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Statistical Sensitivity Required to Detect Any Potential Difference of Bioavailability Between DL-Methionine and DL-Methionine Hydroxy Analogue in Layers

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Abstract: Two studies were conducted to compare bioavailability of DL-methionine hydroxy analogue-free acid (MHA-FA) relative to DL-methionine (DLM) in layers. The bioavailability was compared for egg production, egg mass, egg weight and feed conversion. In trial 1 five supplemental levels of methionine (0.02, 0.04, 0.06, 0.08 and 0.10%) from DLM or MHA-FA source were added respectively to a basal diet containing 14.97% protein, 0.27% methionine, and 0.24% cystine. Hy-Line W-36 hens (1,760) 37 weeks old were used. Egg production was not improved beyond the first 0.02% added methionine level. Thus, an accurate bioavailability value of MHA-FA relative to DLM could not be obtained, indicating that this test was not statistically sensitive enough to estimate the bioavailability of MHA-FA relative to DLM. In trial 2, 1,920 Hy-Line W-36 hens 53 weeks old were used in a $3\times2\times2$ factorial experiment with three protein levels, two supplemental methionine levels, and two methionine sources. DLM and MHA-FA were compared at different protein and supplemental methionine levels on an equimolar basis. There were no differences ($P > 0.05$) between the 0.02% and 0.04% supplemental methionine level or between DLM and MHA-FA in egg production, egg mass, or feed conversion. Because no difference in egg production, egg mass and feed conversion between 0.02% and 0.04% methionine could be detected and that difference should be greater than the difference between DLM and MHA-FA, no potential differences between DLM and MHA-FA could be detected. There was a difference ($P < 0.05$) in egg weight between the two supplemental methionine levels. Although the average egg weight of hens fed DLM was not higher ($P > 0.05$) than that of hens fed MHA-FA, the difference was calculated to indicate that the bioavailability of MHA-FA might be 88.9% on a molar basis or 78.2% on a weight basis.

Key words: Bioavailability, DL-Methionine, layers, methionine hydroxy analogue

Introduction

In most poultry diets methionine is the first limiting amino acid, followed by lysine, threonine, and tryptophan or isoleucine. Methionine is crucial to the production of meat and eggs, and the synthesis of enzymes and hormones. Hence, a deficiency of methionine is often a roadblock to growth and egg production. This means that an adequate quantity of methionine is necessary for most practical poultry feeds to obtain optimum performance.

In the commercial poultry industry soybeans, which have high protein content including methionine, can be used. However, the cost of increasing methionine level from natural methionine sources is typically higher than from synthetic methionine sources. Therefore, it is a common practice to supplement diets with synthetic methionine sources, such as DL-Methionine (DLM), or methionine hydroxy analogue-free acid (MHA-FA).

A large number of experiments have been conducted in broilers, layers, and turkeys to determine the bioavailability of MHA-FA relative to DLM. Some of the literature showed that there were no differences between the bioavailability of DLM and MHA-FA (Waldroup *et al.*, 1981; Scott, 1987; Han *et al.*, 1990; Harms and Rusell, 1994; Dibner, 2003). Some researchers reported lower bioavailability for MHA-FA relative to DLM (Thomas *et al.*, 1991; Esteve-Garcia and Llaurado, 1997; Wallis, 1999; Lemme *et al.*, 2002, Drew *et al.*, 2003). Although many studies have been conducted with broilers, only a few experiments have been conducted to compare the bioavailability of DLM and MHA-FA in laying hens. Reid *et al.* (1982) reported that there was no difference between DLM and MHA-FA based on the slope-ratio analysis on egg output (g egg/bird/day), but the bioavailability of MHA-FA was higher relative to DLM according to the difference in average egg production. Van Weerden and Schutte (1984) showed that performance of hens fed MHA-FA was distinctly inferior to that of hens fed DLM. Scott (1987) reported that there was no difference between DLM and MHA-FA on egg production, egg weight and egg mass, but the performance of hens receiving low

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levels of L-methionine was superior to the performances of hens receiving equivalent dietary levels of DLM and MHA-FA. Harms and Russell (1994) used a basal corn-soybean diet that was deficient in methionine to estimate the bioavailability of MHA-FA relative to DLM. These results showed the bioavailability of MHA-FA was 93% according to the slope-ratio regression technique of egg content and 100% according to the comparison of the mean methionine intake per g egg content on equimolar basis.

Table 1: Composition and calculated analysis of basal diet (Trial 1)

Ingredient	(%)
Corn (Crude protein, 8%)	68.47
Soybean meal (Crude protein, 48%)	18.92
Limestone	7.07
Hard shell	2.00
Dicalcium phosphate	1.66
Poultry oil	0.97
NaCl	0.42
Vitamin premix[1]	0.25
Mineral premix[2]	0.25
Calculated Analysis	
Crude Protein	14.97
ME, kcal/kg	2863
Total phosphorus	0.59
Available phosphorus	0.40
Ca	4.00
Na	0.18
Methionine	0.27
Methionine+Cystine	0.51
Lysine	0.75
Tryptophan	0.17

[1]Provided per kilogram of diet: vitamin A (as retinyl acetate), 8,000 IU; cholecalciferol, 2,200 ICU; vitamin E (as dl-α-tocopheryl acetate), 8 IU; vitamin B_{12}, 0.02 mg; riboflavin, 5.5 mg; D-calcium pantothenic acid, 13 mg; niacin, 36 g; choline, 50 mg; folic acid, 0.5 mg; vitamin B_1 (thiamin mononitrate), 1 mg; pyridoxine, 2.2 mg; d-biotin, 0.05 mg; vitamin K (menadione sodium bisulfate complex), 2 mg.
[2]Provided per kilogram of diet: manganous oxide, 65 mg; iodine (ethylene diamine dihydriodide), 1 mg; ferrous carbonate, 55 mg; copper oxide, 6 mg; zinc oxide, 55 mg; sodium selenite, 0.3 mg.

The objective of our first trial was to determine the bioavailability of MHA-FA relative to DLM using exponential or slope-ratio model (Littell *et al.*, 1997). The second trial was designed using three protein levels (15.06, 16.18 and 17.44%) and two methionine levels (0.02 and 0.04%) to determine if any potential difference in the bioavailability between DLM and MHA-FA could be detected.

Materials and Methods

Both experiments lasted for 10 weeks. In Trial 1 a total of 1,760 Hy-Line W-36 hens, (37 weeks old) were randomly allocated to 440 cages (40.6 cm × 45.7 cm) with 4 birds per cage. Five adjoining cages consisted of a group, and then the eighty-eight groups were randomly assigned to 11 dietary treatments. The basal diet was formulated to meet nutrient requirements for Hy-Line Variety W-36 (Anonymous, 2000), with the exception of total protein and amino acids (Table 1). The metabolizable energy content for the basal diet was 2863 kcal/kg. Five supplemental levels of methionine (0.02, 0.04, 0.06, 0.08, and 0.10%) were added to the basal diet on an equimolar basis. The DLM was calculated as 99.7% and MHA-FA was calculated as 88% (Table 2). In Trial 2 a total of 1,920 Hy-Line W-36 birds (53 weeks old) were randomly divided into 480 cages (40.6 cm × 45.7 cm) with 4 birds per cage. Five adjoining cages consisted of a group, and then the ninety-six groups were randomly assigned to 12 dietary treatments. The 3×2×2 factorial experimental design was shown in Table 3, and the ingredient and nutrient composition of diets was shown in Table 4. The metabolizable energy content of all diets was 2844 kcal/kg, and diets were adequate in all other nutrients except for protein (Anonymous, 2000).

All birds were housed in a building with controlled temperature (day and night temperatures at approximately 30 and 18°C, respectively), ventilation, and lighting (14~16 hr/day), but without control of relative humidity. Hens were supplied with feed and water ad libitum. Egg production was recorded daily; egg weight and feed consumption were recorded weekly, whereas egg specific gravity was measured monthly. Egg weight was measured using all eggs produced during two consecutive days in each week. Egg specific gravity was determined monthly using all eggs produced during two consecutive days with a floatation method (Holder and Bradford, 1979). Mortality was checked daily and feed consumption was adjusted accordingly. Body weight was obtained by weighing four birds per group at the end of the experiment. Egg mass and feed conversion (g feed/g egg) were calculated from egg production, egg weight, and feed consumption. Data was analyzed with the general linear models procedure (PROC GLM) in SAS/STAT software (SAS Institute, 2000).

Results

Trial 1: During the 70-d experimental period, total mortality was 0.85% (15 out of 1,760 hens). Mortality was not affected ($P > 0.05$) by treatment. Feed consumption was adjusted for mortality. Feed intake varied among treatments, and there was no increase as supplemental methionine level increased after the 0.02% level (Table 5). Egg production and egg mass were not different ($P > 0.05$) among treatments after the first level, except that egg mass of the birds fed DLM at the highest supplemental methionine level (0.113%) was higher

Table 2: Experimental Design (Trial 1)

Treatment	Protein (%)	Lys (%)	Met+Cys (%)	M+C/L[1] Ratio	Addition of DLM[2] (%)	Addition of MHA-FA[3] (%)
1	14.97	0.75	0.510	68.0	0.0000	
2	14.97	0.75	0.533	71.1	0.0201	
3	14.97	0.75	0.555	74.0	0.0401	
4	14.97	0.75	0.578	77.1	0.0602	
5	14.97	0.75	0.600	80.0	0.0802	
6	14.97	0.75	0.623	83.1	0.1003	
7	14.97	0.75	0.533	71.1		0.0227
8	14.97	0.75	0.555	74.0		0.0455
9	14.97	0.75	0.578	77.1		0.0682
10	14.97	0.75	0.600	80.0		0.0909
11	14.97	0.75	0.623	83.1		0.1136

[1]M+C/L means methionine plus cystine vs lysine.
[2]DL-Methionine (DLM) was calculated as 99.7% methionine in the commercial product.
[3]Based on a liquid DL-methionine hydroxy analogue-free acid (MHA-FA) content of 88% methionine in the commercial product.

than some other levels. Egg weight and feed conversion were inconsistent between DLM and MHA-FA. When the data was analyzed using the slope-ratio model, no performances responded significantly to supplemental methionine. Since no progressive response was obtained with added methionine after the first supplemental level, the exponential analysis was not performed. Therefore an accurate bioavailability value of MHA-FA relative to DLM was not obtainable.

Trial 2: During the 70-d experimental period, total mortality was 1.09% (21 out of 1,920 hens). Mortality was not affected (P > 0.05) by treatment. Feed consumption was adjusted for mortality.

Feed consumption, body weight, and egg specific gravity: On average, birds consumed approximately 90 g of feed per hen per day during the 70-d study (Table 6). Factorial analysis for feed consumption, egg specific gravity, and body weight showed that there were neither interaction effects (P > 0.05) nor main effects (P > 0.05) of protein, methionine source and methionine level (Table 6). Therefore the bioavailability of MHA-FA compared to DLM could not be detected based on these criteria.

Egg production, egg mass, and egg weight: There were no interactions (P > 0.05) among protein level, methionine source, and supplemental methionine level for egg production, egg mass, and egg weight (Table 7). Therefore, only main effects of the three factors were presented. Effects (P < 0.05) of protein levels were obtained for egg production, egg mass, and egg weight. At the 15.06% protein level, egg production and egg mass were lower (P < 0.05) than egg production and egg mass at the 16.18% and 17.44% protein level. There was no difference (P > 0.05) in egg production and egg mass between the 16.18 and 17.44% protein level. The average egg weight at the 15.06% protein level (58.19 g) and egg weight at 16.18% protein level (58.20 g) were closer (P > 0.05), but both of them were lower than egg weight at 17.44% protein level (58.99 g) (P < 0.05).

There were no differences (P > 0.05) in egg production and egg mass between 0.02 and 0.04% methionine level and between DLM and MHA-FA. Since the differences between 0.02 and 0.04% methionine levels could not be detected, no potential difference between DLM and MHA-FA could be found, because the difference between 0.02 and 0.04% levels would be much greater than any potential difference between DLM and MHA-FA.

There was a difference (P < 0.05) in egg weight between 0.02 and 0.04% methionine level. There was no significant difference in egg weight between DLM and MHA-FA, but egg weight for DLM were higher than that for MHA-FA (P>0.05). Because the difference between 0.02 and 0.04% methionine level was statistically detected, the difference between DLM and MHA-FA was taken into consideration. The 100% ((0.04%-0.02%)/0.02%=100%) methionine difference between 0.02 and 0.04% level represents 0.64 g egg weight difference (58.78 g for 0.04% methionine level vs 58.14 g for 0.02% methionine level). Based on that difference, it can be calculated that 0.08 g egg weight difference between DLM and MHA-FA (58.50 g for DLM and 58.42 g for MHA-FA) represents a 12.5% methionine difference between DLM and MHA-FA. The 12.5% methionine difference between DLM and MHA-FA indicates that the bioavailability of MHA-FA is 88.9% (Since (DLM-MHA-FA)/MHA-FA=12.5%, so MHA-FA=88.9%DLM) on a molar basis and 78.2% (88.9%×88%=78.2%) on a weight basis compared to DLM.

Feed Conversion: There was an interaction (P < 0.05) among protein level, methionine source and methionine

Table 3: Experimental Design (Trial 2)

Treatment	Protein (%)	Met Level (%)	Met Source	Addition of Met source (%)
1	17.44	0.02	DLM[1]	0.0200
2	17.44	0.02	MHA-FA[2]	0.0227
3	17.44	0.04	DLM	0.0400
4	17.44	0.04	MHA-FA	0.0454
5	16.18	0.02	DLM	0.0200
6	16.18	0.02	MHA-FA	0.0227
7	16.18	0.04	DLM	0.0400
8	16.18	0.04	MHA-FA	0.0454
9	15.06	0.02	DLM	0.0200
10	15.06	0.02	MHA-FA	0.0227
11	15.06	0.04	DLM	0.0400
12	15.06	0.04	MHA-FA	0.0454

[1]DL-methionine, was calculated as 99.7% methionine in the commercial product.
[2]DL-methionine hydroxy analogue-free acid, was calculated as 88% methionine in the commercial product.

Table 4: Ingredient composition and calculated analysis of the experimental diets (Trial 2)

Ingredient	The basal diet 1-4 for treatment	The basal diet for treatment 5-8	The basal diet for treatment 9-12
Corn (Crude protein, 8%)	61.36	65.36	68.92
Soybean meal (Crude protein, 48%)	25.35	22.00	22.00
Limestone	5.11	5.12	5.13
Hard shell	4.00	4.00	4.00
Dicalcium phosphate	1.50	1.51	1.52
Poultry oil	1.80	1.12	0.51
NaCl	0.39	0.39	0.39
Vitamin premix[1]	0.25	0.25	0.25
Mineral premix[2]	0.25	0.25	0.25
Calculated Analysis			
Crude Protein	17.44	16.18	15.06
ME, kcal/kg	2844	2844	2844
Ca	4.00	4.00	4.00
Available phosphorus	0.38	0.38	0.38
Methionine	0.30	0.29	0.27
Methionine+Cystine	0.60	0.57	0.54
Lysine	0.92	0.83	0.75
Tryptophan	0.21	0.19	0.17

[1]Provided per kilogram of diet: vitamin A (as retinyl acetate), 8,000 IU; cholecalciferol, 2,200 ICU; vitamin E (as dl-α-tocopheryl acetate), 8 IU; vitamin B_{12}, 0.02 mg; riboflavin, 5.5 mg; D-calcium pantothenic acid, 13 mg; niacin, 36 mg; choline, 50 mg; folic acid, 0.5 mg; vitamin B_1 (thiamin mononitrate), 1 mg; pyridoxine, 2.2 mg; d-biotin, 0.05 mg; vitamin K (menadione sodium bisulfate complex), 2 mg. [2]Provided per kilogram of diet: manganous oxide, 65 mg; iodine (ethylene diamine dihydriodide), 1 mg; ferrous carbonate, 55 mg; copper oxide, 6 mg; zinc oxide, 55 mg; sodium selenite, 0.3 mg.

level in feed conversion. Therefore, the data were presented according to 15.06, 16.18 and 17.44% protein level. There were no interactions (P > 0.05) between methionine levels and methionine sources, and there were no differences (P > 0.05) between 0.02 and 0.04% methionine levels and between DLM and MHA-FA on the three protein levels (data was not shown). With the same reason, since the differences between 0.02 and 0.04% methionine levels could not be detected based on feed conversion, no potential difference between DLM and MHA-FA could be found, because the difference between 0.02 and 0.04% levels would be much greater than any potential difference between DLM and MHA-FA.

Discussion

One hundred and sixty hens comprising eight replicate groups were used for each treatment in both studies, which were much more than other experiments in layers (Reid *et al*., 1982; Scott, 1987; Harms and Ressell, 1994). Relative small standard errors for most of the criteria were obtained in both studies compared to those in previous experiments. In Trial 1 the birds did not respond progressively to the increment supplemental methionine levels, indicating that no significant

Table 5: Influence of supplemental methionine on performances (Trial 1)

Supplemental met level (%)	Met Sources	Feed consumption (g/day)	Egg production (%)	Egg mass (g/hen/day)	Egg weight (g)	Feed conversion (g feed/g egg)
0.00		86.7±1.0[e]	81.0±1.6[b]	46.33±0.77[c]	57.25±0.37[e]	1.87±0.01[a]
0.02	DLM[1]	90.2±1.0[abc]	83.3±0.7[ab]	48.59±0.66[ab]	58.37±0.46[cd]	1.86±0.02[abc]
0.04	DLM	89.7±0.7[abcd]	83.6±1.1[ab]	48.68±0.76[ab]	58.26±0.030[cd]	1.84±0.02[abcd]
0.06	DLM	89.2±0.9[bcd]	81.7±1.1[ab]	48.28±0.71[b]	59.12±0.23[abc]	1.85±0.02[abcd]
0.08	DLM	88.2±0.7[cde]	82.2±1.1[ab]	48.00±0.83[bc]	58.41±0.26[bcd]	1.84±0.02[abcd]
0.10	DLM	90.4±0.8[abc]	84.2±0.5[a]	50.09±0.18[a]	59.51±0.45[a]	1.81±0.02[cd]
0.02	MHA-FA[2]	90.6±0.4[ab]	83.5±0.4[ab]	48.74±0.24[ab]	58.39±0.28[cd]	1.86±0.00[ab]
0.04	MHA-FA	91.8±0.9[a]	83.7±0.6[a]	49.62±0.44[ab]	59.29±0.25[ab]	1.85±0.01[abcd]
0.06	MHA-FA	88.4±1.1[bcde]	83.9±0.4[a]	48.82±0.33[ab]	58.19±0.15[d]	1.81±0.03[bcd]
0.08	MHA-FA	88.9±0.3[bcde]	83.3±0.9[ab]	48.65±0.54[ab]	58.46±0.30[bcd]	1.83±0.02[abcd]
0.10	MHA-FA	87.7±0.6[de]	83.0±0.9[ab]	48.76±0.52[ab]	58.74±0.15[abcd]	1.80±0.03[d]

[abcde] means the means with different superscripts at the same supplemental Met level differ significantly between DLM and MHA-FA, P<0.05. [1]DL-methionine; [2]DL-methionine hydroxy analogue-free acid.

Table 6: Influence of protein level, methionine source and level on feed consumption, body weight, and egg specific gravity (Trial 2)[1]

Factor		Feed consumption (g/hen/day)	Body weight (kg)	Egg specific gravity
	15.06	89.8±0.4	1.47±0.02	1.0801±0.0002
Protein	16.18	89.6±0.6	1.46±0.02	1.0802±0.0002
Level (%)	17.44	90.3±0.6	1.51±0.02	1.0800±0.0002
		NS	NS	NS
Met	DLM[2]	90.1±0.5	1.48±0.02	1.0802±0.0002
Source	MHA-FA[3]	89.7±0.4	1.48±0.02	1.0800±0.0002
		NS	NS	NS
Added Met	0.02	89.5±0.4	1.46±0.02	1.0803±0.0001
Level (%)	0.04	90.2±0.4	1.50±0.03	1.0799±0.0002
		NS	NS	NS

[1]Mean ± SE. [2]DL-methionine. [3]DL-methionine hydroxy analogue-free acid. NS means P > 0.05.

Table 7: Influence of protein level, methionine source and level on egg production, egg mass, and egg weight (Trial 2)[1]

Factor		Egg production (%)	Egg mass (g/hen/day)	Egg weight (g)
	15.06	77.1±0.5[a]	44.83±0.37[a]	58.19±0.23[a]
Protein	16.18	79.9±0.5[b]	46.46±0.34[b]	58.20±0.26[a]
Level (%)	17.44	79.8±0.7[b]	47.07±0.43[b]	58.99±0.22[b]
		**	**	*
Methionine	DLM[2]	78.7±0.5	46.02±0.35	58.50±0.17
Source	MHA-FA[3]	79.1±0.5	46.22±0.33	58.42±0.23
		NS	NS	NS
Methionine	0.02	78.3±0.5	45.77±0.36	58.14±0.22
Level (%)	0.04	79.1±0.5	46.47±0.31	58.78±0.16
		NS	NS	*

[1]Mean ± SE. [2]DL-methionine. [3]DL-methionine hydroxy analogue-free acid. NS means P > 0.05; * means P < 0.05; ** means P < 0.01; means P<0.05; ** means P<0.01.

differences between DLM and MHA-FA could be detected. In Trial 2 some differences (P < 0.05) among the three protein levels were obtained for egg production, egg mass, and egg weight. This indicated the experiment was well conducted and the effect of protein level could be detected. However, no significant differences (P > 0.05) between 0.02 and 0.04% methionine level were observed for feed consumption, egg production, egg mass, feed conversion, body weight, and egg specific gravity. The reason that the differences between 0.02 and 0.04% methionine level were not statistically detected was that laying hens were not sensitive enough to supplemental methionine. In this case, any potential difference between DLM and MHA-FA could not be detected, since any potential difference between DLM and MHA-FA would be less than the difference between 0.02 and 0.04% supplemental methionine levels.

Significant differences ($P < 0.05$) between 0.02 and 0.04% methionine levels were observed for the average egg weight. This indicated that egg weight was a more sensitive criterion than egg production, egg mass, and body weight. From this experiment, although no significant difference for egg weight between DLM and MHA-FA was observed, the difference between egg weight for DLM and for MHA-FA could be calculated to indicate that the bioavailability of MHA-FA was 88.9% on a molar basis and 78.2% on a weight basis. These results agreed with our previous study (Liu *et al.*, 2004). In some previous studies (Reid *et al.*, 1982; Scott, 1987; Harms and Russell, 1994) with laying hens, which were conducted on an equal molar basis, it was concluded that DLM and MHA-FA had the same bioavailability when no significant difference between DLM and MHA-FA was obtained. However, the results might only indicate the sensitivity of laying hens to added methionine was not enough to detect any potential difference between DLM and MHA-FA. This may account for some previous inconclusive or inconsistent experiments with layers.

In a further study the bioavailability of MHA-FA was assumed as 65% on a weight basis (or 74% on a molar basis) before conducting the experiment (Danner and Bessei, 2002). It was concluded that MHA-FA had only 65% bioavailability of DLM on a weight basis (or 74% on a molar basis) when no significant difference between DLM and MHA-FA was detected. However, the result might also only indicate the sensitivity of laying hens to methionine was not enough to detect the difference between DLM and over adding MHA-FA when the effectiveness of MHA-FA is assumed as only 65% relative to DLM.

The results indicated that a lack of sensitivity and big variations are largely responsible for the difficulty in detecting any potential difference between DLM and MHA-FA in laying hens. Recently, researchers in The Netherlands (Jansman *et al.*, 2003) conducted a literature study and evaluation of previous papers and reported the following: the average bioavailability of MHA-FA compared to DLM was 73% on a weight basis or 83% on a molar basis in laying hens, and 68% on a weight basis and 77% on a molar basis in broilers. The authors also indicated that the estimate of the bioavailability of MHA-FA for layers was less accurate than the estimate for broilers, which was expressed in the large confidence interval, and recommended that more research work is needed in laying hens regarding this topic.

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