Development of a production process for a candidate BSA reference material.

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Objectives

Development of Bovine Serum Albumin Certified Reference Material.
Development of a candidate production process & its analysis.

Summary

Here we present the development of the production process for a candidate CRM of BSA using fast protein liquid chromatography (FPLC) and a comparison between our candidate and a BSA CRM of NIM. The candidate produced has high purity (≥ 99.1%). Sufficient purity and quality were achieved to continue scale-up and certification efforts in future works.

Background

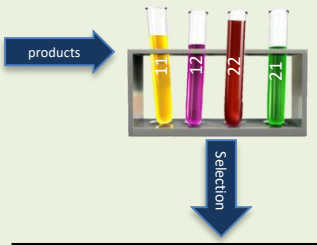
BSA is a universally accepted standard for total protein quantification.
It`s target application as a daily working standard is for "quantification of total serum proteins" and also proteins Biotechnology productions, in colorimetric methods.
INTI, INMETRO and CENAM, work in this project, under the support of Inter-American Metrology System (SIM).
This development will provide a CRM useful for regional calibration.
This is the starting point for Protein CRM production and certification of Latin American & Caribbean countries according ISO NORMS 17.034.

PRODUCTION

Process selection

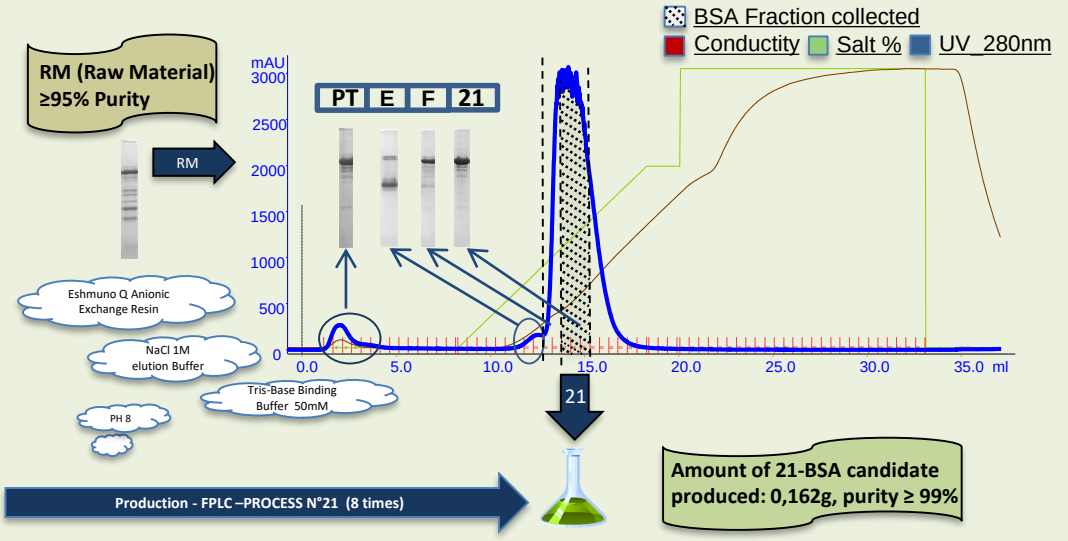
Four different purification processes were tested using a combination of three different raw materials (plasma BSA Products "around 95% purity"), and two different anionic exchange resins.

Fast Pressure Liquid Chromatography (FPLC)



Process N°	HPLC Purity		Yield	cost (x)
	BSA%	RSD%	(gP/IR)	
N° 11 BSA	98,82	0,03	63,42	1,00 x
N° 12 BSA	99,10	0,18	65,22	1,50 x
N° 22 BSA	99,25	0,32	20,02	9,91 x
N° 21 BSA	99,13	0,17	39,08	1,01 x

FPLC production process N° 21



ANALYTICS

Identity of the candidate

The majority of the sequence was found; this was a key step to characterize the candidate and for the quantification of BSA by amino-acid analysis. Also, BSA was identified.

BSA coverage sequence detected 87,52 %

MKWVTFISLLLLFSSAYSRGVFRRDTHKSEIAHRFKDLGEEHFKGLV
LIAFSQYLQQCPFDEHVKLVNELTEFAKTCVADESHAGCEKSLHTLF
GDELCKVASLRETYGDMADCCEKQEPERNECFLSHKDDSPDLPKLK
PDPNTLCDEFKADEKKFWGKYLYEIARRHPYFYAPELLYYANKYNG
VFQECCQAEDKGACLLPKIETMREKVLASSARQRLRCASIQKFGER
ALKAWSVARLSQKFPKAEFVEVTKLVTDLTKVHKECCHGDLLECAD
DRADLAKYICDNQDTISSKLKECCDKPLLEKSHCIAEVEKDAIPENLP
PLTADFAEDKDVCKNYQEAKDAFLGSFLYEYSRRHPEYAVSVLLRLA
KEYEATLEECCAKDDPHACYSTVFDKLKHLVDEPQNLIKQNCDQFE
KLGEYGFQNALIVRYTRKVPQVSTPTLVEVSRSLGKVGTRCCTKPES
ERMPCTEDYLSLILNRLCVLHEKTPVSEKVTKCCTESLVNRRPCFSAL
TPDETYVPKAFDEKLFTFHADICTLPDTEKQIKKQTALVELLKHKPKA
TEEQLKTVMENFVAFVDKCCAADDKEACFAVEGPKLVVSTQTALA

DETECTED FRAGMENTS
NO DETECTED FRAGMENTS
} SEQUENCE USED FOR BSA QUANTIFICATION BY ISOTOPIC DILUTION
SIGNAL FRAGMENTS NOT INCLUDED IN THE MATURE PROTEIN

Bottom-up proteomic, HPLC-ESI-Orbitrap:
Hydrolysis: BSA-Trypsin (1:200) over night.
HPLC: Gradient acetonitrile:water-TFA 0,1%.
Column: HPLC-Phenomenex C18, 90A Column.
Detector: Orbitrap (resolution 60.000).
Analysis software: Skyline.
Missed cleavages: 0.
Precursors charge: 1, 2.
Ion type looked: Precursors.

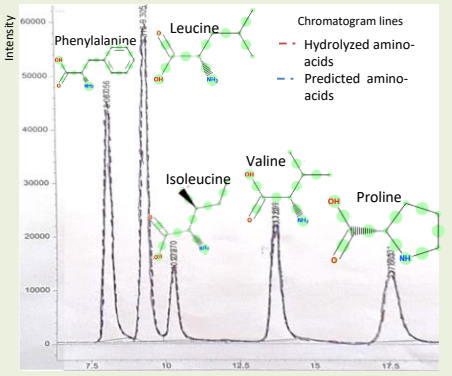
BSA quantification by amino acid analysis ID-MS

The overlapped chromatograms between amino-acids predicted and amino-acid hydrolyzed (- - and - -) and a low variation coefficient between replicates ≤ 2% are an additional clue that correlated with the high purity previously found.

Sample	Reference Value	Exp. Uncertainty.
21-BSA Candidate	17,98 mg/g	2.9 %

Hydrolysis condition: 3 days 160° C.
Amount of Protein Hydrolyzed: ~70,8 µg of candidate.
Detection system: HPLC (HILIC Column)-Single Quadrupole detector.

Reference Value: Was calculated with five different amino-acids.
Expanded Uncertainty: coverage factor of 2,04 and with a confidence of 95% (t-student).



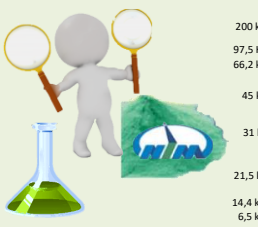
COMPARISON

Purity & DDA analysis

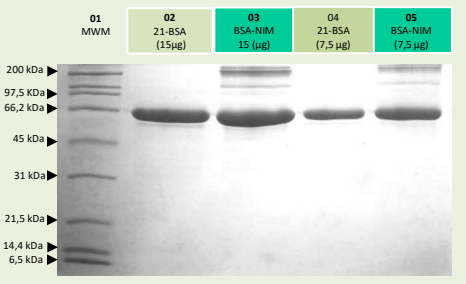
Sample	HPLC-UV-Purity, BSA	RSD %	MS- Purity, BSA	RSD%	N° proteins identified by DDA
21.-BSA	99,13%	0,17	99,49%	0,02	28
BSA-NIM	99,26%	0,01	99,96%	0,01	13

The purity of the candidate by mass spectrometry or by HPLC-UV was ≥ 99% and the same for CRM from NIM.

More traces of proteins on average appeared in the Candidate, by Bottom up proteomics, blasted against Bovine plasma proteins.



Protein profile by SDS-PAGE



A smaller amount of BSA aggregates were found in the candidate in comparison with the reference material.
(Shown in lanes 02 and 04 where there is only one 66Kda BSA band). On the other hand, NIM-CRM, lanes 03 and 05, has molecular weight bands higher than 66 Kda.

Analysis & conclusion

21-BSA candidate was chosen between four candidates. On this fast screening, 3 point were achieved for the selection of the candidate. **1. High Purity** (≥ 99%)**, 2- High production yield** more than 30g of candidate per liter of resin used. **3- Low cost and local production of the raw material** used to produce the candidate (see production).

In all cases, the candidate showed purity ≥ 99,1% (see analytic and comparison tables and SDS-PAGE figure), regardless of the method used. Also, on top of the amino acid analysis a low standard deviation was found between 5 different amino acids. **This Candidate under development has therefore been highly purified.**

Other plasma proteins were detected in the candidate (by DDA analysis blasted against bovine plasma proteins). **To move forward with this project, less than 1% of impurities was consider acceptable**. **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.

Inter- comparison

In order to compare amino-acid analysis results, we are interested on performing a ID-MS comparison with other NMIs, during 2019. Contact the author.

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