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A CRITICAL APPROACH OF THE CALCULATION PROCEDURES TO BE USED IN DIGESTIBILITY DETERMINATION OF FEED INGREDIENTS FOR RABBITS

VILLAMIDE M.J.[1], MAERTENS L.[2], CERVERA C.[3], PEREZ J.M.[4], XICCATO G.[5]

[1] Dpto. Producción Animal, E.T.S.I. Agrónomos, Universidad Politécnica, 28040 MADRID, Spain
[2] Centre for Agricultural Research - Ghent, Department of Animal Nutrition and Husbandry, Burg. Van Gansberghelaan 92, 9820 MERELBEKE, Belgium
[3] Dpto de Ciencia Animal. Universidad Politécnica de Valencia, 46071 VALENCIA. Spain
[4] INRA, Station de Recherches Cunicoles, BP 27, 31326 CASTANET-TOLOSAN Cedex, France
[5] Dept. Scienze Zootecniche, Università di Padova, 35020 LEGNARO (Padova), Italy

ABSTRACT : The effect of differences between tested ingredient and basal diet dry matter content and the effect of premix inclusion on the digestible nutrient content of tested feedstuffs are analysed. The calculation procedures to be followed in digestibility evaluation assays of feed ingredients for rabbits are also described by means of examples. The impact of dry matter correction on the estimation of the digestible nutrient content of tested ingredient increases with increasing of differences between the dry matter content of basal diet and tested ingredient, and between the nutrient content of basal and test diet, and with decreasing substitution rate, varying from 0.05% to 9%. Premix correction increases the digestible nutrient content of tested ingredient proportionally to the premix addition (calculated on dry matter basis) and independently of the substitution rate and of the kind of feedstuff evaluated. Nevertheless, the complete correction (both by dry matter and premix) sometimes has a compensatory effect, when corrections act in opposite way.

RESUME : Présentation critique des méthodes de calcul devant être suivies pour l'évaluation de la digestibilité des matières premières alimentaires, chez le Lapin.
Les auteurs ont analysé les conséquences des différences de composition entre la matière première testée et le régime de base servant à l'étudier, ainsi que les effets de la prise en compte du taux d'inclusion du prémix, dans l'estimation de la digestibilité des éléments nutritifs de cette matière première. La procédure de calcul qu'il convient de suivre pour la mesure de la digestibilité d'une matière première est décrite à l'aide d'exemples. L'effet de la correction pour la teneur en matière sèche de la matière première étudiée est d'autant plus grand que cette teneur s'éloigne de celle de l'aliment de base, ou que s'accroît l'écart de teneurs en nutriments digestibles entre l'aliment de base et l'aliment expérimental contenant la matière première étudiée. L'effet de la correction pour la teneur en matière sèche s'accroît aussi avec la réduction du taux d'incorporation de la matière première étudiée. Selon les situations, cette correction pour la teneur en matière sèche, a un impact qui représente de 0,05% à 9% des teneurs en éléments digestibles estimées. La correction liée à la prise en compte du prémix (lorsqu'il est incorporé à taux fixe dans tous les régimes expérimentaux) accroît la valeur de la digestibilité attribuée aux nutriments de la matière première étudiée, de manière proportionnelle au taux d'incorporation de ce prémix (calculé sur la base de la matière sèche), quel que soit le type de matière première ou le taux de substitution entre le régime de base et la matière première étudiée.. Toutefois, la correction complète (matière sèche et prémix ensemble) a quelquefois un effet compensateur, quand les corrections agissent de manière opposée..

1. INTRODUCTION

Nutritive evaluation assays of feed ingredients in rabbits involve a complicated design (multi-substitution level) as a consequence of the high number of feed ingredients and their different nature (energy and protein concentrates, fibrous sources). Furthermore, rabbits are sensitive in terms of digestive disorders to nutrient imbalance (LEBAS *et al.*, 1998). Therefore, digestibility of only a very limited number of feedstuffs (e.g. alfalfa meal) can be determined directly, thus using these feedstuffs as sole feed in a digestibility assay.

The substitution method requires complicated calculation procedures and difficult statistical analyses. Moreover in some papers about this subject, a description of the calculation procedures used to estimate the digestible nutrient content of feedstuffs and their variation (if mentioned) are simplified, not being possible to estimate from those of the diets. Efforts have been done to standardise both methodology and calculation procedures (PEREZ *et al.*, 1995a; VILLAMIDE, 1996) but there exist still a lot of confusion especially in view of assaying raw materials.

An example of the complexity of factors that influence the results of nutritive evaluation assays was demonstrated in an inter-laboratory study (VILLAMIDE, 1998). The same data-set was sent to 4 laboratories asking to calculate the digestible energy content of a single raw material, which has been evaluated using 4 substitution rates (0, 15, 30 and 45%). The obtained values ranged from 13.17 to 16.62 MJ/kg dry matter (DM) with the most important differences being for the lowest substitution rate (17% of difference between extreme values) and with the highest agreement for the regression estimations (3%).

Variability of the results can be ascribed to calculation and manufacturing procedures as well as statistical analyses. Related to the former points, diet's formulation and preparation are done on an air-dry basis, so that differences in ingredients dry matter should not have an important effect on the actual level of inclusion. Vitamin-mineral premix can be included at a constant rate in all the experimental diets (to avoid deficiencies in extreme diets) or only in the basal diet, which implies important differences in the calculation procedures. Likewise, the chemical composition of the experimental diets could be a problem if they differ from the theoretical (additivity principle) because the calculated or the determined composition could be used. Another important source of variation is the use of a statistical test of outlier data.

The purpose of this approach is to analyse the influence of differences in ingredients dry matter content and of premix inclusion on the digestible nutrient content of feeds and to describe the calculation procedures to be followed in digestible nutrients evaluation assays of feed ingredients for rabbits. Other points as differences between analysed and calculated chemical composition of diets, or the elimination of outlier data are also briefly discussed. They aim to contribute to increase harmonisation in feed ingredient evaluation.

Table 1a : Example of composition of experimental diets (% air dry basis) and dry matter content of ingredients

	Basal Diet	Test Diet	% Dry matter
Basal Diet	100	70	90.0
Tested ingredient	0	30	85.0

Table 1b : Correction of the inclusion level by dry matter content

	Basal Diet	Corrected level of inclusion in the basal diet	Test Diet	Corrected level of inclusion in the test diet
Basal diet	100 x 0.90=90	$\frac{90}{90}=1$	70 x 0.90 =63	$\frac{63}{88.5}=0.712$
Tested ingredient	0 x 0.85 = 0	$\frac{0}{90}=0$	30 x 0.85 =25.5	$\frac{22.5}{88.5}=0.288$
Total DM content	90 +0 = 90		63+ 25.5 =88.5	

2. CORRECTION FOR DRY MATTER CONTENT OF INGREDIENTS

2.1. Premix as a single ingredient of basal diet

Diets are manufactured by weighing the ingredients on an air-dry basis. The dry matter content of ingredients is relatively constant, usually varying from 0.84 to 0.92, reaching greater dryness for some minerals. Therefore, the dry matter content is usually not taken into account in diet formulation, because the variability in dry matter content is usually small. However, in substitution assays where the substitution level has a great importance on the results of the tested ingredient, the actual substitution level on dry matter has to be calculated. So, a sample of the basal diet and the tested ingredient should be taken to analyse its dry matter content when the ingredients are dosed.

As an example in Table 1a and 1b, the composition of two experimental diets used in a digestibility assay are presented, when manufactured and when the correction by dry matter content was done, respectively.

Therefore, the actual inclusion level of the tested ingredient, when the different dry matter content of basal diet and of tested ingredient are taken into account, is 28.8% instead of 30% as was designed and manufactured.

The new substitution rate (corrected by dry matter content) of the tested ingredient can be calculated from the following equation :

$$SRc = \frac{SR}{(1-SR)\frac{DM_{BD}}{DM_{TI}} + SR} \qquad \text{(equation 1)}$$

being SRc the corrected substitution rate (0.288 in the example), SR the not corrected substitution rate (0.30 in the example) and DM_{BD}, DM_{TI} the dry matter content of basal diet and tested ingredient, respectively.

Following with the above mentioned assay, these experimental diets were distributed *ad libitum* to 10 rabbits according to the European methodology for the determination of diet's digestibility (PEREZ *et al.*, 1995). Results of this digestibility assay (Table 2) are expressed in digestible nutrient contents per kg and not as digestibility coefficients because for the other calculations it is not possible to operate with percentages established over different basis.

Table 2 : Results of the digestibility assay (mean ± standard deviation)

Nutrients	Basal Diet	Test Diet
Digestible Energy (MJ/kg DM)	10.04 ± 0.21	10.46 ± 0.23
Digestible Crude Protein (g/kg DM)	125 ±1.4	135±1.5

Table 3 : Calculation of the digestible nutrients content of the tested ingredient

Digestible Energy (MJ/kg DM)	$\frac{(10.46-0.712x10.04)}{0.288}=11.50$
Digestible Crude Protein (g/kg DM)	$\frac{(135-0.712x125)}{0.288}=159.7$

2.1.1. Calculation of the digestible nutrients contained in the tested ingredient (TI)

Assuming the additivity principle, for one specific nutrient such as digestible energy (DE) or digestible crude protein (DCP), the digestible nutrient content (DNC) of the test diet is the sum of the contributions of basal diet and tested ingredient in proportion to their relative inclusion rate in the test diet:

DNC_{TD} = (1-SRc) x DNC_{BD} + SRc x DNC_{TI} (equation 2)

where DNC_{TD}, DNC_{BD} and DNC_{TI} are the digestible nutrients contained in the test diet, in the basal diet and in the tested ingredient, respectively.
From this equation, the digestible content of one nutrient in the tested ingredient can be calculated as follows:

$$DNC_{TI} = \frac{DNC_{TD} - (1-SRc) \text{ x } DNC_{BD}}{SRc} \quad \text{(equation 3)}$$

When this equation 3 is applied to data of Table 2 according to the corrected substitution rate calculated in the Table 1b, the digestible nutrients content of the tested ingredient is obtained as follows (Table 3).

The corresponding results without dry matter correction are 11.44 MJ/kg and 158.3 g/kg for DE and DCP, respectively. This implies an error of -0.5% and -0.9% in energy and protein evaluation, respectively. The impact of the dry matter correction, however, increases

1. with increasing differences in dry matter content of basal diet and tested ingredient,
2. with increasing differences in digestible nutrient content between basal and test diets, and
3. with decreasing substitution rate.

An estimation of the maximum effect of dry matter correction on the energy value of ingredients has been done for different classes of feedstuffs taking in consideration their normal substitution rates and the design of basal diet. Fats or oils are ingredients usually evaluated at low substitution rates (from 3 to 12%), with high differences in energy value between basal and test diets (from 0.77 to 3.14 MJ DE/kg DM) and also with large differences in dry matter content between ingredient and basal diet (99 *vs* 85-90%). In this case, the effect of dry matter correction leads to much lower values (from 6 to 9%). That is to say, when the substitution rate of fats are not corrected by their dry matter content, their estimated energy value are over-evaluated from 6 to 9% (*i.e.* 36.5 MJ DE/kg DM without dry matter correction, but only 33.5 MJ after dry matter correction). For fibrous feedstuffs, the dry matter correction produce maximum differences from 2 to 8%. For energy and protein concentrates, maximum differences range between 0.4 to 1.5% and for feedstuffs of medium digestible energy value the impact of dry matter correction is usually between 0.05 and 0.6%.

2.1.2. Calculation of standard errors of the digestible nutrients content of ingredients estimated by difference

The standard error (SE) of values estimated by difference can be calculated from the equation proposed by VILLAMIDE (1996), in which the variance of both diets (VAR_{TD}, VAR_{BD}) is weighed by the substitution rate and taking into account the number of data used in each diet digestibility measure (n_{BD} and n_{TD}) :

$$SE_{TI} = \frac{1}{SRc}\sqrt{\frac{VAR_{TD}}{n_{TD}} + (1-SRc)^2 \frac{VAR_{BD}}{n_{BD}}} \quad \text{(equation 4)}$$

Applying this equation to the standard deviation of data shown in Table 2, we obtain the following standard errors for digestible nutrient content of the tested ingredient (Table 4).

As a consequence of the uncertainty produced by the extrapolation from 28.8% (SRc) to 100%, these standard errors are much higher (4.3 times) than the standard errors of the diets, *i.e.*
$\frac{0.21}{\sqrt{10}} = 0.066$ and $\frac{1.4}{\sqrt{10}} = 0.44$ as SE of digestible

Table 4 : Calculation of the standard errors of digestible nutrients contained in the tested ingredient

	Digestible Energy	Digestible Crude Protein	Number
VAR_{BD}	$0.21^2 = 0.044$	$1.4^2 = 1.96$	10
VAR_{TD}	$0.23^2 = 0.053$	$1.5^2 = 2.25$	10
SE_{TI}	$\frac{1}{0.288}\sqrt{\frac{0.044}{10}+(1-0.288)^2\frac{0.053}{10}} = 0.30$	$\frac{1}{0.288}\sqrt{\frac{2.25}{10}+(1-0.288)^2\frac{1.96}{10}} = 1.98$	

Table 5a : Composition of the experimental diets (% air dry basis) and dry matter content of ingredients

	Basal Diet	Test Diet	% Dry Matter
Basal	98.0	68.6	90.0
Tested ingredient	0	29.4	85.0
Premix	2.0	2.0	95.0

Table 5b : Correction for the differences in dry matter content

	Basal Diet	Corrected level of inclusion in the basal diet	Test Diet	Corrected level of inclusion in the test diet
Basal	98 x 0.90=88.2	$\frac{88.2}{90.1}$=0.979	68.6x0.90 =61.7	$\frac{617}{88.6}$=0.697
Tested ingredient	0 x 0.85 = 0	$\frac{0}{90.1}$=0	29.4x 0.85 =25	$\frac{25}{88.6}$=0.282
Premix	2 x 0.95 = 1.9	$\frac{1.9}{90.1}$=0.021	2 x 0.95=1.9	$\frac{1.9}{88.6}$=0.021
Total	88.2+1.9=90.1		61.7+25+1.9=88.6	

Table 5c : Level of inclusion of the basal diet and of the tested ingredient (%) without premix

	Basal Diet	Test Diet
Basal	$\frac{97.9}{97.9}$=1	$\frac{69.66}{0.979}$=71.2
Tested Ingredient	0	$\frac{28.2}{0.979}$=28.8

Table 5d : Correction of the digestible nutrients content of diets with a fixed premix inclusion

	Basal Diet	Test Diet
Digestible Energy (MJ/kg DM)	$\frac{10.04}{0.979}$=10.26	$\frac{10.46}{0.979}$=10.684
Digestible Crude Protein (g/kg)	$\frac{125}{0.979}$=127.7	$\frac{135}{0.979}$=137.9

energy and digestible protein of the basal diet, and

$\frac{0.23}{\sqrt{10}} = 0.073$ and $\frac{1.5}{\sqrt{10}} = 0.47$ as SE of digestible energy and digestible protein of the test diet

2.2. Premix inclusion at a fixed level in all experimental diets.

The premix includes many essential nutrients (*e.g.* vitamins and micro-minerals), which should be added to the diets to avoid possible deficiencies. Especially when non-classical ingredients have to be evaluated and at high inclusion level, nutrient deficiencies can result.

When the tested ingredient is incorporated at a low level (*e.g.* less than 10%) the premix is generally included in the basal diet itself, and is "diluted" in proportion of the substitution level in the test diet. In this case, no specific correction is needed for the premix effects, because they are included in the basal diet effects.

On the contrary, when the premix is included in the basal diet as an ingredient and if the tested ingredient has a low content of some minerals and vitamins, the experimental diets obtained at high substitution rates result in a proportional decrease of some essential nutrients. Therefore, in this case, the addition of a premix as a constant supply to all experimental diets can be justified.

The dry matter content of the premix is usually different from the basal diet. Besides the correction for the different dry matter content of ingredients, a second correction for the premix level must also be included in order to calculate the exact inclusion level of the tested ingredient and premix. Table 5a and 5b show, as an example, the composition on an air-dry basis corrected for the different dry matter content of the ingredients and premix when the premix is included at a fixed level.

*2.2.1. **Dry matter corrections** (actual ingredient composition in dry matter. Table 5b)*

2.2.2. Premix correction

A premix that includes only minerals and vitamins does not contribute to gross energy, crude protein and/or fibre. Therefore, we can assume that the dietary digestible nutrient content comes from the basal diet and tested ingredient. Consequently, the digestible nutrient content has to be corrected for the fixed premix inclusion (2.1%, Table 5b) as is shown in Table 5c and 5d.

Therefore, the substitution rate is not affected by the premix inclusion after correction by premix. However, the determined digestible nutrient content of experimental diets (both basal and test diets) are penalised by the inclusion of a premix, which does not supply energy or protein. As a consequence, the digestible nutrients content of these diets (table 2) should be corrected in the same way as for the inclusion level.

Table 6 : Calculation of the digestible nutrients content of the tested ingredient

Digestible Energy (MJ/kg DM)	$\frac{(10.684-0.712x10.257)}{0.288}=11.74$
Digestible Crude Protein (g/kg DM)	$\frac{(137.9-0.712x127.7)}{0.288}=163.1$

Table 7 : Effect of the different corrections on the feeding value of the tested ingredient

	Totally corrected	Corrected for air dry premix	Corrected for Dry Matter	Not corrected for DM or premix
Digestible Energy	**11.74** (=100)	11.67 (99.4)	11.53 (98.21)	11.43 (97.4)
Digestible Crude Protein	**163.1** (=100)	161.6 (99.1)	160.4 (98.4)	158.3 (97.0)

2.2.3. Digestible nutrients content of the Tested ingredient (TI)

As in the previous example, equations 2 and 3 can now be used to calculate the digestible nutrient content of the tested ingredient. Based on the corrected data (Table 5d) this value is presented in Table 6.

When comparing these data to those of Table 3, a difference of 2.1% (corresponding to the premix inclusion) can be observed between both series of results. Therefore, the premix correction increase the digestible nutrient content of the tested ingredient proportionally to the premix addition (on DM) and independently of the substitution rate and of the kind of feedstuff evaluated. Nevertheless, the complete corrections (both for dry matter and premix) sometimes have a compensatory effect, when both corrections act in an opposite way. The most obvious example is again in fat evaluation where the dry matter correction causes an important decrease in its estimated energy value (from 6 to 9%, due to its high dry matter content). On the other hand, the premix correction increases its estimated energy value, resulting in lower effect of the 2 successive corrections (4 - 7% with premix included at 2%, instead of 6 - 9% with only the dry matter correction).

As a summary of the preceding corrections, the results obtained after different types of corrections are presented in Table 7. The highest difference (2.6 and 3%) is obtained when comparing totally corrected values vs. not corrected for dry matter and premix inclusion. However, higher differences will be obtained with lower substitution rates or if greater differences in dry matter content of ingredients and premix occurs, and/or if the difference between the digestible nutrient content of the experimental diets is larger.

2.3. Calculation of standard errors of the nutrient digestible content of ingredients estimated by multilevel assays

Instead of a single inclusion level in the basal diet, different (3 or more) substitution rates can be used to assay the digestible nutrient content of the tested ingredient by extrapolation. In this case, the corrected values of both substitution levels and premix should be included in the regression model using all individual data (that is to say those corresponding with each rabbit). A simplified example (with the mean value/diet) is shown in Table 8, referring to the values mentioned in Tables 5c and d for basal and test diet 30.

Table 8 : Data set to be included in the regression equations to estimate the DE and DCP content of the tested ingredient by extrapolation.

Diet	Substitution rate	DE corrected	DCP corrected
Basal diet	0	10.257	127.7
Test diet 10	0.098	10.398	131.5
Test diet 20	0.197	10.545	134.4
Test diet 30	0.288	10.684	137.9

The obtained equations according to the formula :

DNC = a + b x SRc, are :

DNC	Intercept (a)	b	Probability	r^2	MSE_{reg}
Digestible Energy	10.254	+ 1.502	0.0001	0.999	0.34
Digestible Crude Protein	127.81	+ 34.77	0.0015	0.997	2.9

When extrapolating these linear equations to 1 (100%) of substitution rate, the digestible energy and the digestible crude protein of the tested ingredient are estimated (table 9).

The standard error of the extrapolated values can be calculated from the following equation (VILLAMIDE, 1996):

$$\text{SE (extrapolated value)} = \sqrt{MSE_{reg}\left[\frac{1}{N}+\frac{(1-SR_m)^2}{\Sigma SR_i^2-(\Sigma SR_i)^2/N}\right]}$$

(equation 5)

where MSE_{reg} is the mean square error of regression; SR_m, SR_i = mean and individual substitution rate of tested ingredient, and N = number of total data.

This equation is simplified when the data are balanced among treatments:

$$\text{SE (extrapolated value)} = \sqrt{\frac{MSE_{reg}}{n}\left[\frac{1}{4} + \frac{(1-1.5sr)^2}{5sr^2}\right]}$$

(equation 6)

Where sr is the first substitution rate.

When applying the data of Table 8 and the regression analyses to this equation, the SE of the extrapolated values are those shown in Table 9

Table 9 : Mean and standard error of the extrapolated digestible nutrients contents

	Extrapolated Value	Mean	SE	SE
Digestible Energy (MJ/kg DM)	10.254 + 1 x 1.502=	11.756	$\sqrt{\frac{0.34}{10}\left[\frac{1}{4}+\frac{(1-1.5x0.098\ )^2}{5x0.098_2}\right]} =$	0.047
Digestible Crude Protein (g/kg DM)	127.81 + 1 x 34.77 =	162.58	$\sqrt{\frac{2.9}{10}\left[\frac{1}{4}+\frac{(1-1.5x0.098\ )^2}{5x0.098_2}\right]} =$	2.11

3. ANALYSED OR CALCULATED CHEMICAL COMPOSITION

The chemical composition of the test diet can be calculated using the analysis data of the basal diet and the tested ingredient and taking into account its inclusion level. Theoretically, this calculated composition and the chemical analysis should be identical, but due to processing, sampling and/or analysis errors, sometimes important differences are observed. Thus the chemical composition of the test diet never agrees completely with the expected values.

However, the analysed or determined chemical composition of the test diet should be used because it takes into account the possible losses or variations in diet manufacturing. It is recommended before starting the *in vivo* digestibility trial, to compare the analysed with the calculated value. If discrepancies exist, the actual substitution rate and the analyses should be checked (also the analyses of basal and tested ingredient, i.e. NDF content of barley if barley is the tested ingredient). If the difference between the analysed and the calculated value exceeds two times the analytical tolerance, a new batch of the experimental diet should be prepared.

An exception can be made for raw materials, which provoke segregation problems during manufacturing (*e.g.* fats and molasses). From the chemical analyses of basal and test diets, the exact substitution rate could be recalculated if the most, and the more repeatable chemical analyses agreed.

The importance of the chemical analyses on the dietary digestibility has been stressed in several ring-test (PEREZ *et al*, 1995b; XICCATO *et al.*, 1996). One point of error in dietary chemical composition implies the same point of error in its digestible nutrient content. However, as digestible nutrient contents are lower than total, the impact of analytical errors is relatively much higher on digestible nutrients. This problem becomes greater when using the substitution method for feed ingredient evaluation, and depending on the inclusion level (VILLAMIDE, 1996). Therefore, further efforts have been done to harmonise the analytical methodologies (EGRAN, 2000).

4. ELIMINATION OF OUTLIER DATA

The effect of using an outlier test to eliminate data is even more important than that of the corrections made by dry matter content or premix inclusion. In fact, the most important differences obtained between laboratories in the example described in the introduction corresponded to the elimination of 3 outlier data of the set of 40 rabbits. In the European Reference method for *in vivo* determination of diets digestibility in rabbits (PEREZ *et al.*, 1995a), exclusion of rabbits from the digestibility assay is mentioned in case of anomalous biological data, but not on digestibility results. However the simplicity of this recommended method prevent us from using some biological data of importance for digestibility assay (*i.e.* intake and excreta evolution). Since intake and excretion are recorded in the same period, a rabbit with an apparent normal behaviour can produce outlier data (e.g. very low intake on the first day and a very high intake on the last day of the balance trial).

Therefore, the use of outlier tests for dry matter digestibility values that detects possible errors in the collection of data seems convenient. This implies the exclusion of all other digestibility values obtained by the same rabbit because all the other digestibility coefficients are obtained based on dry matter digestibility. However, individual divergent data of other nutrients may not be considered as outlier but as normal biological variability. It this case it can be recommended to repeat the analysis to detect eventually analytical errors.

5. CONCLUSIONS

Correction by dry matter content of ingredients to get the actual substitution rate has an effect on the estimated digestible nutrient content of a tested ingredient that vary from 0.05% to 9%. The impact of dry matter correction increases with increasing differences between the dry matter content of basal diet and of the tested ingredient, between the nutrient content of basal and test diet and with decreasing substitution rate.

Premix correction increases the digestible nutrient content of tested ingredients proportionally to the premix addition (calculated on DM basis) and independently of the substitution rate and of the kind of feedstuff evaluated. Nevertheless, the complete correction (both by dry matter and premix) sometimes has a compensatory effect, when corrections act in opposite way.

The chemical composition of diets and tested ingredients must be determined accurately, because one point of error in chemical composition implies one point in the digestible nutrient content making the impact of analytical errors relatively higher on digestible nutrients.

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