




# Faeces Collection and Procedure for Determination of Apparent Total Tract and Ileal Digestibility Using Indigestible Markers

Marion Girard , Sonja de Vries, Emma Ivarsson, Virág Ács, Tamme Zandstra, Nikkie van der Wielen, Carolien De Cuyper, Helle Nygaard Laerke, and Sam Millet

## Abstract

To accurately formulate diets that meet pigs' nutritional requirements, the digestibility of energy and nutrients of feed ingredients should be estimated. Apparent total tract digestibility (ATTD) and apparent ileal digestibility (AID) are two important indicators for evaluating the nutritional content of feed ingredients and diets. The present guideline proposes a standard operating procedure (SOP) for measuring ATTD and AID during the slaughter procedure using an indigestible marker. The SOP outlines how to prepare the necessary materials, diets, and animals for the experiment, as well as how to collect, process, store, and analyse faecal and ileal samples from individual pigs, including how to calculate the ATTD and AID of a diet. The procedure is customised for pigs weighing  $\geq 15$  kg housed in groups or individual pens. Unlike the total collection method, which requires total digesta collection, the index marker method allows partial digesta or faeces sampling. This approach reduces the need for individual housing in metabolic cages, as pigs can be housed in groups. The procedure does not include details on the method of euthanising the animal for AID determination. Each

**Supplementary Information:** The online version contains supplementary material available at [https://doi.org/10.1007/978-3-032-24899-2\\_6](https://doi.org/10.1007/978-3-032-24899-2_6).

M. Girard (✉)  
Swine Research Group, Agroscope, Posieux, Switzerland  
e-mail: [marion.girard@agroscope.admin.ch](mailto:marion.girard@agroscope.admin.ch)

S. de Vries · T. Zandstra  
Animal Nutrition Group, Wageningen University and Research (WUR),  
Wageningen, The Netherlands

E. Ivarsson  
Department of Applied Animal Science and Welfare, Swedish University of Agricultural  
Sciences (SLU), Uppsala, Sweden

researcher must comply with the ethical rules in force in the country in which the study is conducted.

---

**Keywords**

Apparent ileal digestibility · Marker · Pig · Total tract digestibility

---

## 6.1 Introduction

To meet the maintenance and production needs of pigs while minimising feed costs and reducing the environmental impact of pig production, an appropriate supply of dietary energy and nutrients is crucial (Zhang and Adeola 2017). To accurately formulate diets that meet these nutrient requirements, the digestibility of energy (ATOL\_0001828) and the nutrients (ATOL\_0001231) of feed ingredients should be estimated (Kong and Adeola 2014). The determination of the nutrient and energy digestibility of feedstuffs is a simple method for characterising the availability of nutrients in the ingredients included in pig diets (Sauer and Ozimek 1986).

Apparent total tract digestibility (ATTD) (ATOL\_0001232) and apparent ileal digestibility (AID) are two important indicators for evaluating nutritional or energy value in feed ingredients and diets. Accurate estimation of dietary energy (EOL\_0002011) is an essential prerequisite in pig nutrition because energy represents the main feed cost and is used to predict feed intake (ATOL\_0000772) and, subsequently, the requirements for other nutrients (Noblet and Van Milgen 2004; Kil et al. 2013).

Although the net energy (EOL\_0001935) reflects the actual available energy for pigs, the digestible energy (EOL\_0001933) and the metabolisable energy (EOL\_0001934) are easier to estimate because the methodology for evaluating these indices uses faeces or faeces and urine rather than calorimetry. Therefore, ATTD is still widely used to determine the energy values of feed ingredients or diets. However, the site of digestion and absorption of amino acids in pigs is the

---

V. Ács

Department of Farm Animal Nutrition, Hungarian University of Agricultural and Life Sciences (MATE), Kaposvár, Hungary

N. van der Wielen

Animal Nutrition Group, Wageningen University and Research (WUR), Wageningen, The Netherlands

Nutritional biology group of the Division of Human Nutrition and Health, Wageningen University and Research (WUR), Wageningen, The Netherlands

C. De Cuyper · S. Millet

Animal sciences Unit, Flanders Research Institute for Agriculture, Fisheries and Food (ILVO), Merelbeke-Melle, Belgium

H. N. Laerke

Department of Animal and Veterinary Sciences, Aarhus University, Tjele, Denmark

small intestine. Amino acids that reach the large intestine are degraded or converted into other amino acids or metabolites by fermentation and, therefore, do not provide essential amino acids to the pig. Thus, the evaluation of amino acid digestibility (ATOL\_0001247) is based on the collection of digesta samples from the end of the ileum.

AID can be determined by estimating the amount of amino acids ingested and the amount present in the ileum. However, digesta samples from the ileum contain not only undigested feed protein but also the undigested protein of endogenous origins, such as undigested digestive enzymes, proteins from desquamated epithelial cells, mucus, or bacterial proteins. Ileal endogenous losses may be separated into basal losses, which are not influenced by feed ingredient composition, and specific losses, which are induced by feed ingredient characteristics, such as dietary protein content (EOL\_0000124), levels and types of fibre, and anti-nutritional factors. The correction of the AID values for both basal and specific endogenous losses leads to true ileal digestibility (ATOL\_0001258). However, reliable procedures to routinely measure specific endogenous losses are not yet available. Given that the basal endogenous losses are related to the total dry matter intake (ATOL\_0005395), it is possible to correct the AID values for the excretion of undigested basal endogenous protein, thereby yielding standardised ileal digestibility values. The determination of endogenous losses remains a matter of debate. Table values can be used to assess these losses, which reduces the number of animals required and the experimental costs. However, evidence shows that these losses are study-specific and may vary according to the experimental conditions, and thus, table values may not reflect the losses associated with a specific context and individual animal (Blok et al. 2022). Hence, the use of nitrogen-free feed is recommended in all studies to determine endogenous losses (Blok et al. 2022; Stein et al. 2007).

Two methods are used for *in vivo* digestibility studies: the total collection method and the index marker (IM) method. The classic total collection is laborious and time-consuming because it requires a quantitative collection and record of feed intake and total tract faeces excretion to determine the difference between the components in ingested feed and excreted faeces. Failing to fully collect all faeces can cause potential errors, which may result in the overestimation of digestibility. To facilitate total collection, metabolic cages (EOL\_0002023) are primarily used for this method. After 5–21 days of adaptation to cages and feeds (Lyu et al. 2018; Zhao et al. 2018), a total collection of faeces is carried out for 4–6 days (Zhang and Adeola 2017). Total collection is considered the gold standard method for determining digestibility, but it requires a labour-intensive recording of feed intake and faeces output (Kong and Adeola 2014). The IM is a less labour-intensive method (Kavanagh et al. 2001) and allows partial sampling of faeces and feed. This method does not require metabolic cages (EOL\_0002023) if the goal is solely to measure digestibility. However, IM requires precise and accurate chemical analysis of indigestible markers. Various indigestible markers have been proposed, such as titanium dioxide ( $\text{TiO}_2$ ), chromium oxide ( $\text{Cr}_2\text{O}_3$ ), ferric oxide ( $\text{Fe}_2\text{O}_3$ ), and acid-insoluble ash (AIA or silicon dioxide [ $\text{SiO}_2$ ]). A proper indigestible marker should be: (Brestenský et al. 2017; Zhang and Adeola 2017):

- (a) Totally indigestible and non-absorbable
- (b) Non-toxic to the host and its digestive tract
- (c) Able to track the component of interest through the digestive tract
- (d) Easily analysed accurately and precisely
- (e) If possible, a compound/substance that is naturally present in the feed

---

## 6.2 Goal and Scope of the Procedure

The present guideline proposes an SOP to measure ATTD and AID by the slaughter procedure using an indigestible marker. It describes how to prepare the materials, the diets, and the animals for the experiment (Sect. 6.3 and 6.4), how to collect, process, store, and analyse faecal and ileal samples (Sects. 6.5.1 and 6.5.2) from individual pigs, and how to calculate ATTD and AID of a diet (Sect. 6.6).

This procedure is adapted to pigs weighing  $\geq 15$  kg housed in groups or individual pens. For AID determination, this procedure does not describe the method for euthanising the animal. Each experimenter must comply with the ethical rules in force in the country in which the trial is being carried out.

---

## 6.3 Materials and Equipment

### 6.3.1 Faeces Collection on Live Pigs

- Laboratory/examination gloves (powder-free).
- Nitrile or vinyl: preferred to latex as they are less allergenic.
- Water-resistant felt-tip pen or freezer-proof labels.
- Spoons or plastic shovel: to collect a certain quantity of faeces.
- Plastic or aluminium foil trays/plates, small buckets, or plastic bags to collect faeces.
- Cotton swab: to stimulate defaecation (ATOL\_0000492) in small pigs (if needed).
- Styrofoam container with ice: to transport the sub-samples (if needed).
- Pig scale (accuracy 100 g): to weigh the pigs (if needed for the experiment).

### 6.3.2 Ileal Digesta Collection after Slaughter

- Laboratory gloves/examination gloves (powder-free).
- Nitrile or vinyl: preferred to latex as they are less allergenic.
- Water-resistant felt-tip pen or freezer-proof labels.
- Plastic container with lid: to collect ileal content (at least 200 mL capacity).
- Pig scale (accuracy 100 g): to weigh the pigs (if needed for the experiment).
- Styrofoam container with ice: to transport the sub-samples (if needed).
- Dissection material for ileum collection (tie-wraps, medical lancet, medical pliers, scissors, for instance).

### 6.3.3 Sample Processing and Storage

- Inert recipient (~2–5 L) for pooling the faeces (option 1 in Sect. 6.5.1.4).
- Mixer, if faecal (e.g. paddle or hand-held kitchen mixer) or digesta (e.g. stirring plate) samples are pooled (option 1 in Sect. 6.5.1.4).
- Plates or metal trays for freezing the samples. They should also be resistant to drying.
- Certified electric scale (accuracy 100 mg): to weigh the quantity of faeces and ileal content (if dry matter (DM) needs to be determined).
- Freezer (–20 °C/–80 °C): before freeze-drying the digesta, it is important to ensure that it is thoroughly frozen.
- Freeze-drier or oven.
- Grinder: to grind freeze-dried and dried faeces, ileal, and feed samples.

---

## 6.4 Prerequisites and Preparation of the Experiment

### 6.4.1 Preparation of the Feed and the Indigestible Marker

#### 6.4.1.1 Choice of the Indigestible Marker

As mentioned earlier, a marker must meet certain criteria, such as being completely indigestible and non-absorbable, non-toxic, able to pass through the digestive tract at a constant rate with the digesta, easy to analyse, and preferably being naturally present in the feed. Many studies have compared different markers and identified unique advantages and disadvantages. The choice of marker depends on various factors, such as local legislation, the purpose of the study, or the analytical methods available in the laboratory (de Vries and Gerrits 2018). Two commonly used markers (SiO<sub>2</sub> and TiO<sub>2</sub>) are described in Table 6.1. The limiting factor that determines the inclusion level of the marker is its quantification in the feed rather than its quantification in the faeces and digesta, where it is highly concentrated. To be reliably quantified, the marker must be incorporated into the feed in sufficient quantity to reduce measurement inaccuracies when levels are close to the detection and quantification limits of the analytical method. In addition, the marker should minimise confounding effects of trace quantities of the substance naturally present in feed ingredients (e.g. in the case of AIA), without being too concentrated in the digesta to avoid interference with digestive processes.

#### 6.4.1.2 Feed Preparation and Analysis

- The feed including the marker can be offered in dry (mashed, flour, pelleted) or liquid form.
- In the case of leftovers in the feeders, it is important to check that there is no segregation between the marker and the diet.
- In trials in which the ATTD of phosphorus is determined, the basal diet should consist of raw materials without endogenous phytase activity.

**Table 6.1** Chemical composition of two commonly used markers and their inclusion levels in a diet

Applied marker	Inclusion level range in diet (as-fed basis)	Remarks
Silicon dioxide (SiO <sub>2</sub> ; AIA)	0.50–1.5%	The high inclusion level of AIA may impair digestive processes (passage rate) for highly digestible diets, where the concentration of AIA gets very high in ileal content. A larger amount of sample is required for chemical analysis. Intrinsic and added AIAs may have uneven particle sizes, which can lead to improper mixing and differential passage rates. To ensure consistent particle size and even distribution, a source of fine-particle AIA or an inert material, such as Celite™ is recommended (Goddard and McLean 2001).
Titanium dioxide (TiO <sub>2</sub> )	0.10–0.40%	Too high inclusion level (≥ 0.50%) may affect palatability and faecal consistency. Environmental and health issues: Under current EFSA regulations, the use of TiO <sub>2</sub> (and other external markers that are not registered as feed additives, including Cr <sub>2</sub> O <sub>3</sub> ) as an additive is prohibited (EU, 2021/2090), but its use as a tracer in scientific experiments can be authorised by the local authority based on EU directives (87/153/EEC and 83/228/EEC). Animals monitored with TiO <sub>2</sub> must not re-enter the food chain. TiO <sub>2</sub> may interact with phosphorus (Kuboki et al. 2012).

- Depending on the nutrients of interest in the study, the chemical composition of the diet, such as DM, crude ash, crude protein, crude fibre, ether extract, and minerals, may be analysed before starting the trial.

#### 6.4.1.3 Feed Allowance and Feeding Frequency

- The feed can be provided *ad libitum* or restricted. Feeding the pigs restricted but close to *ad libitum* to avoid feed residues is recommended, especially if feed is not pelleted, and to avoid diverging growth rates between treatment groups. Moreover, as feeding level may affect digestibility, different diets must be compared at the same feeding level. However, for group-housed pigs, *ad libitum* feeding may be more convenient to avoid competition.
- It is suggested that the feeding level should be approximately 2.8 times the maintenance energy requirement or approximately 4% of live body weight (ATOL\_0000351) (Zhang and Adeola 2017; CVB 2005), given that nutrient digestibility could be significantly influenced by low feed intake (Moter and Stein 2004).
- In cases of restricted feeding, the feed allowance should be provided twice daily in two equal meals, with a minimum interval of 8 h between meals, or even in three equal meals for young pigs.
- If pigs are feed-restricted, the daily feed allowance may be calculated using the following equation (Eq. 6.1; Everts 2015):

$$\text{Daily feed allowance (kg)} = \frac{LW^{0.60} \times 750 \times FL}{NE_{\text{diet}}} \quad (6.1)$$

where:

- LW is the live weight measured at the beginning of a specific research period (e.g. adaptation or collection, see Sect. 6.4.3 and 6.5.1.2).
- $LW^{0.60}$  is the live weight to the power of 0.60.
- 750 is the energy for maintenance in kJ NE/day per kg live weight to a power of 0.60 (Everts 2015).
- FL is the feeding level; this coefficient should be approximately 2.3–2.8 times the maintenance level for net energy (NE).
- $NE_{\text{diet}}$  (in kJ/kg) is the estimated net energy content of the diet.

## 6.4.2 Animals and Housing

### 6.4.2.1 Housing Conditions

- Animals can be either housed in groups or individually, must have *ad libitum* access to water for the entire duration of the experiment, and must be identifiable (ear tag or number).
- No straw or other bedding should be allowed in the pens in the second phase of the adaptation period or in the collection period (please refer to Sect. 6.4.3 for the definition of the phases of the adaptation period).
- The pigs may have access to inedible and non-destructible enrichment materials (EOL\_0001921, e.g. metal chains with hard plastic attributes or large balls) during the whole experiment to encourage exploratory behaviour. However, some enrichment materials, such as jute products containing cellulose, should be avoided.

### 6.4.2.2 Animals

- Faeces or ileal digesta can be collected from all sexes (castrated boars, boars, or females) and from all physiological stages (piglets, fattening pigs, sows). If samples are taken from young animals, the challenge is to obtain enough material for analysis; otherwise, pools of samples from several animals should be made.
- If this SOP is used to determine the nutrient values of a test ingredient for feeding tables, please refer to the specific feeding system guidelines for the recommended weight of the animals at the time of measurement.
- It is important to define the experimental unit before the start of the trial. Depending on the configuration of the facility, animals kept in a group in the same pen may either receive the same dietary treatment (e.g. pen with one feeder) or different dietary treatments (e.g. pen with several individual electronic feeders, each dispensing one feed or pen with one individual electronic feeder dispensing multiple feeds). Thus, the experimental unit may be the pen or the animal, respectively.
- The number of replicates depends on the experimental unit (animal or pen), the variable studied (ATTD or AID and the nutrient of interest), and the differences between treatments that the experiment aims to detect (power calculation). A

greater number of replicates is required for diets with high fibre content (Pedersen et al. 2010). To determine the number of replicates, please refer to the guidelines of De Cuyper et al. (2025a, 2025b). The CVB (2005) protocol recommends that the standard deviation of the ATTD of organic matter (OM) should be less than 1.5%. Typically, four to six replicates are used for standard diets.

- For animal welfare (ATOL\_0000765) reasons, it is important to socialise pigs. The purpose of socialisation is to accustom pigs to human contact, ensuring they remain calm during tasks such as pen cleaning and sample collection. Interactions between pigs and humans involve acoustic, visual, tactile, and chemical sensory channels. The animals must become familiar with people's smells and voices, and they must allow themselves to be petted. Socialisation can begin one or two days after the pigs have become accustomed to their new environment, that is, on days 2 or 3 of the first adaptation phase (see Sect. 6.4.3 for details of the first phase).

### **Important**

This is an example of a socialisation protocol performed in different steps at Wageningen University:

1. Whenever you enter the room, talk to the pigs or whistle to help them become accustomed to your presence and the sound of your voice.
2. Enter the pen and talk to each pig. Do not make any sudden movements. Just sit and talk to the pig to let it get used to you being in the pen in a non-threatening manner. Spend 5–10 min with each pig.
3. Enter the pen, and after 2 min of sitting and talking, extend your hand for the pig to sniff and explore. Extend your arm to pet the pig. Let the pig get used to this, and then, try keeping your hand on its back. The pig will probably run around the pen. Do not remove your hand until the pig stops moving.
4. Get closer to the pig, and keep your hand on its back for longer. Start giving the pig scratches on its back, behind its ears, and on its belly. You can also bring a container to collect faeces to help them get used to it.
5. Optional: If the pigs are still small, you can try holding them (wear earplugs). This is often the most difficult step, as they will often scream. Do not release the pig unless it stops squealing. Being quiet is the key to being released.
6. Optional: Keep trying to hold the pigs every day until they submit to being held without squealing. From this point onwards, they should always allow you to hold them and rub their belly and back.

Typically, each day, the next step is attempted. Some pigs may take longer to socialise. Others may take less time. If they are quicker, some steps can be combined and performed in one day. During the adaptation period, you can reward the pigs with treats, such as fruit (apples or dates) or chocolate raisins, after completing a step. As pigs like playing, you can also freeze ice blocks in buckets containing pelleted feed and give the pigs these ice blocks during the socialisation period. Then during the measurement/sampling process, you can continue to provide the ice blocks, but without any feed in them.

### 6.4.3 Adaptation Period to the Diet

A period of adaptation must precede each collection period to adapt the animals to the new diet and environment. This period is also important for achieving homogeneous mixing of the marker in the digestive tract. The duration of the period can vary according to the diet composition, the feed allowance (restricted or *ad libitum*), and whether pigs need to adapt to a new environment. This adaptation period can be divided into two parts:

- *First phase:* This first phase is recommended if pigs need to be housed in a new environment, are housed individually, need to be fed restrictedly during the collection period, or if a feed change needs to take place gradually. This first phase of adaptation lasts at least 5 days.
- *Second phase:* This second phase adapts the intestinal physiology to the new feed and allows the marker to reach a steady excretion rate from the digestive tract. This phase begins when the animals have received only the diet to be studied. For example, at least 5 days were required before the marker excretion stabilised after feeding a corn-soybean meal-based diet containing chromic oxide ( $\text{Cr}_2\text{O}_3$ ) in Jang et al.'s (2014) study. However, the duration of this second phase may vary according to the type of marker and the composition of the studied diet, particularly the digestible non-starch polysaccharide content. The adaptation period must be extended for diets containing a high content of low-digestible compounds, such as dietary fibres, to avoid a discrepancy in the intestinal passage rate (Clawson et al. 1955). According to the CVB (2005) protocol, 5 days are necessary for diets containing less than 50 g/kg DM of digestible non-starch polysaccharides, but this period may extend to more than 10 days for diets containing more than 150 g/kg DM of digestible non-starch polysaccharides or even to 14 days when determining the digestibility of phosphorus. It is therefore advisable to have a second phase adaptation period of at least 5 to 7 days for low-fibre diets and of at least 14 days for high-fibre diets or the determination of phosphorus digestibility.

### 6.4.4 Preparation of the Material for Faeces Collection

- The collection plates must be identifiable and weighed before the experiment if DM needs to be determined (Supplementary Material 6.1 for an example of a recording sheet).
- If pigs are housed in groups, a second person may be needed to keep other pigs from disturbing the pig or the person taking the sample.

## 6.5 Description of the Specific Procedures

### 6.5.1 Procedure for Determining Apparent Total Tract Digestibility by Faeces Collection on Live Pigs

#### 6.5.1.1 Weighing Dates

- Animals should always be weighed at the same time of day.
- The following main weighing dates may be considered:
  - At the start of the adaptation period, especially if animals are fed restrictedly, to adjust the amount of feed.
  - 1 to 3 days before the end of the adaptation period (to avoid stress (ATOL\_0002301) during the collection period), especially if animals are fed restrictedly, to adjust the amount of feed
  - At the end of the collection period.

#### 6.5.1.2 Duration of the Collection Period

- It is important to collect faecal samples over several days before pooling them together to obtain more robust results. It is also important to ensure that each collection day is equally represented. A sample collection period that preferably lasts 3–5 days is recommended.
- Pooling grab samples over two consecutive days has been shown to result in greater accuracy and lower variations than a single grab sample (Jang et al. 2014). According to Moughan et al. (1990), a sampling period of 5 days allows the precision of IM for determining DM digestibility to approach that of the total collection method. More precise determination of nutrient digestibility with five collection days was also reported by Agudelo et al. (2010) and Brestenský et al. (2017), whereas Jang et al. (2014) did not observe any improvement by pooling faeces collected across 2–5 days. A sample collection period that preferably lasts 3–5 days is recommended.

#### 6.5.1.3 Faeces Collection.

- Faeces must be collected at least once a day, preferably at the same time every day, using a plate, a small bucket, or a bag.
- Wear laboratory gloves during sampling. If the experimental unit is:
  - The pig: change gloves between pigs
  - The pen: change gloves between pens
- Samples must be collected immediately after defaecation on the floor or directly from the rectum. If the pen is the experimental unit, collect faeces from at least 2 animals per pen.
  - Defaecation is induced by gently stimulating the final 3–6 cm of the rectum (depending on the animal's size) with slow, circular movements of the index finger. Alternatively, a cotton swab can be used to collect faeces rectally on pigs weighing less than 20 kg. It is important to insert the finger or cotton

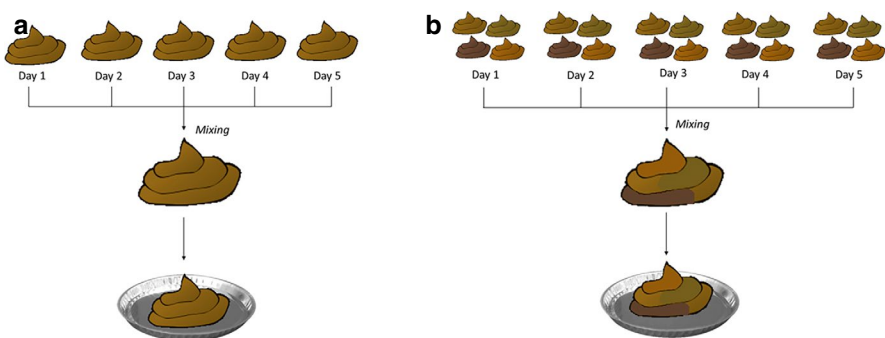
swab gently and not abruptly. The sample can be taken without restraining the animal, but if it moves too much, it can be held briefly by a second person. Sampling takes a few seconds to a few minutes (less than 2 min).

- If faeces are collected from the floor, only collect the part that has not touched the floor.
- If AIA is used as a marker, it is important to collect the sample rectally to avoid contamination with AIA from the environment.
- After collection, faecal samples must be frozen immediately or stored at 4 °C for a maximum of 4 hours before being frozen.
- The quantity depends on the duration of the collection period; the more collection days, the smaller the quantity to be collected per day. The DM of faeces typically varies between 190 and 280 g/kg; thus, it is advisable to obtain approximately 500 g of faeces per experimental unit at the end of the collection period.
- If there is no defaecation at the time of sampling, returning a little later or omitting this sample is recommended. However, it is important to have at least three collection days per experimental unit.
- Make sure that all containers holding faeces are labelled correctly before storage.

#### 6.5.1.4 Faeces Homogenisation and Storage

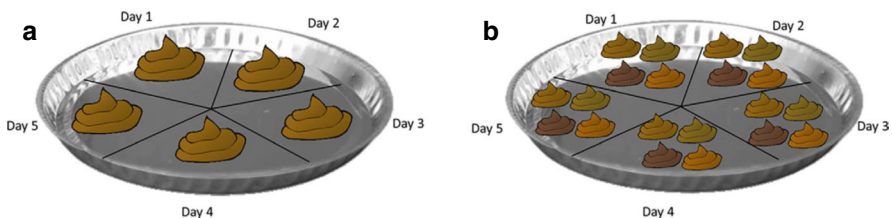
Following collection, there are two ways to proceed for homogenisation and storage, as described below:

- Option 1: Pooling at the end of the collection period (Fig. 6.1).
  1. Immediately after collection, transport the faecal samples on ice in the Styrofoam container, freeze them, and store them at –20 °C until the end of the collection period.



**Fig. 6.1** Pooling at the end of the experimental collection period. Defrosted faeces samples from either the same pig (individual level; a) or from several pigs of the same pen (pen level; b) are pooled before freeze-drying

2. Once all faeces samples have been collected and frozen, defrost the samples in closed buckets or bags by leaving them at 4 °C for a maximum of two days to minimise microbial activity.
  3. After defrosting, pool the samples from each experimental unit (animal or pen) within each experimental collection period by adding the same quantity of faeces per day from the same animal/pen in a suitable container. If the pen is the experimental unit, the faeces of at least two animals per pen should be pooled.
  4. Homogenise the pooled sample using a mixer to ensure a representative sample. Clean the mixer between samples.
  5. A subsample of approximately 250–500 g of homogenised pooled faeces is then taken and placed on a tray or plate suitable for freeze-drying or oven-drying. The exact quantity of the subsample is recorded before freeze-drying or oven-drying if DM needs to be determined. If this subsample is to be freeze-dried, freeze it at –20 °C (it could also be pre-frozen at –70 °C). Samples intended to be dried in an oven should be processed immediately. A second subsample may be collected and frozen as a backup.
- Option 2: Daily pooling during the experimental collection period (Fig. 6.2).
    1. On the first day of the experimental period, labelled plates resistant to freeze-drying or oven-drying (in aluminium or resistant plastic for instance) are tared (one plate per experimental unit), and the weight of the empty plates is recorded. Shortly after collection, a certain amount of faeces is weighed. A total of 100 g per experimental unit per day is optimal. If the experimental unit is the pig, 100 g of faecal sample is weighed. If the experimental unit is the pen, a lower amount of faeces is required, and the faeces of at least two animals per pen are collected to reach 100 g. If a large number of faecal samples are to be collected, one person can collect the faeces, while another weighs them and places them on the plates. As collecting all the samples may take some time, the plates can be kept cool in a freezer or in a Styrofoam container filled with ice. All the plates are then frozen and stored at –20 °C.



**Fig. 6.2** Daily pooling throughout the experimental collection period of freshly collected faecal samples from either the same pig (individual level; a) or from several pigs of the same pen (pen level; b) before freeze-drying

2. On the subsequent day of the experimental period, the plates containing the frozen faeces from the previous day are taken out of the freezer and kept in a Styrofoam container filled with ice to avoid defrosting. Fresh faeces are collected. The plate containing the frozen faeces from the same experimental unit as the previous day is tared. The same quantity of freshly collected faeces as the previous day is added to this tared plate, that is, 100 g per experimental unit, for instance. The exact weight of the fresh faeces for the new day is recorded. If a large number of faecal samples are to be collected, one person can collect the faeces, while another weighs them and places them on the plates. As collecting all the samples may take some time, the plates can be kept cool in a freezer or a Styrofoam container filled with ice. All the plates are then returned to the freezer at  $-20\text{ }^{\circ}\text{C}$ .
3. Step 2 is repeated for the remaining collection days.
4. At the end of the experimental collection period, if DM needs to be determined, the weights of the faeces collected over the days are added to obtain the total weight of the faeces in the plate.

#### 6.5.1.5 Feed Collection and Storage

- Representative samples of feed must be collected during the experimental collection period to analyse the chemical composition and the indigestible marker content. Ideally, a feed sample should be collected on each collection day and then pooled at the end of the collection period for chemical analyses.
- Individual feed intake may be recorded according to the purpose of the experiment, but this record is not mandatory in the IM method.

#### 6.5.1.6 Laboratory Analysis

- The pooled faecal samples, that is, either pooled at the end of the experimental collection period (Sect. 6.5.1.4 Option 1 and Fig. 6.1) or pooled daily during the experimental collection period (Sect. 6.5.1.4 Option 2 and Fig. 6.2), should be freeze-dried or oven-dried until reaching a constant weight and then weighed if the DM content needs to be determined. Afterwards, the freeze-dried or oven-dried samples are ground in order to pass through a 0.5 to 1 mm grid according to the subsequent gross chemical analysis (Kavanagh et al. 2001; Battelli et al. 2020; Prawirodigdo et al. 2021).
- Feed samples must be oven-dried (48 h at  $60\text{ }^{\circ}\text{C}$ ) or freeze-dried if the moisture content exceeds 15% ( $w/w$ ), then ground to pass through a 0.5 to 1 mm grid.
- Accredited and/or validated methods must be used to determine the DM and indigestible marker content in both lyophilised/oven-dried feed and lyophilised/oven-dried faecal samples.
- Depending on the purpose of the experiment, the nutrients of interest (e.g. gross energy, phosphorus, and calcium) are also determined in the faeces and the feed using accredited and/or validated methods.

## 6.5.2 Procedure for Determining Apparent Ileal Digestibility by Ileal Digesta Collection after Slaughter

### 6.5.2.1 Recommendations before Slaughter

- The slaughter technique has been shown to be an alternative to simple T-cannulation and a suitable technique for measuring nitrogen (ATOL\_0001829) and amino acid digestibility in pigs receiving meat and bone meal diets (Donkoh et al. 1994). However, this technique, which provides an instantaneous 'picture', may result in more variations than cannulation, which samples over a longer period (Donkoh et al. 1994; Pedersen et al. 2010). Furthermore, it is more challenging to collect the requisite quantity for analysis from a single animal using the slaughtering technique.
- The slaughter day can take place the week following the end of the adaptation period.
- Animals are weighed on the day of slaughter.
- Unlike normal slaughter conditions, animals should not be fasted to ensure that sufficient ileal content is collected. Therefore, 48 to 72 h prior to dissection, the meal frequency can be increased to four to six meals evenly distributed at regular intervals, and on the day of dissection, pigs are fed once every hour and receive at least six meals (one meal is 1/12th of the daily allowance) to approach steady-state digesta passage as described by van Erp et al. (2020). Alternatively, on the day of slaughter, the morning meal may be divided into several portions, and euthanasia must then take place a minimum of 6 h after the first portion is consumed, and a maximum of 1 h after the last portion.
- Containers for collecting ileal content should be resistant to freeze-drying, and weighed if DM determination is needed.

### 6.5.2.2 Ileal Sample Collection and Storage

- The collection of ileal content occurs post-mortem.
  - Only one pig should be euthanised at a time, and the contents of the ileum should be collected immediately afterwards.
- Make sure that all containers holding ileal content are labelled correctly.
- A qualified person should perform the anaesthesia or stunning (e.g. electro-stunning, CO<sub>2</sub>, captive bolt pistol) before the euthanasia of the pigs.
  - If the sole purpose of the experiment is to collect content from the ileum, a stunning method may be employed. However, if additional samples need to be taken from different parts of the intestine, or if intestinal morphology or kinetics are to be studied, then anaesthesia must be used.
- After euthanasia, the whole digestive tract is carefully collected immediately.
  - To prevent the movement of the digesta upstream, we recommend tying one string at the beginning of the duodenum (in close proximity to the stomach) and one at the termination of the ileum, in the immediate vicinity of the caecum, prior to the unfolding of the entire small intestine.

- The beginning of the caecum is identified, and then the mesentery is gradually and carefully removed until the small intestine is unfolded.
  - The ileum is located in the last 4–5% of the small intestine. As the length of the small intestine varies according to the age of the pig (from 7 m at weaning to over 16 m for an adult pig; Laerke and Hedemann 2012), as well as between pigs of the same age, we recommend that the entire length of the small intestine be unfolded and measured, and the contents should be collected from the final 5% (anterior to the ileocecal valve), omitting the last 5–10 cm from the ileocecal valve.
- The content (digesta) is collected from the final 35 or 80 centimetres (excluding the last 5–10 cm close to the ileocecal valve) in the case of piglets or adult pigs, respectively, within 10 min after euthanasia in identified containers resistant to freeze-drying.
  - To optimise the freeze-drying process, the contents of the ileum in the freeze-drying-resistant container are spread to a maximum height of 2–3 cm. If the height is excessive, a dry ‘crust’ may form and prevent the sample from freeze-drying completely. The sample could also be pre-frozen at  $-70\text{ }^{\circ}\text{C}$  before the freeze-drying process.
  - If DM determination is needed, the containers should have been previously tared, and the digesta weight of the experimental unit is recorded with a 0.1 g precision scale.
  - Since chemical analyses are performed on freeze-dried samples and the DM of ileal samples varies between 70 and 140 g/kg, collecting at least 250 g of ileal content per experimental unit is recommended. When the experimental unit is the pen, the ileal contents of several animals from the same pen (at least two animals) are mixed in the same container. Ideally, the same quantity should be combined per animal within the same experimental unit. When the experimental unit is the pig, the ileal content of a single animal may not be sufficient. In such instances, it is necessary to take the entire ileal content of a second animal that has received the same treatment and combine it with the initial sample to create a pool.
- Immediately after sampling the experimental unit, the container with the digesta is kept cool in a Styrofoam container filled with ice. Once all samples have been collected and processed, they are transported on ice before being frozen and stored at  $-20\text{ }^{\circ}\text{C}$  or  $-70\text{ }^{\circ}\text{C}$ .

### 6.5.2.3 Feed Collection and Storage

Samples of feed must ideally be collected during the last days prior to slaughter, particularly if the feed is not pelleted, and pooled to analyse the chemical composition and the indigestible marker content.

### 6.5.2.4 Laboratory Analysis

Once frozen, the ileal content samples must be freeze-dried (generally for 72 h) until they reach a constant weight and then weighed if the DM content needs to be determined.

Please refer to Sect. 6.5.1.6 for the grinding of freeze-dried ileal samples, the processing of the feed samples, and the determination of the different DMs and indigestible marker content in the feed and the ileal samples.

Depending on the purpose of the experiment, the nutrients of interest (e.g. specific minerals and specific amino acids) are also determined in the faeces and the feed using accredited and/or validated methods.

## 6.6 Calculation Method for Digestibility

The ATTD of nutrient  $N$  is calculated from the concentration of the nutrient in the feed ( $N_{feed}$ ) and in the faeces ( $N_{faeces}$ ) and the concentration of the indigestible marker in the feed ( $M_{feed}$ ) and the faeces ( $M_{faeces}$ ) with the formula (Eq. 6.2; Adeola 2001):

$$ATTD_N (\%) = 100 - \left[ 100 \times \frac{M_{feed}}{M_{faeces}} \times \frac{N_{faeces}}{N_{feed}} \right] \quad (6.2)$$

$M_{feed}$ ,  $M_{faeces}$ ,  $N_{feed}$  and  $N_{faeces}$  are expressed in g/kg DM.

The AID of nutrient  $N$  is calculated from the concentration of the nutrient in the feed ( $N_{feed}$ ) and the ileum ( $N_{ileum}$ ) and the concentration of the indigestible marker in the feed ( $M_{feed}$ ) and the ileum ( $M_{ileum}$ ) (Eq. 6.3):

$$AID_N (\%) = 100 - \left[ 100 \times \frac{M_{feed}}{M_{ileum}} \times \frac{N_{ileum}}{N_{feed}} \right] \quad (6.3)$$

$M_{feed}$ ,  $M_{ileum}$ ,  $N_{feed}$  and  $N_{ileum}$  are expressed in g/kg DM.

Due to variations in the analysed concentration of the marker that cannot be explained by dosing errors, it is advisable to check whether the analysed values of the test diets are within the analytical margin of error. If this is the case, it is advisable to take an average value for all diets with the same anticipated marker concentration as those fed to the animals. In the case of AIA, as intrinsic AIA content may differ among test ingredients and thus diets, taking an average AIA value per diet is recommended.

## 6.7 Compliance with the 3R Principles

The determination of apparent total tract and ileal digestibility using indigestible markers complies with the 3R principles. However, compliance with European, national, and local ethical rules and regulations should always be ensured. The use of the present SOP contributes to reducing stress and discomfort in pigs. In both procedures described, the animals may be housed in groups, unlike other procedures that require metabolic cages or ileal cannulated pigs. The pigs may also have

inedible and non-destructible enrichment material to enable explorative behaviour during the trial.

The determination of ATTD using an indigestible marker is minimally invasive because faeces can be easily collected from the rectum of the animals in their pens or when defecating. Socialising and habituating pigs to human contact during the adaptation period are key steps in reducing animal stress and refining faecal collection during the experimental period. Furthermore, the ATTD method can be used in cross-over studies, reducing the number of animals required in an experiment, as faeces can be collected from the same pig at different collection periods. However, for some nutrients, such as amino acids, ileal digestibility must be determined, and ileal digesta must be collected. Post-mortem collection can be an alternative to cannulation, and the collection of ileum samples may be combined with the collection of other organs and tissues (liver, large intestine, etc.).

---

## 6.8 Conclusions

Assessing the nutrient digestibility of feed ingredients and diets is a prerequisite for optimising diet formulation and thereby pig growth and health (ATOL\_0000928). It is also an effective tool for tackling environmental issues aimed at lowering nitrogen and phosphorous excretions. Unlike the total collection method, which requires total digesta collection, the IM method described in this SOP allows partial digesta sampling. This method reduces the need for individual housing in metabolic cages during the experimental period, as pigs can be housed in group pens, which is more in line with their natural behaviour. Collecting ileal samples post-mortem can also replace the use of cannulated pigs, but different diets cannot be tested on the same animal.

Several compounds can be used as indigestible markers. However, indigestible markers containing heavy metals, such as chromium oxide or titanium dioxide, can cause environmental and suspected health issues. As an alternative, AIA can be used but must be included at a higher level due to the sensitivity of the analytical method used to determine insoluble ash content as well as to ensure proper mixing of the different particles between intrinsic and added AIAs.

**Acknowledgements** The authors would like to thank Professor Ruurd Zijlstra (University of Alberta, CA) and Dr. Karl Schedle (Boku University, AU) for reviewing and improving earlier versions of this SOP.

---

## References

- Adeola O (2001) Digestion and balance techniques in pigs In: Lewis AJ, Southern LL (eds) Swine nutrition, 2nd edn. CRC Press, Washington, pp 903–916
- Agudelo J, Lindemann M, Cromwell G (2010) A comparison of two methods to assess nutrient digestibility in pigs. *Livest Sci* 133:74–77. <https://doi.org/10.1016/j.livsci.2010.06.029>

- Battelli M, Rapetti L, Rota Graziosi A et al (2020) Use of undigested NDF for estimation of diet digestibility in growing pigs. *Animals* 10(11):2007. <https://doi.org/10.3390/ani10112007>
- Blok M, Spek W, Bikker P (2022) P104 basal endogenous amino acid losses in the digestive tract are determined by experimental conditions, a meta-analysis. *Anim Sci Proc* 13(2):188–189
- Brestenský M, Nitrayová S, Heger J et al (2017) Chromic oxide and acid-insoluble ash as markers in digestibility studies with growing pigs and sows. *J Anim Physiol Anim Nutr* 101(1):46–52. <https://doi.org/10.1111/jpn.12503>
- Clawson A, Reid J, Sheffy B et al (1955) Use of chromium oxide in digestion studies with swine. *J Ani Sci* 14:700–709. <https://doi.org/10.1093/ansci/14.3.700>
- CVB (2005) CVB (Centraal Veevoederbureau) Protocol voor een fecale verteringsproef met groeiende intacte vleesvarkens. <https://www.cvbdiervoeding.nl/pagina/10622/protocollenaspx>
- De Cuyper C, Ampe B, Westin R et al (2025a) Opinion paper: improving experimental design in animal research – seven key principles. *Animal* 19(12):101695. <https://doi.org/10.1016/j.animal.2025.101695>
- De Cuyper C, Westin R, Millet S et al (2025b) PIGWEB guidelines for experimental design. Zenodo. <https://doi.org/10.5281/zenodo.15371453>
- de Vries S, Gerrits WJJ (2018) The use of tracers or markers in digestion studies. In: Moughan PJ, Hendriks WH (eds) *Feed evaluation science*. Wageningen Academic Publishers, pp 275–294
- Donkoh A, Moughan PJ, Smith WC (1994) Comparison of the slaughter method and simple T-piece cannulation of the terminal ileum for determining ileal amino acid digestibility in meat and bone meal for the growing pig. *Anim Feed Sci Technol* 49(1–2):43–56. [https://doi.org/10.1016/0377-8401\(94\)90080-9](https://doi.org/10.1016/0377-8401(94)90080-9)
- Everts H (2015) Energy requirement for maintenance in growing pigs (CVB-documentation report; no nr 57). WUR Livestock Research Dept Animal Nutrition. <https://edepot.wur.nl/375603>
- Goddard JS, McLean E (2001) Acid-insoluble ash as an inert reference material for digestibility studies in tilapia, *Oreochromis aureus*. *Aquaculture* 194(1–2):93–98. [https://doi.org/10.1016/S0044-8486\(00\)00499-3](https://doi.org/10.1016/S0044-8486(00)00499-3)
- Jang YD, Lindemann MD, Agudelo-Trujillo JH et al (2014) Comparison of direct and indirect estimates of apparent total tract digestibility in swine with effort to reduce variation by pooling of multiple day fecal samples. *J Ani Sci* 92(10):4566–4576. <https://doi.org/10.2527/jas.2013-6570>
- Kavanagh S, Lynch P, O'Mara F et al (2001) A comparison of total collection and marker technique for the measurement of apparent digestibility of diets for growing pigs. *Anim Feed Sci Technol* 89:49–58. [https://doi.org/10.1016/S0377-8401\(00\)00237-6](https://doi.org/10.1016/S0377-8401(00)00237-6)
- Kil D, Kim B, Stein HH (2013) Feed energy evaluation for growing pigs. *Asian Australas J Anim Sci* 26:1205. <https://doi.org/10.5713/ajas.2013.r.02>
- Kong C, Adeola O (2014) Evaluation of amino acid and energy utilization in feedstuff for swine and poultry diets. *Asian Australas J Anim Sci* 27(7):917–925. <https://doi.org/10.5713/ajas.2014.r.02>
- Kuboki Y, Furusawa T, Sato M et al (2012) Interaction between titanium and phosphoproteins revealed by chromatography column packed with titanium beads. *Biomed Mater Eng* 22(5):283–288. <https://doi.org/10.3233/BME-2012-0718>
- Lærke HN, Hedemann MS (2012) The digestive system of the pig (chapter 5) In: Bach Knudsen KE, Kjeldsen NJ, Poulsen HD, Jensen BB (eds) *Nutritional physiology of pigs*. Online publication, Videncenter for Svineproduktion: Foulum Denmark
- Lyu ZQ, Huang CF, Li YK et al (2018) Adaptation duration for net energy determination of high fiber diets in growing pigs. *Anim Feed Sci Technol* 241:15–26. <https://doi.org/10.1016/j.anifeeds.2018.04.008>
- Moughan PJ, Smith WC, Schrama J et al (1990) Chromic oxide and acid-insoluble ash as faecal markers in digestibility studies with young growing pigs. *N Z J Agric Res* 34:85–88. <https://doi.org/10.1080/00288233.1991.10417796>
- Moter V, Stein HH (2004) Effect of feed intake on endogenous losses and amino acid and energy digestibility by growing pigs. *J Ani Sci* 82(12):3518–3525. <https://doi.org/10.2527/2004.4.82123518x>

- Noblet J, Van Milgen J (2004) Energy value of pig feeds: effect of pig body weight and energy evaluation system. *J Ani Sci* 82:229–328
- Pedersen C, Vinyeta E, Gerritsen R (2010) Comparison of the slaughtering and the T-cannulation methods on ileal digestibility of nutrients in pigs. In: Crovetto GM (ed) energy and protein metabolism and nutrition. Wageningen Academic Publishers, EAAP Scientific Series 127(1):411–412
- Prawirodigdo S, Gannon NJ, Leury BJ et al (2021) Acid-insoluble ash is a better indigestible marker than chromic oxide to measure apparent total tract digestibility in pigs. *Anim Nutr* 7(1):64–71. <https://doi.org/10.1016/j.aninu.2020.07.003>
- Sauer WC, Ozimek L (1986) Digestibility of amino acids in swine: results and their practical applications. A review. *Livest Prod Sci* 15(4):367–388. <https://doi.org/10.1016/j.aninu.2020.07.003>
- Stein HH, Sève B, Fuller MF et al (2007) Invited review: amino acid bioavailability and digestibility in pig feed ingredients: terminology and application. *J Ani Sci* 85(1):172–180. <https://doi.org/10.2527/jas.2005-742>
- van Erp RJJ, de Vries S, van Kempen TATG et al (2020) Feed intake patterns nor growth rates of pigs are affected by dietary resistant starch despite marked differences in digestion. *Animal* 14(7):1402–1412. <https://doi.org/10.1017/S1751731119002945>
- Zhang F, Adeola O (2017) Techniques for evaluating digestibility of energy amino acids phosphorus and calcium in feed ingredients for pigs. *Anim Nutr* 3(4):344–352. <https://doi.org/10.1016/j.aninu.2017.06.008>
- Zhao J, Zhang S, Xie F, Li D, Huang C (2018) Effects of inclusion level and adaptation period on nutrient digestibility and digestible energy of wheat bran in growing-finishing pigs. *Asian Australas J Anim Sci* 31:116–122. <https://doi.org/10.5713/ajas.17.0277>

**Open Access** This chapter is licensed under the terms of the Creative Commons Attribution 4.0 International License (<http://creativecommons.org/licenses/by/4.0/>), which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license and indicate if changes were made.

The images or other third party material in this chapter are included in the chapter's Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the chapter's Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder.

