

## The IRMM/IFCC myoglobin standardisation project: providing a model

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<http://irmm.jrc.ec.europa.eu/>

<http://www.jrc.ec.europa.eu/>



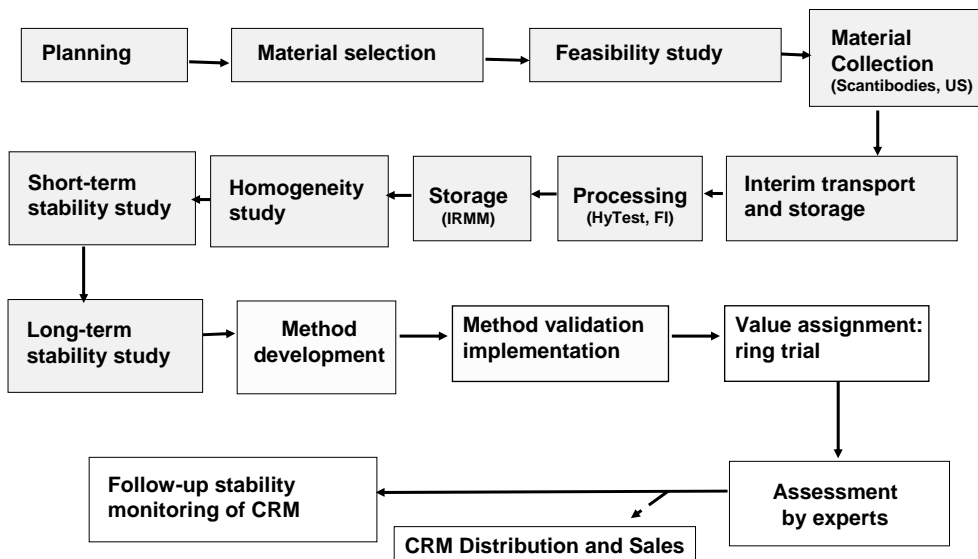
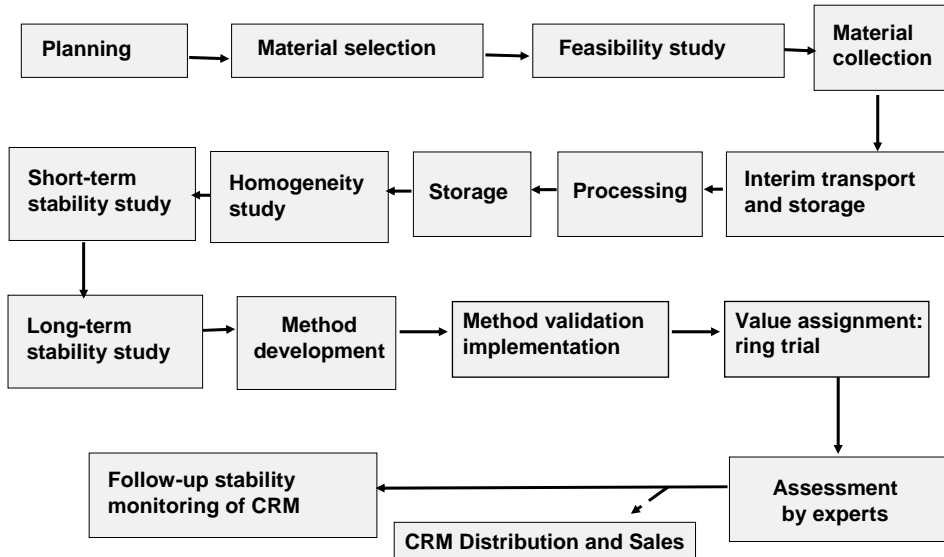
**Material: human serum spiked with HyTest human heart  
myoglobin**

**Targeted concentration: 500-600 µg/L**

**Form: lyophilised from 2 mL serum**

**Parameter to be certified: myoglobin content expressed in  
mol of peptide / L of reconstituted material**

**Amount: 2500 glass vials**



**Integra 800**  
(turbidimetry)

Mean: 850.7 µg/L  
SD: 10.1 µg/L  
CV: 1.2 %



**Homogeneous** ( $F < F_{crit}$ )

No analytical trend

Dilution of sample to fit in linear range

*Autosampler cooled at 2-8 °C*

*Run time 10 min*

**Elecsys 2010**

(electrochemiluminescence)

Mean: 582.02 µg/L  
SD: 8.7 µg/L  
CV: 1.5 %



**Not homogeneous** ( $F > F_{crit}$ )

**Analytical trend**

*Autosampler cooled at 15 °C*

*Run time 15 min*

N=15, n=2

**Method: Elecsys 2010** (*best linear range*)

→ **correction for analytical trend**

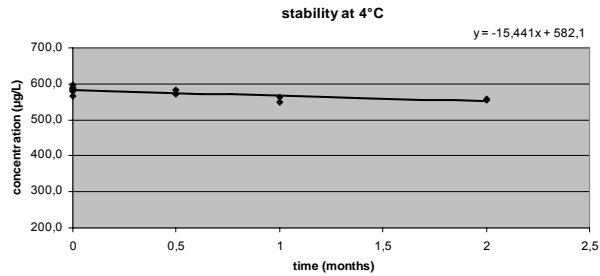
**Reference temperature: -70 °C**

**Tested temperatures:**

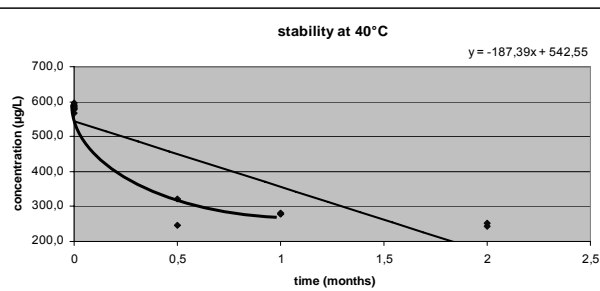
**Short-term 0-2 months: 4 °C, 40 °C**

**Long-term 0-48 months: -20 °C, 18 °C**

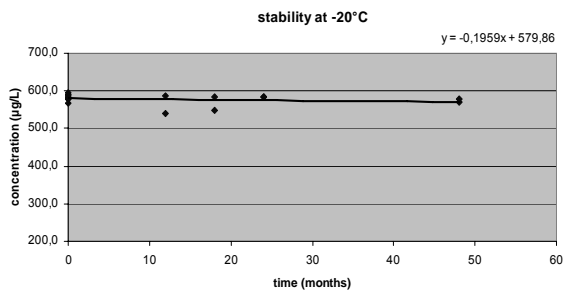
**2 units per temperature and per time, in duplicate**



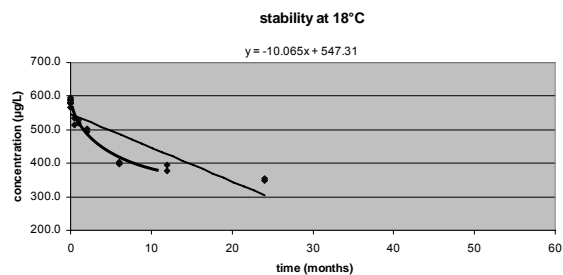
Significant  
slope



Significant  
slope



No significant  
slope



Significant  
slope

**Method: Integra 800** (*no analytical trend*)

**1. Non reconstituted CRM:**

1. Short-term stability: stable for 8 days at 4 °C & 18 °C;  
unstable for 1 h at 60 °C → dispatch conditions
2. Reference samples: stability in liquid N<sub>2</sub> vapour comparable  
to -70 °C → stability monitoring
3. Lyophilised comparable to frozen material → stabilised form

**2. Reconstituted CRM:**

1. Stable for 48 h at 4 °C, 6 h at 20 °C → use on the bench
2. Stable for 1 h at 37 °C → enzymatic digestion

**Development of a characterisation method based on  
standard addition of myoglobin and internal standard  
(IRMM - Univ. Saarland- Univ. Tübingen- Univ. Berlin)**

***Journal of Proteome Research 2006, 5, 414-421***

**Principle:**

1. Separation of myoglobin from most abundant proteins by  
anion-exchange LC
2. Myoglobin fraction digested with Trypsin
3. Myoglobin peptides analysed by capillary LC-(ESI)MS-MS
4. Quantification based on peak area ratios of peptides from  
myoglobin and internal standard

**1. Enzymatic digestion:**

- **No peptide detected**  
→ **Experimental conditions modified: 19h, recovery ~ 57 %**
- **Unsuitable internal standard (horse myoglobin):**
  - **digestion recovery ~32 %**
  - **digestion kinetic horse < human myoglobin**

**Thus, need for:**

- **Alternative internal standard (labelled human myoglobin)**
- **Shortened digestion time (stability 1h at 37 °C)**
- **Validated enzymatic digestion**

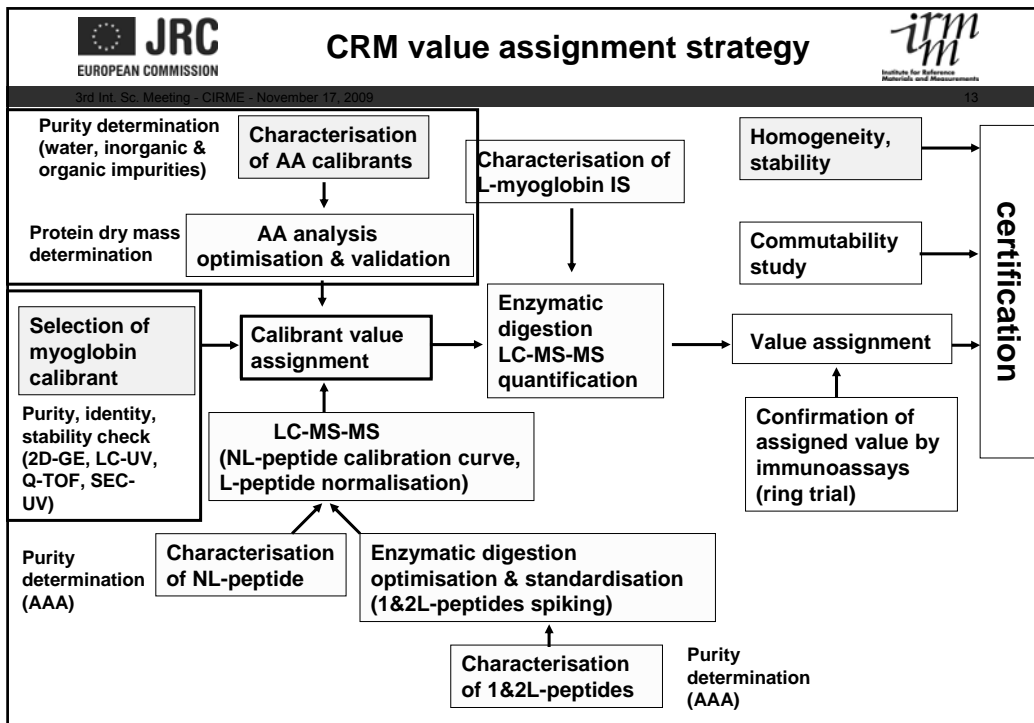
**2. Calibrant:**

**Purified human myoglobin selected**

- **Search for impurities: Q-TOF, LC-UV, 2D GE-MS, SEC-UV**
- **Stability of working solutions checked**
- **Value assignment by AAA and MS (on-going)**

**Thus, need for:**

- **Validated in-house AAA and MS methods**
- **AA calibrants characterised for AAA**
- **Peptide calibrants characterised for MS**
- **Intercomparison with laboratories for AAA and MS**



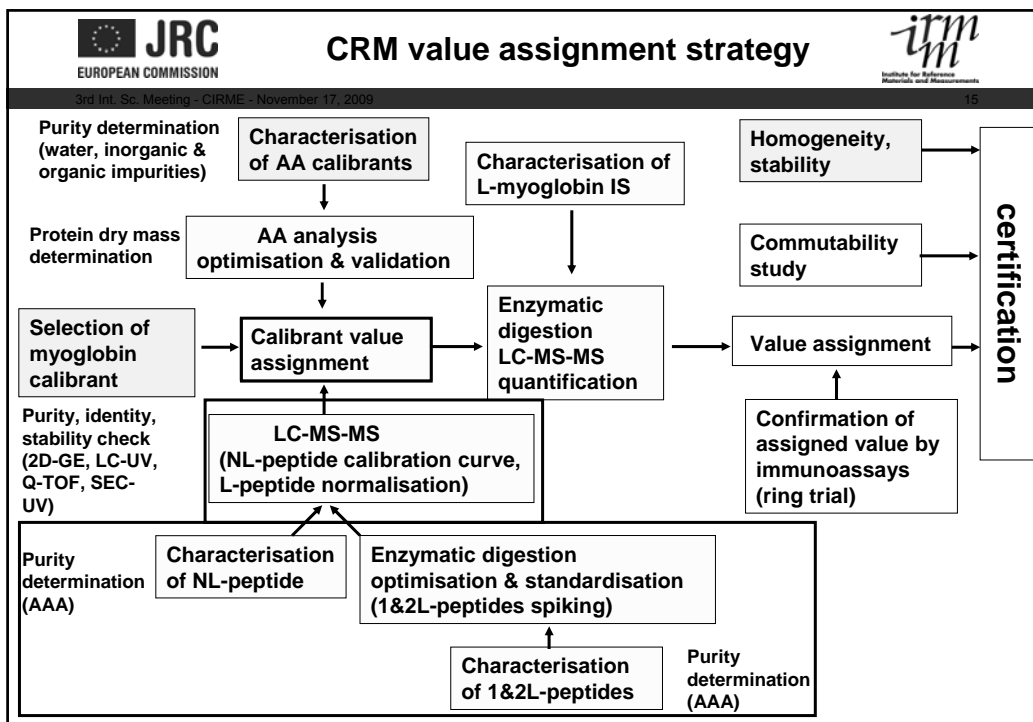
**JRC** EUROPEAN COMMISSION **Amino acid analysis: current work** *irm* Institute for Reference Materials and Measurements

3rd Int. Sc. Meeting - CIRME - November 17, 2009 14

**Amino acid analysis is used for the characterisation of human myoglobin and the target myoglobin peptide and internal standards**

**The method has been optimised at IRMM**

- Validation is on going
- Trueness is verified by a second primary method (dry mass determination)



**JRC** EUROPEAN COMMISSION **Enzymatic digestion: current work** *irm* Institute for Reference Materials and Measurements

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**Standard enzymatic digestion conditions:**

- More than 24 h digestion to reach plateau
- Need for additional spiking of trypsin

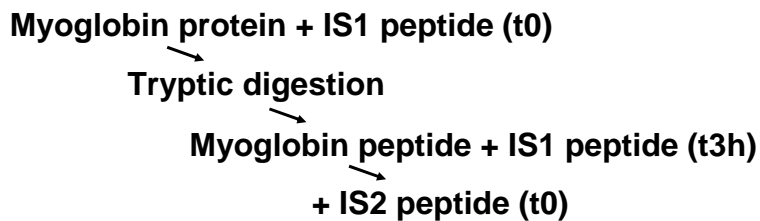
**Peptide degradation during digestion results in underestimation of the degree of completion of the digestion and therefore biases the overall myoglobin quantification**

**Therefore need to speed up the digestion and follow up the degradation of the forming peptide**

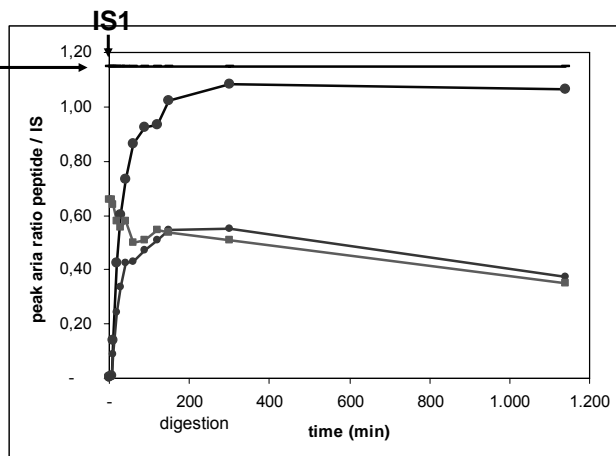


**IRMM approach:**

- We are in the process of optimising digestion conditions to reduce the time needed for the digestion
- We use two internal standards to estimate the degree of completion of digestion and the degree of degradation of the forming peptide



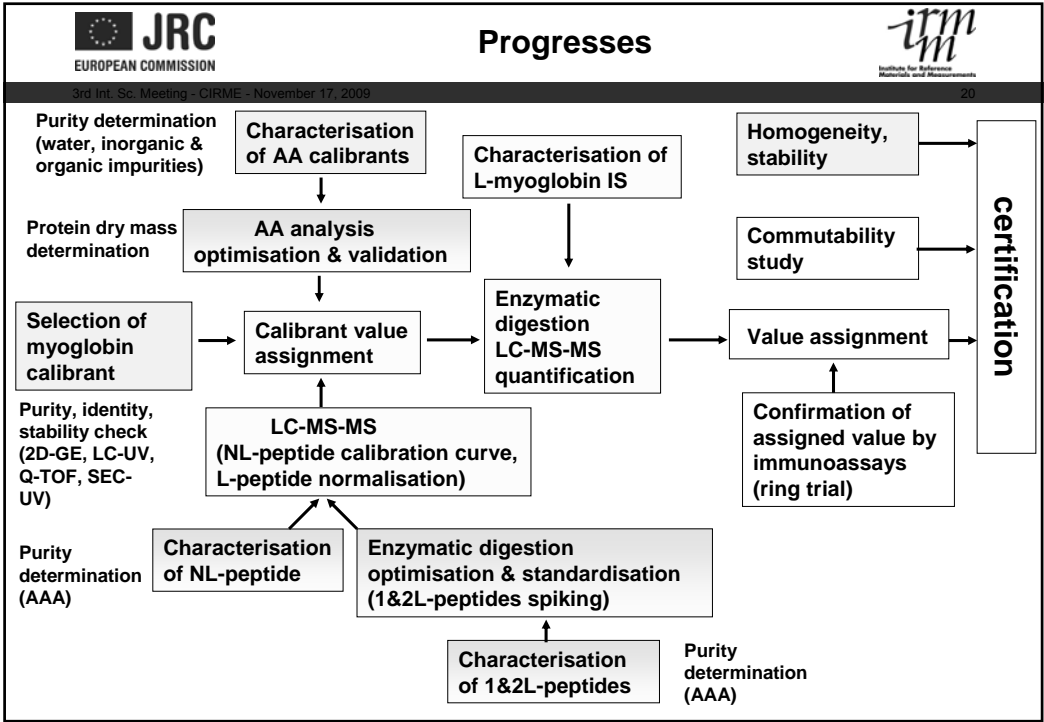
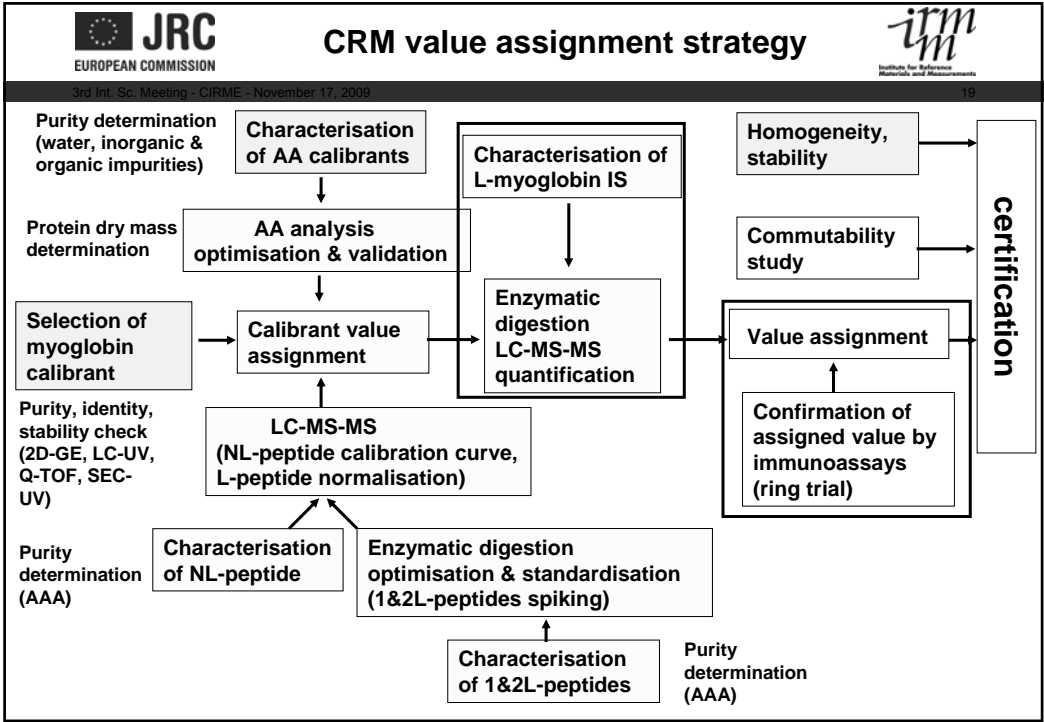
Theoretical amount of myoglobin peptide for 100 % recovery



IS1 / IS2  
 Degradation IS1 observed  
 Same degradation as Myoglobin peptide

Myoglobin peptide / IS1  
 Plateau reached

Myoglobin peptide / IS2  
 Plateau not reached  
 Degradation observed



**A CRM for myoglobin, more than a effort towards standardisation of myoglobin measurement system:**

- **Also provides a model approach for protein quantification with SI traceable result and certified uncertainty**
- **Implies full characterisation of calibrants and traceability chain at different stage of standardisation**
- **Involves orthogonal techniques for value assignment**

**Thank you !**