



Improved protein extraction methods for immunological detection of PAP in feed.

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EU Project Objective

To develop a more appropriate sample extraction method for use with Neogen Lateral Flow Device (LFD) method and validate the improved method through in-house and inter lab studies.

How does the LFD work?

- Single step lateral flow immunochromatographic assay.
- The protein extracted is wicked through a reagent Zone containing antibodies conjugated to coloured particles.
- If a specific protein is present in the sample, it will be captured by the Ab conjugate and migrates to test zone where the Ag-Ab complex concentrates and forms a visible line.
- Control zone: a coloured immune complex present in the reagent zone is migrated and captured by a control Ab present in control zone forming a visible line.

Dipstick technology

Advantages

- Easy to use.
- Appropriate to screen large set of samples.

Disadvantages

- Test sensitivity: 1%
- Sample viscosity delays migration.
- High mineral contents or vitamins in samples: either inconclusive or false positive results.

WP 2 Objectives

The specific objectives of VLA are:

- To improve the test sensitivity and detection of species specific proteins.
- To avoid inconclusive/false positive results.

Methods

VLA has evaluated two protein extraction methods:

- Salting out proteins using anhydrous ammonium sulphate (AAS).
- Decalcification of bone fragments into their mineral and protein constituents - with EDTA free acid.

Method-1

- Salting out: This method based on the principle that proteins are less soluble at high salt concentrations.
- Salt concentration for protein precipitation differs from protein to protein.
- Dialysis.
- Proteins salting out method is routinely used in our lab for CIE and ELISA methods.

- In ELISA method Ammonium sulphate was used 45% in Primary and 75% in Secondary precipitation.
- In CIE method Ammonium sulphate was used 50% in Primary and 93% in Secondary precipitation.
- A 40 grams of feed sample was cooked in 360 ml of PBS.
- A range of primary and secondary ammonium salt precipitations levels were tested to obtain specific protein.

Results

- Proteins obtained by salting out are not encouraging.
- Pure meat meal expressed prozone phenomenon.
- MBM samples rendered at 133, 137 and 141°C detected but rendered at 145°C failed at 1% level.
- Kit method failed on samples rendered at 137, 141 and 145°C .
- Salting out using CIE method failed.

- Out of a range of Ammonium Sulphate salt precipitations tried, 35%/65% primary and 75%/95% secondary were shown to be the best percentages to detect ruminant proteins at 0.5 - 1% level contamination respectively.
- All other precipitations were not significant to gain accurate target protein band.

Method - 2

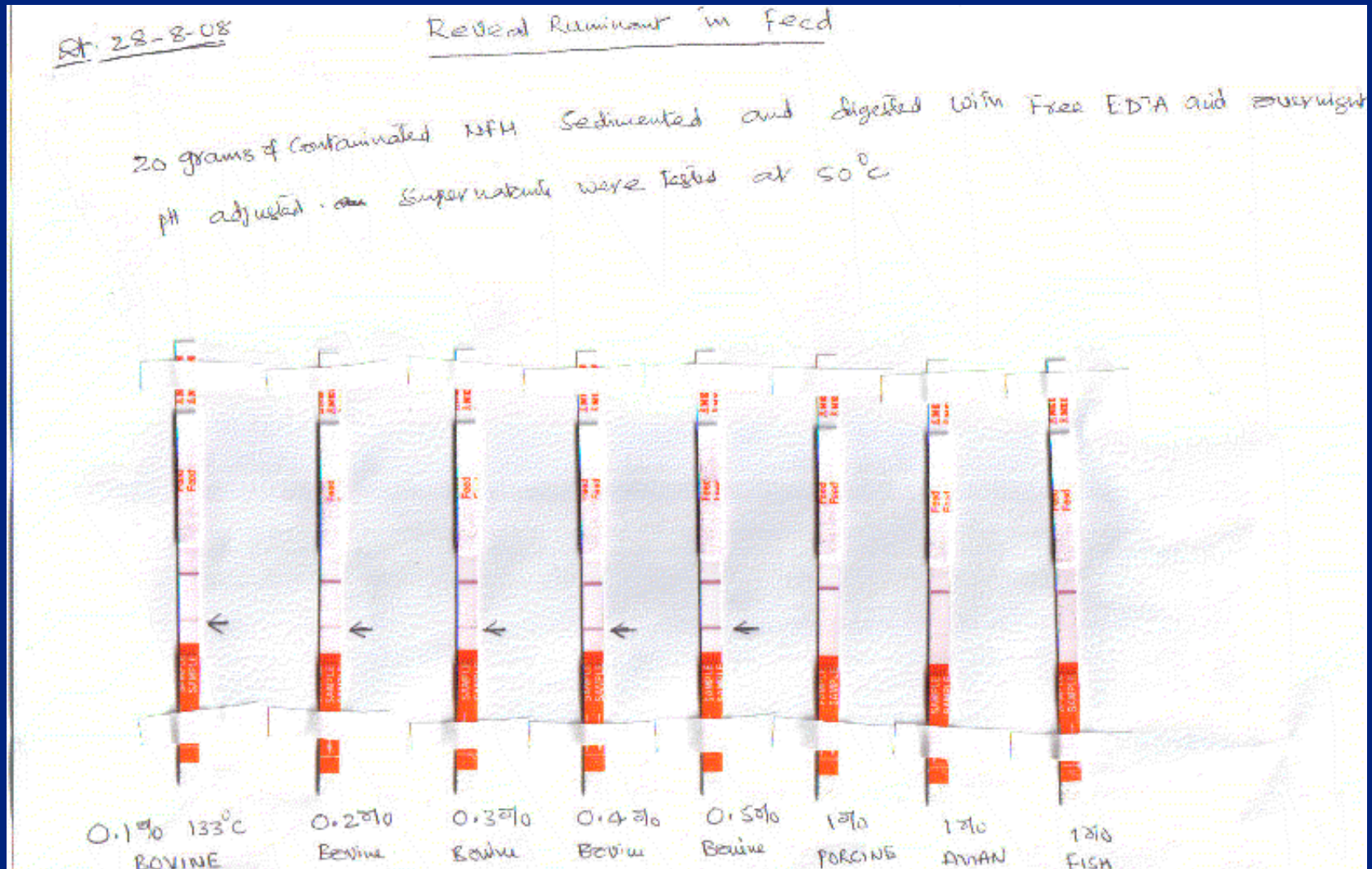
Decalcification of bone fragments - with EDTA free acid.

- Proteins (Troponin) are covalently bonded to mineral structures within the bone.
- The mineral structure of the bone could potentially protect the protein molecules during the rendering process.
- Recovering proteins by decalcifying the bones with EDTA free acid was developed by VLA.

- 20 grams of sample was sedimented with TCE as per the EU MAT method.
- The sediment was washed with 15 ml of 1:1 acetone and butanol.
- The dried sediment was digested O/N with EDTA free acid (1:3) in deionised water (2 ml to 100 mg of sediment) by rocking at 160 RPM.
- Following day 4ml of PBS was added and adjusted the pH of the mixture to 7.5 to 8 with 20N NaOH.
- The mixture was allowed to settle for 15 minutes and supernatant was collected.
- The supernatant was diluted 1:4 and tested at 50 °C for 10 minutes for the presence of ruminant protein.

Results

- 8 feed samples spiked with 0.1% to 0.5% bovine MBM along with 1% porcine, 1% Avian and 1% fish meal samples were tested.
- All the spiked samples with ruminant MBM were detected positive and all the non-ruminant samples detected negative.
- The intensity of band gradually increased according to the level of contamination showing the sensitivity.



The Neogen kit's limit of detection is at 1-2% level whereas our improved method is able to detect at 0.1% level.

- Negative feed matrix spiked with 0.1, 0.2, 0.3, 0.4, 0.5, 0.75 and 1% of Bovine and ovine samples rendered at 133 °C and Bovine sample rendered at 144 °C were tested along with 0.5% porcine, avian and Fish meal samples.
- All the ruminant samples spiked above 0.2% levels were correctly identified.

EDTA METHOD

Product #8100

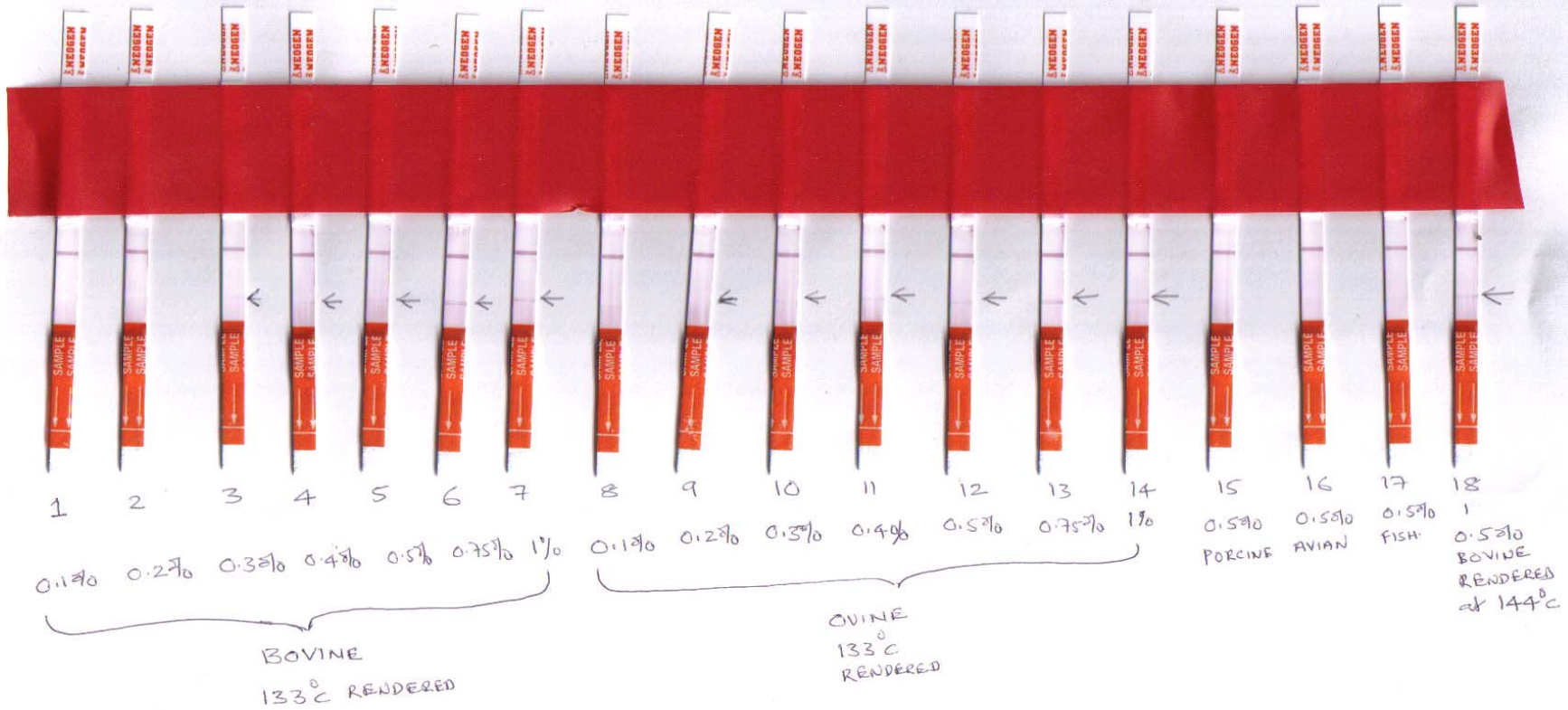
Reveal

@ 50°C incubation

for Ruminant Feed
Extraction Solution

Date Prepared
Expiry Date

28/7/09
A. [Signature]



- Further a 25 blind samples spiked with various concentrations of ruminant MBM were tested using the EDTA extraction method and all the samples were identified correctly.
- The PAP in PAP samples tested have not produced expected results . The possible reason could be due to steric hindrance (crowding of specific and nonspecific antigen i.e. troponin) on proximity and orientation for antigen and antibody complex formation.

Summary

- Of all the methods tested, bone digestion method with EDTA free acid shown to be the best.
- The proteins hidden in bone sustain high temperatures during rendering process.
- These proteins also escape the chemical insults during the rendering process.
- This sample extract is suitable for MS analysis to identify bone proteins for speciation.

- In house validation studies are in progress. Based on the results produced from interlab studies the method will be further modified and validated for its routine use.

Thank you

