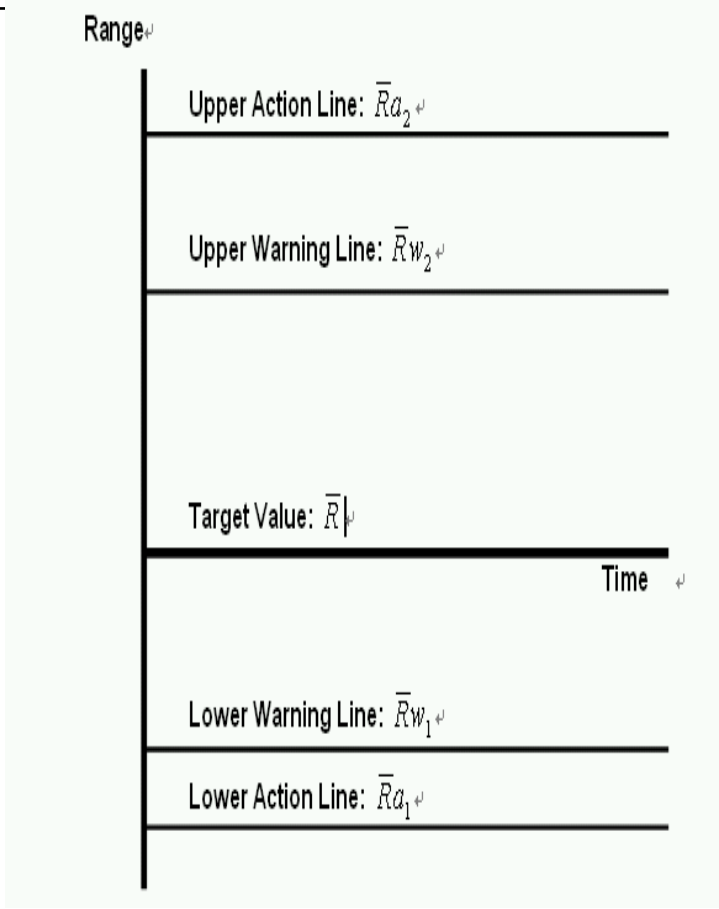


Table 2: Suggested maximum RSD as a function of analyte concentration.

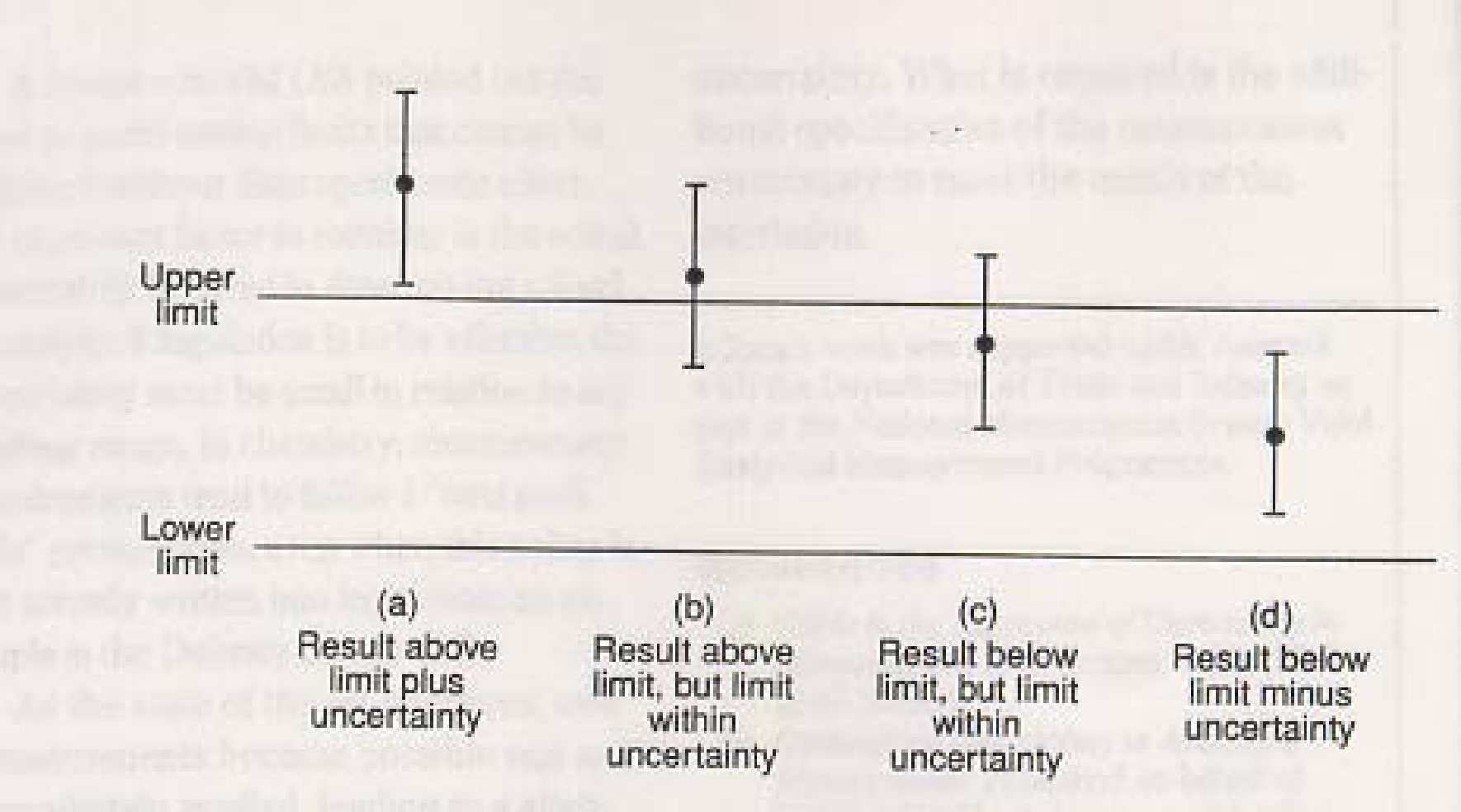
Analyte (%)	Analyte ratio	Unit	RSD (%)
100	1	100%	1.34
10	10^{-1}	10%	1.89
1	10^{-2}	1%	2.68
0.1	10^{-3}	0.1%	3.79
0.01	10^{-4}	100 ppm	5.36
0.001	10^{-5}	10 ppm	7.58
0.0001	10^{-6}	1 ppm	10.72
0.00001	10^{-7}	100 ppb	15.16
0.000001	10^{-8}	10 ppb	21.44
0.0000001	10^{-9}	1 ppb	30.32

These RSD are based on the modified Horwitz equation which suggests that:

$$\mathbf{RSD < 2^{(1 - 0.5 \log C)} \times 0.67}$$

Where C is the concentration of the analyte expressed as a decimal fraction (i.e. 0.1, $1x10^{-6}$ etc.)

结果可靠性与不确定度关系



单一样本的测试 - I

- 一定时间内结果稳定性? ————》 统计控制图

- 如果试验结果不能提供充足数据建立统计控制图：
  - 平行分析次数
  - 添加回收、线性关系

单一样本的测试 - II

- 建立 CD值：critical difference.

- CR＝f * σ * sqrt(2).

  - f (CR factor) depends on the probability level to be associated with the critical difference and on the shape of the distribution.

单一样本的测试 - III

- the repeatability limit $r=2.8\ \sigma_r$
- the reproducibility limit $R=2.8\ \sigma_R$.

For R and r, the probability level is 95% and we assume an approximately normal distribution.

  - Under these conditions, *f* is 1,96 and *f* sqrt2 is 2,77 (we use a rounded value of 2,8).

分析同一样本的重复性考察

- 对同一样本的三个部位分析结果应符合:

  Cmax-Cmin< 3.31*r/2.8

两个实验室间对同一样本分析结果的比较:

- lab. 1 ⟹ n_1 results giving a mean of y_1
- lab. 2 ⟹ n_2 results giving a mean of y_2
- under repeatability conditions, the SD (y_1-y_2) is:

$$\sigma = \sqrt{\sigma_L^2 + \frac{1}{n_1}\sigma_r^2 + \sigma_L^2 + \frac{1}{n_2}\sigma_r^2}$$

- and the critical difference for $|y_1-y_2|$ is:

$$CD = \sqrt{(2.8\sigma_R)^2 + (2.8\sigma_r)^2\left(1 - \frac{1}{2n_1} - \frac{1}{2n_2}\right)}$$

Part II

- **Technical Material and Preparations: Guidance for generating and reporting methods of analysis in support of pre- and post-registration data requirements**
- 欧盟关于产品登记产品化学的要求 （分析方法部分） **SANCO/3030**

定性和定量分析中需要提供可靠、准确、精确的方法

- In order to generate data for authorisation and post-registration control and monitoring purposes under Directive 91/414 EEC, robust, accurate and precise analytical methods are required.
- Methods are required for the identification and quantification of the active substance in the technical material and formulated product.

——Directive 91/414/EEC

主要内容

- 方法描述 method description;
- 方法验证 Method validation;
- 确证方法 confirmatory techniques;
- 衍生化 derivatisation;
- 非特异性方法 non-specific and common moiety methods.
- 小结 summary of required method validation data.

方法开发和验证是否GLP 需求？

- The development and validation of a method is not subject to GLP, however where the method is used to generate data for safety purposes, for example where the a.s. degrades to toxicologically significant product(s), those studies must be conducted to GLP.
- 研究毒理学代谢时需要按照GLP， 方法开发和验证不一定遵循GLP。
- Commission guideline documents ： 7109/VI/94 (2) and 7017/VI/95 (3).

1 分析方法描述的基本要素

- 原理 principle of the method (including scope and method specificity)
- 方法提要 method summary;
- 仪器与试剂 equipment/reagents (including details of any hazards or precautions required and reagent stability information)
- 标样 、样本储存 full details of standard compound purity where relevant storage of validation samples prior to analysis (where appropriate, details of conditions and period of storage)
- 样本制备 general sample preparation techniques
- 分析过程：提取、仪器、校正曲线、典型图谱、典型质控图谱 analytical procedure (including extract preparation and analytical instrumentation) details of calibration where chromatographic technique used, representative chromatograms, including peak assignments, (control blank(s), analytical standard/matrix standard(s), lowest fortification(s))
- 计算方法、参考文献 calculations； references

方法描述（续）

- Quantification procedures should be described, including detection system calibration, calculation of analyte concentration and any compliance with statistical parameters required.
- Supporting chromatograms/spectra or non-chromatographic data should be clearly labelled. Labelling should include sample description, scale, concentration and identification of all relevant components.
- 定量计算要求： 校正曲线、计算过程、统计分析
- 附图谱要标注， 包括样本、浓度、主要结果和标识

2 方法适用范围与应用

- **Sample extraction and purification techniques**：The use of novel/complex analytical techniques/ instrumentation or hazardous reagents must be justified.
- **Derivatisation**
- **Non-specific and common moiety methods**：Disadvantages：acceptable in exceptional circumstances where there is no other practical means of determining the target analyte, and in these cases, full justification is required.

3、METHODS OF ANALYSIS FOR TECHNICAL MATERIAL AND PREPARATIONS

- 原药： 有效成分与杂质

采取CIPAC、AOAC等官方方法；不需要全部验证各参数， interference 干扰物 < 0.3%. 杂质分析方法的验证。

Mean % recovery: = $\frac{\text{Mean \% content determined x 100}}{\text{Theoretical \% content}}$

This mean % recovery should be within the following ranges:

% active (nominal)	Mean % recovery
>10	98.0 - 102.0
1 - 10	97.0 - 103.0
<1	95.0 - 105.0

$$|t| = \left|(\bar{x} - \mu)\frac{\sqrt{n}}{s}\right|$$

$\bar{x}$ = sample mean

μ = true value

n = no. of samples

s = standard deviation

3.1 Method Validation for the active substance

- 特异性：干扰物< 3% area
- ~~线性： ＋ — 20%：r>0.99：~~ 3×2 or 1*5level
- *Accuracy: 回收率, 需要测定干扰物质影响和方法精密度。*
- *重复性： 至少5个重复， 符合改良的*Horwitz 方程。

- % Analyte Proposed acceptable RSDr
- (Horwitz value x 0.67) %
- RSDR = 2(1-0.5 logC)
- 100 1.34
- 50 1.49
- 20 1.71
- 10 1.90
- 5 2.10
- 2 2.41
- 1 2.68
- 0.25 3.30

3.2 Method Validation for relevant impurities

- 特异性： 证明在有效成分、或者其它杂质存在时可以检出某一杂质
- 线性范围
- 准确性： 回收率
- 精密度
- LOQ

不同含量下 回收率要求

Guideline confidence intervals for % mean recovery from preparations, based on consultation with Industry, are as follows.

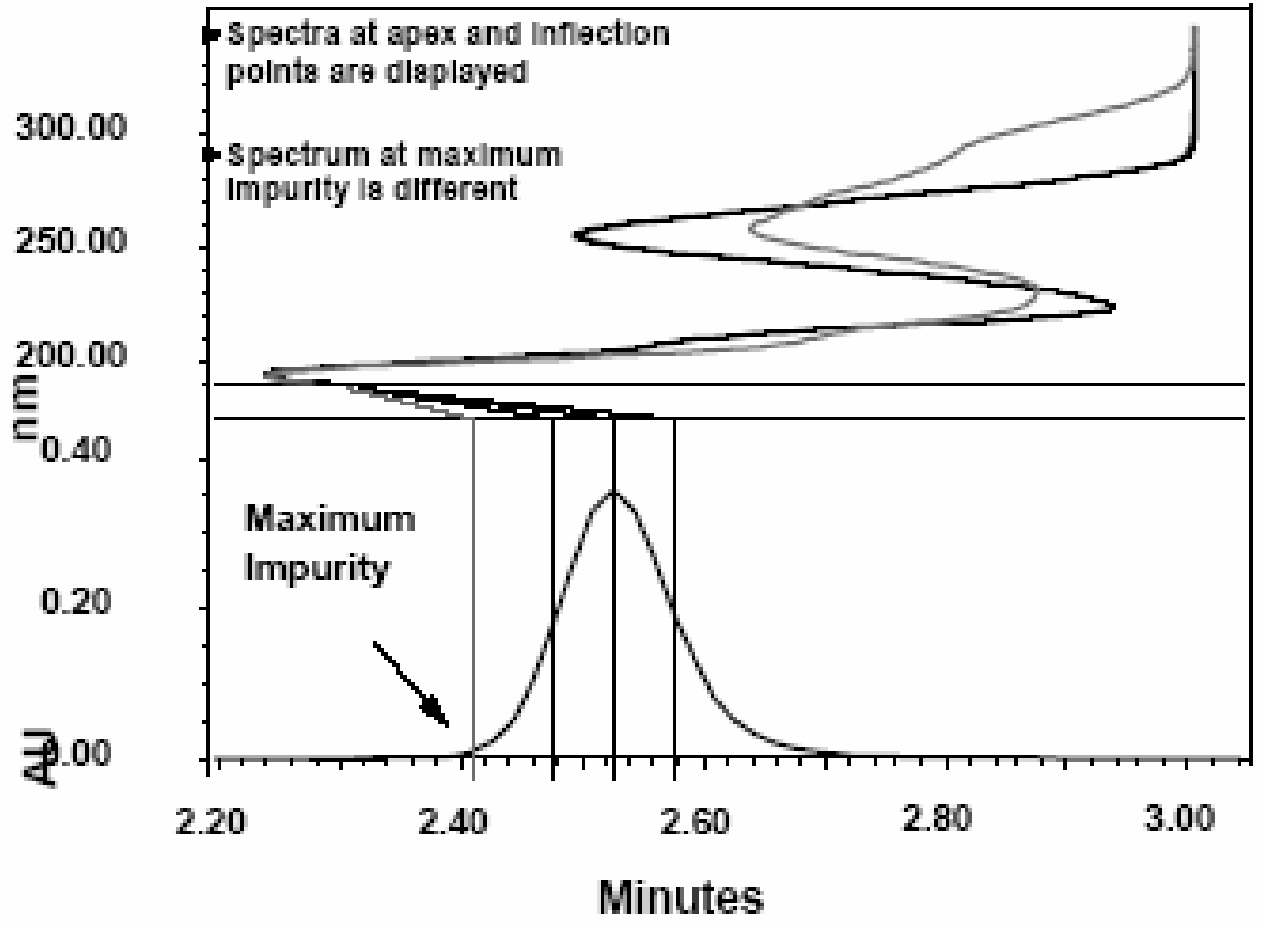
% active (nominal)	mean % recovery	% impurities(nominal)	mean % recovery
>10	98-102	>1	90-110
1-10	97-103	0.1-1	80-120
<1	95-105	<0.1	75-125
0.01-0.1	90-110		
<0.01	80-120		

3.3 Confirmation of analyte identification

毒理学意义杂质、主要杂质：

GC-MS,
HPLC-DAD,
HPLC-MS,
MS-MS,
NMR,
IR
MS: 至少 3个离子 （m/z >100）

Spectra at Various Points on the Peak



内部质量控制中的实例

Q 1

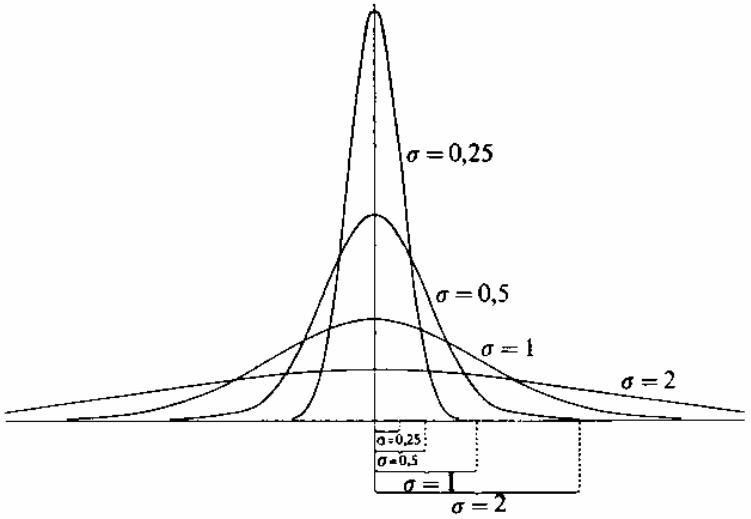
- 对同一样本， 有两组测定，结果如下：
  - A: 0.51; 0.5; 0.53; 0.5; 0.52
- B: 0.49; 0.55; 0.54; 0.44; 0.51
- 求：
- **1**、 各组平均值及其范围
- **2**、比较平均值是否有差异、结果的标准偏差是否有差异？
- **3**、单次测定结果的范围如何？

Q1:

$$\bar{x} = \frac{\sum_i x_i}{n} \qquad s = \left\{ \frac{\sum_i \left(x_i - \bar{x}\right)^2}{\nu} \right\}^{\frac{1}{2}}$$

CV= s/X(average)

V=s2



	A	B
Arithmetic mean	0.512	0.506
Standard deviation	0.013038	0.043932
Variance	0.00017	0.00193
CV	0.025466	0.086822

Q2:

expected range of the estimated mean values

$$\mu = \bar{x} \pm \frac{ts}{\sqrt{n}}$$

- A: 0.495813% - 0.528187 %,
- B: 0.45146 % - 0.56054 %,

- t2α=0.05, ν=4 = 2.776, in 95% of the cases.

Q2: Testing the difference of the mean values

$$t = \frac{\left|\bar{x}_1 - \bar{x}_2\right|}{\sqrt{\frac{s_1^2}{n_1} + \frac{s_2^2}{n_2}}}$$

- t = 0.9759
- t0.05, ν=4 = 2.776
- The null hypothesis is retained: the mean values are not different
- Note: since n1 = n2 = n, the degree of freedom is n-1

Q2:比较平均值是否有差异、结果的标准偏差是否有差异？

- Check the difference of standard deviations with F-test

$$F = \frac{S_1^2}{S_2^2}$$

by definition s12 > s22

If Fcalc < Ftab, P, ν1,ν2 then the difference is not significant.

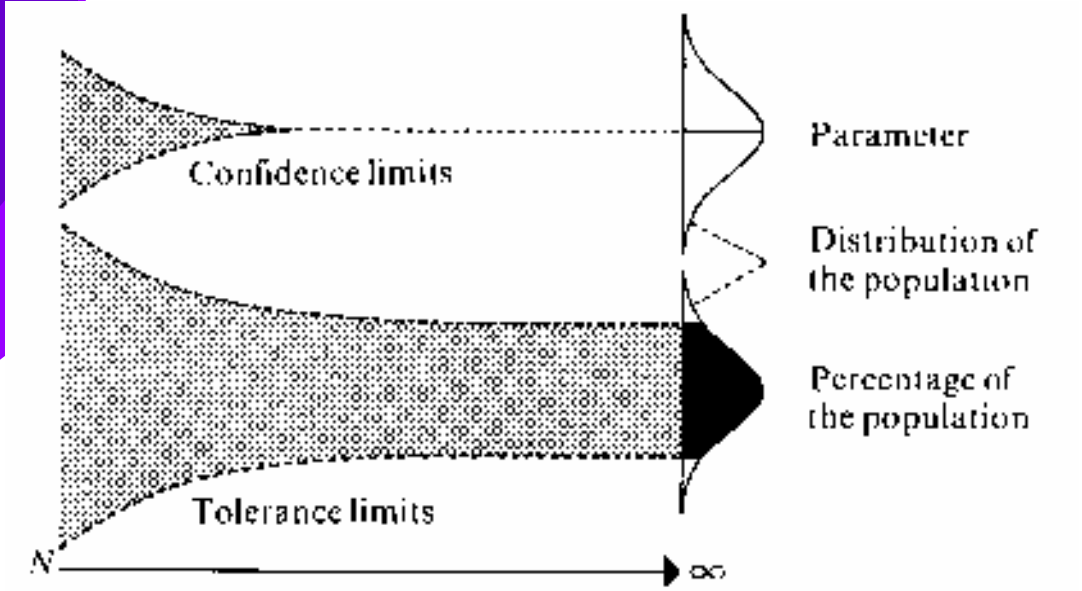
s1 =0.0439; s2 = 0.013

F=0.00193/0.0.00017= 11.35

F0.95,4,4 = 6.39; F0.975,4,4 = 9.6

s1 is significantly different from s2.

Q2: Expected range of individual measurements and their standard deviation



$$x_{\min}^{\max} = \bar{x} \pm ks$$

- At 95% confidence and probability level:
- A max0.578457 %B max0.72992 %
- A min0.445778 %B min0.28287 %
- Read k from Table A 2.3 at N=5: k=5.079

Q3: A, B, C 三人在不同的天数对粉剂的五个部分进行分析， 结果如下：

A d1	B d1	C d1	A d2	B d2	C d2	A d3	C d3
0.43	0.51	0.5	0.51	0.49	0.47	0.49	0.52
0.52	0.5	0.48	0.5	0.48	0.49	0.55	0.52
0.5	0.53	0.48	0.48	0.51	0.51	0.54	0.5
0.49	0.5	0.52	0.49	0.47	0.54	0.44	0.49
0.51	0.52	0.46	0.56	0.49	0.5	0.51	0.5

- **1) A, B, C** 三个人实验的重复性**?**
- Sa,ν=12 0.037193 % ； CV = 0.074189
- ~~Sb,ν=8 0.013964 % CV = 0.027928~~
- Sc,ν=12 0.021370 % CV = 0.042854

- **2)** 如何评价**A B C** 三人的测定**?**

- **3)** 建立实验室内部重复性、再现性**?**
  Sr =？ SR＝？

- **5) Are the Sr and SR values significantly different?**

- 6) 样本是否均匀?

	A d1	B d1	C d1	A d2	B d2	C d2	A d3	C d3
Ave	0.49	0.512	0.488	0.508	0.488	0.502	0.506	0.506
SD	0.035355	0.013038	0.022804	0.031145	0.014832	0.025884	0.043932	0.013416
Var	0.00125	0.00017	0.00052	0.00097	0.00022	0.00067	0.00193	0.00018

Q5: 检查异常数据: 统计分析前

The suspect population is the A d3 measurements:

Dixon test 0.55 0.54 0.51 0.49 0.44

$$r_{10} = (x_n - x_{n-1})/(x_n - x_1) \quad \text{or} \quad (x_2 - x_1)/(x_n - x_1)$$

$r_{10} = 0.454545$

$r_{10,\,0.05}$ 0.642 $r_{10} < r_{10,\,0.05}$ critical

The 0.44 % value is not an outlier

Q5: Outliner test:Grubb's test:

Grubb's test:

$$G'_{lowest} = (\bar{x} - x_1)/s \text{ or } C'_{highest} = (x_n - \bar{x})/s \qquad (3.5)$$

- If the test statistics G is $\leq G_{crit,0.05}$ (5% critical value) the item tested is accepted as correct.
- If $G_{crit,0.05} < G \leq G_{crit,0.01}$ the item is called straggler
- If $G > G_{crit,0.01}$ the item is a statistical outlier.

calculate *s* with all data points, see A11 for critical values

$G'_{lowest} = 1.502$,

$G'_{0.05} = 1.672$

The 0.44 % value is not an outlier

Table A.13

Q5: 三个人的分析水平是否相当？

Apply Cochran test to verify that the 8 sets of measurements may come from the same population:

$$g = \frac{S_{\max}^2}{\sum_{i=1}^{p} S_i^2} \qquad (3.8)$$

g = 0.00193/0.00591 = 0.326565

read critical value from table A13.1 at p=8 (number of data sets, and n=5 (number of replicate measurements): $g_{0.05} = 0.391$

0.391> 0.32

The measurements may come from the same population, that is there is no significant difference between the performance of analysts.

Note: for such comparison, that is comparing the results of a series of replicate measurements, the F-test cannot be applied!

Q5: 实验室内重复性

$$s_p = \sqrt{\frac{(s_1^2 x df_1) + (s_2^2 x df_2) + \ldots + (s_n^2 x df_n)}{df_1 + df_2 + \ldots + df_n}}$$

- The average repeatability standard deviations and corresponding coefficient of variations of the individual analysts (calculated from their measurements made on different days) are:
- SA,ν=12 0.037193 % CV = 0.074189
- SB,ν=8 0.013964 % CV = 0.027928
- SC,ν=12 0.021370 % CV = 0.042854

What is the within laboratory repeatability (Sr) of the method?

It is the average of the variations obtained by all analysts:

Pooled standard deviation

$$S_p = \sqrt{\frac{(s_1^2 x\, df_1)+(s_2^2 x\, df_2)+\ldots+(s_n^2 x\, df_n)}{df_1+df_2+\ldots+df_n}}$$

$$df_p = df_1+df_2+\ldots+df_n$$

The $df = \nu$ of each set of measurement in this case is 5-1=4. The ν_p= 8*4=32!
$S_p = S_r = 0.02718$

What is the within laboratory reproducibility (SR) of the method?

$$s = \left\{ \frac{\sum_i \left(x_i - \bar{x}\right)^2}{n-1} \right\}^{\frac{1}{2}}$$

- The within laboratory reproducibility of the method is the SD of all measurements calculated with eq. 2.5: SR= 0.02632

- Note: Sr ≤ SR!

Q5: 评价室内重复性和室间再现性

Apply F-test to decide (if it is not obvious):

F= 1.0672

Apply two sided test at P = 0.95, read F_{crit} at

$F_{32/39} \sim F_{30/40} = 1.74$ $F_{40/40} = 1.69$

P = 0.9, read F_{crit} at

$F_{32/39} \sim F_{30/40} = 1.54$ $F_{40/40} = 1.51$

The difference is not significant!

样本是否均匀？
OR are the mean values obtained significantly different?

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
A d1	5	2.45	0.49	0.00125
B d1	5	2.56	0.512	0.00017
C d1	5	2.44	0.488	0.00052
A d2	5	2.54	0.508	0.00097
B d2	5	2.44	0.488	0.00022
C d2	5	2.51	0.502	0.00067
A d3	5	2.53	0.506	0.00193
C d3	5	2.53	0.506	0.00018

ANOVA

Source of Variation	*SS*[1]	*df*	*MS*[2]	*F*[3]	*P-value*[4]	*F crit*
Between Groups	0.00336	7	0.00048	0.649746	0.711786	2.312738
Within Groups	0.02364	32	0.000739			
Total	0.027	39				

Q3: 双柱验证－定量分析的例子

- 假设采取内标法测定某杂质，**(CVra = 1.5%).** 对同一个提取液的含量分别进行**3**次测定。

- **CPSIL8CB 0.535 mg/ml：**
- **CPSIL5CB 0.562 mg/ml.**

- Q：两次的结果是否有差异？ －> 是否色谱柱中有干扰物？

Q6

$$t = \frac{\left|\bar{x}_1 - \bar{x}_2\right|}{s_p\sqrt{\frac{1}{n_1} + \frac{1}{n_2}}} \qquad s_p^2 = \frac{(n_1 - 1)s_1^2 + (n_2 - 1)s_2^2}{n_1 + n_2 - 2}$$

- The calculated t value is 4.018,
- $t2\alpha = 0.05$, $\nu = 4$ = 2.776

- The difference is significant (5.9238%). The results indicate the possibility of impurity.