



EURL-AP Standard Operating Procedure

Use of staining reagents

| Experts for edition and revision | |
|---|----------------------------|
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1. SCOPE AND PURPOSE

The purpose of the SOP is to present several staining techniques that may be used for the detection and identification by light microscopy of constituents of animal origin in feed materials and compound feed. This SOP is a complement to point 2.1.3.5. of Annex VI to Commission Regulation (EC) No 152/2009 as lastly amended by Commission Regulation (EU) No 51/2013.

2. SUMMARY

This SOP explains how to use different optional staining techniques allowing operators to better detect and identify by light microscopy the constituents of animal origin in feed materials and compound feed. It presents how to use staining reagents as well as the interpretations that have to be given to the observations. The list of staining techniques presented is not exhaustive: other methods can be found in Makowski *et al.* (2011).

3. VALIDATION STATUS AND PERFORMANCE CHARACTERISTICS

None of the staining techniques presented hereafter are validated. Staining should always been interpreted with prudence.

4. DEFINITIONS

Abbreviations used :

- SOP : standard operating procedure
- NA : not applicable

5. HEALTH AND SAFETY WARNINGS

Cautions should be taken when using staining reagents by avoiding skin contact. Material safety data sheet should be read for each used chemical. Special attention has to be taken in not inhaling vapours of cystine reagent. Norland ® Optical Adhesive 65 should be used with cautions described in the [SOP slide preparation and mounting](#).

6. EQUIPMENT AND MATERIALS

- Classical microscope slides and hollow slides (for >0.25 mm fractions)
- Coverslips (20x20 mm)
- Tweezers
- Fine spatula
- UV source (λ 350-380 nm)
- UV protective glasses
- Gloves
- Alcohol burner or heating plate

7. STEP BY STEP PROCEDURE

7.1. Sample preparation

Representative test portions of different fractions (sediment, flotata or raw material) are prepared in accordance with point 2.1.3.3. of Annex VI to Regulation (EC) No 152/2009.

In case 0.25 mm sieving of fractions was performed use classical slides for the ≤ 0.25 mm fractions and hollow slides for the >0.25 mm fractions. Slides will be prepared according to the recommendations from the [SOP slide preparation and mounting](#) on the EURL-AP website.

7.2. Cystine reagent

- Composition of cystine reagent is described in 2.1.2.1.4.2. of Annex VI to Regulation (EC) No 152/2009.
- This staining applies to the flotata or the raw material.
- It is used as a mounting medium for the detection of cystine-containing constituents (hairs, feathers, horns, etc) which turn black-brown over time. The staining reaction can be accelerated by heating the slide carefully by either a heating plate at 50°C or an alcohol burner. Avoid boiling of reagent: vapours are toxic.
- EURL-AP recommends mounting at least one slide per analysis with the cystine reagent.

7.3. Fehling's reagent

- Composition of Fehling's reagent is described in 2.1.2.1.4.3. of Annex VI to Regulation (EC) No 152/2009.
- This staining applies to the flotata or the raw material.
- It is used as a mounting medium for enhancing the detection of muscle fibres in feed matrices. Muscle fibres are stained pale pinkish-violet. Structural features of muscle fibres such as striation must be considered too.
- EURL-AP recommends mounting at least one slide per analysis with the Fehling's reagent.

7.4. Alizarin Red solution

- Composition of Alizarin Red solution is described in 2.1.2.1.2.1. of Annex VI to Regulation (EC) No 152/2009 and protocol for staining in 2.1.3.5. of Annex VI to Regulation (EC) No 152/2009.
- This staining applies to the sediment only.
- The stained sediment shall be mounted in glycerol or Norland® Optical Adhesive 65. When Norland® Optical Adhesive 65 is used UV protective glasses should be used during UV polymerization process.
- Alizarin Red colours bones, fish-bones, fish scales in bright red-pink. The stain is not specific for the bone but it colours the bone major mineral constituent, hydroxyapatite. It is reported also to react with calcium phosphates (e.g. tricalcium phosphate). Therefore, structural features typical of bones (lacunae, canaliculi) must be considered too for determining a stained particle as from bone origin. The Alizarin Red staining facilitates the screening of bones inside sediments.

7.5. Lugol solution

- Composition of Lugol solution is described in 2.1.2.1.4.1. of Annex VI to Regulation (EC) No 152/2009.
- This staining applies to the flotate or the raw material.
- It is used as a mounting medium for the differentiation of starch from protein. Starch will stain in a deep blue-violet colour while proteins will be stained yellow-orange. The solution may be diluted if required.

7.6. Tetramethylbenzidine – Hydrogen peroxide

- Composition of the reagent is described in 2.1.2.1.4.4. of Annex VI to Regulation (EC) No 152/2009.
- This staining applies to the flotate or the raw material.
- It is used as a mounting medium for helping detecting blood. The staining mixture reacts with blood which immediately becomes blue-green (turquoise) and releases O₂ bubbles. Plasma powder will only be coloured without O₂ release.
- The immediate character of the reaction is relevant for the possible presence of blood and plasma powders. Over time, after 20 min, the mixture turns blue-green even in absence of blood derivate products. However some plant material having peroxidase will react positive (e.g., radish, garlic, carrot, corn, cabbage) but such interference colouring is not immediate.

8. INTERPRETATION OF RESULTS

NA

9. REFERENCES

Commission Regulation (EU) No 51/2013 of 16 January 2013 amending Regulation (EC) No 152/2009 as regards the methods of analysis for the determination of constituents of animal origin for the official control of feed. Official Journal of the European Union, L20, 23.1.2013, 33-43.

Makowski J, Vary N, McCutcheon M, Veys P. 2011. Microscopic analysis of agricultural products. AOCS Press, Urbana, USA

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