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Effect of digestible methionine + cystine concentration on performance, egg quality and blood metabolites in laying hens

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Abstract 1. The estimation of sulphur amino acid requirement is a vital key to providing appropriate nutrition in poultry. The estimation of amino acid requirement depends on what production parameter is taken into consideration for optimisation.

2. A complete randomised block design was performed with 5 treatments and 6 replicates of 8 Hy-line layers (W-36) each from 32 to 44 weeks of age. The blocks were made to have a replicate of each treatment. The dietary treatments were consisted of 5 concentrations of digestible sulphur amino acid (DSAA) at 5.1, 5.6, 6.1, 6.6 and 7.1 (g/kg).

3. Egg production, egg mass, egg weight and feed conversion ratio (FCR) were significantly affected by an increase in DSAA intake. However, feed intake, egg component yield, Haugh unit, specific gravity, eggshell thickness, egg protein and dry matter (DM) were not altered by DSAA intake.

4. A significant increment in plasma high-density lipoprotein was concomitant with a reduction in low-density lipoprotein when DSAA intake was increased. However, triglyceride, cholesterol, uric acid and total protein in plasma were not affected by DSAA intake.

5. The DSAA requirements estimated by the linear broken-line model to optimise egg production, egg mass, egg weight and FCR were 678, 673, 641 and 656 mg/bird.d in the whole experimental period, respectively.

6. The DSAA requirement estimated by the quadratic broken-line model to optimise egg production, egg mass, egg weight and FCR were 4.71%, 7.87%, 8.73% and 7.62% higher than those estimated by linear broken-line fit model in the whole experimental period, respectively.

INTRODUCTION

Energy and protein account for about 85% of feed cost (Gunawardana *et al.*, 2008). Protein and amino acids play a vital role in poultry nutrition to improve egg production and animal welfare and lessen environmental nitrogen pollution (Ji *et al.*, 2014). Efficient utilisation of protein in diets depends on the amount, composition and digestibility of dietary amino acids (Dersjant-Li and Peisker, 2011). Diet formulation based on digestible amino acids not only reduces feed cost and supplies true requirements of birds, but also reduces environmental pollution due to decreased nitrogen excretion. Methionine is the first limiting amino acid in most practical diets of laying hens. Nowadays, addition of synthetic methionine to diets deficient in protein and amino acids is common (Harms and Russell, 1996; Bunchasak and Silapasorn, 2005; Rao *et al.*, 2011).

In order to maintain desirable protein utilisation in birds, synthetic amino acids such as methionine and lysine are added to the diet to balance amino acids. Novak *et al.* (2004) and Liu *et al.* (2005) concluded that addition of lysine and total sulphur amino acids (TSAAs) in diets can improve laying hens productivity, so determination of sulphur amino acid requirements is a vital key to appropriate nutrition for laying hens. According to NRC (1994), the daily requirement of methionine + cystine is 580 mg/bird.d. Advances in genetics, management and health of animals have contributed to laying hens that

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produce larger eggs with longer-lasting peaks of egg production (Solarte *et al.*, 2005). Numerous researchers have determined sulphur amino acid requirements in layers, but there are inconsistent results in the estimation of methionine and TSAA requirements (Schutte and Van Weerden, 1978; Schutte *et al.*, 1983, 1994; Schutte and Jong, 1994; Waldroup and Hellwig, 1995; Harms *et al.*, 1999; Bregendahl *et al.*, 2008). These experiments were conducted in different environmental conditions, with different basal diets, genetic lines, feed intakes, energy levels, several feed ingredients, nest spaces and using different hen ages and numerous estimation methods, all factors that can alter amino acid requirements (Baker and Han, 1994; Leeson *et al.*, 2001; Bregendahl *et al.*, 2008; Rao *et al.*, 2011). Ahmad *et al.* (1997) reported that ranges of 580–660 mg/bird.d TSAA do not affect productive performance. Although Schutte *et al.* (1994) estimated TSAA requirement to 740 mg/bird.d with 440 mg/bird.d methionine in laying hen based on the highest egg production. In addition, Harms and Russell (1996) reported the daily requirement of methionine based on broken-line regression analysis was in the range of 259.4–244.3 mg/bird.d during 20–42 weeks of age. Solarte *et al.* (2005) predicted the amount of methionine + cystine required to achieve optimal egg production, egg weight, egg mass and the best FCR were 658, 681, 664 and 666 mg/bird.d based on the polynomial equations in Leghorn laying hens during 18–35 weeks of age, respectively. Egg yolk and albumen DM and protein contents are modified in response to dietary amino acids and protein (Shafer *et al.*, 1996). In the experiment of Shafer *et al.* (1998) with an increment of methionine intake from 413 to 556 mg/bird.d, egg production, egg weight, albumen and yolk weight, DM, albumen and yolk protein were increased and their findings were in agreement with their previous study (Shafer *et al.*, 1996). Estimation of amino acid requirements depends on what production parameter is taken into consideration for optimisation (Novak *et al.*, 2004). The aim of this experiment was to evaluate the effects of different concentrations of digestible sulphur amino acids (DSAAs) on performance, egg quality and some blood metabolites in laying hens during 32–44 weeks of age.

MATERIALS AND METHODS

Birds and management

This experiment was conducted on the base of comprehensive guides of animal welfare adopted at Ferdowsi University of Mashhad, Mashhad, Iran. A total of 240 laying hens, Hy-line W-36, at 85% egg production (egg/hen/d) were used during the 32–44 weeks of age. Blocks were used to impede the effect of different cage locations and consisted of one replicate from each treatment. All replicate cages were located in an upper row with the exception of 5 replicate cages in the lower row of a California cage system design. Each cage of 40 × 40 × 39 cm (L × W × H) had 4 hens. All birds had free access to drinking water and feed. The house temperature was in the range of 16–18°C and the lighting programme was 16L:8D. Individual hen body weight was recorded at the beginning and the end of the experiment.

Diets and treatments

The experimental diet formulation was based on the recommendations of Hy-line layers (W-36) for the peak of egg production, with the exception of DSAA. The experiment was conducted in a complete randomised block design, with 5 treatments and 6 replicates of 8 birds each. The experimental treatments comprised mash diets containing 5 concentrations of DSAA, namely 5.1, 5.6, 6.1, 6.6 and 7.1 g/kg (Table 1). The diets were produced by taking a batch of the basal diet (lowest DSAA concentration), dividing it into 5 equal portions, adding DL-methionine (99% purity) on top of each portion and mixing to make the 5 diets. Protein, amino acids and digestible amino acids of feed ingredients were analysed using NIR by Evonik Degussa Co. (Tehran, Iran). The experimental period included a 2-week equilibration phase followed by a 12-week of data collection period, which was divided into three consecutive periods of 28-d periods.

Egg production and quality

Egg production (egg mass and hen d egg production) was daily recorded by registering the number and weight of eggs produced in each replicate unit. Feed consumption at the end of each 28-d period was calculated considering the difference of given and leftover feed. Three eggs were randomly picked up from each replicate unit in three consecutive days at the end of each period in order to assess egg quality. Egg width and length were measured by digital caliper (0.01 mm, model 1116–150 Insize Co, Suzhou, China). Egg index was computed by the following formula: egg index = (egg width (mm) /egg length (mm)) ×100.

After weighing each individual egg by digital electronic balance (0.001 g, Model GF 400, A&D Weighing, CA, USA), method of Shafer *et al.* (1998) was used to characterise the egg components. Egg yolk and albumen were separated and measured with a commercial handheld egg

Table 1. *Ingredients and nutrients composition of the basal diet[1] (g/kg)*

Ingredient	Basal diet
Maize meal (crude protein, CP = 74 g/kg)	591.5
Soybean meal (CP = 447 g/kg)	245.3
Calcium carbonate	98.2
Vegetable oil	36.1
Dicalcium phosphate	18.7
Sodium chloride	4.0
Vitamin premix[2]	2.5
Mineral premix[3]	2.5
DL-Met	0.7
L-Thr	0.2
Lys-HCl	0.3
Calculated analysis	
Metabolisable energy (ME) (MJ/kg)	12.1
CP	155.0
Ca	42.0
Available P	4.8
Na	1.8
Digestible met	3.1
Digestible met+cys	5.3
Digestible lys	7.5
Digestible threonine	5.3
Analysed composition	
CP	154.0
Digestible met	2.9
Digestible met+cys	5.1
Digestible lys	7.6
Digestible threonine	5.3

[1] The diets were provided in a way that a batch of basal diet (lowest DSAA concentration) was made and then divided into 5 equal portions, the DL-methionine was added on top of each portion and mixed to make the 5 dietary treatments.

[2]Provides per kg of diet: vitamin A (retinol), 2.4 mg; vitamin D3 (cholecalciferol), 75 μg; vitamin E (DL-α-tocopheryl acetate), 5 mg; vitamin K3 (menadione), 2.2 mg; vitamin B1 (thiamin), 1.5 mg; vitamin B2 (riboflavin), 4.0 mg; vitamin B3 (niacin), 8.0 mg; vitamin B5 (pantothenic acid), 35.0 mg; vitamin B6 (pyridoxine), 2.5 mg; vitamin B9 (folic acid), 0.5 mg; vitamin B12 (cyanocobalamin), 10 μg; vitamin H2 (biotin), 0.15 mg; choline, 468.7 mg.

[3]Provides (mg/kg of diet): Mn (manganese sulphate) 80.0, Fe (iron sulphate) 75.0, Zn (zinc sulphate) 64.0, Cu (copper sulphate) 6.0, Se (sodium selenite) 0.3.

separator and measured. With the use of paper napkins, adhering albumen was removed from the yolk and then the yolk was weighed. The eggshells were washed in water and dried for 48 h followed by weighing and measuring eggshell thickness. Eggshell thickness was measured at three different locations (top, middle and bottom of the egg) using a micrometer (0.001 mm, Digital micrometer, Model 293-240, Mitutoyo Co, Kanagawa, Japan) and the average of the three measurements was calculated as overall eggshell thickness. Albumen weight was calculated by subtracting the yolk and eggshell weight from the whole egg weight. Haugh unit was computed based on the following formula (Gunawardana *et al.*, 2009):

$$\text{Haugh units} = 100 \times \log [\text{albumen height (mm)} + 7.57 - (1.7 \times \text{weight (g)}^{0.037}].$$

These measurements were done less than 6 h after the egg collection. To assess DM and egg protein in the last day of the experiment, two eggs were collected randomly from each replicate unit. The egg yolk and albumen were separated using a commercial egg separator, and after mixing and homogenisation of yolk and albumen, 5–6 g of each sample was placed into aluminium dishes in an oven at a temperature of 105°C for 24 h. The samples were placed in a desiccator after removal from the oven and were weighed immediately (Wu *et al.*, 2005; Li *et al.*, 2013). Crude protein was analysed using the SD-Kjeldahl method (Aoac, 2010; method 992.15).

Blood collection and analysis

To assess the amount of blood lipid metabolites, uric acid and total protein, two birds were randomly selected from each replicate unit and 2.5 ml blood samples were taken, using 5 ml sterile syringes, from the brachial vein after 10 h of feed withdrawal at the end of the experiment (8 h in the dark period and 2 h in the light period) (Abdel-Wareth and Esmail, 2014). All plasma samples were separated and analysed by a multi-test automatic random-access system auto analyser (Cobas Bio, Roche, Basel, Switzerland).

Statistical analysis

The experiment was performed in a completely randomised block design. All data were analysed for normality using SAS software (SAS, 2003) through univariate plot normal procedure. Then data were subjected to analysis using SAS software general linear model (GLM) procedures with Tukey tests. The significant and non-significant differences were considered as $P < 0.05$ and $P \geq 0.05$, respectively. There were no significant differences among the blocks in any of the considered parameters in any of the experimental periods. The DSAA requirements for optimal egg production were determined by utilisation of analysed DSAA intakes using non-linear (NLIN) procedures, through linear and quadratic broken-line regression analysis as described by Robbins *et al.* (2006).

RESULTS AND DISCUSSION

Hen performance

The effects of DSAA intake on feed intake and nutrient intake are shown in Table 2. An increase in the concentration of DSAA did not have any significant impact on feed intake during the first, third and the whole experimental periods. Subsequently, no significant differences were

Table 2. *Effect of digestible sulphur amino acid (DSAA) concentration on productive performance of laying hens during periods 1 (32–36 weeks), 2 (36–40 weeks), 3 (40–44 weeks) and the whole experiment (overall: 32–44 weeks)*[1]

Analysed DSAA concentration (g/kg)	Feed consumption (g/b/d)				ME intake (kJ/b/d)				CP intake (g/b/d)				Analysed DSAA intake (mg/b/d)			
	Period 1	Period 2	Period 3	Overall	Period 1	Period 2	Period 3	Overall	Period 1	Period 2	Period 3	Overall	Period 1	Period 2	Period 3	Overall
5. 1	93.4	99.8[bc]	98.6	97.3	1133	1211[bc]	1196	1180	14.4	15.4[b]	15.2	15.0	479[e]	512[e]	506[e]	499[e]
5. 6	93.6	99.5[c]	101.2	98.1	1136	1207[c]	1228	1190	14.4	15.3[b]	15.6	15.1	527[d]	560[d]	570[d]	552[d]
6. 1	95.5	102.9[ab]	102.0	100.1	1159	1249[ab]	1238	1215	14.7	15.9[a]	15.7	15.4	585[c]	631[c]	625[c]	614[c]
6. 6	97.9	104.3[a]	103.0	101.7	1188	1266[a]	1250	1234	15.1	16.1[a]	15.9	15.7	649[b]	692[b]	683[b]	674[b]
7. 1	96.8	103.9[a]	104.6	101. 8	1175	1261[a]	1269	1235	14.9	16.0[a]	16.1	15.7	690[a]	741[a]	746[a]	726[a]
SEM	1.49	1.02	2.11	2.22	6.6	9.0	9.8	6.4	0.35	0.15	0.49	0.39	7.1	7.6	7.6	7.4
DSAA *P*-value	0.195	0.008	0.301	0.071	0.181	0.007	0.298	0.075	0.197	0.005	0.340	0.081	< 0.001	< 0.001	< 0.001	< 0.001

[a–d]Values in a column with no common superscript letter are significantly different ($P < 0.05$). [1]Data are means of 6 replications of 8 hens each.

observed in intake of any nutrient, with the exception of DSAA, in the periods. However, in the second period, feed intake was significantly affected by the DSAA concentration. An increase in dietary DSAA from 5.1 to 6.6 (g/kg) significantly enhanced feed consumption ($P < 0.05$) from 99.8 to 104.3 g/bird.d. Consequently, nutrient intake was significantly changed during this period. The DSAA consumption, according to the feed consumption data and amino acids analysis, for the birds fed diets containing 5.1, 5.6, 6.1, 6.6 and 7.1 (g/kg) DSAA were 499, 552, 614, 674 and 726 mg/bird.d in the whole experimental period, respectively. There are contradictory results on the effect of TSAA on feed consumption. For example, no significant effect in feed consumption was reported by Shafer *et al.* (1996). But Gomez and Angeles (2009), Bunchasak and Silapasorn (2005) and Solarte *et al.* (2005) observed significant impacts on feed intake with an increase of dietary methionine concentration.

Hen-d egg production was significantly improved with an increase in dietary DSAA concentration in each and the whole experimental periods (Table 2). Egg production was significantly increased from 0.83 to 0.92 (egg/hen d) with an increment in DSAA intake from 479 to 649 mg/bird.d in the first period. By an increase in the DSAA intake from 512 mg to 692 mg/bird.d, egg production significantly improved from 0.83 to 0.92 (egg/hen d) in the second period. Furthermore, egg production was significantly enhanced from 0.82 to 0.89 (egg/hen d) with an increment in the DSAA consumption from 506 to 631 mg/bird.d in the third period. An increase in the DSAA intake from 499 to 674 mg/bird.d significantly enhanced egg production from 0.83 to 0.91 (egg/hen d) in the whole experimental period. The DSAA requirements for optimisation of egg production were estimated based on broken-line models to 640 and 678 mg/bird.d for the third and the whole experimental periods, respectively. The quadratic broken-line model, however, estimated the DSAA requirements for the third and the whole experimental periods to be 695 and 710 mg/bird.d, respectively. Egg production responses to the dietary treatments in the first and the second periods did not fit either of these two models.

Egg production was significantly improved in response to an increment in sulphur amino acids consumption in numerous studies (Schutte and Van Weerden, 1978; Schutte *et al.*, 1994; Waldroup and Hellwig, 1995; Harms and Russell, 1996; Shafer *et al.*, 1998; Bunchasak and Silapasorn, 2005; Solarte *et al.*, 2005) but in others (Schutte *et al.*, 1983; Shafer *et al.*, 1996) no significant differences were observed by an increase in dietary TSAA. Sulphur amino acid requirement for Lohmann hens fed diets based on maize–soybean meal-sorghum with egg production of 0.87 (egg/hen d) was estimated to be 658 mg/bird.d based on a polynomial equation during 20–38 weeks of age (Solarte *et al.*, 2005). Their estimation was 2.9% and 7.3% lower than those estimated by linear and quadratic broken-line models in hens at 0.87% average egg production during 32–44 weeks of age in the present study, respectively.

Egg mass significantly increased in each period, as well as in the whole experimental period by an increase in dietary DSAA concentration (Table 2). Egg mass was significantly improved from 48.0 to 57.6 and 47.9 to 57.6 g by an increase in DSAA consumption from 479 to 649 and 512 to 692 mg/bird.d in the first and second periods, respectively. Egg mass was increased from 48.1 to 54.1 g with an increment in DSAA consumption from 506 to 625 mg/bird.d in the third period. In addition, egg mass was significantly improved from 48.0 to 57.2 g with the increase in DSAA intake from 499 to 674 mg/bird.d during the whole experimental period. Egg mass responses to dietary DSAA concentration were fitted to the models in the second, third and the whole experimental periods. The DSAA requirements to optimise egg mass based on linear broken-line models were 679, 655 and 673 mg/bird.d in the second, third and the whole experimental periods, respectively. These values were estimated by quadratic broken-line model to be 738, 708 and 726 mg/bird.d for the second, third and the whole experimental periods, respectively.

In numerous experiments, an increase in dietary sulphur amino acids resulted in an enhancement of egg mass (Schutte and Van Weerden, 1978; Schutte *et al.*, 1994; Waldroup and Hellwig, 1995; Harms and Russell, 1996; Solarte *et al.*, 2005; Gomez and Angeles, 2009). Solarte *et al.* (2005) reported a requirement of 664 mg/bird.d sulphur amino acid for Lohmann hens with an average of 51.18 g egg mass during 20–38 weeks of age by using polynomial equations, which is 1.33% and 8.53% lower than those estimated by linear and quadratic broken-line models in the present study.

With an increase in DSAA intake from 479 to 649 mg/bird.d, the FCR was significantly decreased from 1.95 to 1.70 in the first period. Furthermore, with an increase in DSAA consumption from 512 to 692 mg/bird.d, the FCR was significantly decreased from 2.08 to 1.81 in the second period. A significant reduction in FCR from 2.05 to 1.89 was observed with an increase in DSAA intake from 506 to 625 mg/bird.d in the third period (Table 2). The FCR was significantly decreased from 2.03 to 1.78 in response to an increment in DSAA intake from 499 to 674 mg/bird.d throughout the whole experimental period. The DSAA requirements to optimise FCR were estimated by linear broken-line fit models at 671, 643 and 656 mg/bird.d in the second, third and the whole experimental periods, respectively. The quadratic broken-line fit models estimated DSAA requirements at 724, 690 and 706 mg/bird.d in the second, third and the whole experimental periods, respectively. The FCR, with an increase in the concentration of dietary methionine, was decreased significantly in other studies (Schutte and Van Weerden, 1978; Schutte *et al.*, 1983, 1994; Waldroup and Hellwig, 1995; Bunchasak and Silapasorn, 2005; Solarte *et al.*, 2005; Gomez and Angeles, 2009). The estimated TSAA requirement for Lohmann hens for obtaining the best FCR was 665 mg/bird.d based on polynomial equation during 22–38 weeks (Solarte *et al.*, 2005).

The increase in DSAA consumption resulted in a significant improvement in body weight (Table 3). An increase in the DSAA intake from 499 to 674 mg/bird.d significantly increased the body weight gain from 10.20 to 17.58 g for the whole experimental period. This result is in agreement with other studies (Harms and Russell, 1996; Gomez and Angeles, 2009). Harms and Russell (1996) reported that an increase in the methionine concentration from 2.26 to 2.93 (g/kg) in a diet containing 11.78 MJ/kg energy helped to lower reduction in body weight from 213 to 121 g in concomitant with significant improvement in feed intake, egg production and egg mass in Hy-line W36 during 30–36 weeks of age. Furthermore, Gomez and Angeles (2009) observed an increase in TSAA concentration from 1.9 to 5.8 (g/kg) in a diet containing 12.13 MJ/kg energy and 140 g CP/kg which resulted in lesser reduction in body weight, concomitant with significant improvement in feed intake, egg production, egg mass and egg weight in Hy-line W36 during 100–106 weeks of age.

The discrepancy in results of different studies could be due to differences in strain, age, rate of egg production, basal diets, environmental conditions, type of amino acids expression (total or digestible) and estimation model.

Egg weight and quality

Egg weight was significantly affected by DSAA consumption throughout each and the whole experimental period (Table 4). Egg weight was significantly improved from 57.8 to 61.4 g with an increase in DSAA intake from 479 to 585 mg/bird.d in the first period. Egg weight was significantly increased from 57.6 to 61.6 g, with an increment in the DSAA consumption from 512 to 631 mg/bird.d in the second period. An increase in the DSAA consumption from 506 to 683 mg/bird.d significantly improved egg weight from 58.5 to 62.7 g in the third period. Egg weight was significantly improved from 58.0 to 62.6 g with an increase in DSAA intake from 499 to 674 mg/bird.d in the whole experimental period. The DSAA requirements to optimise egg weight based on linear broken-line fit model were 600, 652, 676 and 641 mg/bird.d in the first, second, third and the whole experimental periods, respectively. These values were estimated by quadratic broken-line fit model at 635, 725, 735 and 697 mg/bird.d in the first, second, third and the whole experimental periods, respectively.

Egg weight, in the numerous studies conducted in the first or second laying cycle, was affected by the various concentrations of dietary sulphur amino acids (Schutte and Van Weerden, 1978; Schutte *et al.*, 1994; Waldroup and Hellwig, 1995; Harms and Russell, 1996; Shafer *et al.*, 1996, 1998; Bunchasak and Silapasorn, 2005; Solarte *et al.*, 2005; Gomez and Angeles, 2009). Solarte *et al.* (2005) stated the requirement of 681 mg/bird.d for methionine + cystine based on polynomial regression in Lohmann hens at the average egg weight of 58.61 g. This amount is almost 6.24% higher and 2.29% lower than those estimated by linear and quadratic broken-line fit models for the average egg weight of 60.85 g in the present experiment, respectively.

Albumen, yolk and shell yield of eggs were not affected by DSAA intakes during each and the whole experimental period (Table 4). These results are similar to other reports (Shafer *et al.*, 1996, 1998; Gomez and Angeles, 2009). However, Bunchasak and Silapasorn (2005) observed significant changes in the percentage of albumen and yolk in hens during 24–44 weeks of age. Yolk yield was reduced from 25.01 to 23.76 (g/100 g egg) and albumen significantly increased from 64.95 to 66.19 (g/100 g egg) by an increase in methionine intake from 228 to 294 mg/bird.d. One possible reason for this inconsistent result may be due to the differences in the basal diets because they used low-protein diets with 140 g/kg protein. Also, in their experiment, the percentage of albumen and yolk did not change when hens were fed on diets containing 160 g/kg protein. Accordingly, it can be concluded that the amount of albumen and yolk for hens fed on low-protein diets is affected by the TSAA consumption (Novak *et al.*, 2006).

Egg index, Haugh unit, specific gravity and shell thickness were not affected by the DSAA intake in any or the whole experimental period (data not shown). The average of egg index, Haugh unit, specific gravity and shell thickness was 77.23, 88.69, 1.083 (g/cm^3) and 0.379 (mm). Solarte *et al.* (2005) reported that Haugh unit was significantly improved by an increase in the DSAA consumption from 444 to 630 mg/bird.d in Lohmann hens during 22–38 weeks of age. Their result contradicted the present findings, which may be due to the use of diets with very

Table 3. *Effect of digestible sulphur amino acid (DSAA) concentration on productive performance of laying hens during periods 1 (32–36 weeks), 2 (36–40 weeks), 3 (40–44 weeks) and the whole experiment (overall: 32–44 weeks)*[1]

Analysed DSAA concentration (g/kg)	Rate of lay (egg/hen d)				Egg mass (g egg /hen d)				FCR (feed/egg mass)				Body weight changes (g)
	Period 1	Period 2	Period 3	Overall	Period 1	Period 2	Period 3	Overall	Period 1	Period 2	Period 3	Overall	Overall
5.1	0.83^{c}	0.83^{b}	0.82^{b}	0.83^{b}	48.0^{b}	47.9^{b}	48.1^{b}	48.0^{c}	1.95^{a}	2.08^{a}	2.05^{a}	2.03^{a}	10.20^{bc}
5. 6	0.82^{c}	0.84^{b}	0.86^{b}	0.84^{b}	49.9^{b}	49.6^{b}	52.2^{b}	50.6^{bc}	1.87^{ab}	2.00^{ab}	1.94^{a}	1.94^{a}	9.50^{c}
6. 1	0.85^{bc}	0.88^{ab}	0.89^{a}	0.87^{ab}	51.1^{b}	54.2^{ab}	54.1^{a}	53.5^{ab}	1.83^{b}	1.90^{bc}	1.89^{b}	1.87^{b}	13.21^{b}
6. 6	0.92^{a}	0.92^{a}	0.90^{a}	0.91^{a}	57.6^{a}	57.6^{a}	56.5^{a}	57.2^{a}	1.70^{c}	1.81^{c}	1.82^{b}	1.78^{c}	17.58^{a}
7. 1	0.90^{ab}	0.89^{a}	0.89^{a}	0.89^{a}	55.6^{a}	55.8^{a}	55.5^{a}	55.6^{a}	1.74^{bc}	1.86^{c}	1.89^{b}	1.83^{bc}	18.09^{a}
SEM	0.842	0.759	0.854	0.848	0.52	0.52	0.53	0.52	0.017	0.018	0.018	0.018	0.203
DSAA P-values	0.011	0.031	0.312	0.004	0.001	0.028	0.025	0.002	< 0.001	0.002	0.002	< 0.001	0.030
Estimated requirement (mg/b/d)													
Linear broken line	–	–	640	678	–	679	655	673	–	671	643	656	–
Quadratic broken line	–	–	695	710	–	738	708	726	–	724	690	706	–

[a–d]Values in a column with no common superscript letter are significantly different ($P < 0.05$). [1]Data are means of 6 replications of 8 hens each.

Table 4. *Effect of digestible sulphur amino acid (DSAA) concentration on egg qualitative traits of laying hens during periods 1 (32–36 weeks), 2 (36–40 weeks), 3 (40–44 weeks) and the whole experiment (overall: 32–44 weeks)*[1]

Analysed DSAA concentration (g/kg)	Egg weight (g)				Albumen (g/100 g egg)				Yolk (g/100 g egg)				Shell (g/100 g egg)			
	Period 1	Period 2	Period 3	Overall	Period 1	Period 2	Period 3	Overall	Period 1	Period 2	Period 3	Overall	Period 1	Period 2	Period 3	Overall
5. 1	57.8^{c}	57.6^{b}	58.5^{c}	58.0^{b}	64.8	64.1	64.1	64.3	25.3	26.2	26.5	26.0	9.9	9.6	9.4	9.6
5. 6	60.5^{b}	59.1^{b}	$60.\ 8^{b}$	60.1^{b}	65.0	63.9	63.6	64.2	25.5	26.7	26.8	26.3	9.6	9.6	9. 6	9.6
6. 1	61.4^{a}	61.6^{a}	61.1^{ab}	61.4^{ab}	65.9	64.0	63.7	64.5	24.5	26.6	27.0	26.0	9.6	9.4	9.3	9.4
6. 6	62.6^{a}	62.4^{a}	62.7^{a}	62.6^{a}	66.3	63.3	63.7	64.4	24.3	26.9	26. 8	26.0	9.3	9.7	9.5	9.5
7. 1	61.9^{ab}	62.3^{a}	62.4^{a}	62.2^{ab}	64.6	64.3	63.8	64.2	25.8	26.2	26.7	26.2	9.5	9.5	9.5	9.5
SEM	0.57	0.61	0.60	0.59	0.63	0.62	0.62	0.62	0.34	0.26	0.26	0.25	0.12	0.12	0.09	0.09
DSAA *P*-values	< 0.001	< 0.001	0.039	0.030	0.345	0.589	0.972	0.962	0.437	0.753	0.976	0.963	0.647	0.849	0.989	0.960
Estimated requirement (mg/b/d)																
Linear broken line	600	652	676	641	–	–	–	–	–	–	–	–	–	–	–	–
Quadratic broken line	635	725	735	697	–	–	–	–	–	–	–	–	–	–	–	–

[a–d]Values in a column with no common superscript letter are significantly different ($P < 0.05$). [1]Data are means of 6 replications of 9 eggs each.

low sulphur amino acids in their trial compared to this experiment.

The DM and protein content of yolk, albumen and the whole egg were not affected by the DSAA intake in the whole experimental period (data not shown). The average of DM of yolk, albumen and liquid egg (total egg without shell) was 51.41, 11.84 and 20.99 g/100 g, respectively. The average protein content in yolk, albumen and liquid egg was 17.00, 10.87 and 11.42 g/100 g DM, respectively.

The present observations contradicted the findings of Shafer *et al.* (1998). The main reason for this inconsistency may be due to the differences in methionine intake. In the study of Shafer *et al.* (1998), the experimental diets had high concentrations of methionine, so that the amount of daily total methionine consumption ranged from 413 to 556 mg/bird.d, whereas in the present study the digestible methionine intake ranged from 300 to 420 mg/bird.d.

Blood metabolites

The effect of dietary DSAA on blood metabolites is shown in Table 5. With an increase in DSAA consumption from 499 to 674 mg/bird.d, plasma high-density lipoprotein (HDL) was significantly elevated from 58.1 to 59.2 mg/dl ($P < 0.05$). Furthermore, the level of low-density lipoprotein (LDL) was significantly reduced from 44.8 to 40.8 mg/dl. The balance of dietary amino acids and protein plays an important role in the regulation of cholesterol synthesis (Johnson *et al.*, 1958). There is a lack of information on the effect of sulphur amino acids on blood lipid metabolites of laying hens. It is reported that methionine caused a significant increase in plasma cholesterol, phosphatidylcholine and phosphatidylinositol ethanolamine and the ratio between these two in rats (Hirche *et al.*, 2006). In other cases, TSAA intake significantly increased cholesterol, HDL and blood phospholipids and reduced triglycerides (Sugiyama *et al.*, 1986), or increased HDL and reduced very low density lipoprotein (VLDL) (Oda, 2006). Furthermore, it is reported that cystine has less ability to increase plasma HDL compared to methionine (Oda *et al.*, 1986). In general, sulphur amino acids may increase expression of gene *apo A-I* and can cause an increment in synthesis of HDL in concomitant with increasing expression of *apo CYP7A1*, which can reduce LDL synthesis (Oda, 2006). Because of the diversity in the metabolism of cholesterol and lipoproteins in animal species, the necessity for more comprehensive studies on the effect of sulphur amino acids in cholesterol and lipoprotein metabolism in laying hens is highlighted. Triglyceride, cholesterol, uric acid and total protein in plasma were not significantly affected by graded DSAA intake.

The estimation of DSAA requirement depends on what production parameter is taken into consideration for optimisation. The summary of DSAA requirements for the optimisation of egg production, egg mass, egg weight and FCR estimated by linear and quadratic broken-line fit models is shown in Table 6. The predicted DSAA requirements in the linear broken-line model for optimal egg production, egg mass, egg weight and FCR were about 4.5%, 7.3%, 8.0% and 7.1% lower than those predicted by the quadratic broken-line model in the whole experimental period, respectively, although the optimum values estimated in linear broken-line models were slightly higher compared to those estimated by quadratic broken-line models for egg production (0.91 vs. 0.89 rate of lay), egg mass (56.4 vs. 56.3 g/bird.d) and FCR (1.80 vs. 1.81).

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Table 5. *Effect of digestible sulphur amino acid (DSAA) intake on blood metabolites of laying hens determined at the end of the experiment*[1]

Analysed DSAA concentration (g/kg)	Triglycerides (mg/dl)	Cholesterol (mg/dl)	HDL (mg/dl)	LDL (mg/dl)	Uric acid (mg/dl)	Total protein (g/dl)
5. 1	99	105	58.1[b]	44.8[a]	5.80	6.00
5. 6	98	103	58.5[b]	44.5[ab]	5.65	6.40
6. 1	94	117	59.0[b]	43.4[b]	5.65	6.73
6. 6	89	120	59.2[a]	40.8[c]	5.30	6.42
7. 1	92	121	59.9[a]	40.1[c]	5.60	6.55
SEM	4.3	4.5	0.55	2.75	0.118	0.522
DSAA *P*-values	0.101	0.098	0.036	0.046	0.091	0.646

[a–d]Values in a column with no common superscript letter are significantly different ($P < 0.05$).

[1] Data are means of 6 replications of two blood samples.

Table 6. *Summary of requirements of digestible sulphur amino acid (DSAA) intake for optimisation of egg production parameters estimated by linear and quadratic broken-line regression fit models in laying hens*

Models	Linear broken-line regression analysis						Quadratic broken-line regression analysis					
Parameter	Estimated DSAA requirement[4]	Lower 95% confidence levels	Upper 95% confidence levels	Predicted value[5]	R^2	R^2_{adj}	Estimated DSAA requirement[4]	Lower 95% confidence levels	Upper 95% confidence levels	Predicted value[5]	R^2	R^2_{adj}
Egg production[1] (*weeks*)												
40–44	640	587	768	89.58	0.97	0.95	695	615	775	89.55	0.97	0.93
32–44	678	580	761	91.14	0.94	0.92	710	632	788	89.57	0.87	0.84
Egg mass[2] (*weeks*)												
36–40	679	619	739	56.7	0.97	0.96	738	572	903	56.5	0.93	0.91
40–44	655	615	695	56.0	0.97	0.95	708	628	788	55.9	0.98	0.97
32–44	673	617	730	56.4	0.98	0.96	726	571	880	56.3	0.94	0.92
Egg weight (*weeks*)												
32–36	600	545	657	62.3	0.92	0.91	635	556	714	62.2	0.96	0.94
36–40	652	642	661	62.3	0.99	0.97	725	665	784	62.4	0.99	0.96
40–44	676	599	754	62.5	0.95	0.93	735	602	752	62.5	0.95	0.94
32–44	641	605	677	62.4	0.98	0.94	697	642	852	62.4	0.99	0.95
FCR[3] (*weeks*)												
36–40	671	619	722	1.83	0.97	0.96	724	597	830	1.84	0.96	0.93
40–44	643	579	707	1.85	0.92	0.89	690	549	890	1.85	0.92	0.88
32–44	656	594	717	1.80	0.96	0.93	706	566	845	1.81	0.94	0.90

[1]The egg production responses did not fit the linear and quadratic broken-lines models to estimate the DSAA requirements in first (32–36 weeks) and second (36–40 weeks) periods. [2]The egg mass responses did not fit the linear and quadratic broken-lines models to estimate the DSAA requirements in the first period (32–36 weeks). [3]The FCR responses did not fit the linear and quadratic broken-lines models to estimate the DSAA requirements FCR in the first period (32–36 weeks). [4]Expressed as mg/bird.d. [5]Predicted values by models to obtain optimum egg production (%); optimum egg mass (g/b/d); optimum egg weight (g) and optimum FCR (daily feed intake/egg mass).

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