Development of a production process for a candidate BSA reference material.
This is a regional project.

INMETRO, Brazil, 27-29 June 2017.
**Objective**

Develop a highly-pure traceable BSA preparation and Certify it, according to ISO norms 17.034.

**Uses**

Standardization of Clinical analysis for “quantification of total serum proteins” or total protein content for Biotechnological productions, mainly applied to colorimetric methods.

Images: Word Protein Data Bank, http://www.rcsb.org/pdb/explore/explore.do?structureId=5IFO
Development of a production process for a candidate BSA reference material

Four different processes and products were tested during the development of Candidate RM.

R.M. 1

R.M. 2

R.M. 3

Pre-purified BSA serums. Fraction V or similar fractions.

Purity around 95%

Purity ≥ 98.5%
Process selection-

Four different purification processes tested with IEC

<table>
<thead>
<tr>
<th>Process N°</th>
<th>HPLC Purity</th>
<th>Yield (gP/IR)</th>
<th>cost (x)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSA 98,82</td>
<td>0,03</td>
<td>63,42</td>
<td>1,00 x</td>
</tr>
<tr>
<td>BSA 99,10</td>
<td>0,18</td>
<td>65,22</td>
<td>1,50 x</td>
</tr>
<tr>
<td>BSA 99,25</td>
<td>0,32</td>
<td>20,02</td>
<td>9,91 x</td>
</tr>
<tr>
<td>BSA 99,13</td>
<td>0,17</td>
<td>39,08</td>
<td>1,01 x</td>
</tr>
</tbody>
</table>
FPLC Chromatogram of the Purification Process 21

Impurities detected
<p>| | | | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>N.°</strong></td>
<td><strong>R.T. (min)</strong></td>
<td><strong>Rel. area</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>7,290</td>
<td>5,00</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>7,490</td>
<td>2,62</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>7,910</td>
<td>92,38</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**M2-E** EQ

**M2-F** EQ

**M2-G** EQ

**BSA - 7,910**

**Temperature (°C)**

**R.T. (min)**

**Relative area (%)**
Profile of F. “G” Lot# 1

Correlation with production process
Has the product been protein profiled? SDS-PAGE

### Protein Profile

<table>
<thead>
<tr>
<th>Sample</th>
<th>HPLC-UV Purity</th>
<th>MS Purity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Samples</td>
</tr>
<tr>
<td></td>
<td></td>
<td>BSA %</td>
</tr>
<tr>
<td>R.M.2</td>
<td>97.73</td>
<td>99.49</td>
</tr>
<tr>
<td>Cand. (LOT#1)</td>
<td>99.13</td>
<td>99.96</td>
</tr>
<tr>
<td>Cand. (LOT#2)</td>
<td>99.23</td>
<td></td>
</tr>
<tr>
<td>BSA-CRM NIM</td>
<td>99.26</td>
<td></td>
</tr>
</tbody>
</table>

#### Empai & DDA

<table>
<thead>
<tr>
<th>Sample</th>
<th>MS Purity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BSA %</td>
</tr>
<tr>
<td>G Lot# 1</td>
<td>99.49</td>
</tr>
<tr>
<td>BSA-CRM</td>
<td>99.96</td>
</tr>
</tbody>
</table>

These results were provided by University of Buenos Aires.

Ref. SDS-PAGE 12%, dried with colloidal coomassie. 01: molecular weigh marker, 02,04: BSA CRM NIM 7 and 15 µg per well respectively, 03 and 05: Fraction G lot#1 7 and 15 µg per well respectively.
**162 mg BSA candidate were produced**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Reference Value</th>
<th>Exp. Uncertainty</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSA candidate</td>
<td>17.98 mg/g</td>
<td>2.9 %</td>
</tr>
</tbody>
</table>
Does this result work?

Colorimetric methods -calibrated with BSA Candidate-

Number of analysis: 6 times. 
uncertainty: Intermediate Precision ongoing. 
Does it matter to check if this results are working? I believe that no.
Ongoing project

• **Scale-up Production:** INTI will produce 1 lot of 1000 vials, each one with 2 ml of BSA 7% solution, 150 mg of protein each vial.

  BSA 7% solution as CRM (under development)

• **Characterization & Certification:** will be carried out by NMIs participants
Scale-up Production

20 ml Resin → 0,405 grs.

960 ml Resin → 19,4 grs. (7-9 cycles need to reach 154 gr)
## Characterization & Certification

<table>
<thead>
<tr>
<th>Measurement methods</th>
<th>Quantification total protein</th>
<th>Stability study</th>
<th>Homogeneity study</th>
</tr>
</thead>
<tbody>
<tr>
<td>ID-MS (aa- derivatization)</td>
<td><img src="CENAM.png" alt="CENAM" /></td>
<td><img src="CENAM.png" alt="CENAM" /></td>
<td></td>
</tr>
<tr>
<td>ID-MS</td>
<td><img src="INMETRO.png" alt="INMETRO" /></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HPLC-UV (Purity)</td>
<td></td>
<td></td>
<td><img src="INMETRO.png" alt="INMETRO" /></td>
</tr>
</tbody>
</table>

- Mass balance: on lyophilized, INTI
- Purity RMN (INMETRO & INTI)
- Stability study: HPLC-UV (INTI)
- Stability study: Capillary electrophoresis. (INTI)
There is gap in protein RM's production in our region.

This Candidate under development has therefore been highly purified.

This development sets the basis for the production and certification efforts in our next step.

To achieve the use of the reference material, a commutability study with colorimetric methods should be performed.
Acknowledgements

H. Laiz from SIM P. Gatti & M.A. Cappa, Biotechnology Pilot plant team from INTI. Thank you for your support on the production.

A. Henrion, R. Ohlendorf, C. Arsene, G. O`Connor from Physikalisch-Technische Bundesanstalt, PTB, thank you for your help on the analytics.

W. Liqing From National metrology Institute from China (NIM) for your help on HPLC-UV and BSA CRM.

Informal inter-comparison

In order to compare amino-acid analysis results (ID-MS), we are interested on performing a comparison between others NMIs, during 2019.
¡Muchas gracias!
Thank you!
Vielen Dank!
Merci!
謝 謝 ！
**BSA coverage**

**SEQUENCE DETECTED** 87.52%

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**Bottom-up proteomic, HPLC-ESI-Orbitrap:**

**Hydrolysis:** BSA-Trypsin (1:200) over night

**HPLC:** Gradient acetonitrile:water-TFA 0,1%

**Column:** Phenomenex C18, 90A.

**Detector:** Orbitrap (resolution 60.000)

**Analysis software:** Skyline.

**Missed cleavages:** 0

**Precursors charge:** 1, 2.

**Ion type looked:** Precursors.

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**DETECTED FRAGMENTS**

**NO DETECTED FRAGMENTS**

**SIGNAL FRAGMENTS NOT INCLUDED IN THE MATURE PROTEIN**
DEVELOPMENT OF A CRM OF BSA.

APPLICATION


PROJECT TIMELINE

July 2017 - July 2019

NMIs PARTICIPANTS

CENAM, INMETRO, INTI

FOUNDING

BID

40,000 U$D.
• Workshop, “Protein CRM and Bio-metrology”, 27-29 de June de 2017, Río de Janeiro, Brazil.

• project RG-T2682 IDB, BSA reference material.

• application for second step production further purification and lyophilized BSA CRM development.

• Development of a new proposal for “foot and mouth disease”. DNA reference Material. Fast quantification and detection (Enero 2019). **INTI-INM (Colombia):**
**NETWORKING**

- Short stay as Scientis guest mass spectrometry. Lic. Hugo Amedei- André Henríón.

- Donation lyophilized BSA CRM, personal quotes from Phd. Wu Liqing.

- Personal notes HPLC-UV purity by HPLC-UV.

- Poster & paper to show our work in workshop ""Advances in Metrology in Chemistry and Biology 9-10 April, 2019, Sèvres, France""
600ppm BSA

Confirmed by NIM
Calibration Line:

\[ Y_i = 0.023 \text{mAU/ng} \times X_i - 41.00 \text{mAU} \]

\[ Y_i = 0.002 \text{mAU/ng} \times X_i + 0.245 \text{mAU} \]

\[ r^2 = 0.9985 \]

\[ r^2 = 0.999 \]

Picture 1: area (Mau*min) vs amount of sample (ng of sample) for BSA (Left) & GFP (right).
600ppm BSA + 400 ppm de GFP

Confirmed by FLD Detector
<table>
<thead>
<tr>
<th>No.</th>
<th>Peak Name</th>
<th>Retention Time</th>
<th>Relative Area</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td>7,303</td>
<td>0,38</td>
</tr>
<tr>
<td>2</td>
<td>BSA</td>
<td>7,910</td>
<td>98,82</td>
</tr>
<tr>
<td>3</td>
<td>Component 11</td>
<td>19,923</td>
<td>0,80</td>
</tr>
<tr>
<td></td>
<td>Total:</td>
<td></td>
<td>100,00</td>
</tr>
</tbody>
</table>
INTI - Biotechnology Pilot Plant - Production Capabilities.

Speaker: MS. Hugo Alejandro Amedei

Promote industrial development via innovation & technology transference.

Strengthen the metrology capabilities for setting up traceability & quality of measurements.
Strategic Aims

- **Help Industry** to improve productivity, quality, design, process & products.

- **Improve the metrology** in the region.

- We are focused on developing industries & small businesses.
NATIONAL NETWORK FOR TECHNOLOGICAL INNOVATION

51 Centros de Investigación y Desarrollo

1 - Parque Tecnológico Miguelete
2 - Jujuy
3 - Salta
4 - Tucumán
5 - Santiago del Estero
6 - Catamarca
7 - La Rioja
8 - Córdoba
9 - San Juan
10 - Mendoza
11 - San Luis
12 - La Pampa
13 - Neuquén
14 - Villa Regina
15 - Formosa
16 - Chaco
17 - Corrientes
18 - Misiones
19 - Rafaela
20 - Lácteos
21 - Rosario
22 - Entre Ríos
23 - Textiles
24 - CIRSOIC
25 - Madera y Muebles
26 - Cueros
27 - Cereales y Oleaginosas
28 - Mar del Plata
29 - Micro y Nanoelectrónica
30 - Chubut
31 - Petróleo
32 - Santa Cruz

SECTORIALES
REGIONALES
CERTIFIED REFERENCE MATERIALS AT INTI

Chemistry MRC

- Sodium Chloride (For crioscopy)
- Methanol-water solution (for Chromatography)

SICECAL

MRC related to Milk

- A- D Vitamins
- Skim milk and partially Skimmed milk.
- Milk powder
- Milk powder skimmed
- Serum of Cheese
- Somatic Cells.
- Dulce de Leche (sweet of milk).
REFERENCE MATERIALS AT INTI

- Water-Methanol Solution to **Quantify Methanol by Chromatographyc Methods**.

- **INTI has Mass Calibration and Measurement Capabilities (CMCs) for Quantification of Methanol/water.**
  - Its mass fraction Certified is: 0,500 % ± 0,007%.

*Credit: María Silvina Aued (INTI-Enviroment, Quality and Metrology).*
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<tr>
<td>ICP-MS</td>
<td><img src="Circle.png" alt="Circle" /></td>
<td><img src="Circle.png" alt="Circle" /></td>
<td><img src="Circle.png" alt="Circle" /></td>
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<tr>
<td>SILVER</td>
<td><img src="Circle.png" alt="Circle" /></td>
<td><img src="Circle.png" alt="Circle" /></td>
<td><img src="Circle.png" alt="Circle" /></td>
</tr>
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<td>HPLC-UV (Purity)</td>
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<td><img src="Circle.png" alt="Circle" /></td>
</tr>
<tr>
<td>Moisture</td>
<td><img src="Circle.png" alt="Circle" /></td>
<td><img src="Circle.png" alt="Circle" /></td>
<td><img src="Circle.png" alt="Circle" /></td>
</tr>
<tr>
<td>Ignition</td>
<td><img src="Circle.png" alt="Circle" /></td>
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<tr>
<td>Capillary Electrophoresis</td>
<td><img src="Circle.png" alt="Circle" /></td>
<td><img src="Circle.png" alt="Circle" /></td>
<td><img src="Circle.png" alt="Circle" /></td>
</tr>
</tbody>
</table>
Why 6 was afraid of 7?
Because 7 8 9.
Development of a BSA reference material.

M.S. Hugo Amedei, PAWG- April 2019.
BSA Certified reference Materials

BSA 7% solution, SRM#297

Production of lyophilized BSA CRM