

TECHNICAL NOTE : ATTEMPTS TO HARMONIZE CHEMICAL ANALYSES OF FEEDS AND FAECES, FOR RABBIT FEED EVALUATION

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ABSTRACT : The aim of this document is to describe harmonized procedures of some basic chemical analyses useful for rabbit compound feeds and faeces that have been approved within the six teams forming the EGRAN group. For each criterion (dry matter, ash, energy, nitrogen, fat, fibre), we report a brief definition, its main interest, and some general references used in animal research (ISO

or AOAC standards). We also report the principle of the analytical determination, a brief description of the practical procedures, some suggestions, the among laboratory variability recently measured in a collaborative study on digestibility of feeds for growing rabbit, and the recommended level of tolerance.

RESUME : Cette note décrit les procédures, harmonisées et approuvées par les six équipes formant le groupe EGRAN, utilisées pour l'analyse chimique des aliments composés et des fèces chez le lapin. Pour chaque composants (matière sèche, cendres, énergie, azote, lipides, fibre) nous proposons une brève définition, l'intérêt majeur, et quelques références courantes utilisées en recherche

animale (normes ISO ou AOAC). Nous énonçons aussi le principe de l'analyse chimique, une brève description de la procédure pratique, quelques suggestions, la variabilité entre laboratoires récemment mesurée au cours d'un ring-test de mesure de digestibilité d'aliments pour lapin en croissance, et le niveau de tolérance recommandé.

1 - INTRODUCTION

The in-vivo determination of nutrient digestion implies two steps : 1) the determination of dry matter faecal digestibility, and 2) the determination of nutrient concentrations in feeds and faeces. In the area of rabbit nutrition, the EGRAN (GIDENNE, 1999) obtained significant progress for the first step, harmonising the methodology for measuring the faecal digestibility of dry matter (PEREZ *et al.*, 1995). This method was officially recognised by the Italian Scientific Association of Animal Production (ASPA) (XICCATO *et al.*, 1996a).

With respect to the second step, various methods are used to analyse the basic components of feeds and faeces. In fact, even for very simple criteria, such as dry matter determination, several procedures are commonly used, even "officially" approved (international standards such as ISO, or national standards such as AFNOR in France or AOAC in USA). However, operative conditions in most of the research laboratories may be very different due to the particular types of samples or instruments.

Nevertheless, the standardization of methods would serve as a common basic methodological platform for collaborative studies, cooperation and comparison of studies carried out by different research

teams. The use of reference harmonized methods would be useful for at least three reasons : 1) as a quality tool within a processing operation; 2) to assess the effectiveness of production and processing treatments where there may be an interest in being able to compare results between laboratories or countries; 3) as a research tool in nutrition studies. Therefore, the harmonization of analytical methods has been performed in many scientific areas, e.g. to assess the physical characteristics of meat (HÖNIKEL, 1998). In the field of rabbit research, the harmonization of methods to evaluate the characteristics of meat has already been studied (OUHAYOUN and DALLE ZOTTE, 1996).

In the field of rabbit nutrition, the necessity to use harmonized procedures for chemical analyses in collaborative digestibility experiments was highlighted (XICCATO *et al.*, 1996b) to reduce the variability of results (especially for fibre and fat digestibilities) among teams (Table 1). For these reasons, EGRAN decided to elaborate a document to be useful in further collaborative studies on rabbit nutrition, detailing the practical conditions for chemical analysis of rabbit compound feeds and faeces.

We hope that this description for harmonized chemical analyses will be useful to other scientists working in the field of rabbit nutrition.

For each criterion, we report a brief definition, its main interest, and some general references used in animal research (ISO or AOAC standards). We also report the principle of the analytical determination, a brief description of the practical procedures, some suggestions with regards to the technique, the among laboratory variability measured recently in a collaborative study on digestibility of feeds for the

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Table 1 : Among laboratory variations (CV_L, %), without harmonization of the procedures.

Reference	Sample	DM ⁽¹⁾	ASH	CP	CF	FAT	NDF	ADF	ADL	GE
XICCATO <i>et al.</i> , 1996b	Feeds	0.5	2.1	2.5	5.4	17.8	4.7	2.0	7.5	0.7
	Faeces	0.9	1.3	4.2	2.5	24.7	1.4	2.1	7.2	0.8
EGRAN, 2001	Feeds	0.5	2.2	5.0	3.2	16.6	4.2	4.0	11.1	0.6
	Faeces	0.6	2.9	6.6	1.9	22.7	2.1	2.9	5.7	1.5

(1) : DM= dry matter; CP= crude protein; CF= crude fibre; NDF= neutral detergent fibre; ADF= acid detergent fibre; ADL= acid detergent lignin; GE= gross energy.

growing rabbit (Table 1) and the recommended level of tolerance.

2 - GENERAL NOTES

Before chemical analysis, compound feeds are ground at 1 mm. Faeces are pre-dried (DM > 85%) according to PEREZ *et al.* (1995) and submitted to analysis after grinding at 1mm. The samples are kept in plastic bottles with a double seal in a dry place.

For each criterion assayed in a digestibility trial, three replicates for feed samples and two replicates for faecal samples are required. The lower number of replicates for faeces is partly due to the higher homogeneity of this matrix. A higher number of replicates for feeds is required because the analyses are performed on only one sample of feed compared to 8 to 12 samples of faeces (according to PEREZ *et al.*, 1995). Therefore, in a digestibility assay, an analytical error on a feed sample has a greater impact than on a faecal sample.

The level of tolerance (defined as maximum difference between replicates) for each criterion is stated on the basis of the among-laboratory variability recorded in previous collaborative studies performed without harmonization of procedures (Table 1). When the level of tolerance (e.g. mean of two replicates for faeces) is out of range, 2 or 3 further replicates should be performed.

3 - THE CRITERIA

3.1 - DRY MATTER "DM"

Definition : residue obtained after a total evaporation of water by heating the sample

Interest : it is used to correct the value of chemical analysis in order to express them on the same DM basis.

General references : MAPA, 1993a; AOAC 1995a; ISO, 1999a.

Note : The AOAC procedure recommends to dry 2 g of sample for 5 h at 95-100°C.

The analysis of dry matter concentration is of particular importance, because of the impact of this measurement on all other chemical components. A relatively high quantity of sample is required (3 g vs 2 g e.g. in AOAC procedure)

3.1.1 - Basic procedure

- Sample weight "W1" : minimum 3 g (weight precision : ± 0.0001 g)
- Filling must not exceed 2/3 the volume of the capsule (use dried and cooled capsules)
- Drying till constant weight of sample "W2", i.e. minimum duration: overnight (≈ 16 h)
- Temperature : minimum 103° and maximum 104°C
- Cooling : immediately after drying in a desiccator (not overloaded)

3.1.2 - Calculation

$$\text{Dry matter (\%)} = (W2/W1) \times 100$$

3.1.3 - Variability and tolerance

- The among laboratory variability observed without harmonization is about 0.5% for feeds and 0.8% for faeces (Table 1).
- Tolerance : 1.0 point as absolute maximum difference between replicates

Note : despite storage in a double sealed plastic bottle, it is good practice to repeat the DM determination if the other analyses are performed after more than one month.

3.2 - ASH

Definition : mineral residue obtained by incineration of sample

Interest : to calculate the concentration in organic matter "OM" = DM - Ash.

General references : ISO, 1978; MAPA, 1993b; AOAC 1995b.

Note : the AOAC procedure (Official method 942.05) recommends to ash 2 g of sample for 2 h at 600°C.

3.2.1 - Basic procedure

- Use the same "capsule + sample" used for dry matter determination (sample weight before ashing = W2, the dry weight of sample, if using the same sample used for DM determination)
- Pre-ashing step : sometimes practised according to equipment
- Ashing temperature : 550°C
- Ashing duration : 3 h minimum with "white ash" a final control
- Transfer "capsule + ashing sample" into a pre-heated (103°C) oven for one hour
- Cool in a desiccator and weigh soon after reaching room temperature (sample weight after ashing = W3).

3.2.2 - Calculation

$$\text{Ash (\% DM*)} = (W3/W2) \times 100$$

* : if ash is determined on the same sample used for DM (see 3.1)

3.2.3 - Variability and tolerance

- The variability observed among laboratories without harmonization is about 2.2% for feeds and faeces (Table 1).
- Tolerance : 0.5 points as absolute maximum difference between replicates

3.3 - ENERGY VALUE "GE" (GROSS ENERGY)

Definition : Amount of heat evolved by the total combustion of a unit of weight of sample.

Interest : to calculate the dietary energy value

General references : ISO, 1998 (no AOAC reference).

3.3.1 - Basic procedure

- Calibration of adiabatic bomb with benzoic acid dried 1 h at 70°C : 5 tablets minimum per combustion bomb (Coefficient of variability between replicates : < 0.5%)
- Sample preparation : pellet or packed in a thin paper sheet such as cigarette paper (avoid a powder form)
- Sample weight : minimum 1 g (W0, precision ± 0.0001 g)
- Respect adiabatic conditions : use the same initial water temperature that was used for calibration (maximum 30°C for all the determinations). The cooling water temperature has to be at least 6°C beyond the working temperature
- Check that complete combustion of the sample has occurred
- Note the calorific value (CV, kJ) of sample combustion after correction for other heat sources (wire, cotton thread, paper).

3.3.2 - Variability and tolerance

- The among laboratory variability observed without harmonization is about 0.7% for feeds and 1.2% for faeces (Table 1).
- Tolerance : 0.2 MJ as absolute maximum difference between replicates.

3.3.3 - Calculation

$$\text{Gross Energy (MJ/kg)} = CV/W0$$

3.4 - CRUDE FIBRE (also known as Weende method)

Definition : residue obtained after the acid and basic hydrolysis of a sample. It corresponds to a mixture of the main part of cellulose and of some parts of lignin, cutin, suberine and other fractions of the cell wall. The proportions of these constituents vary according to the type of raw materials or complete feed analysed.

Interest : useful criteria for quality control of raw materials in a feed mill, as it is a low cost, robust and easily reproducible method. It could be included in equations for predicting digestible energy concentration. It also could be used for a gross evaluation of the lower digestible fibre fraction in a rabbit feed.

General references : Henneberg and Stohmann, 1864; ISO, 1981; AFNOR 1993; MAPA, 1993c; AOAC, 1995c.

3.4.1 - Instruments and equipment

- System equipped with a semi-automatic filtration system and glass crucibles (P.2)

3.4.2 - Reagents

- H₂SO₄ solution : 1.25% or 0.255 N (12.5 g of H₂SO₄ (96-98%, d=1.84) diluted to 1000 ml)
- NaOH solution : 1.25% or 0.313 N (12.5 g of NaOH diluted to 1000 ml)
- Acetone

3.4.3 - Basic procedure

- Weigh 1 g of sample (W0, precision ± 0.0001 g) in a clean and dried crucible
- If ether extract is higher than 5%, wash with acetone (20 ml minimum), 3 times of 3 minutes each
- Transfer the crucibles to the hot extraction unit
- Add 150 ml of H₂SO₄ (0.255 N) and heat to boiling
- Adjust heat and boil for 30 minutes
- Filter the solution and wash with boiling distilled water at least 2 times
- Add 150 ml of NaOH (0.313 N) and heat to boiling
- Adjust heat and boil for 30 minutes
- Filter the solution and wash with hot distilled water at least 2 times

- Transfer the crucibles to the cold extraction unit and wash with acetone 3 times
- Oven-dry the crucible at 103°C (minimum 3 h)
- Transfer the crucibles in a desiccator and weigh them (W1) after cooling.
- Ash the sample residues in the crucibles at 550°C (minimum 3 h)
- Transfer the crucibles while still hot (but under 250°C) into an oven for 1 h (103°C)
- Cool into a desiccator and weigh them (W2).

3.4.4 - Calculations

$$\text{Crude Fibre (\%)} = ((W1-W2)/W0) \times 100$$

3.4.5 - Variability and tolerance

- The among laboratory variability observed without harmonization is about 4.5% for feeds and 2.2% for faeces (Table 1).
- Tolerance : 1.2 points as absolute maximum difference between replicates.

3.5 - SEQUENTIAL DETERGENT FIBRE DETERMINATION

(also known as sequential Van Soest method)

Definition : The procedure determines three fibre residues, after successive hydrolysis on the same sample using respectively : a neutral detergent (solubilization of intra-cellular constituents and pectins, residue = NDF) then an acid detergent (solubilization of hemicelluloses, residue = ADF) and finally an acid hydrolysis (solubilization of cellulose, residue = ADL).

Interest : It allows fractionation of the main constituents of the cell wall (hemicelluloses, cellulose, lignin). It is useful to evaluate fibre composition in feeds. It is used as criteria for dietary fibre requirements in order to prevent digestive troubles in rabbits, and to predict the digestible energy content of feed or feedstuffs

The well-known procedure of Van-Soest was initially designed to directly evaluate lignocellulosic (ADF) content of grasses and hay (Van Soest, 1963), and was approved by AOAC (1995d). It is now used more widely for concentrated feedstuffs and feeds. Robertson and Van Soest (1981) have described the sequential procedure. It was then subjected to many modifications (Jeraci and Van-Soest, 1990), such as pre-hydrolysing steps using amylolytic or proteolytic enzymes for high starch or high protein feeds (Van-Soest *et al.*, 1991). The complete sequential procedure for a concentrate feed, with a semi-automatic filtration system apparatus, based on enzyme pre-treatment, neutral and acid detergents and then final acid hydrolysis was described later (Dorleans, 1985) and approved by AFNOR in 1997 (AFNOR 1997a).

General references : Van Soest, 1963; Van Soest and Wine, 1967; Robertson and Van Soest, 1981; Dorleans, 1985; Van Soest *et al.*, 1991; AOAC, 1995d; Giger-Reverdin, 1995; AFNOR, 1997a.

3.5.1 - Instruments and equipment

- System equipped with a semi-automatic filtration system, and glass crucibles (P.2)

3.5.2 - Neutral Detergent Fibre "NDF"

Reagents

Neutral detergent solution :

- | | |
|---|---------|
| • Distilled water | 1 L |
| • Sodium lauryl sulphate | 30 g |
| • Disodium ethylenediaminetetraacetate (EDTA) | 18.61 g |
| • Sodium borate decahydrate | 6.81 g |
| • Disodium hydrogen phosphate, anhydrous | 4.56 g |
| • Triethyleneglycol | 10 ml. |

Check pH to range 6.9 to 7.1

Heat-resistant Amylase 3306 Sigma (see note*)

Acetone

Basic procedure

- Weigh 1 g of sample (W0, precision ± 0.0001 g) in a clean and dried crucible
- If the ether extract is higher than 5%, wash with acetone (20 ml minimum) in the cold extraction unit, 3 times of 3 minutes each
- Transfer the crucibles into the hot extraction unit
- Add 100 ml of neutral detergent solution and 50 µl of the heat-resistant amylase (only for feeds, not for faeces) and heat to boiling
- Adjust heat and boil for 60 minutes (moderate and constant degree of boiling)
- Filter the solution and wash with boiling distilled water twice
- Transfer the crucibles to the cold extraction unit and wash with acetone at least twice
- Oven-dry the crucible at 103°C for 6 hours minimum
- Transfer the crucibles in a desiccator and weigh them (W1) after cooling

*According to Giger-Reverdin (1995), the enzyme A 3306 from Sigma is insensitive to EDTA (Van Soest, *et al.* 1991), differently from other amylases whose alpha 1-6 activities is inactivated by EDTA in neutral detergent solutions (Cherney *et al.*, 1989).

3.5.3 - Acid Detergent Fibre "ADF" (Sequential procedure)

Reagents

Acid detergent solution

- | | |
|--|------|
| • Sulphuric acid solution 1N (49.04 g/l) | 1 L |
| • Cetyl trimetilamonium bromide | 20 g |
| • acetone | |

Basic procedure

- Disperse the NDF residue (add distilled water in the crucible)
- Add 100 ml of acid detergent solution and heat to boiling
- Adjust heat and boil for 60 minutes (moderate and constant degree of boiling)
- Filter the solution and wash with boiling distilled water twice
- Transfer the crucibles to the cold extraction unit and wash with acetone twice
- Oven-dry the crucible at 103°C for 6 hours minimum
- Transfer the crucibles in a desiccator and weigh them (W2) after cooling

3.5.4 - Acid Detergent Lignin "ADL" (Sequential procedure)**Reagents**

ADL Solution :

- Sulphuric acid 72% (w/w)

Check the density of the solution $d=1.634$ **Basic procedure**

- The room temperature must be 20-22°C
- Place the crucibles in a plate
- Fill up (3/4) with ADL solution
- Place a glass stick in the crucible, stir every hour and refill with the ADL solution if it is necessary
- Three hours after the initial addition of ADL solution, filter and wash with hot distilled water at least 6 times until the pH is neutral
- Oven-dry the crucible at 103°C overnight
- Transfer the crucibles in a desiccator and weigh them (W3) after cooling
- Ash the sample residues in the crucibles at 550°C for 3 h minimum
- Transfer the crucibles, while still hot (but under 250°C), into an oven (103°C) for 1 h
- Cool into a desiccator and weigh them (W4) after cooling.

3.5.5 - Calculations for sequential procedure

$$\text{NDF (\%)} = ((W1-W4)/W0) \times 100$$

$$\text{ADF (\%)} = ((W2-W4)/W0) \times 100$$

$$\text{ADL (\%)} = ((W3-W4)/W0) \times 100$$

3.5.6 - Variability and tolerance

- The among laboratory variability observed without harmonization is about
 - for feeds : 4.5% for NDF, 3.0% for ADF and 9.5% for ADL
 - for faeces : 1.7 % for NDF, 2.5% for ADF and 6.5% for ADL (Table 1).
- Tolerance : 1.5 points as absolute maximum difference between replicates.

3.6 - CRUDE PROTEIN "CP"

Definition : Total nitrogen included in proteins, peptides and non-peptidic compounds

Interest : It provides an estimate of the protein content of a sample, using the general coefficient 6.25 to transform total nitrogen into crude protein.

General reference : AFNOR, 1977; MAPA, 1993d; AOAC, 1995e,f; ISO, 1997.

3.6.1 - Instruments and equipment

- semi automated Kjeldahl method

3.6.2 - Reagents

- H₂SO₄ (minimum 95%)
- Catalyst (without Hg or Se, no pollutant - e.g. Merck 108076, Thompson & Capper Ltd AA15)
- Na₂SO₄
- Alkali solution (NaOH 40%)
- Receiver solution :
 - dissolve 100 g of Boric Acid in 10 l distilled water (1% solution)
 - add 100 ml bromocresol green solution (100 mg in 100 ml methanol)
 - add 70 ml methyl red solution (100 mg in 100 ml methanol)
 - add 5 ml NaOH 1N.
- HCl 0.1-0.2N solution (f=1)

3.6.3 - Basic procedure

- Sample size : 1 g (W0, precision ± 0.0001 g), use weighing boat or weighing paper (also transferred to the digestion tube)
- Include three blank tubes in addition to the tubes with samples (with paper if weighing paper is used)
- Add 15 ml of H₂SO₄
- Add the catalyst
- Submit tubes to digestion at 400°C for 60 minutes (digestion is slower without Hg or Se)
- Cool and add 50 ml of distilled water
- Conditions for distillation : in order to improve the steam generation add 2.5 g of Na₂SO₄ in 5 l generator water
- Use a minimum 50 ml of alkali solution
- Use 25 ml of the receiver solution
- Use a minimum distillation volume of 75 ml
- Titrant the distilled sample (T1) using HCl 0.1-0.2N (M) as titrant solution and correcting by mean value of blank tubes (T0)

3.6.4 - Calculation

$$\text{Crude Protein (\%)} = [(T1-T0) \times 1.4 \times M] / W0 \times 6.25$$

3.6.5 - Variability and tolerance

- The among laboratory variability observed without harmonization is about 4.0% for feeds and 5.5% for faeces (Table 1).
- Tolerance : 0.8 points as absolute maximum difference between replicates.

3.7 - ETHER EXTRACT "EE" (CRUDE FAT)

Definition : Residue obtained after intense washing of sample with ether. It corresponds to esters of fatty acids and of various more or less complex alcohols.

Interest : important criteria affecting the energy level of a feed.

General references : M.A.P.A., 1993e; AOAC, 1995h; AFNOR, 1997c; ISO, 1999b.

3.7.1 - Instruments and equipment

Principle : Soxhlet method with previous acid hydrolysis.

Soxhlet extractor :

- Tecator Soxtec System HT - Extraction Unit (or equivalent apparatus)
- Acid-hydrolysis system - Hydrolysing Unit (or equivalent apparatus).

3.7.2 - Reagents

- HCl 3N - pro analyse
- Petroleum ether (boiling range 40-60°C)

3.7.3 - Basic procedure

- Put 3 g of sample (precision : ± 0.0001) (W0) in a glass tube
- Add about 1 g celite and 100 ml HCl 3N
- Put the tube in the acid-hydrolysis system and boil for 60 minutes
- Add 100 ml cold water
- Filter automatically through a glass thimble and wash with hot water (about 50-60°C) until neutrality (about 5 times x 50 ml)
- Put the thimble in an oven and dry overnight at 60°C
- Put the thimble in the extraction unit with a dried and weighted aluminium cup (W1) and add 40 ml petroleum ether
- Boil with the thimble immersed in ether during 30 minutes. The extraction should continue for another 60 minutes with the thimble hanging above the solvent surface
- Put the aluminium cup with extract in oven at 60°C overnight
- Weigh the cup (W2) after cooling in a dessicator at room temperature.

3.7.4 - Calculation :

$$\text{Ether extract (\%)} = ((W2-W1)/W0) \times 100$$

3.7.5 - Variability and tolerance

- The among laboratory variability observed without harmonization is about 17% for feeds and 23% for faeces (Table 1).
- Tolerance : 1 point as absolute maximum difference between replicates.

3.8 - STARCH

At the present, no harmonization was performed among laboratories, although this criteria is of interest in the prediction of the nutritive value of a feed for rabbits, and in the prevention of digestive troubles in young rabbits. For this reason, some general references are provided here.

General reference

1 - Polarimetric method :

ISO, 2000. *Animal feeding stuffs -- Determination of starch content -- Polarimetric method*. Reference number 6493.

AOAC, 1995. *16th edition, vol. I, chapt 4, p22. (§4.7.03) Official method 920.40 - Direct acid hydrolysis (Section 7.080), 13th ed.*

2 - Enzymatic method :

AFNOR, 1997d. *Animal feeding stuffs. Determination of starch content. Enzymatic method. NF V18-121 (Status : certified standard). Ass. Fr. de Normalisation, Paris, France.*

AFNOR, 1996. *Native starches. Determination of starch. Enzymatic method. NF V03-606 (Status : certified standard). Ass. Fr. de Normalisation, Paris, France.*

Using enzymatic procedures, the variability observed among laboratory without harmonization was about 20% for feeds and 48% for faeces (with a very small concentration of starch in faeces) in a recent collaborative study (EGRAN, 2001).

3.9 - PERSPECTIVES IN CHEMICAL ANALYSIS OF FEEDS AND FAECES

The analytical techniques are rapidly changing according to the availability of new instrumentation. For instance, inside the EGRAN laboratories, some new analytical procedures are performed for the determination of some chemicals, as described below.

3.9.1 - Fibre determination

Recently a new procedure, the fibre bag technique, has been developed to accelerate and facilitate the determination of crude fibre, NDF and ADF. This

technique uses bags (special paper) instead of glass crucibles for fibre determinations and analyses of a large number of samples at one time (Ankom company, Faiport, NY, Komarek *et al.*, 1994a ; Komarek *et al.*, 1994b).

Neutral Detergent Soluble Fibre "NDSF" : a simple method was recently described to evaluate the NDSF, such as pectic polysaccharides, β -glucans and fructans that are extractable with hot aqueous solutions (Hall *et al.*, 1997). NDSF could be determined prior NDF extraction.

3.9.2 - Crude protein determination

The total nitrogen content may be measured not according to the Kjeldahl technique but using the combustion method (method of Dumas). This procedure is referenced and officially approved (AOAC, 1995g; AFNOR, 1997b).

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