5. Feed analyses and digestion methods

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Feed characteristics are determined via chemical analyses and digestion methods. Specific NorFor methods for determining parameters such as DM, sCP, iNDF and the *in sacco* methods are fully described in this chapter. Tables 5.1 and 5.2 present an overview of the feed analysis and digestion methods, respectively.

5.1 Feed analyses

5.1.1 Dry matter in roughage

Dry matter is defined as the proportion of the sample remaining after drying to a constant weight at a defined temperature, and after compensation for the loss of volatile compounds in some feeds (*e.g.* silage). The DM value can be determined either by a single- or two-step method. The single-step method is used when only DM is required, while the two-step method is recommended when the sample is used for further chemical analyses. The drying temperature of 60 ºC is chosen to be consistent with the NDF method, described by Mertens (ISO 16472:2006 IDT), and is also used for sample preparation before other chemical, *in vitro* or *in sacco* analyses. Note that in concentrates DM is determined at a temperature of 103 ºC, as described in European Commission Regulation EC No. 152/2009.

In the single-step procedure, roughage samples should be dried to constant weight at 60 ºC and thereafter weighed hot or kept in a desiccator until weighing. The uncorrected DM is calculated as:

$$DM_{uncorrSinglestep} = \frac{Dry\_weight}{Fresh\_weight} \cdot 1000 \qquad 5.1$$

where $DM_{uncorrSinglestep}$ is the uncorrected dry matter before compensation for volatiles, g/kg; Dry_weight is the sample weight after drying, g; and Fresh_weight is the sample weight before drying, g.

Volatiles lost during drying are added to the uncorrected DM as described in Equations 5.5, 5.6 and 5.7.

In the two-step procedure, the dried sample after the first drying step (DM1) should be equilibrated in room temperature for a minimum of four hours and weighed, so DM1 can be calculated as:

$$DM1_{Twostep} = \frac{Equilibrated\_weight}{Fresh\_weight} \cdot 1000 \qquad 5.2$$

where $DM1_{Twostep}$ is the DM1 obtained from the two-step procedure, g/kg; Equilibrated_weight is the dry sample weight after 4 h equilibration in air, g; and Fresh_weight is the sample weight before drying, g.

Table 5.1. Recommended NorFor feed analysis methods.

Parameter	Abbrev	Unit	Reference method	NorFor method
Dry matter in concentrate	DM	g/kg	EC No. 152/2009	
Dry matter in roughage	DM	g/kg		See Section 5.1.1
Ash	Ash	g/kg DM	EC No. 152/2009	
Crude protein	CP	g/kg DM	EC No. 152/2009 or Dumas	See Section 5.1.2
Soluble CP	sCP	g/kg CP		See Section 5.1.3
Ammonia nitrogen	NH_3N	g N/kg N	Free choice of MgO-method or Autoanalyzer (Broderick and Kang, 1980)	
Individual AA[1]	AA_j	g/100g CP	EC No. 152/2009	
Crude fat	CFat	g/kg DM	EC No. 152/2009	
Individual FA[2]	FA_j	g/100 g FA	CEN ISO/TS 17764-1:2007 CEN ISO/TS 17764-2:2007	
Neutral detergent fibre	NDF	g/kg DM	ISO 16472:2006 IDT	
Starch	ST	g/kg DM	Spectrophotometric method or the plate count method described by Bach Knudsen (1997) and Bach Knudsen *et al.* (1987)	
Lactic, propionic, butyric, formic acids and alcohol (ethanol)	LAF, PRF, BUF, FOF, ALF	g/kg DM	HPLC or GC	
Sugar	SU	g/kg DM	EC No. 152/2009	
Calcium	Ca	g/kg DM	ICP or free choice of method	
Phosphorus	P	g/kg DM	EC No. 152/2009 or ICP	
Magnesium	Mg	g/kg DM	ICP or free choice of method	
Potassium	K	g/kg DM	ICP or free choice of method	
Sodium	Na	g/kg DM	ICP or free choice of method	
Chloride	Cl	g/kg DM	EC No. 152/2009 or ICP	
Sulphur	S	g/kg DM	ICP or free choice of method	
Iron, Copper, Manganese and Zinc	Fe, Cu, Mn, Zn	g/kg DM	EC No. 152/2009 or ICP	
Other micro minerals		g/kg DM	Free choice of method	
Vitamin A	VitA	IU/kg DM	EC No. 152/2009 or Jensen *et al.* (1998)	
β-carotene	b-car	IU/kg DM	EC No. 152/2009 or Jensen *et al.* (1998)	
Vitamin D	VitD	IU/kg DM	Any appropriate method is acceptable	
Vitamin E	VitE	IU/kg DM	EC No. 152/2009 or Jensen *et al.* (1999)	

[1] The amino acids that can be reported in the NorFor feed tables are alanine, arginine, asparagine, cysteine, glutamine, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine and valine.

[2] The fatty acids that can be reported in the NorFor feed tables are FA<C12 (the sum of fatty acids with less than 12 carbons), C12:0, C14:0, C16:0, C18:0, C18:1, C18:3, C20:5, C22:6 and RFA (sum of residual fatty acids).

Table 5.2 Recommended NorFor digestion methods.

Parameter	Abbrev.	Unit	Type of method	Method description
Organic matter digestibility	OMD	%	*In vivo* and *in vitro* methods	See Section 5.2.1
Potential degradable CP	pdCP	g/kg CP	*In sacco* method	See Section 5.2.2
Indigestible CP	iCP	g/kg CP	Mobile bag technique	See Section 5.2.4
Degradation rate of CP	kdCP	%/h	*In sacco* method	See Section 5.2.2
Potential degradable NDF	pdNDF	g/kg NDF	*In sacco* method	See Section 5.2.3
Indigestible NDF	iNDF	g/kg NDF	*In sacco* method	See Section 5.2.3
Degradation rate of NDF in concentrates	kdNDF	%/h	*In sacco* method	See Section 5.2.2
Soluble ST	sST	g/kg ST	*In sacco* method	See Section 5.2.3
Potential degradable ST	pdST	g/kg ST	*In sacco* method	See Section 5.2.3
Indigestible ST	iST	g/kg ST	Mobile bag technique	See Section 5.2.4
Degradation rate of ST	kdST	%/h	*In sacco* method	See Section 5.2.3

After DM1 determination the sample can be ground. The second DM (DM2) determination in the ground sample should also be performed at 60 ºC for 16 h when it is used to prepare samples for chemical analyses. The sample should then be weighed while still warm or kept in a desiccator until weighing (DM2), then

$$DM2_{Twostep} = \frac{Dry\_weight}{weight\_before\_drying} \cdot 1000 \qquad 5.3$$

where $DM2_{Twostep}$ is the DM2 obtained from the two-step procedure, g/kg; Dry_weight is the sample weight after the second drying, g; and weight_before_drying is the sample weight before the second drying, g.

Uncorrected DM in the two-step procedure is calculated as:

$$DM_{uncorrTwostep} = \frac{DM1 \cdot DM2}{1000} \qquad 5.4$$

where $DM_{uncorrTwostep}$ is the uncorrected DM before compensation for volatiles, g/kg; DM1 is the DM in first step, Equation 5.2; and DM2 is the DM in the second step, Equations 5.3.

Since DM is intended to be the water free proportion of the feed, volatile compounds lost during drying should be added to the uncorrected DM. These compounds include lactic acid, VFA, lower alcohols and ammonia. In drying at 60 ºC, lower alcohols are assumed to be completely lost, large proportions of ammonia and VFA are lost, but only a small amount of lactic acid is lost (Porter and Murray, 2001). The losses of VFA and lactic acid increase with decreasing pH. Table 5.3 shows the correction factors for the losses, which result in the equations below.

Final DM for silage with a pH lower than 5 is calculated as (Porter and Murray, 2001):

$$DM_{corr} = DM_{uncorr} + \begin{pmatrix} LAF \cdot (0.45 - 0.09 \cdot pH) + ACF \cdot (1.5 - 0.223 \cdot pH) \\ + PRF \cdot (1.4 - 0.182 \cdot pH) + BUF \cdot (1.9 - 0.272 \cdot pH) \\ + ALF + NH_3N \cdot 0.6 \end{pmatrix} \quad 5.5$$

Final DM for silage with a pH higher than 5 is calculated as:

$$DM_{corr} = DM_{uncorr} + \begin{pmatrix} ACF \cdot (1.5 - 0.223 \cdot pH) + PRF \cdot (1.4 - 0.182 \cdot pH) \\ + BUF \cdot (1.9 - 0.272 \cdot pH) + ALF + NH_3N \cdot 0.6 \end{pmatrix} \quad 5.6$$

where DM_{corr} is the corrected and final dry matter, g/kg; DM_{uncorr}, Equations 5.1 and 5.4, g/kg; LAF is the amount lactic acid in feed, g/kg uncorrected DM; ACF is the amount of acetic acid in feed, g/kg uncorrected DM; PRF is the amount of propionic acid in feed, g/kg uncorrected DM; BUF is the amount of butyric acid in feed, g/kg uncorrected DM; ALF is the amount of lower alcohols in the feed, g/kg uncorrected DM and NH_3N is ammonia nitrogen, g/kg uncorrected DM.

Table 5.3 Correction of DM for loss of volatiles, g/kg

Volatile compound	pH	Factor for equations
Lactic acid	Only if pH < 5	0.45-0.09×pH
Acetic acid	For all pH	1.5-0.223×pH
Propionic acid	For all pH	1.4-0.182×pH
Butyric acid	For all pH	1.9-0.272×pH
Lower alcohols	For all pH	1
Ammonia nitrogen	For all pH	0.6

We have also developed a simple equation, which corrects for losses of volatiles when not analyzed for in silage samples. The equation was developed from Norwegian grass silage samples that were analyzed for fermentation products and estimated losses of volatiles. When DM < 700 g/kg, uncorrected DM of silage should be corrected for loss of volatiles by the equation:

$$DM_{corr} = 0.99 \cdot DM_{uncorr} + 10 \quad 5.7$$

where DM_{corr} is the corrected and final DM compensated for losses of volatiles, g/kg; and DM_{uncorr} is the uncorrected DM, Equations 5.1 and 5.4.

The simple procedure to recalculate the chemical composition of a sample on a corrected DM basis for the two-step procedure is illustrated in Equation 5.8:

$$X_{corr} = X_{uncorr} \cdot 1000 \cdot \frac{DM_{uncorr}}{DM2 \cdot DM_{corr}} \quad 5.8$$

where X_{corr} is the chemically analyzed parameter, *i.e.* NDF, CP, ST etc., g/kg corrected DM; X_{uncorr} is the analyzed parameter per kg prepared sample, g/kg DM1; DM_{uncorr} is the uncorrected DM, Equations 5.1 and 5.4; DM2 is the DM measured in the second step, Equation 5.3; and DM_{corr} is the DM corrected for volatile loss, Equation 5.5, 5.6 and 5.7.

5.1.2 Crude protein

For CP determination, in addition to nitrogen content analyzed by Kjeldahl or Dumas × 6.25 as shown in table 5.1, ammonia losses during drying should also be taken into account. If ammonia is not analysed in fresh samples, it is assumed that 60% of the ammonia is emitted during drying (see Section 5.1.1):

$$CP = \left(\frac{N}{DM2} + NH_3N \cdot 0.6 \right) \cdot \frac{DM_{uncorr}}{DM_{corr}} \cdot 6.25 \qquad 5.9$$

where CP is the crude protein, g/kg corrected DM; N is the amount of nitrogen analysed, g/kg DM1; NH_3N is the ammonia analysed in the fresh sample, g/kg uncorrected DM; DM2 is the DM measured in the second step, Equation 5.3; DM_{uncorr} is the uncorrected DM, Equation 5.1 or 5.4; DM_{corr} is the DM corrected for volatiles, Equation 5.5, 5.6 or 5.7.

5.1.3 Soluble crude protein

The procedure to determine sCP in all types of animal feeds is described in Table 5.4. A dried and milled sample is extracted in a borate-phosphate buffer (pH 6.75) at 39 ºC for 1 hour. After centrifugation, the sCP in the supernatant is determined using the Kjeldahl or some other suitable method for total nitrogen determination. For silage samples the content of NH_3N should be corrected for losses during drying, and in the calculation of sCP.

Table 5.4 Description of the method to determine the soluble CP (sCP) (modified from Hedqvist and Udén, 2006)

Item	Procedure
Sample preparation	The samples are dried as specified for the NorFor samples and ground by a hammer mill to pass a 1 mm sieve. Avoid heating during grinding. See note 1.
Reagents	Only use recognized analytical grade reagents. Water: Distilled or deionised water. Mono-sodium dihydrogen phosphate monohydrate ($NaH_2PO_4 \cdot H_2O$) (CAS-No. 10049-21-5). Di-sodium tetraborate decahydrate ($Na_2B_4O_7 \cdot 10\ H_2O$) (CAS-No. 17.48-96-4). Borate-phosphate buffer (modified from Licitra *et al.*, 1996), pH 6.75 ± 0.05. Dissolve 12.2 g of sodium dihydrogen phosphate and 8.91 g of sodium tetraborate in 900 ml of water. Check the pH with a pH-meter and if necessary adjust the pH. Dilute with water in a 1000 ml volumetric flask. Prepare fresh buffer solution daily. Sulphuric acid, ρ_{20} 1.84 g/ml. Catalyst: Kjeltabs CF 5 g ($CuSO_4 \cdot 5H_2O$: approximately 0.10 g Cu per tablet, Thompson and Capper Ltd.) or equivalent. Titrate for the auto-burette in the Kjeltech apparatus, with for example 0.1 M HCl.
Equipment	Analytical balance (capable of weighing to the nearest 1 mg). Centrifuge test tubes, 50 ml, with lids. Dispenser or pipette 50±0.5 ml. Water bath, thermostatted at 39±0.5 °C (or incubating chamber, 39±0.5 °C). Glass rods. Centrifuge suitable for the centrifuge tubes and capable of spinning at 3,000 g (given values of g are for the bottom of the test tubes). Pipette 20±0.2 ml. Kjeldahl equipment or other equipment for total nitrogen determination in liquids. Heating block suitable for digesting the samples.

pH-meter, calibrated and capable of measuring pH to the nearest 0.01 pH unit.

Procedure

Weigh approximately 1.5 g of the test sample to the nearest 1 mg in a centrifuge tube (see note 2).
Add 50±0.5 ml borate-phosphate buffer, pre-heated to 39 ºC, to the samples (see note 3).
A blank sample of 50 ml borate-phosphate buffer should be included in each series of samples.
To hydrate the sample, mix it gently (*e.g.* with a glass rod) then place the lid on the tube and shake the sample thoroughly.
Incubate in a water bath or an incubating chamber at 39±0.5 °C for 1 h±5 minutes, and shake the tubes manually every 15 minutes.
Centrifuge the tubes at 3,000 ·g for 10 min (see note 4).
Pipette 20±0.2 ml of the supernatant and transfer to Kjeldahl tubes.
Add salt/catalyst and the appropriate volume of sulphuric acid to the tubes according to the standard procedure in the lab. Some feed samples foam extensively when the acid is added. Foaming during digestion in the Kjeldahl analysis can be reduced if the acidified samples are allowed to stand at room temperature for 1-2 hours or overnight.
Increase the temperature of the digestor stepwise, to prevent foaming of the samples. Do not include the time it takes to reach working temperature in the total digestion time.
Analyse the nitrogen content by Kjeldahl distillation.
Calculate the content of soluble crude protein.

Calculations

The content of sCP per kg CP, for all samples in which the ammonium nitrogen content is zero, can be calculated as:

$$sCP = \frac{(V_1 - V_0)\cdot c \cdot 14.007 \cdot 6.25 \cdot V_2}{m \cdot CP \cdot V_3} \cdot 1000 \qquad 5.10$$

For silage samples, which have to be corrected for a 60% loss of ammonium nitrogen during drying, the equation is:

$$sCP = \frac{\frac{(V_1 - V_0)\cdot c \cdot 14.007 \cdot 6.25 \cdot V_2}{m \cdot V_3} + \frac{0.60 \cdot NH_3N \cdot 1000 \cdot 6.25}{DM1}}{CP_{uncorr} + \frac{0.60 \cdot NH_3N \cdot 1000 \cdot 6.25}{DM1}} \cdot 1000 \qquad 5.11$$

where sCP is the soluble crude protein g/kg CP; V_0 is the volume of HCl used for titration of a blank sample, ml; V_1 is the volume of HCl used for titration of sample, ml; V_2 is the volume of added buffer, ml ; V_3 is the volume of pipetted extract, ml; c is the concentration of titrant (mol/l); m is the sample size, g; CP_{uncorr} is the CP in pre-dried sample, g/kg DM1; 14.007 is the molar weight of nitrogen, g/mol; 6.25 is the factor for converting nitrogen content to crude protein; DM1is the DM in first step, Equation 5.2; and NH3N is ammonia nitrogen g/kg fresh sample (see note 4).

[1] The particle length of the ground material should be verified regularly according to EU regulations for animal feed analysis (EC No. 152/2009). All the material should be able to pass through a sieve with a quadratic square mesh of 1·1 mm. Heating of samples during grinding should be avoided.

[2] 1.5 g sample and 50 ml buffer are recommended. If using the common 50 ml centrifuge tubes from Falcon, NUNC, Greiner etc. the tubes will be very full when using 50 ml of buffer and the shaking might cause problems. In these circumstances we recommend using 1.2 g sample and 40 ml of buffer. Depending on the facilities in the laboratory other multiples with this sample:buffer ratio could be used, e.g. 3 g of sample and 100 ml buffer.

[3] Analytical steps involving sulphuric acid addition should be performed in sequence without any interruption.

[4] Some insoluble particles (containing trapped air), particularly from forage may float on the surface after centrifugation, but if the supernatant is carefully pipetted the insoluble matter will not cause contamination. The particles may be removed with a spoon or a paper

tissue. If they still cause problems the supernatant can be carefully poured into a beaker through a tea-strainer and then pipetted

[5] For samples containing measurable amounts of ammonium it is necessary to correct the sCP for loss of sCP as ammonia during drying. This loss is currently set to 60%.

5.2 Digestion methods used to predict digestion of nutrients

5.2.1 Organic matter digestibility

The reference method for OMD is based on sheep fed at maintenance (EAAP, 1969). In roughage OMD can also be determined from different *in vitro* analysis, which has been calibrated against the *in vivo* method. In NorFor three *in vitro* methods are available, *i.e.* VOS (ruminal fluid digestible organic matter), IVOS (*in vitro* organic matter digestibility) and EFOS (enzyme digestible organic matter). These methods are briefly described below.

The VOS method was developed in Sweden and described by Lindgren (1979; 1983; 1988). A 0.5 g dried sample is incubated at 38 ºC for 96 h in a solution formed by mixing 49 ml buffer and 1 ml rumen fluid. Incubation residues are then combusted to determine the VOS digestibility coefficient of OM. The OMD *in vivo* is calculated from the VOS value. For forage with more than 50% grass or a whole crop of maize or cereals, and hence less than 50% leguminous plants on a DM basis, the OMD is calculated as follows (Lindgren, 1983):

$$\mathrm{OMD} = -2.0 + 0.90 \cdot \mathrm{VOS} \qquad 5.12$$

For forage samples containing more than 50% leguminous plants (Lindgren, 1983):

$$\mathrm{OMD} = 23.0 + 0.62 \cdot \mathrm{VOS} \qquad 5.13$$

where OMD is the calculated *in vivo* digestibility of organic matter, % of OM; and VOS is the digestibility of organic matter *in vitro*, % of OM.

The IVOS method is based on the method presented by Tilley & Terry (1963). Samples are incubated for 48 h in rumen fluid, followed by 48 h digestion by pepsin and HCl), the only major modification being that residues are combusted to determine OM digestibility. *In vivo* OMD can be calculated from IVOS using the equations of Møller *et al*. (1989).

For grass, clover grass, legumes and silages of grass, clover grass, legumes and small grain whole crops OMD is calculated from:

$$\mathrm{OMD} = 4.10 + 0.959 \cdot \mathrm{IVOS} \qquad 5.14$$

For maize whole crop silage the equation used to calculate OMD is (Søegaard *et al.*, 2001):

$$\mathrm{OMD} = 6.73 + 0.950 \cdot \mathrm{IVOS} \qquad 5.15$$

where OMD is the calculated *in vivo* digestibility of organic matter, % of OM; and IVOS is the digestibility of organic matter *in vitro*, % of OM.

The digestibility of fresh whole crop cereals (barley, wheat, maize), straw and concentrates can be determined using the EFOS method (Weisbjerg and Hvelplund, 1993). The EFOS method begins with a 24 h pepsin-HCl treatment of

the sample, after which the sampled is heated to 80 ºC for 45 min, treated for 24 h with an enzyme mixture at 40 ºC, and then incubated for a further 19 h at 60 ºC.

For fresh whole crops of wheat, barley and maize the equation used to calculate OMD *in vivo* is (Søegaard *et al.*, 2001):

$$OMD = 20.4 + 0.727 \cdot EFOS \quad 5.16$$

For straw the following equation should be used (Hvelplund *et al.*, 1999):

$$OMD = 22.0 + 0.752 \cdot EFOS \quad 5.17$$

For concentrate mixtures, the equation presented by Weisbjerg and Hvelplund (1993) should be used:

$$OMD = 5.38 + 0.867 \cdot EFOS \quad 5.18$$

where OMD is the calculated *in vivo* digestibility of organic matter, % of OM; and EFOS is the digestibility of organic matter *in vitro*, % of OM.

5.2.2 *In sacco* rumen degradation of crude protein, NDF and starch

The *in sacco* technique is used for determining kdCP in roughage and concentrate, kdNDF in concentrate and kdST in roughage and concentrate. It is also used to determine pdCP, sST, pdST and iNDF. The *in sacco* method has several weaknesses, *e.g.*, particle losses, microbial contamination of feed residues, different ruminal environment outside vs. inside the bag and pre-treatment of feed samples (Nozière and Michalet-Doreau 2000). Therefore, an important task for the NorFor feed table and analysis group was to standardize critical parts of the *in sacco* procedure to minimize between-laboratory variation. The NorFor *in sacco* standard protocol, based on the work of Madsen *et al*. (1995), are presented in Table 5.5.

Table 5.5. Standard in sacco procedure in NorFor for determining feed degradation characteristics, modified from Madsen et al. (1995).

Item	Procedure
Animals and diet	Non-lactating dairy cows of the Nordic dairy cow population. Cows are fed at maintenance level and the diet consists of hay, straw and concentrate. The hay and straw to concentrate ratio is 67:33. The CP content of the diet should be higher than 120 g/kg DM. The concentrate should contain a minimum of three sources of protein. Daily ration is divided into two or more meals of equal size with an adaptation period of 14 days. If animals have been on pasture or fed diets or feeding levels totally different from the standard, the minimum adaptation period is 21 days.
Replication	Three cows are required for the determination of each feed parameter, except for iNDF determinations, where two cows are needed. The number of bags per animal is not specified. There is no need to replicate the number of days.

Sample preparation	Preferably the samples should be freeze-dried, but oven drying at 45 °C is also acceptable. For NDF determination, a drying temperature of 60 °C is recommended. The samples should be ground in a mill with a screen size of 1.5 mm. Cutter mill is preferable but a hammer mill is also acceptable. Sample size should be 1.0-2.0 g of dried sample depending on the bag surface area.
Bags	Bag size refers to internal dimensions when the bag is sealed and mounted on the carrying device to be used. There should be 10 mg sample/cm^2 when samples of the required size are placed in the bag. The internal length:internal width ratio should preferably be 1:1.3 (and thus 8.1·6.2 cm for 1.0 g samples and 11.4·8.8 cm for a 2.0 g sample). Bags should have round corners. The pore size should be 38 µm. Recommended bag material is polyester of the model Saatifil PES 38/31 manufactured by Saatitech S.p.A (22070 Veniano, Como, Italy). Any method can be used to seal the filled bags, but the standardized internal bag length must not be altered. Currently, the bags are mounted on a rubber stopper in Denmark and Iceland. In Norway the bags are closed with a rubber band, while in Sweden the open end of the bag is inserted through a slit and strapped. The bags may be re-used for incubations for a maximum of 20 runs.
Incubation interval	When determining kdCP, the incubation times should be 0, 2, 4, 8, 16, 24 and 48 h, while for roughage and concentrates with low degradation rates the time should be extended up to 96 h. As a rule, concentrates in which less than 80% of total N has disappeared after 48 h should be incubated for 96 h. When determining kdNDF the incubation times should be 2, 4, 8, 16, 24, 48 and 96 h. The 0 h is omitted from the calculation. When determining the starch degradation rate the incubation times should be 0, 2, 4, 8, 16, 24, 48 and 72 h.
Incubation conditions	The bags should be pre-soaked prior to incubation (including 0 hr bags) for 20 minutes at 39 ºC in tap water without agitation. The bags that will be incubated for 2, 4 and 8 h should be inserted simultaneously 15-30 minutes prior to morning feeding. For bags that will be incubated for longer times the insertion time is not specified. The inserted bags should be placed in the ventral rumen. The bag attachment device should allow the bags to be squeezed by rumen contractions.
Rinsing	Bags removed from the rumen, and 0 h bags, must first be rinsed in cold tap water with no squeezing or manipulation before machine washing. Bags may be machine-washed immediately or freeze-stored after the cold tap water rinsing and thawed before machine washing. Bags incubated for all times, 0 to 96 h, should be washed. It is preferable to use identical washing machines and identical washing programs. Use a washing program without spinning and a water temperature of 25 ºC. At present, stomacher treatment is allowed after the machine-washing to reduce microbial contamination of roughage samples; this procedure is used in Denmark. After rinsing the residues are quantitatively removed from the bags and analyzed for chemical constituents. Alternatively, bags including the residues are dried in an oven at 45 ºC for 48 h and then weighted after equilibrated in room temperature. Residues are analyzed for chemical constituents.
Residue analysis	Analyze the remaining samples for nitrogen, NDF or starch. There is no specified quantitative analysis of the residues from each cow or analysis of pooled residues from emptied bags.

Calculations

Calculations should be conducted by non-linear curve fitting, by applying the least squares method to untransformed values. Degradation profiles should currently be fitted without a lag phase.

Calculation of kdNDF

The curve fitting function for NDF degradation is:

$$NDFD_t = \frac{NDF - NDF_t}{NDF} \quad 5.19$$

where NDFDt is NDF degraded at time t, g/g; NDFt is the remaining amount of NDF at time t, g; NDF is the amount of NDF in the bags prior to ruminal incubation, g. If the incubation residue at time t is too small for analysis, then the NDF content from the previous time should be used. Fit the equation according to Ørskov and McDonald (1979):

$$NDFD_t = NDFD_{curvefit} \cdot \left(1 - e^{-kdNDF_{96} \cdot t}\right) \quad 5.20$$

where NDFDt is the degraded NDF fraction at time t; NDFDcurvefit is the asymptotic value of pdNDF obtained from the curve fitting and kdNDF96 is the degradation rate of NDF. There is a restriction that NDFD96 should be ≤ 1. When iNDF at 288 h (Section 5.2.3) is available, report NDFD at 288 h as pdNDF and correct kdNDF according to:

$$kdNDF = \frac{NDFD_{curvefit} \cdot kdNDF_{96}}{1000 - iNDF} \quad 5.21$$

where kdNDF is the corrected degradation rate for NDF, $NDFD_{curvefit}$ is the asymptotic value of degraded NDF obtained from the curve fitting and iNDF is the indigestible NDF fraction estimated from the 288 h ruminal incubation, Equation 5.27.

Calculation of kdCP and pdCP

The curve fitting for crude protein degradation is applied to data on degraded fractions at times 0, 2, 4, 8, 16, 24, and 48 h, and if available 96 h. If the incubation residue at time t is too small for the analysis, CP content from the previous time should be used:

$$CPD_t = \frac{CP - CP_t}{CP} \qquad 5.22$$

where CPD_t is the CP degraded at time t, g/g; CP_t is the remaining amount of CP at time t, g; and CP is the amount of crude protein in the bags prior to ruminal incubation, g. Ruminal CP degradation is fitted to the following equation:

$$\mathrm{CPD_t} = \mathrm{CPD_0} + \mathrm{CPD_{curvefit}} \cdot \left(1 - \mathrm{e}^{-\mathrm{kdCP} \cdot \mathrm{t}}\right) \qquad 5.23$$

where CPD_t is the CP degraded at time t, CPD_0 is the intercept or an estimate of solubility of the CP at time 0 h, $CPD_{curvefit}$ is the asymptotic value of the insoluble but degradable proportion of crude protein obtained from the curve fitting, and kdCP is the degradation rate of pdCP. Restriction conditions are $0 \leq CPD0 \leq 1$, $0 \leq CPD96 \leq 1$ and $0 \leq (CPD0+CPD96) \leq 1$.

CP particle losses should be corrected for according to Weisbjerg et al. (1990):

$$\mathrm{pdCP} = \left(\mathrm{CPD_{curvefit}} + \left(\mathrm{CPD_0} - \mathrm{sCP}\right) \cdot \frac{\mathrm{CPD_{curvefit}}}{1 - \mathrm{CPD_0}}\right) \cdot 1000 \qquad 5.24$$

where pdCP is the potential degradable protein fraction, g/kg CP; $CPD_{curvefit}$ is the asymptotic value of the potentially degradable fraction obtained from the curve fitting, CPD_0 is the in sacco soluble fraction at time 0 and sCP is the soluble crude protein, analysed according to Section 5.1.3. There is no correction for microbial contamination, except for the possible stomacher treatment.

Calculation of kdST, sST and pdST

The curve fitting for starch degradation is applied to data on degraded fractions at times 0, 2, 4, 8, 16, 24, 48 and 72 h. If the incubation residue at time t is too small for analysis, the ST content from the previous time should be used:

$$STD_t = \frac{ST - ST_t}{ST} \qquad 5.25$$

where STD_t is the ST degraded at time t, g/g; ST_t is the remaining amount of ST at time t, g; ST is the amount of ST in the bags prior to ruminal incubation, g. ST degradation is fitted with the following equation:

$$\mathrm{STD_t} = \mathrm{STD_0} + \mathrm{STD_{curvefit}} \cdot \left(1 - \mathrm{e}^{-\mathrm{kdST} \cdot \mathrm{t}}\right) \qquad 5.26$$

where STD_t is the ST degraded at time t, STD_0 is the intercept or an estimate of solubility of ST at time 0 h, $STD_{curvefit}$ is the asymptotic value of the insoluble, but degradable proportion of ST obtained from the curve fitting, and kdST is the degradation rate constant of pdST. Restriction conditions are $0 \leq STD0 \leq 1$, $0 \leq STD72 \leq 1$ and $0 \leq (STD0+STD72) \leq 1$.

STD_0 and $STD_{curvefit}$ in Equation 5.26 are the same as soluble starch and potentially degradable starch, g/kg ST.

5.2.3 Indigestible NDF

The method for determining iNDF involves incubating feed samples *in sacco* for 288 h in the rumen, and essentially follows the method described in Section 5.2.2 for determining *in sacco* NDF degradation. Feed samples of 2 g are incubated in bags with 10-15 µm pores and 100-200 cm^2 effective surface area, equivalent to 10-20 mg sample/cm^2. For the iNDF determination the polyester cloth Saatifil PES 12/6 (Saatitech S.p.A., 22070 Veniano, Como, Italy) with pore size 12 µm and open surface area of 6% is recommended. Each determination should be performed on at least two animals.

The iNDF content is calculated as:

$$\text{iNDF} = \left(\frac{\text{NDF}_{288}}{\text{NDF}} \right) \cdot 1000 \qquad 5.27$$

where iNDF is the total indigestible NDF fraction in the feed, g/kg NDF; NDF_{288} is the amount of NDF in the bag remaining after 288 h of ruminal incubation, mg; and NDF is the amount of NDF in the bag before ruminal incubation, mg.

For measuring iNDF in roughage in commercial feed laboratories near infrared spectroscopy (NIRS) calibrations have been developed (Nordheim *et al*., 2007).

5.2.4 Indigestible crude protein and indigestible starch

The reference method for determining iCP and iST is the MBT technique. The procedure, based on the work of Madsen *et al*. 1995), is described in Table 5.6.

Table 5.6 The standard mobile nylon bag procedure for determining intestinal digestibility of rumen undegraded protein and starch, modified from Madsen et al. (1995).

Item	Procedure
Animals and diet	Duodenal fistulated cows fed at maintenance level or at production level (Michalak *et al*., 2003). When cows are fed at maintenance level the diet is the same as for the ruminal *in sacco* procedure (Table 5.5).
Replications	Minimum two replicates per cow.
Nylon bags	The bag's pore size should be 11-15 µm. The bag surface area should be 6·6 cm.
Samples	Concentrate sample size is 10 to 15 mg per cm^2 of the bag's surface area, approximately 1 g. Roughage sample size is 5 to 7 mg per cm^2, approximately 0.5 g. The samples should be pre-incubated in the rumen. Concentrate samples should be incubated in the rumen for 16 hours and roughage for 24 hours.
Pre-incubation	Step 1. Place the sample bag in 0.004 M HCl solution at pH=2.4 for 1 h. Step 2. Place the sample bag in a pepsin/HCl solution (100 mg pepsin per litre of 0.004 M HCl solution) for 2 h at 40 ºC in a shaking water bath.
Incubation in the duodenum and collection from faeces.	After pre-incubation the bags are introduced to the duodenum through a duodenal cannula. When determining the iCP fraction the bags are collected from the faeces, while when determining the AA profile of digestible protein the bags are collected from an ileal cannula.
Washing	Rinse the bags with tap water. Then wash the bags in a sieve basket in

	cold running water for two hours as described by Hvelplund *et al.* (1992). Alternatively the bags can be washed in a washing machine using the same procedure as for the rumen bags (Table 5.5).
Calculation	Indigestible crude protein is calculated as: $$iCP = \left(\frac{N_{MBT}}{N}\right) \cdot 1000 \quad 5.28$$ where iCP is the indigestible crude protein in the feed, g/kg CP; N_{MBT} is the nitrogen content in the bag residue that has passed through the small intestine, g; and N is the amount of nitrogen in the weighed sample, g.
	Indigestible starch is calculated as: $$iST = \left(\frac{ST_{MBT}}{ST}\right) \cdot 1000 \quad 5.29$$ where iST is the indigestible starch in the feed, g/kg ST; ST_{MBT} is the starch content in the residue bag that passed through the small intestine, g; and ST is the amount of starch in the weighed sample, g.

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