SZENT ISTVÁN UNIVERSITY

ABSORPTION OF NATURAL CAROTENOIDS AND THEIR TISSUE DISTRIBUTION IN CHICKEN

THESIS OF PH.D. DISSERTATION

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Gödöllő

2011

Data of the doctoral school

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1. PRELIMINARIES AND OBJECTIVES

As a response to the technological requirements of industrialized livestock production man gradually got further and further away from the natural forms of livestock production during the past decades. Today however, natural methods in animal husbandry are gaining legitimacy. These methods are spreading mainly in farms with small number of livestock, but their impact is increasingly felt in industrialized livestock production as well. Re-introduction of almost forgotten farm animals (mangalica, native chicken species) into breeding processes, and the use of food products originating from these species bear potential benefits. Due to the natural methods of animal husbandry and the genetic background, their body composition is more favourable than in case of intensive farming, in many aspects (KISS et al, 2003).

The origin and type of natural additives used in feedstock cannot be irrelevant in implementing natural animal farming, especially when antioxidant properties of natural substances are known. Limitations in the use of artificial feedstock additives, therefore of dyes as well, can be projected. Colouring materials originating from natural sources may even remain applicable in human foodstuff (CFR 2002). This means that carotenoid-containing side products of canning processes of tomatoes, paprika, pumpkin are expected to be used in animal nutrition. (STRAND et al., 1998, MLODKOWSKI and KUCHTA 1998, LAI et al., 1996, HEIDLAS et al., 1996).

Research in carotenoids is gradually getting into the centre of attention, and has become one of mainstream areas. This is in close correlation with positive effects they have on both human and animal nutrition: antioxidant and cytoprotective effect, reducing LDL oxidation, reducing the risk of atherosclerosis, decreased chance of the development of prostate, breast and lung cancer, improved immune response and cell to cell communication, etc. Compounds responsible for these effects should be analyzed more thoroughly during the production and consumption of goods of animal origin.

During the research, absorption and tissue distribution of natural carotenoids were investigated in different age groups of chickens.

Following the accession to the EU, high level of protection of animals during production of goods of animal origin became accentuated. Therefore, besides rational nutrition, special attention is to be given to low stress feeding which means the lowest possible amount of antibiotics used, and giving priority to natural ingredients over synthetic feedstuff.

The relationship between carotene uptake and the risk of certain diseases raises many questions. Whether carotenoids are protective factors by themselves, or are they effective only in combination with other factors, as well as if the level of carotenoids detectable in foodstuff and/or in tissues are indicators of certain processes should be determined. The issue of absorption and

tissue distribution is particularly important, as the majority of carotenoids have their own activity, and only a small number of them express pro-vitamin effect. From among the numerous factors of carotenoid metabolism - which may vary even between individuals – the physical and chemical characteristics of certain carotenoids in foodstuff, the differences in absorption that is partly influenced by the above, and the possible transformation and/or packing into the transport molecular complex in the intestinal epithelial cells, is what later determines the tissue uptake.

The objectives of the analyses of carotenoid metabolism, a special field of fat metabolism in chicken, were the following:

- Understanding the impact of lycopene supplement on carotenoid and lipid metabolism of laying hens and its absorption into eggs.
- Studying absorption of lycopene, lutein and β-criptoxanthin supplement in newly hatched chicks during their 0-72 hours of age.
- Determination of lycopene, β-carotene and lutein absorption in laying hens.
- Studying the storing of natural carotenoids and their distribution in tissue.

2. MATERIALS AND METHODS

2.1. Examination of lycopene, lutein and β-criptoxanthin absorption in day old chicks during their 0-72 hours of age (1st experiment)

The experiment was carried out on 63 newly hatched chicks during their first 72 hours of life. To study the absorption, natural carotenoids were provided *per os* to the examined chicks.

The treated animals (63 pieces – 7 animals per group) received the following supplements: 5 ppm lutein [0,152 g Capsantal EBS 40 NT: tagetes (*Tagetes erecta*) extract with active substances of: 40 g/kg yellow xanthophyll: 0,8% ß-carotene (BC), 1,5% criptoxanthin (BCX), 82,0% trans-lutein (LU), 4,0% trans-zeaxanthin (ZX), 11,7% other carotenoids], and 5 ppm lycopene (0,012 g tomato paste). In all cases, the active substance was mixed with 125 µl of sunflower oil, and 500 µl of water. The emulsion prepared this way was given to the chicks through their beaks, using a pipette. Drinking water was provided continuously, while apart from the supplement, the animals could not get any other nutrition.

Treated chicks were sacrificed and bled in the 0th, 2nd, 4th, 6th, 8th, 24th, 36th, 48th, and 72nd hour (7 chicks each time) *lege artis.* Following the clotting of blod, serum was extracted via centrifugation, yolk sac and liver were prepared. The carotenoid profile of the samples was determined using HPLC technique by normal phase isocratic elution on Rocket Platinum column (Alltech Inc.) preceding direct extraction.

As addition to the above experiment, another 42 newly hatched chicks received 3,846 mg of Redivivo™ Lycopene 5,2% TG/P (DSM Nutritional Products) lycopene mixed with 0,5 ml water with a syringe directly into the yolk sac.

These chicks were sacrificed and bled in the 0th, 24th, 48th and 72nd hours (10 chicks on average each time), and blood, sac and liver samples were gathered. Carotenoid profile was determined using a HPLC by normal phase isocratic elution preceeded by direct extraction.

2.2. Absorption of lycopene, β-carotene and lutein in hens (2nd experiment)

Following a one-time *per os* administration of 20ppm carotenoid to laying hens (nearly one year old, already laying *bovans nera* hybrids*; 8* animals/treatment), the detectable kinetics of carotenoids (0-48 hours) was determined in the blood plasma using HPLC.

The supplement was calculated based on known average daily feed consumption and literature data: 20 ppm carotenoid/animal (357 mg lutein CWS/S-TG 5,6%; 192,3 mg β-carotene 10,4%; 384,6 mg Redivivo lycopene 5,2%; DSM Nutritional Products). The two combinations of

this were: the 20 ppm carotenoid/animal was made of 1/3 (6,6 ppm lutein + 6,6 ppm β-carotene + 6,6 ppm lycopene), and 3 x 20 ppm carotenoid/animal (20 ppm lutein + 20 ppm β-carotene + 20 ppm lycopene) was the dosage.

At the beginning of the experiment on sodium EDTA after the 0^{th}, 6^{th}, 12^{th}, 18^{th}, 24^{th}, 30^{th}, 36^{th}, 42^{nd}, and 48^{th} hours blood sampling was done (app. 3ml), while eggs were collected continuously. Carotenoid (lutein+zeaxanthin, β-cryptoxanthin, lycopene, β-carotene) and retinoids (retinol, retinyl palmitate) profile determination was carried out of the samples using HPLC technique.

To determine the antioxidant effect of carotenoids, iron reduction testing (FRAP) was carried out. Also, the Thiobarbituric Acid Reactive Substances (TBARS) level of red cell hemolysate was determined.

2.3. The impact of lycopene supplement on carotenoid and lipid metabolism of laying hens and its incorporation into eggs (3^{rd} experiment)

The experiment was carried out in a laying barn furnished with batteries for 14 000 hens. The barn was filled with *hy-line brown* laying hybrids. 6 hens were placed in one compartment. The hens were fed via an external feeding pan filled automatically using a feeding belt, and received water from an automated drinking pan. Feeding pans enough for 4 compartments were placed on the belts and were filled twice a day with the experimental feedstuff, meaning basically, that hens received the feedstuff *ad libitum*. The eggs of the seaparately fed hens could be collected separately using a collecting flap placed vertically onto the egg collecting belt. Samples gathered from hens other than the treated ones were used as control samples.

The experimental feed was prepared taking into account the composition of feedstuff given to hens at the laying barn, which did not contain yellow dye (Carophyll® Red – DSM Nutritional Products with canthaxanthine content). One of the laying groups consumed this feed (group LY_0). Another two experimental groups were given this same feed without canthaxanthine, except that the feed of the LY_5 group contained 5g, and the LY_{10} group's feed 10g of Redivivo™ Lycopene 5% TG/P (DSM Nutritional Products) per kg. Their feed contained 250mg and 500mg lycopene/kg respectively.

All three groups (overall 72 hens) received feed without Carophyll for the first two weeks. 10 eggs and 10 blood samples (5ml/hen) were collected from each group, including from the control group, at the end of the first two weeks. The groups (LY_0, LY_5 és LY_{10}) were given the experimental feed following the sampling. 10 eggs were collected from each group for 4 weeks at the end of each week of the treatment. Blood samples were taken from 10 hens from each group at the end of the fourth week.

The lipide concentration of egg yolk and serume, the colour intensity of the egg yolk, and the carotenoid and retinole concentration of blood was analyzed. Thiobarbituric Acid Reactive Substances (TBARS) level of egg yolk were determined to estimate the antioxidant effect of carotenoids.

2.4. Hatching technology

The chicks used in the 1st experiment originated from eggs bought from the Research field of the Animal Husbandry and Animal Research Institute in Gödöllő, all from the same stock (origin of parents: yellow Hungarian type). These eggs were hatched in ME3M (MainoEnrico-Ariano Di Maino Roberto C.S.N.C.) type incubator at the laboratory of the Faculty for Animal Physiology and Animal Health.

2.5. Biological Sampling

The preparation of samples varied according to the pressure/sensitivity of the chromatography system used for carotenoid and retinoid analysis [normal phase: a.) NP-HPLC, or reversed phase: b.) RP-HPLC].

Preparation of plasma/serume for carotenoid and retinoid analysis

Samples were stored on -20°C. After melting and vortex mixing of the samples, 250 µl was added to a 4ml centrifuge pipe, and 250 µl of 10% ascorbic acid and 500 µl ethanol was added to it. 1000 µl hexane was added after a 30-second vortex mixing, and continued the mixing for another 30 seconds. The clear supernatant after a 10-minute centrifugeing:

a.) was directly used for NP-HPLC analysis.

b.) 400µl was put into an Eppendorf tube with a pipette for RP-HPLC analysis, and was evaporated with N_2 stream (for app. 4-5 minutes). Prior to injecting onto the HPCL column, the residue was added to 100 µl ethanol-dioxane 1:1 mixture, and after a short vortex mixing, 150 µl of acetonitrile was added.

Preparation of liver for carotenoide and retinoide analysis

The liver was removed during the dissection of the bled chicken, weighed and stored on -20°C until use. After melting, 1,5ml of 10% ascorbic acid was added to 0,3 grams of the sample. During vortex mixing, it was homogenized with a glass rod, and 3ml of extraction mixture (10:6:6:7: hexane: acetone: abs. ethanol: toluol) was added. The clear supernatant after a 10-minute centrifugeing:

a.) was directly used for NP-HPLC analysis.

b.) 400µl was put into an Eppendorf tube with a pipette for RP-HPLC analysis, and was evaporated with N_2 stream (for app. 4-5 minutes). Prior to injecting onto the HPCL column, the

residue was added to 100 µl ethanol-dioxane 1:1 mixture, and after a short vortex mixing, 150 µl of acetonitrile was added.

Preparation of sac for carotenoide and retinoide analysis

The sac was removed during the dissection of the bled chicken, weighed and stored on -20°C until use. After melting, 1,5ml of 10% ascorbic acid was added to 0,3 grams of the sample. During vortex mixing, it was homogenized with a glass rod, and 3ml of extraction mixture (10:6:6:7: hexane: acetone: abs. ethanol: toluol) was added. The clear supernatant after a 10-minute centrifugeing:

a.) was directly used for NP-HPLC analysis.

b.) 400µl was put into an Eppendorf tube with a pipette for RP-HPLC analysis, and was evaporated with N_2 stream (for app. 4-5 minutes). Prior to injecting onto the HPCL column, the residue was added to 100 µl ethanol-dioxane 1:1 mixture, and after a short vortex mixing, 150 µl of acetonitrile was added.

2.6. Analitical methods

2.6.1. High Pressure Chromatography HPLC

2.6.1.1. Isocratic Normal Phase High-pressure Chromatography (NP-HPLC)

Carotenoide and retinoide composition of egg yolk, sac, liver and serum samples in the 1st experiment were measured using normal phase HPLC method (BIESALSKI et al., 1986; BÁRDOS, 1988; MATUS et al., 1994), defining characteristic carotenoide profiles.

The HPLC equipment comprised of the following units:

- High pressure pump (Jasco PU-980 Intelligent HPLC Pump, Japan)
- UV-Vis photometer with flow-through cuvette (ISCO V4 - Akron, USA)
- Rocket Platinum-CN analytical column with a front column (100A 3µ 53 mm x 7 mm) (Alltech, USA)
- Barspec Data Software Package System Version No. 1.26 (Barspec Systems, Inc. Izrael)

Separation parameters were as follows:

- The induced sample amounted to 20 ml,
- Eulating liquid: n-hexane/methanol (98:2). Both solvents are HPLC quality (Reanal, Budapest)
- The elution flow rate and pressure were 1,51 ml/min, 50 bar,
- Detection wavelength in case of retinoids was 325, carotenoids 450nm, and lycopene 505nm.

2.6.1.2. Isocratic Reversed Phase High-pressure Cromatography (RP-HPLC)

Carotenoide and retinoide composition of egg yolk, sac, liver and serum samples in the 2nd and 3rd experiment were measured using reversed phase HPLC method (KERTI ÉS BÁRDOS, 2006).

The clear extract (20 µl) gathered at the end of the sampling described in previous sections was injected onto C18 Rocket Platinum column (100A 3µ 53 mm x 7 mm) (Alltech, USA), that contained a PU-980 pump and UV-2077 4λ 4 channel detector (Jasco, Japan) connected to the HPLC system. The mobile phase was prepared with a 1% mixture of acetonitrile:tetrahydrofuran:methanol:ammonium acetate (684:220:68:28). The flow rate was 1ml/minute, and chromatogrammes to tocopherole were determined at 290nm, retinoide 325nm, carotenoids 450nm, and lycopene 505nm. The analyses were determined with a Chrompass program based on standards and concentrations.

2.6.2. Triglyceride determination

Triglyceride content of the serum was measured by enzyme colorimetric (GPO-POD) method (Reanal, Budapest). Triglyceride is hydrolized by the lypoproteine-lipase (LPL). The resulting glycerine is transformed into glycerine-3-phosphate by the glycerol kinase (GK) in the presence of ATP. The reaction of glycerine-3-phosphate oxygen and glycerine-3-phosphate-oxidase (GPO) results in the formation of hydrogene peroxide, which, in the presence of peroxidise enzyme (POD) and colouring agent gives a colored oxidation product, the amount of which is proportionate to the triglyceride content of the sample, and is measurable by photometry.

2.6.3. Determination of cholesterine/HDL cholesterine

Certain lipoids of the egg yolk and serum samples were also determined, and the cholesterine (CH) concentration using enzymatic (CHOD-POD) colorimetric test (Reanal, Budapest) was done, and following the light lypoproteid fraction precipitation the value of HDL cholesterine fraction was measured as well.

Following the cholesterine esterification with fatty acids, cleavage was done using cholesterol esterase. As a result of a catalytic reaction through cholesterine oxidase (CHOD) hydrogen peroxide was formed. This hydrogen peroxide, with the help of peroxidise enzyme (POD) and a coloring agent, formed a colored oxidation product determinable by photometre due to the fact that it was proportionate to its overall cholesterine content.

VLDL, LDL, kilomicron (portomicron) fractions were precipitated from the plasma using a phosphorus-wolfram-acid-magnesium reagent. The HDL fraction was contained in the supernatant, the cholesterine content of which was measured using Reanal cholesterine (CHOD-POD) diagnostic reagent set.

2.6.4. Measuring the Ferric Reducing Ability (FRAP)

At low pH values the ferry-tripiridil-triazine (Fe^{III} TPTZ) complex is reduced to ferro (Fe^{II} TPTZ) form in relation to the level of oxidant substances. The latter gives an intensive blue colouring measurable at 593 nm (BENZIE and STRAIN, 1996).

Stock solutions

A: 300 mmol/l acetate puffer (3,1 g Na-acetate $^{*}3H_2O$+16 ml acetic acid ad 1l)
B: 10 mmol/l TPTZ (2,4,6-tripyridyl-s-triazin) dissolved in 40 mmol/l HCl
C: 20 mmol/l ferri-chloride ($FeCl_3{}^{*}6H_2O$)
D: 1000 µmol/l ferro-sulphate ($FeSO_4{}^{*}7H_2O$)

Reagent solution

A+B+C (10:1:1) prepared daily according to number of samples

Standard solution

Gained by diluting the D stock solution

Concentration (µmol/l)	D-stock solution dilution
1000	Original D-stock solution
750	1,5 ml ad 2,0 ml
500	1,0 ml ad 2,0 ml
250	0,5 ml ad 2,0 ml
100	0,2 ml ad 2,0 ml

Implementation

20 µl sample (EDTA plasma) + 600 µl reagent ▲OD_{593nm} 20 sec.

2.6.5. TBARS measurement

TBARS levels (expressed in nmol MDA/g egg yolk) showing the oxidative stability of egg yolk was measured using the methodology described by DORMAN et al. (1995). 0,1 g of egg yolk sample was homogenized in 0,9ml of 1,15% KCl solution. To 0,5 ml of this homogenized substance, 1,5 ml of 20% acetic acid (pH 3,5), 1,5 ml of 0,8% tibarbitur acid solution (diluted in 1,1% Na-dodecyl-sulphate), and 0,5 ml of 0,8% distilled water was added. Following its homogenisation, it was incubated in water of 100°C for 30 minutes. After cooling, 5ml of butanol was added to each sample, and homogenized again, then centrifugated on 1500 rotation/minute for 10 minutes. Photometric measuring of the butanol phase was carried out with butanol blank solution on 532 nm. To express the measured values in MDA (malondialdehyde) concentration, standard measurement values of an ethanol solution of tetraetoxipropane (malonaldehid-bis-dimethyl-acetate) were used.

2.6.6. Measuring the coloring of egg yolk

2.6.6.1 Objective colour measurement

The objective measurement of surface colors is done by comparing the light returned to the sensor after white light was flashed for a moment, and the coordinate system compares the surface luminousity (L*), its system of red-green (a*) and yellow-blue (b*) with the CIELab points (Special Chem). The manual measuring device we used (Micromatch™; Sheen Ltd) compares the flashed surface's color to this same CIELab spectrum. This device is used in industrial production on surfaces, but for measuring and determining the color of food additives as well. Measurement with this device is quick and objective. Data can be stored and transferred to a computer. The measuring lense of the device was fastened to a broken egg yolk using a suitable dish into which egg yolk was placed. With a slight pressure, the whole surface of the measuring lense could be fixed to the yolk, therefore error factors (yolk breaking, light from sides) could be completely eliminated, providing thus objective coloration measuring (SZABÓ et al, 2007a).

The colour of egg yolk was determined using a manual reflexing target photometer (Micromatch™; Sheen Ltd.) with colorimetric method by comparison to the CIELab scale and a method adapted on site (SZABÓ ET AL., 2007a).

2.6.6.2. YCF

Comparison to the Yolk Colour Fan color spectrum is a semi-quantitative method. The color scale consists of 15 – easily recognized colors by humans – from ligh yellow to orange red. Its use is based on direct comparison with egg yolk, while values are given according to their correspondence with those of the scale.

2.7. Statistical evaluation

Results of the experiments were graded with ANOVA test (Turkey) Prism 5 program for Windows on $p<0,05$ level (GRAPHPAD SOFTWARE). During the evaluation of the analysis average calculation (x), deviation estimate (±s), and the calculation of significance level was also used. The latter, with the so called Student type two-sample t-test. Graphs were prepared using *Microsoft Excel 6.0.*

3. RESULTS

3.1. Examination of lycopene, lutein and β-criptoxanthin absorption in day old chicks during their 0-72 hours of age (1st experiment)

Lycopene was not detected in serum, or in the liver carotenoids. At the 24th hour the lycopene level in sac was decreasing, while in liver and the serum it still showed increase. The carotenoide profile development in the control chicks was similar to that of the experimental chicks. As the result of an intensive transport the bowel part that plays an active role in carotenoid absorption was tamponated with own sac material rich in carotenoids hindering thus the effective absorption of carotenoids administered *per os*.

Gradual increase was found in case of lycopene injected into sac, while no significant increase could be observed in blood and liver.

3.2. Absorption of lycopene, β-carotene and lutein in hens (2nd experiment)

Compared to the status before starting the experiment, both methods of administration resulted in significant increase in concentration of the administered carotenoid in case of all three analyzed carotenoids (lutein, β-carotene, lycopene). Based on sampling time the analysis showed that the presence of the analyzed carotenoids in the plasma were one-phased. The highest concentration level of carotenoids (β-karotin és likopin) was reached in the 6th hour following administration, and in the 12th hour in case of luteine. After reaching the highest value, and in lack of additional carotenoids administered, carotenoid concentration gradually dropped back to the original level.

Significant ($p<0,05$) deterioration from the other combined administration could be observed only in case of β-carotene and lycopene individual carotenoid supplements. Furthermore, joint administration of carotenoids, in smaller combined doses ($\sum$ 20 ppm: 6,6 ppm individually) resulted in higher increase in the concentration of all three carotenoids. Higher combined doses ($\sum$ 60 ppm: 20 ppm egyedileg) showed interaction in carotenoid concentrations of plasma.

It was concluded that the presence of non-polar carotenoid (lycopene) worsened the utilization of oxicarotenoids (luteine and zeaxanthine). Also, its antioxidant effect could not be verified via measuring ferrous reduction capacity, but could be detected via TBARS. Therefore, the TBARS levels of red blood cell hemolysate dropped in case of all three carotenoids.

3.3. The impact of lycopene supplement on carotenoid and lipid metabolism of laying hens and its incorporation into eggs (3rd experiment)

Significant drop in the overall carotenoid content of blood was observed during the two weeks of preliminary feeding. In this emptying stage the average CIELab a* values of egg yolk dropped to their quarter value (19,59→4,82) ($p<0,01$). During the next month of the experiment, with smaller variability, the same levels were measured in the LY_0 group. Colour intensity in both groups that received lycopene supplement (LY_5 és LY_{10}) by the week following the emptying stage (3rd week), reached the original value. The starting (original) colour intensity value was reached in the 4th week by the LY_{10} group, and by the 6th week by the LY_5 group.

Lycopene was detectable only in blood from groups that were given feed containing Redivivo™. Still, lycopene concentration of the blood samples of these two groups did not reflect double difference in dosage. Retinol concentrations of blood plasma remained on the same level throughout the experiment.

From week 3 to week 6, significant difference was observed in the objective color measurements (CIELab) and in the results of chemical analysis of the overall carotenoid content between the LY_0 group that received feed without lycopene, and the two groups that were given feed containing lycopene (LY_5 és LY_{10})

At the closure of the experiment the overall cholesterine concentration of the serum was significantly less ($p<0,05$) in the L_{10} group as compared to the other laying hens in the barn. The overall cholesterine measured in egg yolk, and the HDL-cholesterine and triglyceride concentrations in serums did not differ between the groups.

The antioxidant content of egg yolk is inversely proportional to TBRAS level. This is in close connection with the fact that the presence of lypidperoxidation processes in lack of carotenoids show an increase.

4. NEW SCIENTIFIC RESULTS

1. In the early post embrional age (newly hatched age, from hathing to app. the 3rd day, that is, to the 72nd hour) carotenoid absorption occurs exclusively from the sac, which shows that the missing lycopene carotenoids from the sac could not be detected in the carotenoide of blood fraction even in case of *per os* administration of lycopene.

2. In the case of laying hens, significant increase in all three analyzed carotenoid (lycopene, β-carotene, lutein) concentrations were detected, both when administered individually, and as a feed supplement, demonstrating their absorption. Still, despite of the same dosage (concentrations) differences were onserved in blood, that indicates there is interaction among carotenoids.

3. The level of increase of a given carotenoid concentration in blood is likely to be related to molecular structure (polarity, that is, solubility), its activities in the bowel, and then, its rate of incorporation into portomicron.

4. The two nonpolar carotenoids (lycopene, β-carotene) administered *per os* reached their highest concentration in blood in the 6th hour, while oxicarotenoid in the 12th hour following its absorption in laying hens. Due to its longer period of increase, lycopene worsens the utilization of oxicarotenoids.

5. Egg colouring ability of lycopene was proven both with objective colour measurements and with an analytical method (HPLC). Antioxidant effect was detectable via the TBARS method.

5. CONCLUSIONS AND SUGGESTIONS

5.1. Examination of lycopene, lutein and β-criptoxanthin absorption in day old chicks during their 0-72 hours of age (1st experiment)

The carotenoide dose administered during the experiment was calculated according to an already proven dose that has a coloring effect (LEESON és CASTON, 2004). The dose we administered was calculated based on the above, taking into consideration that it should be enough for a day. Examining standard carotenoids it can be concluded that lycopene with 80, beta carotene with 90, beta cryptoxanthine with 102-108 and luthein+zealathine with 180 sec. retention time can be registered on the chromatograms. From the administered carotenoids, lycopene is not found in the sac naturally. Based on the acquired data lycopene was not detectable in the serum, nor in the liver carotenoids of newly hatched chicks. Additionally, it is higly possible that the level of *per os* administered, otherwise major oxicarotenoids (luteine and zeaxanthine) did not increase in the serum nor in liver on a scale proportionate to the doses applied.

In newly hatched chicks, this is further complicated due to the absorption of the sac contents. The effect we observed indicates that there are two ways of material absorption from the sac after hatching. The content of the sac can be emptied to the intestines through the yolk stalk. This lasts about 72 hours. Later, the accumulated lymphatic cells partially close the passage. It took two hours for the vital dye initiated into a newly hatched chick on a no food diet to dye to bowel contents from the gizzard to the cloaca. That is, the sac entered the migdut through the yolk stalk where the antiperistaltic and peristaltic movements helped conduct the dyed material. (FEHÉR and GYŰRŰ, 1971). In hens (not chicken) transport from sac to blood prior to hatching and up to 72 hours afterwards was observed. The transport from the sac to the intestines took 120 hours after hatching (in chicken this was 72 hours). Secretion took place primarily in the ileum proximalis, and by a reflux, emptied into the small intestine and the gizzard. After hatching, the antiperistaltic movement increased, and secretion lasted longer than in chicken. In the distal small intestine sac was not utilized around hatching (NOY és SKLAN, 1998).

In our case, the first 24 hours of intensive sac→jejunum→duodenum→ventricle transport resulted in covering the section of the bowel responsible for carotenoids absorbtion with sac material rich in carotenoids. This prohibited the absorption of carotenoids applied *per os*.

Kinetics of lycopene injected directly into the sac of a newly hatched chick proves that it cannot be found in feedstuff, but when injected directly into the sac, it appears, but it does not when applied *per os*. The conclusion is that lycopene is a kind of indicator, and may even be a

carotenoide absorbtion model that could be detected in newly hatched chiks when injected into the sac. It reaches the egg in hen and there it has a biological activity, that is, it is a potentially enriched feedstuff. As lycopene is utilized this way, by depositioning in the egg, its importance is multiplied in regard to various food supplements, side products (grape tomatoes, tomato grit), and therefore has a value in human nutrition taken with egg, due to its previously mentioned beneficial effects.

5.2. Absorption of lycopene, β-carotene and lutein in hens (2nd experiment)

Compared to the status before starting the experiment, both methods of administration resulted in significant increase in concentration of the administered carotenoid in case of all three analyzed carotenoids (lutein, β-carotene, lycopene). Based on sampling time the analysis showed that the presence of the analyzed carotenoids in the plasma were one-phased. The highest concentration level of carotenoids (β-karotin és likopin) was reached in the 6th hour following administration, and in the 12th hour in case of luteine. After reaching the highest value, and in lack of additional carotenoids administered, carotenoid concentration gradually dropped back to its original level. Differences observed in accumulation and storing of various carotenoids confirms the presence of selective absorption and transporting processes dependant on polarity.

Not all observed carotenoids have provitamine activity. Our results confimed that only β-carotene supplementation was followed by significant increase in blood retinoid concentration. Retinyl palmitate concentrations in plasma varied both in β-carotene and combined application. As the result of metabolitic utilization and deposition the levels dropped back to starting levels after the end of the experiment.

Higher concentration increase of all three carotenoids was observed in case of joint application, in smaller combined doses (∑ 20 ppm: 6,6 ppm individually). Higher combined dosage (∑ 60 ppm: 20 ppm individually) resulted in interaction between plasma carotenoid concentrations. As the result of competition among carotenoids, saturation of binding and/or transport lypoproteine complexes may have occurred, and the presence of apolar carotenoide (lycopene) hindered the utilisation of oxicarotenoids (lutheine and zeaxanthine).

The analysis was planned out in order to gain detailed information on laying hens during a 48 hour sampling time and applying various amounts of different single dosage carotenoids.

Presumably, various interactions take place among different carotenoids during absorption, eg. racing when entering the mycella, competing for binding sites, carotenoid exchange among lypoproteins after absorbtion, and prohibition of carotenoide cleavage.

5.3. The impact of lycopene supplement on carotenoid and lipid metabolism of laying hens and its incorporation into eggs (3rd experiment)

5% lycopenecontent microcapsulated factory product was used in the study. (Redivivo™ Lycopene 5% TG/P, DSM Nutritional Products). This same product used in laying hens is said to be effective only if mixed with feedstuff after treatment with water hydrolysis on 60°C (OLSON et al., 2008). In the case of this study, the absorption proved to be effective without the above treatment, and the proof for that is the lycopene concentration measured in the experimental groups' blood and egg yolk, as well as the intensive yellow color of the egg yolks.

It can be senn from the egg yolk, that the applied concentration dyed the yolk not red, but intensive yellow, despite the fact that the absorptional spectrum of lycopene falls into the red colour.

The a* results of photometric color measurements carried out according to CIELab standards were found to be in significant correlation with results measured using photometry following a degreasing extraction, as well as with the more common, but susceptible to the evaluator's subjective color scale (Yolk Colour Fan, DSM Nutritional Products) (SZABÓ és mtsai., 2007). Colour intensity of egg yolk, and lycopene concentration levels reflected the higher, more significant level of differences in the applied feedstuff additives (LY_5 250 and LY_{10} 500 mg/feedstuff kg). This result is comparable to the carotenoide absorption and storing analyses described earlier, meaning that smaller the carotenoide concentration in the applied feed, the higher the absorption, and the effectiveness of utilization (feedstuff → blood, blood → skin) (NA et al., 2004). The LY_5 resulted in 4,35 ± 0,91, LY_{10} in 5,57 ± 1,38 μg/g lycopene concentration in egg. In an egg yolk of 17-19 g this corresponds to ~80-100 μg. Even though these are less than in tomato and tomato products (LUGASI et al., 2004), the possibility that lycopoene distributed in the biological medium of ehh yolk is more effective than that of plant origin cannot be excluded.

This lycopene containing food could be enriched with lycopene enriched egg production. Lycopene mixed with food for laying hen, apart from the foodstuff's own carotenoide content provided adequate coloring as required by the market even after two weeks of application. This feed supplement does not only meet the subjective consumer's demand, but has a functional effect as well. Consumers' requirements vary in terms of egg yolk coloring. The color of eggshell varies according to species/hybrids and does not show correlation with other internal values of the egg, like the yellow color of the egg yolk. Today, the most popular color of egg yolk falls between 11 and 12 of the internationally accepted colour scale (Yolk Colour Fan). This color can be achieved with 250 mg lycopene/kg concentration, therefore higher doses of lycopene application are not required.

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