Uncertainty Estimation on the Quantification of Major Proteins in Milk by Liquid Chromatography

Contribution to a Chemical Reference Measurement System

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Content

Introduce… the Objectives / ..the Analytical Problem

1. **Specify the Measurand**

   Basic Statistics

2. **Identify Uncertainty Sources**

   Introduce Stated References / Calibrants

3. **Quantify Uncertainty**

4. **Combine Uncertainties / Report Uncertainty**

   Conclusions

Relevant Guidelines

"**Guide to the Expression of Uncertainty in Measurement**", (GUM), ISO, 1995 (JCGM 100:2008)

"**Quantifying Uncertainty in Analytical Measurement**", EURACHEM/CITAC Guide, 2nd Ed., 2000

"**The Fitness for Purpose of Analytical Methods**", EURACHEM, 1998

Definitions

Measurement Uncertainty (MU)

“non-negative parameter characterizing the dispersion of the **quantity values** being attributed to a **measurand,** based on the information used”

VIM, 2008.

Why?

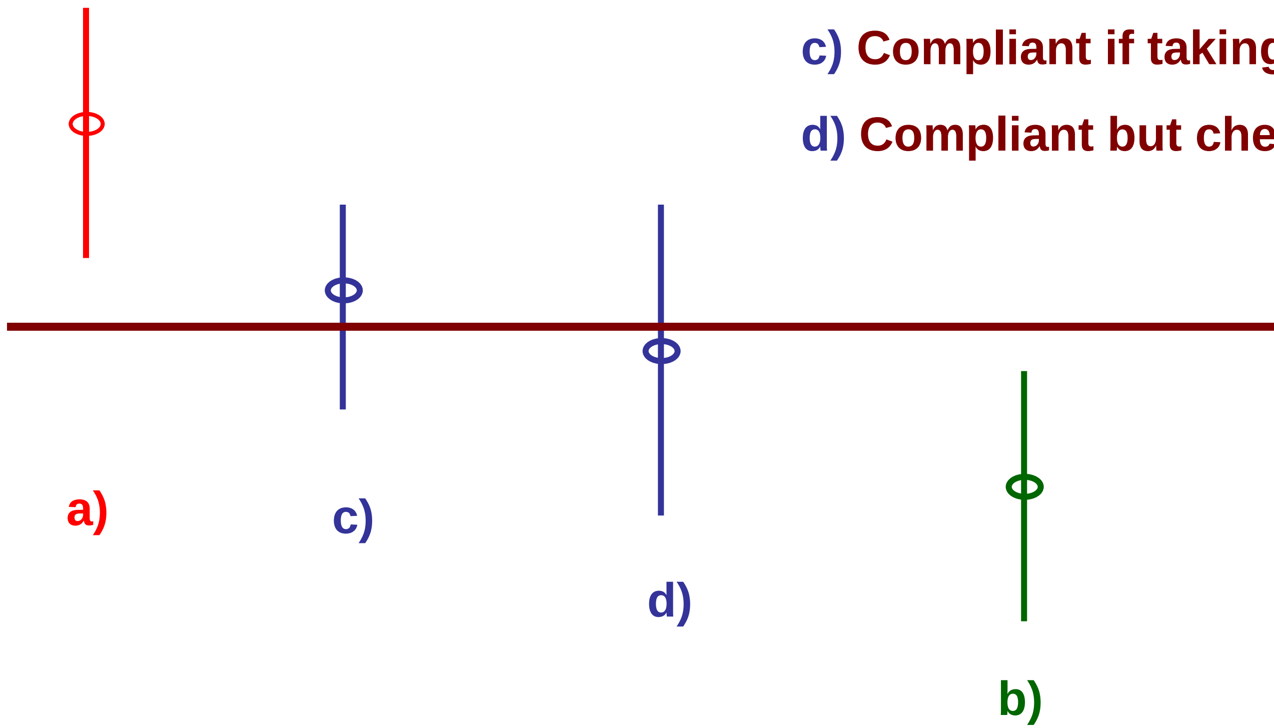
- ✓ Compliance with legal limits (or contractual)
- ✓ Confidence in analytical results
- ✓ Quality Assurance
- ✓ Demonstrate "*Fitness for purpose*"
- ✓ Traceability - Measurement...
  Comparability/Transferability

Compliance with legal limits

Result ± MU (expanded U ~ 95 %)

c) Compliant if taking MU into account

d) Compliant but check for future sample



Upper (legal) control limit

a) Result exceeds the limit plus U (non-compliant)

b) Compliant

c,d) Result exceeds/is below the limit by less than U (requires discussion in light of any agreement with the user of the data)

Traceability

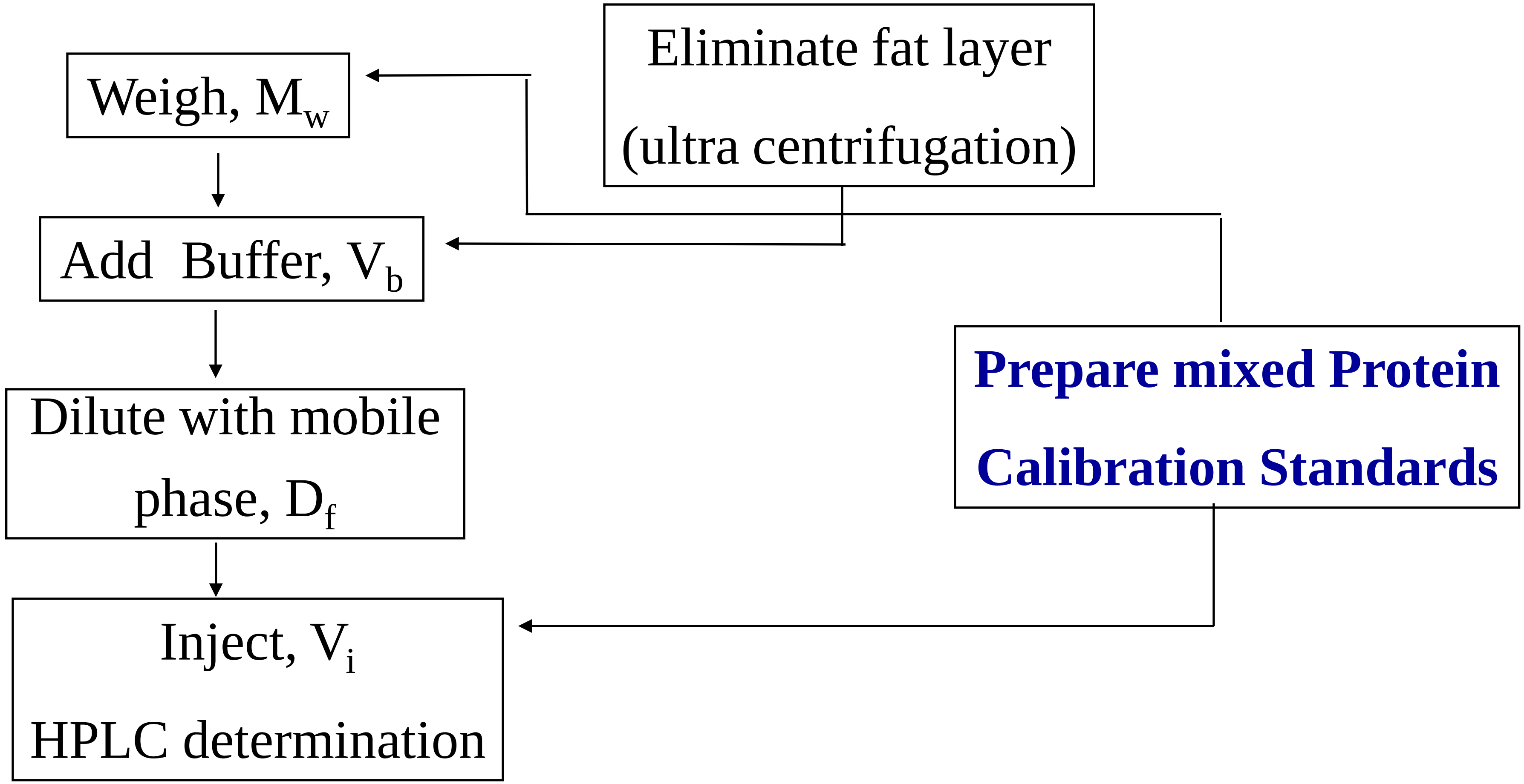
"property of a **measurement result** whereby the result can be related to a reference through a documented unbroken chain of **calibrations,** each contributing to the **measurement uncertainty**"

VIM: 2008

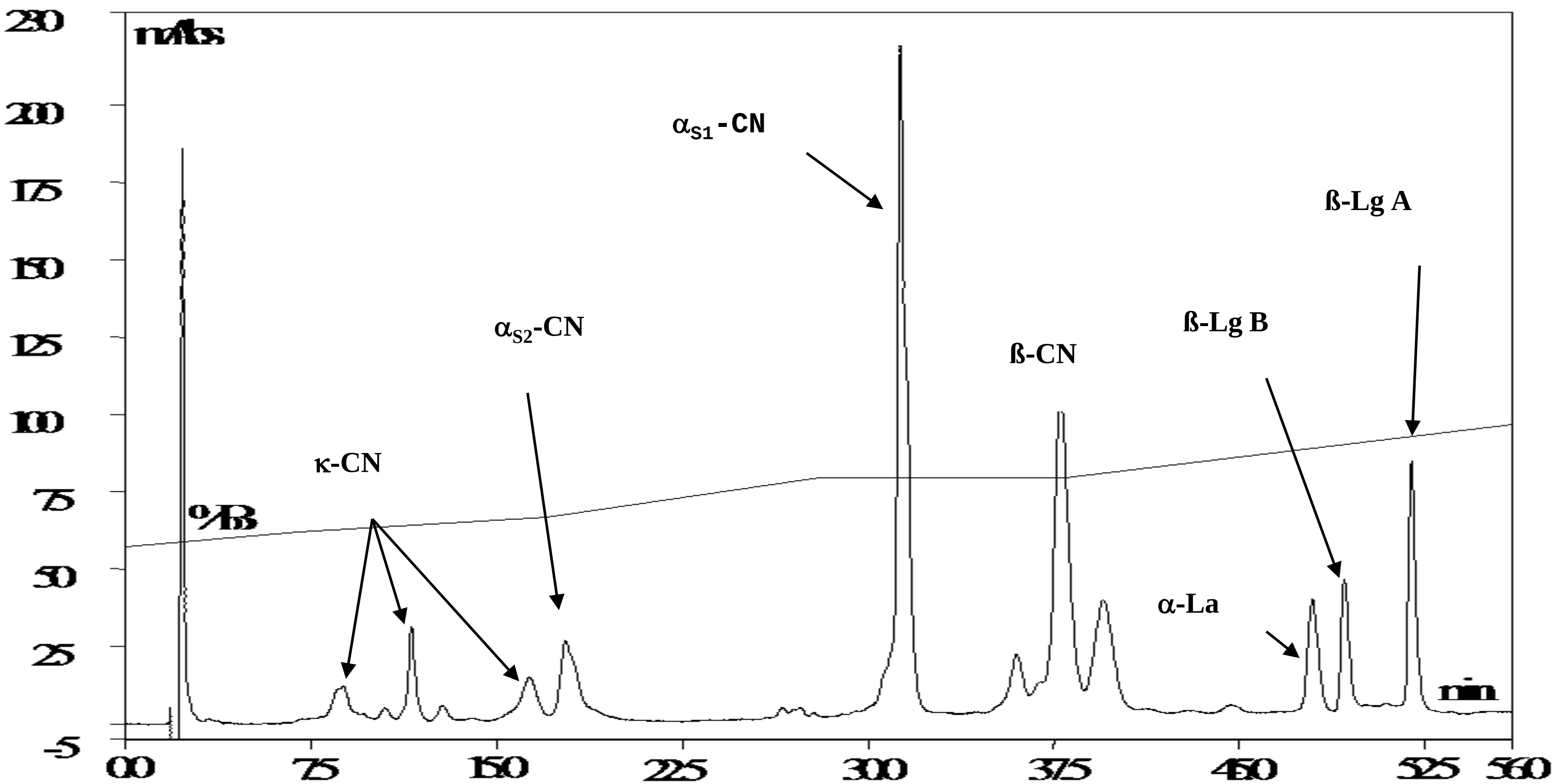
Experimental

Powdered Samples

Liquid Samples



Separation of Major Milk Proteins by Reversed Phase Ion-pair HPLC



Typical chromatographic profile for raw bovine milk

1. Specify the Measurand

Protein Concentration (C)

$$C = M_0\ 1000 / M_i$$

where M_i = injected mass of sample, depends on...

$M_i = M_w\ V_i\ 1000\ D_f / V_b$ **(powdered milk samples)**

$M_i = D\ V_s\ V_i\ 1000\ D_f / (V_b + V_s)$ **(liquid milk samples)**

Combining...

$C = M_0\ V_b / (M_w\ V_i\ D_f)\ .EF$ **(in µg mg^{-1})**

Combined Standard Uncertainty

Y = a + b

$$u_c(Y) = \sqrt{u(a)^2 + u(b)^2}$$

Y = a / b

$$\frac{u_c(Y)}{Y} = \sqrt{\left(\frac{u(a)}{a}\right)^2 + \left(\frac{u(b)}{b}\right)^2}$$

Uncertainty Budget / Law of Uncertainty Propagation

Basics (2)

Estimating Uncertainties of Type A:

Evaluation of uncertainty by statistical analysis

Estimating Uncertainties of Type B:

Evaluation of uncertainty by other means than statistical analysis

Only type A uncertainties can be improved

...by more measurements

2. Uncertainty Sources

Uncertainty Component	Value	Unit	u(x)	Distribution	n	Obser.
Weighing, M_w	≈ 1.5	mg	0.32 %	Normal	6	
Volumetric measurements (V_s, V_b, V_t)	(Gravimetry)	µl	Various	Normal	15	Done for each dispensed volume
Injection, V_i	20.0	µl	0.15 %	Triangular	-	Type B
Dilution, D_f	0.333	-	0.25 %	Combining		
Interpolated, M_0	-	µg			6	Regression
Protein Purity	1		6.5 % (of C)	Rectangular	-	
Injected mass (Standards, M_i)	1	µg	0.3 % (of C)	Rectangular	-	
Analytical Recovery (R_m)	1.04	-	0.025	Normal	70	On MCRM

Calibrants / Traceability

Purified Caseins:

κ-CN C-0406 (> 80 % by electrophoresis)

α-CN C-6780 (~ 85 % by electrophoresis), contains both α_{S1}-and α_{S2}-CN

ß-CN C-6905 (> 90 % by PAGE)

Purified Whey proteins:

α-La L-5385 (~ 85 % by PAGE)

ß-Lg B L-8005 (> 90 % by PAGE)

ß-Lg A L-7880 (> 90 % by PAGE)

Individual solutions are prepared and calibration curves (total peak area Vs. injected mass of protein) are established based on a mixed standard solution (mimics a real sample).

BCR 063R - Skimmed Milk Powder

(certified for trace elements including Total Nitrogen)

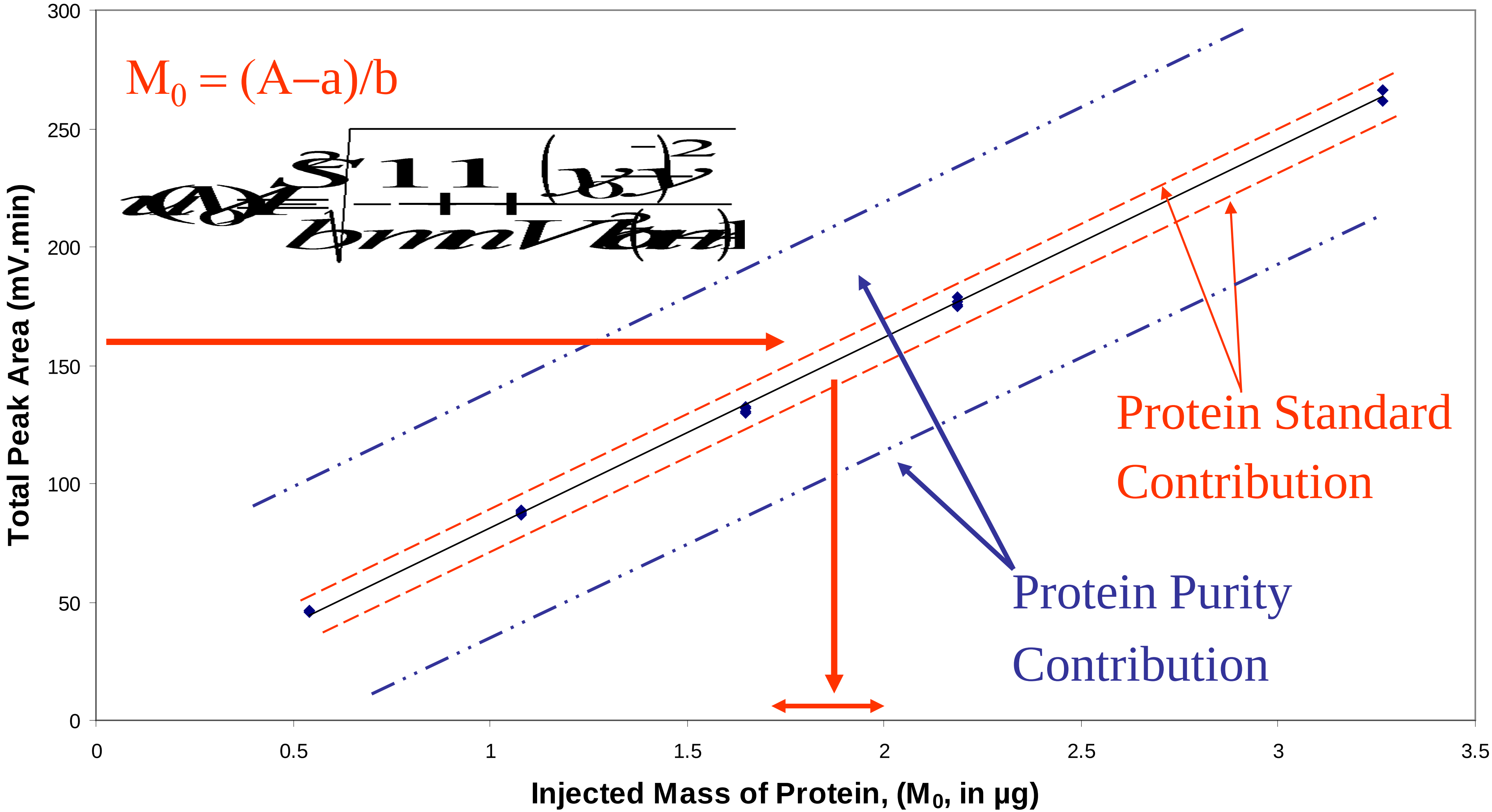
- **Matches** a real milk powder
  (analyte concentration and matrix composition)

- **Is Representative for the investigated samples**

- **Total Protein content can be estimated with stated uncertainty - (Method Validation and Assessment of MU, Uncertainty in Analytical Recovery)**

Estimating MU (Regression)

Uncertainty Estimation on the Regression



Uncertainty Contributions for a Single Major Protein: κ-CN

Uncertainty Component	Interpolated Mass (M_0 in μg)		C (μg mg^{-1})	Range	u(x)	u(x)/x	Obser.
		1.990	48.11		0.01	0.65 %	Nominal
Injected Mass Standard Protein	Lower limit	1.978	48.11	0.49/2	0.25/√3	0.3 %	Rectangular Distribution
	Upper limit	1.998	48.60				
Protein Purity	100 % purity	2.21	53.76	11.19/2	5.6/√3	6.5 %	Rectangular Distribution
	Lower Purity	1.75	42.57				

Other Sources of Uncertainty

- **Analytical Bias. 12 independent preparations of matrix CRM (BCR) (n=6, N=71): +15.20 µg mg⁻¹**

- **Mean method Analytical Recovery R_m = 1.04, S_{obs} = 0.05**

$$u(R_m) = R_m \sqrt{\left(\frac{S^2_{obs}}{nC^2_{obs}}\right) + \left(\frac{u_{CRM}}{C_{CRM}}\right)^2} = 0.025$$

- **Significant t-test is performed, if:**

$$\left|R_m - 1\right| / u(R_m) > t_{crit}$$ **: R_m differs from 1**

t_{crit}(N-1, 0.05) = 1.99 > t_{cal} = 1.6 → no correction applied

- **Sample treatment steps all Combined in the above procedure.**

4. Combining Uncertainties (1)

Assuming no correlation between input quantities we have:

$$u_c{}^2(y) = \sum \left(\frac{\delta f}{\delta x_i} \right)^2 \cdot \left(u(x_i) \right)^2 \qquad \text{(Eqn. 1)}$$

Where,

$$y = f(x_1, x_2, x_3, .., x_i)$$

Combining Uncertainties (2)

On a computer . . .

$$\frac{\delta f}{\delta x_i} = \frac{f(x_i + h) - f(x_i)}{h}$$

if now

$$h = u(x_i)$$

Combining Uncertainties (3)

we get . . .

$$\frac{\delta f}{\delta x_i} = \frac{f(x_i + u(x_i)) - f(x_i)}{u(x_i)}$$

this we put into Eqn. 1

Combining Uncertainties (4, Kragten*)

$$u_c^{\,2}(y) = \sum \left(\frac{f(x_i + u(x_i)) - f(x_i)}{u(x_i)} \right)^2 \cdot \left(u(x_i) \right)^2$$

which can be simplified to:

$$u_c^{\,2}(y) = \sum \left(f(x_i + u(x_i)) - f(x_i) \right)^2$$

* J. Kragten, *Analyst*, 1994, **119**, 2161-2166

Uncertainty Budget (single protein)

	Value	u(x)	u(x)/x	M_w	Injection	Dilution	V_b	M_o	Purity	M_i
M_w	6.18	0.01978	0.32	6.19978	6.18	6.18	6.18	6.18	6.18	6.18
Injection	20	0.03	0.15	20	20.03	20	20	20	20	20
Dilution	0.33356	0.00083	0.25	0.33356	0.33356	0.33439	0.33356	0.33356	0.33356	0.33356
V_b	1002.9	1.10319	0.11	1002.9	1002.9	1002.9	1004	1002.9	1002.9	1002.9
M_o	1.99	0.01294	0.65	1.99	1.99	1.99	1.99	2.00294	1.99	1.99
Purity	1	0.03147	3.15	1	1	1	1	1	1.03147	1
M_i	1	0.00145	0.15	1	1	1	1	1	1	1.00145
C (µg mg^{-1})	48.41	1.6	3.25	48.254	48.336	48.287	48.461	48.723	49.931	48.478
			Diff	-0.154	-0.073	-0.121	0.053	0.315	1.523	0.070
			Squares	0.024	0.005	0.015	0.003	0.099	2.320	0.005
Uncertainty Contribution:				1.0	0.2	0.6	0.1	4.0	93.9	0.2

Concentration: 48.41 ± 3.1 µg mg^{-1} (U, k=2)

$$C = M_0 V_b /(M_w V_i D_f)$$

Total Protein (TP) Content

Group	Protein	C	u(C)	(% P)	G (%)	U
Whey	α-La	8.0	0.5	2.0	8.3	0.5
	ß-LG B	8.3	0.3	2.0		
	ß-LG A	17.5	0.5	4.3		
Caseins	κ-CN	48.4	1.6	11.8	91.7	5.4
	α_{S1}-CN	177.6	6.0	43.4		
	α_{S2}-CN	20.7	1.0	5.1		
	ß-CN	128.9	5.1	31.5		
TP ± U (k=2)		409.4 ±	16.3	μg mg^{-1}	REU =	4%

Total Protein : TP = $\sum_i C_i$

$$u_c(TP) = \sqrt{\sum_i u_c^{\,2}(C_i) + u^2(R_m)}$$

Certified N = 62.3 μg mg^{-1} ⇨ TP = 397.5 ± 10.2 μg mg^{-1}

Conclusions (1)

Measurement Uncertainty demands ...

- ✓ **Clear statement for the measurand**
- ✓ **Identification and quantification of All conceivable sources of uncertainty**
- ✓ **Major uncertainty sources Should be re-evaluated**
- ✓ **The Method of Estimation May Affect Uncertainty Values**

Conclusions (2)

Measurement Uncertainty leads to ...

- ✓ **Better understanding of the method**
- ✓ **Continuous improvement through Good Chemistry/QA**
- ✓ **Higher Reliability on the analytical results**
- ✓ **Quality (on the measurement) is demonstrated**

Technical requirements towards;

- Personnel (competence, training, responsibilities, etc)
- Accommodation
- Test, Calibration and Method Validation
  - ✓ Using CRM/RMs for instrument, method calibration and IQC
  - ✓ Participation in ILCs
  - ✓ Systematic assessment of all influencing factors
  - ✓ Measurement uncertainty
- Equipment (calibration, acceptance limits)
- Quality control (acceptance limits)
- Traceability

ISO 17025: 2005

5.4.6 …Shall apply a procedure for MU…shall at least attempt to identify all the components of uncertainty and make a reasonable estimation

- **ISO-GUM (bottom-up approach)**
- **From Validation (top-down approach)**

Consider as much as possible all conceivable uncertainty sources!

MU from Method Validation (1)

Using collaborative method validation studies:

- Precision (as S_R at different concentration levels)
- Estimation of laboratory bias and method trueness (CRMs)
- Estimate the effect of any additional factors

(Assessment of the MU based on scientific understanding of the theoretical principles of the method)

Use A Well Balanced Experimental Design!

MU from Method Validation (2)

Design your experiments trying to mimic real routine conditions… Use different…

1 - Operators,

2 - Time,

3 - Instruments, (identical) chromatographic columns,

4 - Standard (stock) solutions, response curves

Use RM/CRM which matches routine materials

Apply analysis of variance (ANOVA)

Statistical grounds for your S_R estimation!

(ISO 21748:2004) S_R ~ MU (collaborative study)

MU from Method Validation (3)

13 independent sample preparations. Effect of time, stock solutions, response curves, detector response, etc.

M_i	Total Protein Concentration, (in µg mg^{-1})						TP_{obs}	$u_c(TP_{obs})$
41.11	379.23	377.04	372.90	372.17	375.33	374.85	376.09	11.91
45.77	445.93	427.14	407.69	437.40	421.46	428.01	427.89	12.91
45.90	385.84	387.58	398.04	393.03	390.20	390.41	390.64	11.34
41.97	375.98	419.82	415.30	408.86	375.03	397.90	399.06	12.16
42.04	410.80	410.80	393.20	416.98	386.77	392.01	402.00	12.31
47.56	403.07	408.12	417.79	411.06	416.95	430.19	414.42	15.32
38.51	380.68	380.16	378.86	377.82	380.94	386.39	380.90	11.39
43.10	378.89	379.58	383.29	386.08	379.12	383.29	381.39	11.37
42.51	394.97	398.73	397.55	394.50	392.14	390.03	394.54	10.71
39.45	378.96	393.41	393.92	397.21	397.97		392.44	11.68
44.70	385.68	414.99	402.68	400.00	401.57	391.95	399.77	11.93
37.45	441.12	438.72	456.07	454.74	440.32	443.79	445.66	12.78
43.17	365.53	369.47	369.47	375.03	379.66	379.43	373.40	10.10
Total N = 77							**398.32**	**12.00**

Expanded uncertainty for total protein content ~ 24 µg mg^{-1} (k = 2)

MU from Method Validation (4)

Protein	S_r (a)	r (a)	S^2_{Be}	S_I (a)	R_w (a)	n	$u_c(x)$
κ-CN	1.1	3.1	2.3	1.9	5.2	6	1.6
α_{S2}-CN	2.0	5.5	26.4	5.5	15.3	6	1.1
α_{S1}-CN	2.9	8.1	32.4	6.4	17.7	6	10.2
ß-CN	4.4	12.1	217.3	15.4	42.6	6	5.3
α-La	0.9	2.4	0.3	1.0	2.9	6	1.2
ß-Lg B	0.8	2.2	2.0	1.6	4.5	6	2.0
ß-Lg A	1.5	4.2	10.4	3.6	9.8	6	1.4
TP_{obs}	9.3	25.6	423.9	22.6	62.6	6	12.0
Group Protein (%)	0.4	1.2	0.6	0.9	2.4	6	11.6 [b)] 2.7 [c)]

k-CN (u_c) = 1.6 µg mg^{-1} (bottom-up). S_I ~ MU = 1.9 µg mg^{-1}

Using Horwitz values $\sigma_H = 0.02\ c^{0.8495}$ = 1.5 µg mg^{-1} (48.4.10^{-3})