CHAPTER 5
Classical microscopy: improvements of the qualitative protocol

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SUMMARY

The monitoring of the presence of animal proteins is based on the detection by a microscopic method. The current official description is published in Regulation 152/2009/EC, which is based on Directive 2003/126/EC. This method is presented here in outline as well as in more detail, also with some recommendations which would provide further optimization. Several additions pertaining to the extraction of muscle fibres, hair and feather filaments, handling of fat and oil samples, and spot tests for the detection of specific ingredients are presented. Several experiments are discussed describing improvements of the method. A meta-analysis of proficiency tests indicates that 10 grams of material for starting the sedimentation procedure, and using as much material as possible for examination would optimise the performance of the microscopic method. The latter situation can be achieved by the compulsory examination of the entire sediment using a stereo microscope. A careful choice of an appropriate embedding agent directed to have an optimal viscosity is necessary to optimise the visibility of structures in the particles. The visibility is also influenced by applying different colouring methods.

Keywords. Animal proteins, meat and bone meal, fishmeal, Microscopy method, identification

5.1 INTRODUCTION

The detection of prohibited animal proteins by using microscopic protocols has a long history. Currently microscopic detection is still the only accepted method which is validated in the European Union. Additional methods are accepted in order to improve the identification of the materials found, e.g. DNA identification (PCR), and protein identification (ELISA). The strength of the microscopic method is the combination of a relative short time for the detection of animal proteins, the low level of detection and a well established distinction between prohibited (e.g. meat meal, meat and bone meal) and legally allowed (e.g. blood meal, milk powder) products of animal origin.

The development of the screening method with classical microscopy started in the nineties of the twentieth Century with the development and publication of Directive EC/88/1998, stimulated by the research of the International Association for Feedingstuff Analysis, Section Feedingstuff Microscopy. As a result of on-going research, partly in the framework of the European funded project STRATFEED, a new protocol was published in Directive 2003/126/EC. Further developments are coordinated by the European Union Reference Laboratory (formely CRL) for animal proteins. In the mean time, The European Commission decided to publish all methods for feeding stuff analysis in one Regulation. Annex VI of Regulation 152/2009/EC contains a protocol identical to the repealed Directive 2003/126/EC.

This chapter will describe the application of the official method which is published in Regulation 152/2009/EC, the experiments which indicate the prime parameters for improving the performance of the method, and possible further harmonisations. Based on the results of experiments, ring trials and literature research, the official protocol will be presented for the microscopic detection of animal proteins, with a series of recommended modifications and additions. The use of markers during examination and evaluation of the materials detected will be presented and discussed in chapter 6. Special attention will be given to dedicated protocols for the detection of specific ingredients.

5.2 METHOD DESCRIPTION

5.2.1 Optimised general method

The basic method for microscopic detection of animal proteins is described in Regulation 152/2009/EC, Annex VI. This Annex contains the unchanged description of the method as published in Directive 2003/126/EC. The next outline and detailed description of the method are based on this text and on improvements proposed from the results of several experiments. Some aspects of the method outlined here are additions to or modifications of the official method, which are mentioned as recommendations. Amounts of material randomly selected from the original or laboratory sample for sieving or sedimentation can be referred to as analysis sample.

5.2.2 General outline

This general overview is meant to give a first indication of the procedure including recommendations.

Sample preparation:

1. Label glassware and work organized in order to avoid confusion.

2. Take a look and smell. These first impressions might give valuable information for further observations. Some crushed material (mortar) can be examined using a binocular.
3. Take preferably 100 grams of material randomly from the total sample to form a representative laboratory sample. Grind these 100 grams by using a mill; homogenization is also achieved in this way.
4. Sieve at least 5 grams (analysis sample) and make two fractions: larger than 250 µm (fraction A) and smaller than 250 µm particle size (fraction B).
5. Make a sediment in tetrachloroethylene (TCE) from at least 5 grams (analysis sample) of ground sample material. An amount of 10 grams is highly recommended. Sieve the dried sediment: larger than 250 µm (fraction C) and smaller than 250 µm particle size (fraction D). Additionally you may dry the flotation and sieve it: larger than 250 µm (fraction E) and smaller than 250 µm particle size (fraction F).
6. Clean all material carefully (mill, glassware and sieves).

Observations:

7. Use a stereo microscope for looking at the coarse fractions (A, C and E); try to describe the particles or take a first decision (colour, transparency, crispness).
8. Make a slide of the fine sediment fraction (D) from step 5 in a suitable embedding agent and make observations. Two additional slides shall be made when fish meal is present. Try to confirm the first decision of step 7.
9. Take the fine sieve fraction (B) from step 4 or the fine flotation fraction (F) from step 5 and make a slide in Fehling or in Sodium hydroxide. Try to confirm the conclusion of steps 7 and 8.
10. If necessary, compare your observations with information from literature and from decision support systems (such as ARIES). Further information on the evaluation of your observations is given in Chapter 6.
11. Draw the final conclusion and report.

5.2.3 Detailed description

The recommended additions to the protocol of Regulation 152/2009/EC are indicated in *italics*. The research results leading to these additions are discussed in the paragraph state of the art.

Sample preparation:

1. Take portions of the total sample for getting a random laboratory sample of 50 grams of material. *Recommendation*: select at least 100 grams for the laboratory sample**.**
2. It is recommended to carry out a short organoleptic examination. The look and smell of the material will give a first impression of the composition, the nature and possible contamination of the sample. Pelleted material might be crushed by means of a mortar. For example, hairs and/or feathers might presumably be examined at first sight. Fish material can be observed by its smell, even at relatively low contamination levels.
3. Reduce the size of the particles of the laboratory sample with an appropriate equipment. A mill can be applied with a drum with mesh size of 2 mm. This will result in particles with a size of approx. 1,5-1,7 mm as maximum. Alternatively a mortar can be used when a sample contains more than approx. 15 % of fat.
4. Take at least 5 grams of the ground sample (analysis sample) and sieve it. *Recommendation*: the use of a sieve with a mesh size of 250 µm will result in two fractions of approximately the same amount. These fractions are: **A**: with particles larger than 250 µm and **B**: particles smaller than 250 µm. A sieve with mesh size of 355 µm can be used as well.
5. Take at least 5 grams of the ground laboratory sample (analysis sample) for the sedimentation procedure. *Recommendation*: based on research experiments and ring tests, an amount of 10 grams is highly preferred. In the case of pure animal meal or fish meal 2 grams would be sufficient. Place the material in a chemical separation funnel or a conical bottomed beaker in at least 50 ml Tetrachloroethylene (TCE). *Recommendation*: in the case of having 10 grams selected for the analysis sample, an amount of 100 ml of TCE might be necessary to apply.
   - Chemical separation funnel: close the funnel with a stop. Shake firmly for several times. Leave the funnel for 3 minutes and open the tap just as long as necessary to release the sediment. Close the tap and shake for a second time. After 3 minutes open the tap again shortly to release the final amount of sediment. *Recommendation*: the walls of the funnel can be rinsed with TCE in order to wash down all the material.
   - Conical bottomed beaker: stir firmly for several times making a shape of an "8". Wait for several (up to 5) minutes, and decant the flotate carefully. After decanting and pouring off most of the TCE, release the sediment from the glass. *Recommendation*: the decanting and pouring off procedure should be carried out very carefully in order to avoid contamination of the sediment with flotate material. The walls of the glass should be cleaned before collecting the sediment.

Collect the sediment on a Petri dish or on an hour glass. Dry overnight if necessary. The sedimentation time might have an influence on the colour of the bones since fat is extracted by the TCE treatment. The sediment contains the particles with a specific density higher than 1.62, i.e. mainly bone particles, fish scales, egg shells and minerals. If quantification is required, the sediment must be weighed before any further preparation or treatment is applied. *Recommendation*: the result of the weighing should be stored as absolute weight and as percentage of the weight of the original sample. In this way there is always a connection to the original amount of material used for sedimentation, which is especially important when a deviating amount of material has been used.

Sieve the sediment if a lot of coarse fragments are present in the sediment. *Recommendation*: a sieve with a mesh size of 250 µm can be used. Two fractions result: **C**: with particles larger than 250 micron and **D**: particles smaller than 250 µm. The main part of the feed sample remains floating on the TCE. *Recommendation*: this floating part can be examined additionally to fractions A and B. The included particles are generally the same as in fractions A and B except for the bones, but a clearer view might occur because the flotation is largely defatted after the sedimentation in TCE. Sieve a part of the floating remnants with a mesh size of 250 µm. Two fractions result: **E**: with particles larger than 250 micron and **F**: particles smaller than 250 µm.

The fractions must be stored in closed jars or bottles that are clearly labelled, in order to avoid loss of material and/or contamination. All equipment, especially mill, drum, glassware and sieves must be cleaned thoroughly with a brush and high pressure air. It is highly recommended to use a brush with artificial (nylon) hairs, to avoid any contamination with animal hairs. The outline of the method is illustrated in figure 5.1.

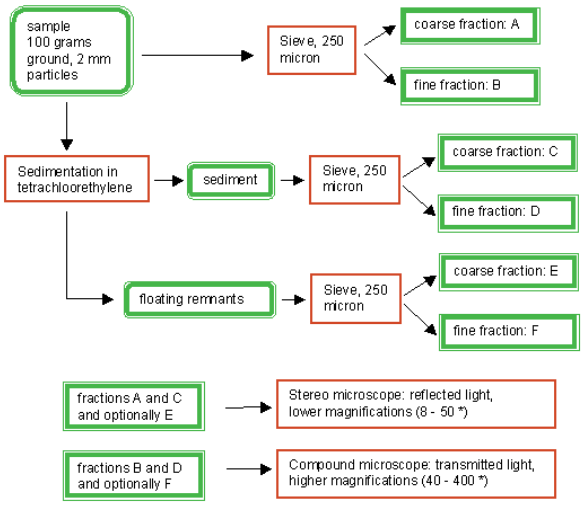

Figure 5.1. Outline of the basic steps of the method for microscopic detection of animal proteins in animal feeds. Several recommendations to the basic protocol are included. Source: van Raamsdonk et al., 2004.

Examination:

According to Regulation 152/2009/EC, the examination of the fractions A and C and, if prepared, fraction E by using a stereo microscope at lower magnification (8 - 50 X) under reflected light is compulsory. *Recommendation*: observe as much material as possible; especially the coarse sediment fraction (C) can easily be observed fully in this stage. The advantage of this first examination is that the representative fraction of the 5 grams or more of material is observed in its entirety. Any presence of animal proteins can be observed, which enhances the low detection level of the microscopic method. Features to be examined:

Fraction A: presence and morphology of fragments: bones, scales, minerals, feather filaments, muscles, etc.

Fraction C: presence and morphology of fragments; identify type, especially of bones and egg shells.

Additional tests for both fractions:

- Hydrochloric acid: apply some drops of 3N HCl to some particles on an hour glass. The response is the production of CO_2, visible as effervescence of calcium (bones, egg shells, minerals). Bones will react but to a lesser extent than the calcium carbonate particles.
- Silver nitrate: five gram of silver nitrate is dissolved in 100 ml water. A color response will be visible from phosphates (yellow) and from chlorides (white) after applying some drops of solution to mineral particles.
- Potassium ferricyanide: three grams of potassium ferricyanide is dissolved in distilled water, which is finally added to 100 ml. Sulphate salts are detected after applying some drops of the solution to mineral

particles. Specific colour reactions can be recognised from copper sulphate (reddish brown), iron sulphate (blue), cobalt sulphate (green or turquoise), and manganese sulphate (milky white).

Fraction E (optional): presence and morphology of fragments, especially hairs, feather filaments and muscles.

The **fraction B** and **D** and, optionally **fraction F** will be examined at higher magnifications (100 - 400 X) by means of a compound or research microscope with transmitted light. For fraction D (the fine fraction of the sediment) a portion as large as possible should be used for preparing one or more slides. A suitable amount of sediment material will be accommodated on a slide and a suitable embedding agent is applied. According to Regulation 152/2009/EC paraffin oil or glycerol is appropriate with a moderately high viscosity: 68 - 81 [mPa.s]. Although not indicated in the Regulation, additionally immersion oil or Norland adhesive can be applied. Using a higher or lower viscosity than mentioned in the Regulation will effect the visibility of the lacunae and especially on the visibility of the canaliculae. The amount of material used for preparing one slide should be chosen in such a way that the particles are lying free from each other. Overlap of particles should be avoided, but if a too small amount of material is used for a slide, more slides have to be prepared and examined. An optimal amount results from experience. The material is mixed in the embedding agent before the preferably large cover glass (e.g. 26 x 50 mm) is placed on top of the mixture for mounting. It is recommended to use a cover glass instead of examining the slide and mixture without covering, in order to avoid an uneven surface between the embedding agent and the air. The application of a cover glass would enhance the recognition of particles.

Possible observations:

Fraction B: basic examinations in water or in a muscle reagent (Fehling) bone morphology, lacunae, muscles, hairs, (hydrolysed) feathers, scales, etc.; with or without polarised light.

Fraction D: basic examinations in a suitable embedding agent (moderate viscosity): bone morphology (lacunae), sand, minerals; with or without polarised light; occasionally starch and plant parts may be present.

Slides should be examined as soon as they are prepared for a proper visibility of the canaliculae, if present (see Chapter 6).

Fraction F (optional): basic examinations in Sodium hydroxide or in Fehling: especially muscles, hairs and (hydrolysed) feathers.

It is recommended to make photographs of (examples of) positively identified particles, either originating from fish or from terrestrial animals for documentation and future approval.

The viscosity or fluidity determines the speed upon which the embedding agent enters the fragments and especially the lacunae of the bone fragments. In the case of moderately high viscosity (60 – 150 mPa.s) it takes some time before the air in the lacunae and the canaliculae is replaced by the embedding agent. See Chapter 6 for more details. Paraffin oil is a natural, diverse product and the viscosity will vary from brand to brand. The company Norland produces a whole series of embedding agents with a large diversity of viscosities. A slide prepared with a Norland embedding agent can be made permanent by applying ultraviolet light. A permanent slide can be examined any time and is very useful for future reference. Be careful when applying ultraviolet light, and use the appropriate laboratory tools, equipment and safety measures (glasses).

Regulation 152/2009/EC states that when the presence of fish meal is mentioned at the label or when fish meal is found, at least three slides of fraction D need to be examined, although the preferred amount of material used to prepare these slides is not indicated. It is recommended to use a substantial amount, which is possible using the recommended large cover glasses. The use of more than one slide for examination of fraction D is advisable anyway in order to reach the conclusion “animal remains absent” reliably. Especially this part of Regulation 152/2009/EC is under discussion. The reliability of a positive conclusion about the presence of animal proteins when only 5 or less particles are found, is disputable. An acceptable solution to this problem is to grind a second portion of 100 grams, selected from the original sample, take one or more analysis samples for sedimentation, and investigate these analysis samples as an addition to the first investigation. This procedure could be considered as a test for “intra-laboratory repeatability”, since it covers the entire method starting with grinding. If no animal proteins are found in those additional examinations, the possibility of laboratory contamination should not be excluded. The finding of animal proteins in these additional examinations confirms the presence of contamination at a low level.

Agents and additional tests:

- Fehling: this reagent consists of two basic solutions. Reagent I: 6,93 g copper sulphate is dissolved in 100 ml water. Reagent II: 34,6 g potassium sodium tartrate and 10,3 g sodium hydroxide are dissolved in 100 ml water. Equal amounts of reagent I and reagent II are combined for the final solution. The final solution will be used as embedding agent and enhances specifically the visibility of the muscle fibres.
- Lead acetate solution (cystine reagent): Lead(II)acetate trihydrate in a solution of Sodium hydroxide. Ready after preparation for the detection of structures with high content of cystine (keratin in horn, hairs, feathers), with careful heating.

- Lugol (Iodine potassium-iodine): 0.2 gram KI and 0.1 gram I_2 is dissolve in 30 ml H_2O (aqueous solution). A ready-to-use solution can also be bought. Applying some drops of the solution allows a better discrimination of protein and starch; especially suitable for muscle fibre staining. Other concentrations can be applied as well.

The expert system ARIES (2004; 2010) provides further documentation on the preparation and use of these reagents.

5.2.4 Special colour procedure for the sediment fractions

The sediment, before sieving it to get the fractions C and D, can be stained to differentiate more specifically bone fragments from the total of the particles of the sediment. This staining procedure can be applied as follows:

Ingredients: Alizarin Red S, 1,2-dihydroxyanthraquinone-3-sulfonic acid disodium salt, $C_{14}H_6O_7SNa_2$ (CAS-no 93982-72-0), Hydrochloric acid solution 2N (q.v.). Sodium hypochlorite, NaOCl (CAS-no 7681-52-9), also referred to as Eau de Javel, soda bleaching lye or Eau de Labarraque; be aware of the toxic effects.

Preparation: dissolve 1,25 ml 2N Hydrochloric acid in 100 ml water and add 200 mg Alizarin Red to this solution. Can be used up to 6 months after preparation.

Application: after the sediment is separated off, it shall be placed in a glass test tube and rinsed twice with ethylalcohol 96%, each time a vortex should be used and the sediment should be allowed to settle again for approx. one minute. Bleach the sediment with sodium hypochlorite by adding 1 ml to a part of the sediment. Allow the reaction to continue for 10 minutes. Fill the tube with water, let settle the sediment for 2 to 3 minutes and pour the water and the suspended particles off. Rinse the sediment twice with about 10 ml of water (use a vortex, let settle and pour the water off each time). Add 2-3 drops of the Alizarin Red solution to some particles on a slide and stain for 2 - 10 seconds. Rinse the sediment twice with ethyl alcohol 96% and once with acetone. Use each time a vortex and let the sediment settle for one minute before pouring off. The stained sediment is now ready for drying. The bone and fish scale particles turn red. Mineral particles usually do not show a colour reaction. The final sediment after the staining procedure is approximately 60% of the weight of the original sediment. The red colouring could mask gradually the fine structure of the bone particles (van Raamsdonk et al., 2009b). It could be recommended to apply the staining with Alizarin only to the coarse sediment fraction C, and examine at least a part of the fine sediment fraction D without staining.

5.2.5 Special microscopic preparation protocols (muscles, hairs, feathers, fat and oil samples)

Fragments such as muscle fibres and hair filaments usually show up in the flotation rather than in the sediment. These particles are nevertheless of special interest, since further identification of the animal group can be realised. More information on the identification of hairs is presented in Chapter 6, and of muscle fibres in Chapter 13. In the following paragraph the procedure for concentration of muscle fibres is presented.

Muscle fibres, hair and feather filaments, as all particles of vegetable origin, have a lower specific density than the solvent TCE. There is a difference between the specific gravity of the different groups of particles, which provides the possibility to separate these particles using a suited solvent.

Several solvents or mixtures thereof have been tested. A mixture of TCE : heptane in the ratio of 70 : 30 appeared to be sufficient to get a flotation containing predominantly muscle fibres and a pellet or sediment with all other particles including vegetable fragments, minerals and other proteins such as bone fragments. If no sufficient separation can be achieved, mixture of TCE : heptane in the ratio of 75 : 25 or 80 : 20 can be used. As a result more vegetable particles might show up in the flotation, but a concentration of the lighter particles from muscles and hairs can still be achieved.

The procedure is identical to that as described in step 3) of the general protocol, using the TCE and heptane mixture, and a chemical separation funnel.

Fat and oil can contain animal proteins in the form of e.g. bone fragments at low contamination levels. This low level of contamination urges for special treatment, and the (semi-) liquid nature of fat and oil allows a special preparation. In case of more solid products (fat) it should be warmed in e.g. a microwave oven until it is liquid. A certain amount of the oil or liquid fat is taken by a pipette and transferred to a centrifugation tube. Regulation 152/2009/EC states an amount of 40 ml, but a higher amount can be used, provided that it can be contained in the tube. Centrifugation is applied for 10 minutes at 4 000 rpm. If the fat is solid after centrifugation, it should be warmed again until it is liquid. The centrifugation can be applied for a second period of five minutes at 4 000 rpm. A half of the decanted particles are placed on a microscopic slide using a spoon or spatula, embedded in paraffin oil, immersion oil or Norland adhesive, and covered by a cover glass. Observations might include meat fibres, feathers, bone fragments. The other half will be used for a sedimentation. The sediment can be used to identify bone fragments. These two portions of the decanted material are comparable to the combined fractions A/B and to C/D of the official method, respectively.

Concluding remark

A larger explanation of the microscopic method is published in:

- Decision support system ARIES, providing several galleries, information on method alternatives, legislation and several identification trees: ARIES, 2004; 2010.
- Manual of the section Microscopy of the American organisation AOAC (Bates, 1992).
- Feigl (1958), Bates (1992) and ARIES (2004; 2010) present a larger range of and more details on the application of spot tests.

5.3 STATE OF THE ART

The current microscopic method (Regulation 152/2009/EC) contains several steps and actions for preparation of the sample that allow modification within the limits of the Regulation. Since 2003, several interlaboratory studies have been carried out (Overviews in van Raamsdonk et al., 2007; 2008). In some of these studies an inventory for the application of a range of methodological parameters have been organised and method application details have been submitted by the participants.

There is a principle difference between validation studies and proficiency tests. A validation study focuses on the validity of a specific protocol, which has to be applied precisely by all the participants. A proficiency test allows the participants to apply their own modifications of the targeted method. Principally validation studies do not allow to draw conclusions on modifications that might be intended to be an improvement, since such modifications are not allowed. Proficiency tests can not be used as validation of a method, since a precise protocol is not fixed prior to those studies. Further details are given by von Holst et al. (2005). The term "intercomparison study" can apply to both types of studies. The IAG - International Association for Feeding stuff Analysis, Section Feeding stuff Microscopy (international group of microscopy scientists) prefers to use the term "ring test" to their annual proficiency test (van Raamsdonk et al., 2009a). Actually hybrid studies may occur. In some cases an official protocol allows the application of circumscribed alternative choices for some method parameters. If participants to an intercomparison study are asked to apply strictly such a protocol, the results can be referred to as a validation study, while simultaneously the correlation between performance and allowed modifications for those parameters can be investigated. The latter information can be presented as a proficiency test. Since the protocol modifications are the primary focus in a discussion of method improvement, the studies to be discussed in the next paragraphs are indicated as proficiency tests.

In the following meta-analysis the results of two elaborated proficiency tests will be discussed and evaluated in terms of method improvement. A proficiency test was organised in the framework of the European project STRATFEED, which was the first in which the official new method as published in Directive 2003/126/EC was applied. This STRATFEED study was intended to be a validation study, but information on the application of modifications allowed under Directive 2003/126/EC is available (von Holst et al., 2005) In 2009 a proficiency test was organised on behalf of IAG - section Feeding stuff Microscopy, which was the largest study in terms of number of participants (49). These two studies mark the development of the methodology in the framework of the official protocol as published in Directive 2003/126/EC and Regulation 152/2009/EC.

5.3.1 STRATFEED proficiency test (2004)

In the STRATFEED proficiency test samples contaminated with several mammalian meat and bone meals at levels of 0.1% and 0.5%, whether or not in the presence of 5% fish meal have been tested (Holst et al., 2005). Every mixture was represented three times in the sample set. The entire set of test samples comprised 24 samples. Four different parameters extracted from the STRATFEED proficiency test have been analysed further to show possible effects on the performance of the microscopic method.

These parameters are: equipment of grinding (mortar vs. electric mill; number of participants reporting n=23), type of separation funnel (closed [chemical separation funnel or specially designed funnel] vs. open [beaker or champagne glass]; n=24), and the amount of material used for sedimentation (5 vs. 10 grams; n=24). The main difference between an open and a closed separation funnel is the way the sediment is extracted from the solution. In a "closed" funnel, the sediment is extracted from the bottom of the funnel, where it is located after sedimentation. Using an "open" beaker or glass, the flotation and most of the solution has to be poured off first, before the sediment can be collected. The effect of the three parameters is indicated as specificity (effect of false positives, based on samples with 5% fish, no MBM) and sensitivity (effect of false negatives, samples with 5% fish and 0.1% MBM).

An indication can be given of the best performing choice for the amount of material subjected to sedimentation, for the grinding method and for the type of separation funnel (Figure 5.2). An amount of 10 grams and the use of a closed funnel (extraction at the bottom of the glass without the need to pour off the flotation first before separating the pellet or sediment) appeared to give better results. The application of grinding equipment was tested in the framework of the study, i.e. standard compound feed samples were used, and no

conclusion can be drawn when particles of fat-rich materials have to be reduced in size. The use of a binocular can be an advantage for the examination of the entire sediment, as which would decrease the possibility to overlook animal proteins (higher sensitivity). Embedding agents with a low viscosity (0.8-1.5 mPa.s) were applied by only two participants, which imply that a further analysis of this parameter is impossible. In the framework of this study of von Holst et al. (2005) "high" viscosity pointed to viscosities ranging from 60 – 110 mPa.s. Currently embedding agents are applied with viscosities of 1000 mPa.s or higher. Examples of those high viscosities are Norland's Adhesive 65 and 63.

Further analysis of possible improvements will be discussed based on the results of a second proficiency test.

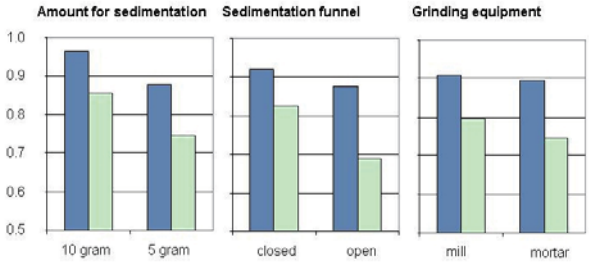

Figure 5.2. Bar diagrams of the effect of changing parameters in the microscopic method. Specificity (effect of false positives, dark bars) and sensitivity (effect of false negatives, light bars) is expressed on the y-axis. Source: Raw data of the STRATFEED validation study (Holst et al., 2005).

5.3.2 IAG proficiency test (2009)

In a proficiency test organised in 2009 under the responsibility of IAG section Microscopy by RIKILT, an inventory was carried with a questionnaire of nine questions on the implementation of the microscopic method (van Raamsdonk et al., 2009a). This test was primarily organised for providing information on the proficiency of the individual laboratories. As a side effect, the results of the inventory provided some information for evaluating the method of Regulation 152/2009/EC. The proficiency test contained four samples, respectively a blank, one sample with 0.1% of animal proteins, one sample with 5 % of fish meal and one sample with both 0.1% of animal proteins and 5 % of fish meal. "Animal proteins" means a contamination with a meat-and-bone meal of cattle containing 70% of bones and 30% of other materials.

The majority of the participants in the proficiency test started the sedimentation procedure with an amount of 10 grams of material (n=41). Also in a majority a chemical sedimentation funnel was used (n=28). Fourteen participants used a staining (alizarin) of the sediment for evaluation. It was not stated if this staining procedure was used as the standard method or only additionally to the examination of unstained material. Examination of the sediment at lower magnifications by using a binocular is requested in Regulation 152/2009/EC, but nine participants reported to skip this part of the procedure. The portion of the sediment used for microscopic examination ranged from 2% to 100%, accommodated on only one slide or up to more than 10 slides. It can be expected that a larger portion of sediment used for examination is correlated with a larger amount of slides, but this appeared to be not the situation. There is an apparent diversity in the way slides are prepared for the microscopic examination. Also a range of seven different embedding agents for the sediment was reported, some of them hardly suited for a good examination of sediment material (e.g. water). All these modifications, except for skipping the examination by using a stereo microscope and using non-suited embedding agents, are allowed in the framework of the Regulation.

The specificity scores for the detection of land animal material in the IAG 2009 proficiency test (blank: 0.96; in presence of fish meal: 0.98) were in the upper part of the range of past tests. Eight participants reported the presence of fish in samples free of fish meal (specificity scores: blank: 0.96; in presence of MBM: 0.88). Usually only traces of fish material were reported. Possibly, in a few cases, plant particles may have been mistaken for animal particles. The use of pictures is necessary for affirming or refuting misinterpretation.

The sensitivity scores achieved in this proficiency test are in the top of the range as obtained in previous tests (van Raamsdonk et al., 2007). The sensitivity score for land animal material in the presence of fish meal was 0.98. The other sensitivity scores were all 1.0. With a coverage of 49 laboratories, including NRLs, other public institutes and private companies, these results indicate that the current monitoring is at a sufficient level in terms of sensitivity.

Considering all the results, in only one case a false negative (misinterpretation of presence) was reported. All other deviating results were false positives (misinterpretations of absence).

The effect of method modifications can only be discussed if a substantial number of participants applied a specific modification. Both the use of sediment staining (Alizarin; n=14) and the lack of examination at lower magnifications (use of stereo microscope; n=9) are applied by significant numbers of applicants. The use of 5 grams for sedimentation was applied only by five participants, whereas other modifications are too diverse to allow any conclusion. The false positives for fish absence were equally distributed among the groups that used a stereo microscope and those

that did not. The specificity score for the latter group was nevertheless lower, because of the smaller number of participants (n=9 vs. n=40). Five out of 11 false positive results were obtained after examining uncoloured sediments, whereas the other six false positives were reported after applying Alizarin staining. It is known that the Alizarin Red staining process reduces considerably the amount of sediment available for slide preparation (Veys et al., 2007b). Some other parameters as used in the inventory, such as the glassware used, the use of a binocular for examination at lower magnifications, and the way the slides are prepared (e.g. embedding agent) show interesting possibilities for improvement. Training of microscopists and the use of well qualified materials for preparing the samples are also important.

5.4 DISCUSSION

The detection of land animal material in the presence of fish material is the corner stone of the performance of the microscopic method, among other methods. Microscopy is a good choice for fast screening of samples and might give a first indication of the nature of the animal proteins (e.g. fish vs. terrestrial animals), if found. Further identifications can be achieved with other methods (see other chapters in this volume). The EU-RL pays attention to further development of, and a good balance between, the different methods.

Theoretical calculations have been carried out for estimating the effect of using the entire sediment or only a part of the sediment material, and of the starting amount of material for sedimentation (van Raamsdonk et al., 2008). Both aspects, using more material for sedimentation (10 g vs. 5 g), and using more material for examination (up to 100% of the sediment material), have an increasing effect on the performance of the microscopic detection (Figure 4.4). Results of the past six years (only results obtained after applying the method of Regulation 152/2009/EC or its predecessor Directive 2003/126/EC are considered) generally indicate lower sensitivity scores than theoretically achievable. In the situation of an absence of fish meal, the sensitivity scores for the presence of terrestrial animals are mainly above 0.95 for all contamination levels between 0.1% and 0.02%. In the presence of 5% of fish meal, however, the first documented study in which a method comparable to that of Directive 2003/126/EC has been used (2003), resulted in a sensitivity score of only 0.44 (Gizzi et al., 2003; calculated on original data; see also Annex XI of Gizzi et al., 2003). A bench mark study, carried out in 2003 with a selected set of nine laboratories resulted in a sensitivity score for 0.1% of animal protein in the presence of 5% fish meal of 0.985. More recent results increased to a sensitivity level of finally 0.98 in the most recent study (2009; see v. Raamsdonk et al., 2007; 2008; 2009a for documentation), which is a very good achievement in a time frame of six years.

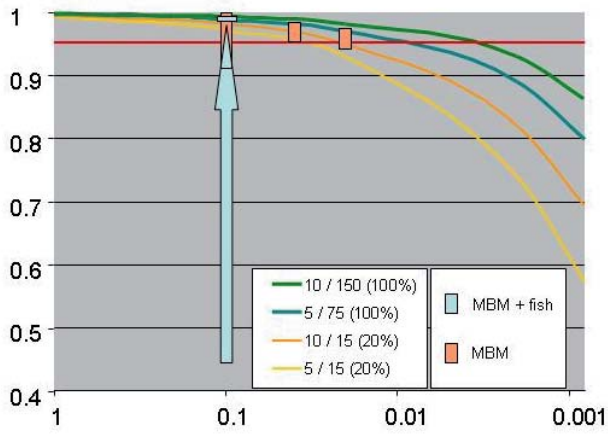

Figure 5.3. Sensitivity scores (y-axis) for different contamination levels of animal proteins of land animals (logarithmic x-axis). Lines: theoretical calculations for 5 or 10 grams of starting material for sedimentation, and the use of 20% or 100% of the sediment material for microscopic examination. Bars: Sensitivity for the detection of animal proteins without (orange) or with the presence (blue) of fish meal at 5%. Blue arrow: increase from 2003 to 2009. The small blue band indicates the level achieved in the bench mark study (2003: 0.98).The desired 0.95 level is indicated by an red horizontal line.

A meta-analysis of proficiency tests in which information on the application of at least some method parameters is collected or which included at least a blind sample containing both fish and terrestrial MBM, is presented in Table 5.1. Two parameters which represent the use of more material for sedimentation, and the use of a stereo microscope allowing to examine more material (up to 100 %), as used in the theoretical calculations, show an improvement. This optimization seems to be related to an improvement of the performance of the microscopic method, as indicated by the sensitivity scores of terrestrial animal material in the presence of fish meal. Data on the portion of material used for preparing slides is only available from very few studies. For comparison, certain parameters for which a negative effect on the performance can be expected, such as a non-suited embedding agent, can not be judged due to the low frequency of application (Table 5.1).

Table 5.1. The relative share of participants applying specific choices of some method parameters (10 grams of material for sedimentation, skipping examination using a stereo microscope, and using non-suited embedding agents for slide preparation) as deducted from the inventories of four proficiency tests. The sensitivity score for the detection of terrestrial animal proteins (0.1 %) in the presence of fish meal (5 %) is given as reference to the performance in the studies mentioned. N.d.: not determined.

parameter	Study: choice	DG-SANCO, 2003	STRATFEED, 2004	CRL, 2006	CRL, 2007	IAG, 2008	IAG, 2009
sedimentation:	10 grams	21%	26%	37%	56%	58%	84%
stereo microscope:	not applied	45%	13%	n.d.	n.d.	36%	18%
embedding agent for sediment:	water, chloral hydrate, or comparable	n.d.	3%	n.d.	n.d.	9%	4%
Sensitivity score for the detection of terrestrial animal material in the presence of fish:		0.44 (n = 22)	0.77 (n = 31)	0.88 (n = 21)	0.84 (n = 25)	n.d. (n=45)	0.98 (n = 49)
Reference		Gizzi et al., 2003; 2004	Von Holst et al., 2004	Veys et al., 2007a	Veys et al., 2007b	van Raamsdonk et al., 2008	van Raamsdonk et al., 2009

The results from the meta-analysis of the proficiency tests as well as from the theoretical calculations indicate that an improvement of the sensitivity is achievable. The most likely parameters to be improved are the starting amount of material for sedimentation and the portion of sediment material used for examination, either by using a stereo microscope and/or by applying a majority of the sediment on one or more slides. The use of a stereo microscope for examination at lower magnification is already stated as compulsory in Regulation 152/2009/EC. The advantage to apply a stereo microscope is not directly supported by the presented results, but it can be assumed to be beneficial. It can frequently occur that the amount of sediment produced is too large for examination in a series of slides at a higher magnification. The only alternative is to examine the entire sediment by using a stereo microscope. If, for example, only 20 % of the sediment is examined, then an amount of sediment of 1 or 2 grams is effectively taken in account and not the original 5 or 10 grams, respectively. This would largely annihilate the achieved improvements of using a larger amount of sediment. The recognition of animal proteins at lower magnifications is different from detection at higher magnifications. Training is an important requirement in this respect. Specificity, as the other aspect of the proficiency of the microscopic method, can and should be improved by training to distinguish fragments of land animals from those of fish or from other sources. Collecting images of (presumed) positive findings is highly recommended to allow retrospective evaluation.

Literally the text of Regulation 152/2009/EC requests to apply exclusively paraffin oil or glycerol as embedding agent. However, a wide range of different paraffin oils is available, and therefore the required range in viscosity is also mentioned (68 – 81 [mPa.s]). It could be reasonable to allow immersion oil as embedding agent, since it is a more stable product than paraffin oil with a comparable viscosity. An embedding agent with a moderate high viscosity (approx. 60 - 150 mPa.s) allows a good examination of the shape of the lacunae and of the visibility of canaliculae. Permanent adhesives of Norland in this viscosity range are readily available.

Glassware allowing extracting the sediment from the bottom, e.g. a chemical separation funnel, eases the prevention of remixing the flotation and the sediment.

Although a contamination level of 0.1% is used most frequently in tests, the use of a contamination level below 0.1% allows evaluating the strength of the microscopic method. From theoretical calculations it can be concluded that below 0.01% chances increase to report false negative results. In one of the most recent proficiency tests (IAG 2008: van Raamsdonk et al., 2008), a contamination level of 0.05% was included, resulting in a sensitivity score of 0.956. The main parameters discussed a proper starting amount of material for sedimentation and the portion of sediment material used for examination, will have specifically a marked effect at these lower contamination levels.

The final results of applying the microscopic detection method for animal proteins, depends on both the proper application of the method, and a good evaluation of the observations. The latter aspect will be discussed further in Chapter 6.

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