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Short communication: Estimation of ileal digestibility in chickens using single- and dual-tracer methods

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ABSTRACT

Fractionation of digesta, as occurs during gastrointestinal transit in chickens, complicates accurate measurements of ileal digestibility using tracers. Dual-tracer methods using separate tracers for solid and fluid digesta phases may improve the accuracy of digestibility measurements when assumptions of the single tracer method are violated. The aim of the present study was to compare the apparent ileal digestibility (**AID**) of nutrients calculated with single- and dual-tracer methods in chickens fed diets varying in particle size, anticipating digesta phase separation in the proximal gastrointestinal tract. A total of 112 Dekalb White (BW: 1.53 ± 0.107 kg) and 112 Bovans Black (BW: 1.79 ± 0.127 kg) 29-week-old laying hens were distributed over 32 pens (seven birds/pen). Within breed, pens were randomly assigned to one of two experimental diets (coarse vs fine oat hulls; n = 8 replicate pens per diet/breed combination). Diets were supplemented with TiO_2 (3 g/kg) and Co-EDTA (2 g/kg). On days 34, 35, or 36, birds were euthanised and digesta from the ileum was collected for tracer and nutrient analyses. Apparent ileal digestibility was subsequently calculated by single- and dual-tracer methods. Although coarse oat hulls were hypothesised to increase the fractionation of solid and fluid digesta phases, no breed or diet × method interactions were found. Using a single tracer method based on TiO_2, AID of nitrogen (**N**) was overestimated by 3%-units ($P < 0.01$) compared with the dual-tracer method, whereas AID estimates of DM, starch, fat, and non-starch polysaccharides did not differ ($P > 0.09$) and precision of all AID estimates was improved. In conclusion, these results show that although from a conceptual perspective, dual-tracer methods are presumed to better account for the variation in flow behaviour of different digesta phases, AID estimates obtained by the commonly used single tracer method using solid-phase tracer TiO_2 were more precise and only marginally differed from estimates obtained by a dual-tracer method using distinct tracers for solid (TiO_2) and liquid (Co-EDTA) digesta phases. Considering technical and economical constraints, the single tracer method may thus be the method of choice in many situations. Only when digestibility of proteins or amino acids is of specific interest, single tracer methods using a solid-phase tracer may not suffice. Nevertheless, for both single- and dual-tracer methods, tracer selection is critical, and the choice of tracers should depend on the nutrient(s) of interest.

Implications

Assays to measure nutrient digestibility to predict the nutritional value of feed ingredients provide critical input for diet formulation. To predict nutrient – particularly protein – uptake, ileal digestibility measurements are preferred, typically performed using the single tracer method, where digestibility is estimated from the ratio between an indigestible tracer and the nutrient of interest in diet and digesta. Our results indicate that the commonly used solid-phase tracer TiO_2 yields sound estimates for most nutrients, except nitrogen. When protein- or amino acid digestibility is of specific interest, the use of an alternative tracer or the dual-tracer method should be considered.

Introduction

Digestibility measurements are a crucial tool in feed evaluation. Particularly to assess the bioavailability of proteins and amino acids of feed ingredients, ileal nutrient digestibility assays are indispensable. Tracers such as Cr_2O_3, TiO_2, or acid insoluble ash are widely applied to measure ileal digestibility in poultry, where digestibility is estimated from the ratio between the indigestible

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tracer and the nutrient of interest – the tracee – in feed and digesta (Sales and Janssens, 2003). Such single tracer methods, typically performed with solid-phase tracers, assume unidirectional flow of digesta and identical flow behaviour of tracer and tracee. Fractionation of digesta, as occurs in the gastrointestinal tract of chickens, however, complicates accurate measurements of ileal digestibility using tracers, particularly for high-fibre diets (de Vries et al., 2014). Methods using combinations of tracers to follow distinct digesta phases and accounting for non-ideal tracer behaviour - i.e., a tracer is not exclusively associated with the digesta phase it is supposed to trace - and unrepresentative digesta sampling, may improve the accuracy of digestibility measurements, when assumptions of the single tracer method are violated. The aim of the present study was to compare the apparent ileal digestibility (**AID**) of nutrients calculated with single- vs dual-tracer methods in chickens fed diets varying in particle size, anticipating digesta phase separation in the proximal gastrointestinal tract (Hetland et al., 2005). The commonly used single tracer method (**single$_{\mathbf{Ti}}$**) using TiO_2 as a tracer – often considered the gold standard (Sales and Janssens, 2003) – was compared with a dual-tracer (**dual$_{\mathbf{TiCo}}$**) method using TiO_2 as a tracer for solid digesta and Co-EDTA as a tracer for fluid digesta. To further evaluate the impact of violations of the assumptions regarding the behaviour of tracer and tracee and digesta sampling, AID estimates for various nutrients were additionally compared with estimates obtained with a single tracer method using the fluid tracer Co-EDTA (**single$_{\mathbf{Co}}$**) and two dual-tracer methods assuming ideal tracer behaviour and representative digesta sampling (**dual$_{\mathbf{B}}$**) or representative digesta sampling only (**dual$_{\mathbf{C}}$**). We hypothesised that (1) the dual-tracer method (dual$_{TiCo}$) can improve AID estimates compared with the commonly used single$_{Ti}$ method, (2) violation of the assumptions regarding the behaviour of tracers and tracee and digesta sampling will lead to inferior AID estimates, and (3) differences in AID estimates obtained by various methods will be particularly noticeable in chickens fed a diet containing coarse oat hulls, presumed to enhance phase separation of digesta.

Material and methods

Experimental procedures are described in detail in Supplementary Material S1. Briefly, a total of 112 Dekalb White and 112 Bovans 29-week-old laying hens were assigned to one of two experimental diets (coarse vs fine oat hulls) consisting of seven birds per replicate pen and eight replicate pens per diet/breed combination. Diets were fed as pellets, with TiO_2 (3 g/kg) and Co-EDTA (2 g/kg) as inert tracers. Birds had *ad libitum* access to feed and water. On days 34, 35, or 36, contents from the ileum were collected by squeezing and pooled per pen. Two subsamples of 15 g per pen were taken, where one was used as such (whole digesta, X) and the other one was centrifuged (10 min at 3 500*g*; Heraeus Megafuge 40R, Thermo Fisher Scientific, Waltham, MA) and decanted after which the residue was collected and weighed (solid digesta, S). Diet-, whole digesta-, and solid digesta samples were analysed for tracer and nutrient concentrations as described in Supplementary Material S1.

Apparent ileal digestibility of nutrient N (AID_N) was calculated using single- and dual-tracer methods as described by France and Siddons (1986), reviewed by de Vries and Gerrits (2018). Briefly, for the single tracer methods, AID_N was calculated using tracer and nutrient concentrations measured in the whole digesta sample (X), as follows:

$$AID_N = \left(1 - \left(\frac{([T]_{diet} \times [N]_X)}{([T]_X \times [N]_{diet})}\right) \times 100\% \quad (1a)$$

where $[T]_{diet}$, $[T]_X$, $[N]_{diet}$, and $[N]_X$ (g/kg) are concentrations of the tracer (TiO_2 or Co-EDTA) and nutrient (N) in the diet and whole digesta sample.

For the dual-tracer methods, AID_N was calculated as:

$$AID_N = \left(\frac{I_N - F_N}{I_N}\right) \times 100\% \quad (1b)$$

where I_N and F_N are intake rate and flow of nutrient N (g/day), and F_N was calculated using TiO_2 as tracer for solid digesta and Co-EDTA as tracer for fluid digesta assuming steady state conditions for chickens having *ad libitum* access to feed (van der Klis et al., 1990). Tracer and nutrient concentrations were analysed in whole digesta (X) and solid digesta (S) and calculated by subtraction of tracer pool sizes for fluid digesta (F).

To obtain $F_{N,}$ for the dual-tracer method accounting for non-ideal tracer behaviour and unrepresentative digesta sampling (dual$_{TiCo}$), a reconstitution factor (R_S), i.e., the number of units of S that must be added to (or removed from) one unit of X to obtain true digesta was calculated as follows:

$$R_S = \frac{\frac{[Ti]_X}{I_{Ti}} - \frac{[Co]_X}{I_{Co}}}{\frac{[Co]_S}{I_{co}} - \frac{[Ti]_S}{I_{Ti}}} \quad (2a)$$

The concentration of nutrient N in true digesta (D) was then calculated as:

$$[N]_D = \frac{([N]_X + R_S \times [N]_S)}{(1 + R_S)} \quad (2b)$$

and the flow of true digesta (F_D) and F_N were subsequently calculated as:

$$F_D = \frac{I_{co} \times (1 + R_S)}{[Co]_X + R_S \times [Co]_S} \quad (3)$$

$$F_{N\_dual\_TiCo} = [N]_D \times F_D \quad (4)$$

For the dual-tracer methods assuming ideal tracer behaviour and representative digesta sampling (dual$_B$) or representative digesta sampling only (dual$_C$), F_N was calculated as described in Supplementary Material S2.

Pen was the experimental unit for statistical analyses. Estimated AID values were compared using a general linear mixed model (PROC MIXED, SAS version 9.4, SAS Institute Inc., Cary, NC), with breed, diet, method, and their interactions as fixed effects. Preliminary analysis revealed no 2- or 3-way interactions ($P > 0.05$) between breed, diet, and method, and hence, interaction terms were omitted from the final model, except for AID of non-starch polysaccharides (**NSP**). Method was modelled as within-subjects random (R-side) effect, assuming an unstructured covariance structure, to account for repeated observations for the five methods within pen. For the sake of brevity, only main effects of methods are presented. Differences among means were tested using type III least squares statistics, using Tukey adjustments for multiple comparisons. Model assumptions and goodness of fit of models were evaluated through the distribution of conditional Pearson residuals, the null model Likelihood Ratio test, and Akaike and Bayesian information criteria. The relation between AID estimated with dual$_{TiCo}$ vs alternative methods was further evaluated by simple linear regression (PROC REG, SAS version 9.4, SAS Institute Inc., Cary, NC) using the model y = a + βx. Where y is AID estimated with alternative method, a is intercept, β is slope, and × is AID estimated with dual$_{TiCo}$. Data are presented as least squares means and pooled relative SD, unless indicated otherwise. Differences among means with $P < 0.05$ were accepted as representing statistically significant differences.

Results and discussion

The aim of this study was to compare estimates of AID of nutrients with the commonly used single tracer method using TiO_2 as tracer, with the dual-tracer method using TiO_2 as tracer for solid digesta and Co-EDTA) as tracer for fluid digesta. One may assume that dual-tracer methods that use distinct tracers to follow solid and liquid digesta phases may result in more accurate AID estimates than single tracer methods, as digesta fractionation can complicate AID measurements when only one tracer is used. Nevertheless, $single_{Ti}$ yielded similar mean AID estimates as $dual_{TiCo}$ ($P > 0.09$) for all nutrients except AID of nitrogen (**N**), which was 3%-units greater ($P < 0.001$) when estimated with $single_{Ti}$ compared with $dual_{TiCo}$ (Table 1). Single tracer methods require analysis of concentrations of nutrients and one tracer in the whole digesta sample, resulting in a smaller analytical error for $single_{Ti}$ and a lower relative SD, compared with $dual_{TiCo}$, where concentrations of nutrients and two tracers are analysed in at least two of the three fractions of digesta (whole, solids, fluids). In the present study, a contrast in digesta phase separation in the proximal gastrointestinal tract was anticipated by including oat hulls (150 g/kg) in coarse vs fine form. Interestingly, no diet × method interactions were found ($P > 0.3$), coinciding with a lack of effect of coarse vs fine oat hulls on gizzard development and passage behaviour of solid and liquid digesta through the proximal gastrointestinal tract (data not shown). Yet, fractionation of digesta and separation of tracers from digesta components may depend on dietary fibre concentration and physicochemical properties of dietary ingredients. However, also in another study (Rezaei Far and de Vries, 2022), where a high-fibre diet (152 g NSP/kg) increased separation between solid and liquid digesta phases in the gizzard-proventriculus complex (56%, $P = 0.006$) and small intestine (199% in fast growing broilers, $P = 0.005$, 17% in slow growing broilers, $P = 0.951$) compared with a low fibre diet (89 g NSP/kg), we could confirm similar findings with no differences between AID estimated with $single_{Ti}$ vs $dual_{TiCo}$ for DM, N, starch, and NSP ($P > 0.10$), regardless of diet.

To further evaluate the impact of violations of the assumptions regarding behaviour of tracer and tracee and digesta sampling, AID estimates for various nutrients were compared with estimates obtained by single- and dual-tracer calculation methods varying in underlying assumptions (Table 1, Supplementary Fig. S3). $Dual_{TiCo}$ accounting for (1) the proportion of nutrients present in the solid and fluid phases, (2) non-ideal tracer behaviour, and (3) unrepresentative digesta sampling was considered as reference method comprising least stringent assumptions. We compared single tracer methods using a solid ($single_{Ti}$) or fluid ($single_{Co}$) tracer, where it is assumed that nutrients of interest behave similar as the tracer used; and two dual-tracer methods assuming ideal tracer behaviour and representative digesta sampling ($dual_B$) or representative digesta sampling only ($dual_C$). Clearly, violation of the assumption of ideal tracer behaviour ($dual_B$) had most impact, resulting in underestimation of AID, particularly at lower AID values. Assumptions regarding similar behaviour of tracer and tracee ($single_{Co}$ vs $single_{Ti}$) and representative digesta sampling ($dual_C$) were less important, although AID estimates of DM and N seemed more sensitive than fat and starch. The eligibility of single tracer methods depends on the match between tracer and tracee, and will typically be less appropriate when nutrients with properties different from the tracer are of interest. $Single_{Ti}$ resulted in overestimation of AID of N compared with the reference method $dual_{TiCo}$, whereas $single_{Co}$ resulted in underestimation of AID of DM, NSP, and to a lesser extent starch and fat. These findings logically follow from the relative distributions of tracers and nutrients over the different digesta fractions (Fig. 1). Although limited to the chemical and physical characteristics – particularly solubility and hydration properties – of the diets used in this study, these data suggest that for single tracer methodology, Co-EDTA may be a more appropriate marker when N is the nutrient of interest than TiO_2, whereas TiO_2 is preferred for other nutrients. Although the high inclusion of oat hulls can be considered rather unusual for poultry diets, the proteins in the diets used in the current study, originating from wheat gluten (>50% of proteins), maize (>25% of proteins), and soybean (>10% of proteins), are considered to reflect commonly used protein properties of rather insoluble nature.

In conclusion, these results show that although from a conceptual perspective dual-tracer methods are presumed to better account for the variation in flow behaviour of different digesta phases, AID estimates obtained by the common $single_{Ti}$ method only marginally differ from estimates obtained by $dual_{TiCo}$ that

Table 1
Apparent ileal digestibility (AID, % of intake)[1] of DM, nitrogen (N), fat, starch, and non-starch polysaccharides (NSP) in laying hens fed diets containing 150 g/kg oat hulls, measured at 34 weeks of age, as calculated with single- or dual-tracer methods[2].

Item	Nutrient				
	DM	N	Starch	Fat	NSP
n[3]	32	32	32	32	32
$Single_{Ti}$	59.3[d]	80.1[d]	97.4[d]	85.6[d]	−11.1[d]
RSD	4.41	3.12	1.33	5.59	89.00
$Single_{Co}$	51.7[b]	76.4[b]	96.9[b]	83.0[b]	−32.1[b]
RSD	9.69	4.60	1.72	6.54	53.71
$Dual_B$	32.5[a]	63.3[a]	95.4[a]	75.5[a]	−81.7[a]
RSD	15.40	9.33	2.60	11.23	24.13
$Dual_C$	57.4[c]	78.6[c]	97.2[c]	84.8[c]	−16.1[c]
RSD	5.12	3.87	1.48	5.91	65.90
$Dual_{TiCo}$	59.5[d]	77.1[b]	97.2[cd]	85.2[cd]	−8.2[d]
RSD	6.58	5.19	1.62	5.82	191.79
Model *P*-value[4]	<0.001	<0.001	<0.001	<0.001	<0.001

[1] Data are presented as Least Squares Means (LSMeans) and relative SD (RSD, %; calculated as pooled SD × 100/|LSMean|).
[2] Commonly used single tracer methods using TiO_2 ($single_{Ti}$) or Co-EDTA ($single_{Co}$), or dual-tracer methods. Digesta flow of combined solid and fluid phases was calculated using methods accounting for non-ideal tracer behaviour and unrepresentative digesta samples ($dual_{TiCo}$) or assuming ideal tracer behaviour and representative digesta sampling ($dual_B$) or representative digesta sampling only ($dual_C$).
[3] Number of replicate pens (seven birds/pen).
[4] Model established *P*-values for the fixed effect of method, using a model including breed, diet, method and their (2- and 3-way) interactions as fixed effects. If non-significant, interaction terms were omitted from the model. Only for AID of NSP a significant breed × method interaction was found ($P = 0.0170$), explained by a non-significant difference between $Dual_C$ and $Dual_{Tico}$ within Dekalb White hens ($P = 0.0305$), whilst a non-significant difference in Bovans Black hens ($P = 0.204$). For all other method comparisons, effects were similar in both breeds.
[a–d] Values within a column with different superscripts differ significantly at $P < 0.05$.

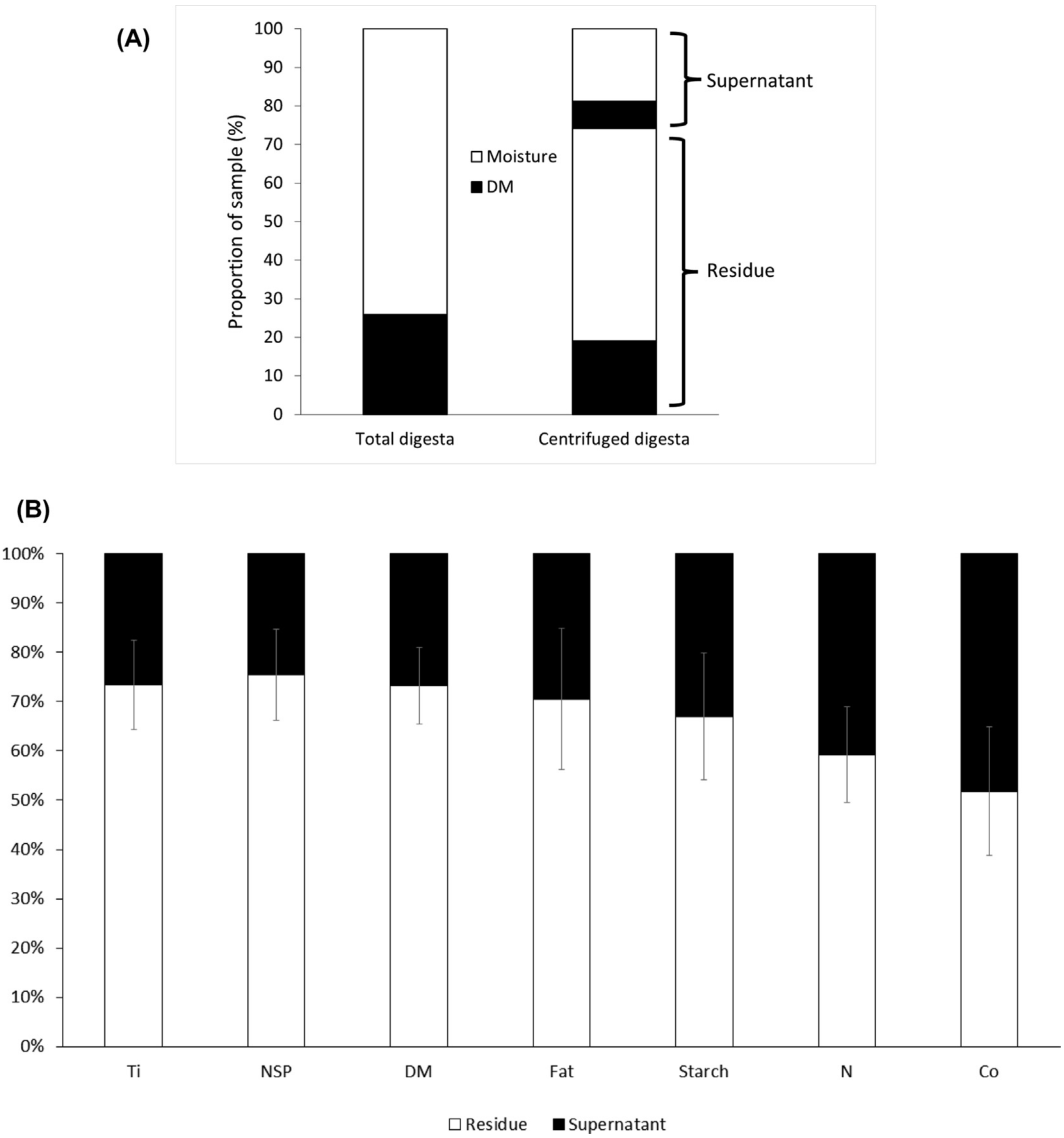

Fig. 1. (A) Relative contribution of moisture and DM to total digesta sample, or residue and supernatant after centrifugation of digesta[1,2]. (B) Distribution of tracers and nutrients over residue and supernatant after centrifugation of digesta[1,2]. Error bars indicate SD. [1]Measured in laying hens fed diets containing 150 g/kg oat hulls at 34 weeks of age. [2]Centrifugation for 10 min at 3 500g. Abbreviations: Co = Cobalt originating from Co-EDTA, N = nitrogen; NSP = non-starch polysaccharides; Ti = titanium originating from TiO_2.

uses distinct tracers for solid and liquid digesta phases. $Single_{Ti}$ resulted in overestimation of AID of N by 3%-units, whereas AID estimates of DM, starch, fat, and NSP did not differ and precision of all AID estimates was improved. Due to the presumed soluble nature of N in the gastrointestinal tract, $single_{Co}$ resulted in more accurate estimates for AID of N, whereas it was inferior to $single_{Ti}$ for other nutrients. Considering technical and economical constraints, the single tracer method may thus be the method of choice in many situations. Only when digestibility of proteins or amino acids is of specific interest, single tracer methods using a solid-phase tracer may not suffice. Nevertheless, for both single- and dual-tracer methods, tracer selection is critical, and the choice of tracers should depend on the nutrient of interest.

Supplementary material

Supplementary material to this article can be found online at https://doi.org/10.1016/j.animal.2023.101010.

Ethics approval

Experimental procedures were approved by the Dutch Central Committee of Animal Experiments under the authorisation number AVD1040020197324.

Data and model availability statement

The data were not deposited in an official repository. Data that support the study findings are available from the authors upon request.

Declaration of Generative AI and AI-assisted technologies in the writing process

The authors did not use any artificial intelligence-assisted technologies in the writing process.

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HB: methodology, writing - review and editing.
AG, WG: conceptualisation, writing - review and editing.

Declaration of interest

AG is employee of Trouw Nutrition. SV, HB, and WG declare no conflict of interest.

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