

**VETERINARY SERVICES ACT
(CAP. 437)**

**Rules on the Analytical Method for the Determination of
Constituents of Animal Origin for the Official Control of
Feedingstuffs, 2005**

IN exercise of the powers conferred by article 25 of the Veterinary Services Act, the Minister for Rural Affairs and the Environment has made the following rules:—

Title and scope.

1. (1) The title of these rules is the Rules on the Analytical Method for the Determination of Constituents of Animal Origin for the Official Control of Feedingstuffs, 2005.

(2) The scope of these rules is to implement the rules found under the European Union Commission Directive 2003/126/EEC on the analytical method for the determination of constituents of animal origin for the official control of feedingstuffs.

How official analysis of feedingstuffs shall be carried out.

2. Where official analysis of feedingstuffs is carried out with a view to officially controlling the presence, identification and, or estimation of the amount of constituents of animal origin in feedingstuffs, in the framework of the coordinated inspection programme in the field of animal nutrition in accordance with the European Union Council Directive 95/53/EC, it shall be carried out in accordance with the provisions of the Schedule to these rules.

Role of laboratories.

3. (1) Laboratories carrying out official controls on the presence of animal constituents in feedingstuffs shall participated periodically in proficiency testing on the analytical methods.

(2) Laboratory personnel carrying out analyses shall receive adequate training.

SCHEDULE

Conditions for the microscopic detection, identification or estimation of constituents of animal origin in feedingstuffs

1. Objective and field of application

These conditions shall be used when detection of constituents of animal origin (defined as products from processing bodies and body parts of mammals, poultry and fish) in feedingstuffs is carried out by means of microscopic examination in the framework of the coordinated inspection programme in the field of animal nutrition in accordance with Council Directive 95/53/EC. Provided that the methods in this Schedule are used in all official tests, a second test may also be carried out using variant or alternative methods, in order to improve the detection of certain types of animal constituents or to specify further the origin of the animal constituents. Furthermore, a variant protocol may be used when examining certain specific animal constituents such as plasma or bones in tallow (see also point 9), provided that these analyses are made in addition to the analyses foreseen in the coordinated inspection programme.

2. Sensitivity

Dependent on the nature of the constituents of animal origin, very small amounts ($< 0,1 \%$) in feedingstuffs can be detected.

3. Principle

A representative sample, taken in accordance with the provisions laid down in Commission Directive 76/371/EEC of 1 March 1976 establishing Community methods of sampling for the official control of feedingstuffs (1) which has undergone suitable preparation is used for the identification. The following protocol is fit for handling feed with low moisture content. Feed with an amount of moisture higher than 14 % shall be dried (condensed) prior to handling. Special feed or feed materials (e.g. fats, oils) need dedicated treatment (see point 9). The constituents of animal origin are identified on the basis of typical, microscopically identifiable characteristics (i.e. muscle fibres and other meat particles, cartilage, bones, horn, hair, bristles, blood, feathers, egg shells, fish bones, scales). The identification has to be done both on the sieve fraction (6.1) and the concentrated sediment (6.2) of the sample.

4. Reagents

4.1. Embedding agent

4.1.1. Chloral hydrate (aqueous, 60 % w/v)

4.1.2. Lye (NaOH 2,5 % w/v or KOH 2,5 % w/v) for sieve fractions

4.1.3. Paraffin oil or glycerol (viscosity: 68-81) for microscopic observations in the sediment

4.2. Rinsing agents

4.2.1. Alcohol, 96 %

4.2.2. Acetone

4.3. Concentrating agent

4.3.1. Tetrachloroethylene (density 1,62)

4.4. Staining reagents

4.4.1. Iodine/potassium iodide solution (Dissolve 2 g potassium iodide in 100 ml water and add 1 g iodine while shaking frequently)

4.4.2. Alizarin Red (Dilute 2,5 ml 1M hydrochloric acid in 100 ml water and add 200 mg alizarine red to this solution)

4.4.3. Cystine reagent (2 g lead acetate, 10 g NaOH/100 ml H₂O)

4.4.4. Iodine/potassium iodide solution (dissolved in 70 % ethanol)

4.5. *Bleaching reagent*

4.5.1. Commercial sodium hypochlorite solution (9.6 % active chlorine)

5. **Equipment and accessories**

5.1. Analytical balance (accuracy of 0,01 g except for the concentrated sediment: 0,001 g)

5.2. Material for grinding (grinding mill or a mortar, especially for feed containing > 15 % fat on analysis)

5.3. Sieve fitted with sieve mesh with square meshes of width of 0,50 mm maximum

5.4. Separation funnel or conical bottomed settling beaker

5.5. Stereomicroscope (minimum 40' magnification)

5.6. Compound microscope (minimum 400' magnification), transmitted light or polarised light

5.7. Standard laboratory glassware

All equipment shall be thoroughly cleaned. Separation funnels and glassware need washing in a washing machine. Sieves need cleaning using a brush with stiff hairs.

6. **Procedure**

Pelleted feeds may be pre-sieved if both fractions are analysed as a separate sample.

At least 50 g of the sample shall be treated (ground with care using the suitable grinding equipment (5.2) if necessary in order to achieve an appropriate structure). From the ground material two representative portions shall be taken, one for the sieve fraction (at least 5 g) (6.1) and one for the concentrated sediment (at least 5 g) (6.2). Colouring with staining reagents (6.3) can additionally be applied for the identification.

In order to indicate the nature of the animal proteins and the origin of the particles, a decision support system such as Aries can be used and reference samples can be documented.

6.1. *Identification of constituents of animal origin in the sieve fractions*

At least 5 g of the sample is sieved through the sieve (5.3) in two fractions.

The sieve fraction(s) with the large particles (or a representative part of the fraction) is applied as a thin layer to a suitable support and screened systematically under the stereomicroscope (5.5) at various magnifications for constituents of animal origin.

Slides made with the sieve fraction(s) with the fine particles are screened systematically under the compound microscope (5.6) at various magnifications for constituents of animal origin.

6.2. *Identification of constituents of animal origin from the concentrated sediment*

At least 5 g (accurate to 0,01 g) of the sample shall be transferred into a separation funnel or conical bottomed settling beaker and treated with at least 50 ml of tetrachloroethylene (4.3.1). The mixture shall be shaken or stirred repeatedly.

— If a closed separation funnel is used the sediment shall be left to stand for a sufficient time (at least three minutes) before the sediment is separated off. Shaking shall be repeated and the sediment shall be left to stand again at least three minutes. The sediment shall be separated off again.

— If an open beaker is used, the sediment shall be left to stand for at least five minutes before the sediment is separated off.

The total sediment shall be dried and subsequently weighed (accurate to 0,001 g). The weighing is only necessary in case an estimation is required. If the sediment consists of many large particles it may be sieved through a sieve (5.3) in two fractions. The dried sediment shall be examined for bone constituents under the stereomicroscope (5.5) and the compound microscope (5.6).

6.3. *Use of embedding agents and staining reagents*

The microscopic identification of the constituents of animal origin can be supported by the use of special embedding agents and staining reagents.

Chloral hydrate (4.1.1): By carefully heating, cell structures can be seen more clearly because starch grains gelatinise and unwanted cell contents are removed.

Lye (4.1.2): Either sodiumhydroxide or potassiumhydroxide clears the material of the feed, assisting the detection of muscle fibres, hairs and other keratin structures.

Paraffin oil and glycerol (4.1.3): Bone constituents can be well identified in this embedding agent because most lacunae remain filled with air and appear as black holes about 5-15 µm.

Iodine/potassium iodide solution (4.4.1): Used for the detection of starch (blue-violet colour) and protein (yellow-orange colour). Solutions may be diluted if required.

Alizarin red solution (4.4.2): Red/pink colouring of bones, fish bones and scales. Before drying the sediment (see section 6.2), the total sediment shall be transferred into a glass test tube and rinsed twice with approximately 5 ml alcohol (4.2.1) (each time a vortex shall be used, the solvent shall be let settle about one minute and poured off). Before using this staining reagent, the sediment shall be bleached by adding at least 1 ml sodium hypochlorite solution (4.5.1). The reaction shall be allowed to continue for 10 minutes. The tube shall be filled with water, the sediment shall be let settle two to three minutes, and the water and the suspended particles shall be poured off. The sediment shall be rinsed twice more with about 10 ml of water (a vortex shall be used, let settle, and the water poured off each time). Two to 10 or more drops (depending on the amount of residue) of the alizarine red solution shall be added. The mixture shall be shaken and the reaction shall be let occur a few seconds. The coloured sediment shall be rinsed twice with approximately 5 ml alcohol (4.2.1) followed by one rinse with acetone (4.2.2) (each time a vortex shall be used, the solvent shall be let settle about one minute and poured off). The sediment is then ready to be dried.

Cystin reagent (4.4.3): By carefully heating, cystin-containing constituents (hair, feathers, etc.) become black-brown.

6.4. *Examination in feed possibly containing fishmeal*

At least one slide shall be examined from the fine sieve fraction and from the fine fraction of the sediment under the compound microscope (see sections 6.1 and 6.2).

Where the label indicates that the ingredients include fishmeal, or if the presence of fishmeal is suspected or detected in the initial examination, at least two additional slides of the fine sieve fraction from the original sample, and the total sediment fraction shall be examined.

7. Calculation and evaluation

Member States shall ensure that the procedures described in this point are used where an official analysis is carried out with a view to estimating the amount (and not simply the presence) of animal constituents.

The calculation can only be made if the constituents of animal origin contain bone fragments.

Bone fragments of terrestrial warm-blooded species (i.e. mammals and birds) can be distinguished from the different types of fish bone on the microscopic slide by means of the typical lacunae. The proportion of constituents of animal origin in the sample material is estimated taking into consideration:

- the estimated proportion (weight %) of bone fragments in the concentrated sediment and
- the proportion (weight %) of bone in the constituents of animal origin.

The estimate has to be based on at least three (if possible) slides and at least five fields per slide. In compound feedingstuffs, the concentrated sediment as a rule contains not only terrestrial animal bone and fish bone fragments, but also other particles of high specific weight, e.g. minerals, sand, lignified plant fragments and the like.

7.1. *Estimated value of the percentage of bone fragments*

$$\% \text{ terrestrial bone fragments} = (S \times c)/W$$

$$\% \text{ fish bone and scale fragments} = (S \times d)/W$$

(S = sediment weight (mg), c = correction factor (%) for the estimated portion of terrestrial animal bones in the sediment, d = correction factor (%) for the estimated portion of fish bones and scale fragments in the sediment, W = weight of the sample material for the sedimentation (mg)).

7.2. *Estimated value of constituents of animal origin*

The proportion of bone in animal products can vary greatly. (The percentage of bone in the case of bonemeals is of the order of 50 to 60 % and in the case of meat meals of the order of 20 to 30 %; in the case of fish meals bone and scale contents vary according to the category and origin of the fishmeal, normally in the order of 10 to 20 %).

If the type of animal meal present in the sample is known, it is possible to estimate the content:

$$\text{Estimated content of constituents of terrestrial animal products (\%)} = (S \times c)/(W \times f) \times 100$$

$$\text{Estimated content of constituents of fish products (\%)} = (S \times d)/(W \times f) \times 100$$

(S = sediment weight (mg), c = correction factor (%) for the estimated portion of terrestrial animal bone constituents in the sediment, d = correction factor (%) for the estimated portion of fish bones and scale fragments in the sediment, f = correction factor for the proportion of bone in the constituents of animal origin in the sample examined, W = weight of the sample material for the sedimentation (mg)).

8. Expression of the result of the examination

The report shall at least contain information on the presence of constituents derived from terrestrial animals and from fishmeal. The different cases shall be reported in the following way:

8.1. *With regard to the presence of constituents derived from terrestrial animals:*

— As far as was discernible using a microscope, no constituents derived from terrestrial animals were found in the submitted sample, or:

— As far as was discernible using a microscope, constituents derived from terrestrial animals were found in the submitted sample.

8.2. *With regard to the presence of fishmeal:*

— As far as was discernible using a microscope, no constituents derived from fish were found in the submitted sample, or:

— As far as was discernible using a microscope, constituents derived from fish were found in the submitted sample.

In case constituents derived from fish or terrestrial animals are found, the report of the examination result, if required, can further indicate an estimation of the amount of constituents detected (x %, < 0,1 %, 0,1-0,5 %, 0,5-5 % or > 5 %), further specification of the type of terrestrial animal if possible and the animal constituents identified (muscle fibres, cartilage, bones, horn, hair, bristles, feathers, blood, egg shells, fish bones, scales).

For the case where the amount of animal ingredients is estimated the correction factor *f* used shall be mentioned.

For the cases where bone constituents from terrestrial animals are identified, the report shall contain the additional clause:

‘The possibility that the above constituents are derived from mammals cannot be excluded.’

This additional clause is not necessary in cases where the bone fragments from terrestrial animals have been specified as bone fragments from poultry or mammals.

9. **Optional protocol for analysing fat or oil**

The following protocol may be used for the analysis of fat or oil:

— If the fat is solid, it is warmed for example in a microwave oven until it is liquid.

— Using a pipette, 40 ml of fat is transferred from the bottom of the sample to a centrifugation tube.

— Centrifuge for 10 minutes at 4 000 rpm.

— If the fat is solid after centrifugation, it is warmed once more in an oven until it is liquid. Repeat the centrifugation for five minutes at 4 000 rpm.

— Using a small spoon or a spatula, one half of the decanted impurities is transferred to a small petri dish or a microscopic slide for microscopic identification of a possible content of animal constituents (meat fibres, feathers, bone fragments). As an embedding agent for microscopy, paraffin oil or glycerol is recommended.

— The remaining impurities are used for sedimentation as described in point 6.2.

