Effects of Sunlight Illumination of Marigold Flower Meals on Egg Yolk Pigmentation

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The effect of sunlight illumination of marigold flower meal on the pigmentation of feed for laying hens and of their resulting egg yolks was evaluated by high-performance liquid chromatography and by subjective and light reflectance colorimetry techniques. Illumination did not have an effect on the identity of carotenoids in the flower meal. Interestingly, illuminated marigold flower meals yielded an oleoresin with a higher *all-trans*-zeaxanthin level, with respect to meals kept in darkness. A higher level of *all-trans*-zeaxanthin was also present in feed pigmented with that oleoresin. Additionally, this pigmented feed showed the best color values and resulted in egg yolks with good color; remarkably, these egg yolks had a low pigment content with respect to control treatments. Thus, sunlight illumination of marigold flower meal showed a positive effect on egg yolk color; the main factors contributing to this effect were not the identity or quantity of carotenoids in laying hen feed or egg yolks, but rather other as yet unidentified marigold component(s) which was (were) somehow influenced by sunlight illumination.

Keywords: *Lutein; carotenoids; color; in vivo pigmentation; marigold; Tagetes*

INTRODUCTION

Egg yolk color is one of the most important factors considered in the evaluation of egg quality in some parts of the world. Food color influences the desire or appetite for foods, and although a better color does not necessarily provide higher nutrition levels, a premium is paid to producers of better pigmented egg yolks. The color of table eggs (whole shelled eggs) should be from moderately light to deep yellow, depending on the geographical zone, while darker breaker eggs (eggs used by the food industry) are preferred for particular processes (De-Groote, 1970; Marusich and Baurenfeind, 1981; Karunajeeva et al., 1984). The natural color of egg yolks from free-laying hens is produced by xanthophylls (oxycarotenoids); due to their wide distribution in plants and bioavailability, lutein and zeaxanthin are the main xanthophylls in egg yolks (Karunajeeva et al., 1984).

At the industrial level, most egg producers use a high-energy concentrated feed having a low pigment content, which must be supplemented with natural pigments; this supplementation should take into account people's concerns and governmental regulations preventing addition of synthetic pigments. Some of the traditional sources of natural xanthophylls are yellow corn, alfalfa, and marigold (*Tagetes erecta*); the latter plant has its area of origin and greatest diversity in Mexico (Neher, 1968). Also, marigold is one of the most important sources of xanthophylls for the local poultry industry; its principal carotenoid is lutein (Avila et al., 1990; Delgado-Vargas and Paredes-López, 1996). About 6−8 mg of xanthophylls/kg of feed is required to reach an acceptable color in table eggs for Mexican consumers (Avila et al., 1990). Despite this small quantity of pigment, it represents a considerable cost for egg producers (Sunde, 1992; Williams, 1992), and various studies have been carried out seeking to improve pigmentation efficiency (Middendorf et al., 1980; Marusich and Baurenfeind, 1981). Middendorf et al. (1980) suggested that by controlling process and storage conditions, its availability is increased and undesirable changes are prevented in xanthophyll extracts of marigold.

Fletcher et al. (1977a,b) studied variations in egg yolk and broiler skin pigmentation by laying hens and broilers, respectively, housed on the floor and in cages. They found differences between housing conditions and suggested that better pigmented egg yolks and skin could be obtained when laying hens and broilers were exposed to higher light intensities. However, some of their experiments were not designed to observe the variability in egg yolk pigmentation as a result of light intensity. They suggested that the difference in pigmentation could be associated with chemical changes from lutein and zeaxanthin to redder pigments such as astaxanthin, although they did not provide evidence.

Fletcher (1981) evaluated the effect of light-exposed feed on broiler skin pigmentation. He fed exposed or nonexposed feed to birds reared in windowless or open-sided pens. He observed that broilers fed the light-exposed feed exhibited higher pigmentation in both housing conditions. Again, he proposed that differences

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could be due to chemical modifications of xanthophylls (oxidation, isomerization) resulting in a higher ratio of red to yellow carotenoids, and therefore certain conditions of feed storage could be beneficial to the pigmentation process. In that regard, several studies have shown metabolic routes in laying hens involving oxidation and reduction reactions that cause the transformation of xanthophylls provided in the feed. The new carotenoids with color tones different from those of their precursors could produce coloration on poultry products better than that expected from the carotenoids in the feed (Hencken, 1992).

Certainly, one of the major technical advances in the poultry industry is the introduction of high-performance liquid chromatography (HPLC) systems to evaluate pigmenting agents, feed, and poultry products, and this information has permitted the preparation of better balanced feeds (Williams, 1992). By using HPLC, it has been possible to elucidate metabolic routes involved in the processing of carotenoids fed to broilers and laying hens (Hamilton, 1992). Delgado-Vargas and Paredes-López (1997) used a C_{30} polymeric column to separate the lutein isomers of marigold oleoresin by HPLC; they showed that light exposure produces an increment in *all-trans*-lutein in a mixture of lutein isomers.

As mentioned above, interesting results have been observed for the effect of light exposure. However, none of the reported studies have monitored the variations in identity and/or quantity of xanthophylls along the pathway starting at the marigold meal and ending at the poultry product(s). Furthermore, it is clear that feed illumination could affect not only carotenoids but other feed components as well (e.g. vitamins, antioxidants, lipids, and proteins) (Tyczkowski et al., 1989; Avila et al., 1990). Thus, because the effect of light exposure of marigold carotenoids and other feed components has not been examined in detail with regard to the quality of poultry end products, we believe that in-depth and simpler studies are necessary to explain the improved pigmentation observed in poultry products caused by illumination.

Our objective in this work was to assess the effect of sunlight illumination of marigold flower meals on egg yolk pigmentation; thus, marigold flower meals were exposed to sunlight illumination or to darkness, and then the carotenoid composition was evaluated in the marigold flower meal, in the oleoresin derived from it, in the feed containing the oleoresin, and in the resulting egg yolks by HPLC. Also, the color of feed and egg yolks was measured by a subjective visual determination (Roche color fan) and/or light reflectance colorimetry.

MATERIALS AND METHODS

Fresh Materials. Fresh marigold (*T. erecta*) flowers were kindly supplied by Industrial Orgánica, S.A., Monterrey, Mexico. Flowers were separated from the receptacles, and petals were dehydrated in a vacuum oven (Forma Scientific, Marietta, OH) to 10% moisture content at 60 °C. Dehydrated marigold was milled through a 0.5 mm sieve by using a Brinkmann mill (Brinkmann, Westbury, NY), and all material was thoroughly mixed. This material is referred to as marigold flower meal (MFM). Commercial plastic bags (12.5 × 8 cm), transparent or black, were each filled with 5 g of MFM, evacuated with a stream of nitrogen, heat-sealed, and stored at 4 °C until experiments or analyses. Moisture content was determined in triplicate according to AOAC methodology (AOAC, 1984). Total pigment content was evaluated by HPLC (Delgado-Vargas and Paredes-López, 1996) in triplicate.

Illumination Treatments of Marigold Flower Meals. Plastic bags containing MFM were divided into three groups as follows: (1) MFM in transparent bags was illuminated in a compartment exposed to sunlight during 12 h daily for 60 days; treated material is hereafter referred to as outside light and temperature-treated MFM (OLT-MFM). (2) MFM in black bags was placed in a compartment covered from light and located right beside the compartment containing transparent bags; material resulting from this control treatment is then referred to as outside darkness and temperature-treated MFM (ODT-MFM). This system was designed in preliminary experiments to ensure that the temperatures in both compartments were the same. Both compartments were connected to compensate for any variations in temperature. Temperature monitoring was carried out during 1 week under conditions similar to those used in the formal experiment. All bags were placed in a single layer in the compartments, and MFM was distributed as a thin layer inside the bags. (3) Other bags containing MFM also used as controls were protected from light and placed at inside room temperature; material so treated is from now on referred to as inside darkness and temperature-treated MFM (IDT-MFM). Every day, all samples were stored overnight at 4 °C and covered from light. Temperatures and illuminances were measured every hour during the illumination period through the 60 days. Samples of treated MFM were analyzed in triplicate by HPLC to evaluate total pigment content (Delgado-Vargas and Paredes-López, 1996).

Oleoresins. OLT-, ODT-, and IDT-MFM were treated with 125 mg of butylated hydroxytoluene (BHT)/kg of meal and extracted three times by boiling with hexane: 100 g of meal with 250 mL of hexane; solids were filtered using vacuum. The extract was collected and concentrated in a rotavapor (Buchi, Laboratoriums-technik, Flawil, Switzerland) at 35 °C under vacuum. The residual solvent was eliminated in a vacuum oven for 9 h. All operations were carried out under dim light conditions, and the resulting oleoresins were stored in dark flasks under N_2 and at 4 °C until use. Resulting oleoresins are hereafter referred to as OLT-, ODT-, and IDT-oleoresin. Total pigment content was evaluated according to the AOAC methodology (AOAC, 1984) and HPLC (Delgado-Vargas and Paredes-López, 1996); all determinations were done in triplicate. Also, a sample of commercial marigold oleoresin (AVELUT, Pigmentos Vegetales del Centro S.A. de C.V., Guanajuato, Mexico) dissolved in vegetable oil was used as another control.

Feed Preparation and Analysis. The basal pigment-free diet (Table 1) was prepared in one large batch and separated into four lots. Each lot was pigmented with one of the oleoresins (OLT-, ODT-, or IDT-oleoresin or AVELUT) by adding 6 mg of xanthophylls/kg of feed. Added xanthophylls contained in the oleoresins were quantified according to the AOAC methodology (AOAC, 1984) as it is done in industry. Correspondingly, the resulting pigmented feeds are referred to as OLT-, ODT-, IDT-, and AVELUT-feed. These feeds were kept in cotton cloth bags at room temperature at the chicken house. Feed samples were collected weekly, placed in black plastic bags, evacuated with a stream of nitrogen, and stored at 4 °C until analyses. Color of feed samples was evaluated by reflectance colorimetry. Pigment analysis of the feeds was carried out by HPLC (Delgado-Vargas and Paredes-López, 1996). All measurements were done in triplicate.

Pigmentation Test on Laying Hens. We used a factorial design of four treatments, with three replicates with 10 birds each. One hundred and twenty Leghorn white laying hens (55 weeks old) were housed in individual wire cages with feed and water available ad libitum. The pigment-free basal diet (Table 1) was fed for 1 week; the pigmented feeds (OLT-, ODT-, IDT-, or AVELUT-feed) were provided during the following 4 weeks. Eggs were collected daily. Egg yolk color was evaluated weekly by subjective measurements from weeks 2−5 and by reflectance colorimetry for weeks 3 and 5. One day per week starting with week 2, egg yolks were collected and freeze-dried (Virtis Co., Gardiner, NY). Dried yolks were milled in a mortar, placed in black plastic bags under nitrogen, heat-

Table 1. Composition of Basal Diet for Laying Hens

	%
Ingredients	
sorghum	62.91
oil meal	21.60
calcium phosphate	1.36
calcium carbonate	10.30
vitamins premix[a]	0.25
minerals premix[a]	0.10
DL-methionine	0.15
vegetable oil	2.87
salt	0.40
antioxidant	0.02
choline chloride 60%	0.04
Calculated Analysis	
ME, kcal/kg	2780.00
CP, %	15.50
calcium, %	4.14
nonphytate phosphorus, %	0.34
lysine, %	0.80
methionine + cystine, %	0.62

[a] Supplied per kilogram of diet: (vitamins) A, 10000 IU; D_3, 2750 IU; E, 10 IU; K-3, 2.2 mg; B_1, 1.25 mg; B_2, 5.0 mg; B_6, 1.25 mg; niacin, 22.5 mg; calcium panthotenic acid, 15.0 mg; B_{12}, 10 μg; biotin, 50 μg; (minerals) Fe, 110 mg; Cu, 12 mg; Zn, 50 mg; Mn, 110 mg; I, 0.3 mg; Se, 0.1 mg.

sealed, and stored in the dark at 4 °C or analyzed immediately by HPLC (Delgado-Vargas and Paredes-López, 1996) in triplicate.

Color Determination. Feed color by reflectance colorimetry: 10 g of feed was placed in a new glass Petri dish (100 × 12 mm) and evenly distributed by pressing to form a uniform surface. The Petri dish was placed on the port of the Hunter-Lab D25 colorimeter (Hunter Lab Co., Reston, VA), and readings were taken at three positions, by rotating the Petri dish 90° each time. Samples were evaluated in triplicate.

Egg Yolk Color by Reflectance Colorimetry. Eggs were broken on a white surface leaving the yolks intact. The sensor of the Minolta Cr-200 colorimeter (Minolta Co., Ramsey, NJ) was placed in contact with the yolk surface, and readings were taken in triplicate.

The reflectance instruments determined three colorimetric parameters: lightness (L), redness (a), and yellowness (b). Using those values, hue ($H = a/b$) and chroma [$C = (a^2 + b^2)^{1/2}$] were calculated.

Egg Yolk Color by Subjective Evaluation. Whites were separated from yolks and discarded. Yolks were placed in a glass Petri dish (100 × 12 mm) over a white background, and color was determined visually by comparison with the Roche color fan 1993. All evaluations were carried out by a person that did not know the origin of samples and in triplicate.

Sample Extractions for HPLC Analyses. Carotenoids were extracted and processed from samples (50 mg of treated or untreated MFM, 50 mg of oleoresin, 4 g of feed, 2 g of freeze-dried yolk) by using an AOAC modified method (AOAC, 1984) as described by Delgado-Vargas and Paredes-López (1997), but some samples were extracted without saponification (i.e. without addition of 40% methanolic KOH).

HPLC Analyses. Pigment separations were carried out with a YMC PACK C_{30} column (YMC Inc., Morris Plains, NJ). The equipment was a Hewlett-Packard (HP) HPLC (Palo Alto, CA) model 1050 equipped with a quaternary pump solvent delivery and degasification system, autosampler, and diode array detector with a built-in Chemstation program in an HP-Vectra 486/66 XM computer. Detection was carried out at 450 nm, and UV spectra were obtained in the range 200−500 nm. Other HPLC conditions were as described by Delgado-Vargas and Paredes-López (1997).

Identification and Quantitation of Pigments. Peaks were identified by using authentic standards (*all-trans*-canthaxanthin, *all-trans*-lutein and its photoisomerization products, and *all-trans*-zeaxanthin) and their spectroscopic characteristics. *all-trans*-Lutein was quantified using an absolute calibration curve, while other lutein isomers were determined as *all-trans*-lutein equivalents. This information was previously described (Delgado-Vargas and Paredes-López, 1996, 1997).

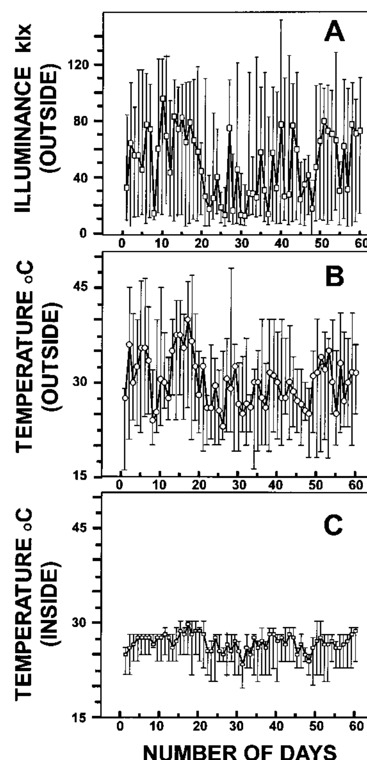

Figure 1. Temperature and illumination conditions observed during treatments: illumination at (A) outside conditions (OLT), (B) outside temperature (ODT and OLT), and (C) inside temperature (IDT). Median values are shown with their corresponding ranges.

Chemicals. Reagents were from Sigma (St. Louis, MO), and solvents were from Merck (Darmstadt, Germany). *all-trans*-Zeaxanthin was obtained from yellow maize seeds (Delgado-Vargas and Paredes-López, 1997).

Statistical Analyses. We used analysis of variance procedures (ANOVA) with Fisher's PLSD multiple-comparison tests (Statview Software, Abacus Concepts, 1991).

RESULTS

Illumination and Temperature Conditions. Figure 1 shows the conditions of sunlight illumination and temperature observed during treatments of MFM. For outside illumination (OLT), we observed median values around 60 klx and illuminances that commonly reached >100 klx (Figure 1A). In Figure 1B, it can be observed that median outside temperatures (OLT and ODT) were generally >30 °C and reached values up to 48 °C. On the other hand, median inside temperatures (IDT) were ∼28 °C, and the upper limit of the range was rarely >30 °C (Figure 1C).

Effect of Illumination and Temperature on Total Pigment Content. The total carotenoid level in OLT-MFM was significantly lower ($P < 0.00005$) than levels observed for ODT- and IDT-MFM. This was reflected in the corresponding oleoresins (Table 2). Total carotenoid contents in MFM or oleoresins were not statistically different ($\alpha = 0.05$) between ODT and IDT treatments. However, both ODT- and IDT-MFM showed a small decrease in relation to the carotenoid content of MFM stored at 4 °C (16451 ± 388 mg/kg) (Table 2).

Table 2. Pigment Content (Milligrams per Kilogram) of MFM Treated during 60 Days and of Their Corresponding Oleoresins[a]

treatment	MFM[b]	oleoresin
outside light and temp (OLT)	4699 ± 109	40947 ± 1873
outside darkness and temp (ODT)	15227 ± 86	120202 ± 7807
inside darkness and temp (IDT)	14924 ± 301	106717 ± 337

[a] All values were determined by HPLC and are the average ± standard error of three determinations. [b] Pigment content of sample stored in bags evacuated with a stream of nitrogen, in darkness and at 4 °C, was 16451 ± 388 mg/kg.

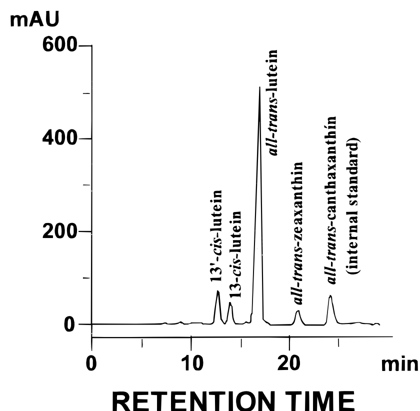

Figure 2. Typical HPLC chromatograms of marigold flower carotenoids. Detection was carried out at 450 nm.

Effect of Illumination on Carotenoid Identity. MFM (OLT-, ODT- and IDT-MFM), their corresponding oleoresins, and pigmented feeds showed similar HPLC chromatograms of carotenoids (Figure 2). Peak identities were established in previous papers (Delgado-Vargas and Paredes-López, 1996, 1997); lutein isomers and *all-trans*-zeaxanthin were the principal carotenoid components in all of the samples.

Carotenoid Composition of Oleoresins. Table 3 shows that OLT- and ODT-oleoresins had lower levels of *all-trans*-lutein and correspondingly higher percentages of 13′-*cis*- and 13-*cis*-lutein than IDT-oleoresin. Also, we observed a higher *all-trans*-zeaxanthin level in OLT-oleoresin than in the ODT- or OLT-oleoresins.

Carotenoid Composition of Feeds. Pigment content and composition in the feeds did not vary during the experiment (ANOVA by storage time, $\alpha = 0.05$). When OLT-, ODT-, and IDT-oleoresins and AVELUT were compared as pigmenting agents, ANOVA carried out on feed pigment contents showed significant differences for 13′-*cis*-lutein, 13-*cis*-lutein, and *all-trans*-zeaxanthin (all with $P < 0.00005$). Because the total pigment content showed little variation among pigmented feeds (Table 4), total pigment content was regarded as constant among the four pigmented feeds. This is important because further inferences are based on a similar level of administered pigment. It is worth mentioning that total pigment levels shown in Table 4 are ∼10.5 mg/kg as evaluated by HPLC; these levels are higher than the amount initially added to feeds (6 mg/kg) because the AOAC method (AOAC, 1984) gives an underestimation with respect to HPLC (Delgado-Vargas and Paredes-López, 1996). Table 4 also shows that 13′-*cis*-lutein was higher in OLT-feed. On the other hand, AVELUT-feed had the lowest levels of 13′-*cis*- and 13-*cis*-lutein and a relatively higher level of *all-trans*-lutein. It is noteworthy that the highest levels of *all-trans*-zeaxanthin were found in OLT- and AVELUT-feeds.

Color of Laying Hen Feeds. Regarding storage time as a factor, color of pigmented feeds was constant throughout the experiment (5 weeks) (ANOVA, $\alpha = 0.05$).

Table 5 shows the effect of oleoresin, used in pigmentation, on the color parameters of feeds. ANOVA revealed a significant effect on all color parameters ($P < 0.00005$). OLT- and AVELUT-feeds were darker because their lightness parameter (L) had lower values. AVELUT-feed showed the highest content of red components (highest a and lowest hue), while OLT-feed had the lowest. However, the content of yellow components for OLT (higher b and hue) was the best. Moreover, chroma showed that the best combination of red and yellow components was for OLT-feed (highest chroma), and this was the best pigmented feed (more yellow than the others).

Egg Yolk Color by Subjective Visual Evaluation. ANOVA showed that egg yolk color was constant from the second to the fifth weeks ($P = 0.07$). We observed a significant effect on the color of fresh egg yolks, caused by the oleoresin used for pigmentation ($P = 0.001$); besides, the variation coefficient was low (CV = 4.6%). Egg yolks with the best subjective color were obtained

Table 3. Pigment Composition (Percent) of Oleoresins Used in Feed Supplementation[a]

	compund			
oleoresin	13′-*cis*-lutein	13-*cis*-lutein	*all-trans*-lutein	*all-trans*-zeaxanthin
outside light and temp (OLT)	12.23 ± 0.07 b	7.85 ± 0.05 a	70.88 ± 0.11 a	9.04 ± 0.01 c
outside darkness and temp (ODT)	12.04 ± 0.02 b	8.42 ± 0.01 b	73.34 ± 0.16 b	6.06 ± 0.03 a
inside darkness and temp (IDT)	11.65 ± 0.03 a	7.91 ± 0.07 a	74.23 ± 0.07 c	6.23 ± 0.03 b
P value	0.0056	0.0062	0.0006	0.0001

[a] All values were determined as area percentage by HPLC and are the average ± standard error of three determinations. Different letters in the same column indicate significant differences.

Table 4. Pigment Content of Laying Hen Feeds (Milligrams per Kilogram)[a]

	compound				
feed	13′-*cis*-lutein	13-*cis*-lutein	*all-trans*-lutein	*all-trans*-zeaxanthin	total pigment
outside light and temp (OLT)	1.1010 d	0.7350 b	8.0330 ab	1.3260 b	11.1960 b
outside darkness and temp (ODT)	0.9400 b	0.6980 b	7.6140 a	1.0550 a	10.3060 a
inside darkness and temp (IDT)	1.0360 c	0.7390 b	8.2380 b	1.0880 a	11.1000 b
AVELUT	0.4930 a	0.3870 a	8.4100 b	1.2900 b	10.5820 ab

[a] All values were determined by HPLC and are the average of at least 15 determinations. Different letters in the same column indicate significant differences.

Table 5. Color Parameters of Laying Hen Feed by Light Reflectance Colorimetry[a]

feed	color parameter				
	L	*a*	*b*	hue (*b*/*a*)	chroma $(a^2 + b^2)^{1/2}$
outside light and temp (OLT)	55.0133 a	3.2467 a	16.7267 c	5.1801 d	17.0407 c
outside darkness and temp (ODT)	55.3067 b	3.3800 b	15.6533 a	4.6384 b	16.0147 a
inside darkness and temp (IDT)	55.5133 c	3.3600 b	16.1467 b	4.8201 c	16.4860 b
AVELUT	54.8733 a	3.5000 c	15.6200 a	4.4795 a	16.0113 a

[a] All values were determined with a Hunter Lab colorimeter and are the average of at least 15 determinations. Different letters in the same column indicate significant differences.

Table 6. Effects of Feeds on Color of Fresh Egg Yolks by Subjective Evaluation[a]

feed	Roche value
outside light and temp (OLT)	5.9 b
outside darkness and temp (ODT)	5.7 a
inside darkness and temp (IDT)	5.9 b
AVELUT	6.3 c

[a] All values were determined with a Roche color fan 1993 and are the average of at least 12 determinations. Different letters in the same column indicate significant differences.

with AVELUT-feed (Table 6). Interestingly, egg yolk colors obtained from OLT- and IDT-feeds were not significantly different.

Egg Yolk Color Measured by Reflectance Colorimetry. The color of fresh egg yolks showed significant differences for all color parameters ($P < 0.03$) as an effect of the different pigmented feeds (Table 7), and low variation coefficients (CV < 6%) were obtained. Egg yolk lightness (*L*) showed a small variation as a result of different pigmented feeds, which has been suggested to be an indication of a similar pigment content (Fletcher, 1981), and *L* values were high (toward the white side). Egg yolk redness (*a*) had negative values, which represent tendencies to greenness; AVELUT- and IDT-feeds produced the highest values. Egg yolk yellowness (*b*) was higher for OLT- and AVELUT-feeds, as could be expected from the pigment compositions of these feeds (Table 4). Hue showed the highest tendencies toward red tones (higher negative value) for egg yolks obtained with AVELUT-feed. Finally, chroma showed the best combination of color parameters (*a* and *b*) for egg yolks resulting from AVELUT- and OLT-feeds (higher chroma values).

Carotenoid Identities in Egg Yolks. Figure 3A shows the HPLC profile of unsaponified pigments of freeze-dried egg yolks. We observed two peaks with retention times of 4 (peak 1) and 16 min (peak 2). The UV−visible spectrum of peak 1 showed maxima at 228, 273, 330.9, 442, and 470 nm and one shoulder at 422 nm.

The chromatographic and spectroscopic characteristics of peak 2 (Figure 3A) correspond to those reported by Tyczkowski et al. (1986) for 3′-oxolutein. Those characteristics include the UV−visible spectrum of peak 2 corresponding to that of lutein; additionally, the retention time was different from those of lutein isomers (Figures 2 and 3B), and peak 2 disappeared after saponification.

Figure 3B shows an HPLC separation of a saponified extract of egg yolk pigments. The principal peaks correspond to those previously observed in MFM, oleoresins, and pigmented feeds (Figure 2). Peak 3 at 5.8 min (Figure 3B) appeared in all saponified extracts of egg yolk pigments. However, its UV−visible spectrum showed only one broad peak at 400 nm.

Carotenoid Contents in Egg Yolks. ANOVA of pigment contents in egg yolks using feeding time (4 weeks) as a factor did not reveal significant effects on contents of lutein isomers ($P > 0.14$). *all-trans*-Zeaxanthin in egg yolks showed a tendency to decrease toward the final weeks, although this was not reflected in the total pigment content ($P = 0.1048$). When pigmented feed (OLT-, ODT-, IDT-, and AVELUT-feed) was the factor analyzed, ANOVA of pigment contents in egg yolks showed a significant effect on all pigment contents ($P < 0.0303$). Table 8 shows the variation in pigment contents of egg yolks obtained from the different feeds. AVELUT-feed produced the highest contents for all pigments in egg yolks. Interestingly, OLT- and ODT-feeds produced the egg yolks with the lowest *all-trans*-lutein and total pigment contents, yet the OLT treatment led to egg yolk color similar to that obtained for the AVELUT egg yolks. Also, 13′-*cis*-lutein, 13-*cis*-lutein, and *all-trans*-zeaxanthin did not show significant variation among OLT, ODT, and IDT egg yolks.

DISCUSSION

From Figure 1, it is clear that we applied treatments on MFM involving extreme conditions which are not commonly used under normal storage practices. These conditions produced a high carotenoid degradation in OLT-MFM samples that resulted from the combination of illumination and temperature. Temperature alone did not have a significant effect on carotenoid contents of MFM or oleoresins, as indicated by the lack of significant differences ($\alpha = 0.05$) between ODT and IDT treatments (Table 2). We assumed that the carotenoid decrease was due to molecular breakdown by exposure to sunlight. Pesek and Warthesen (1988) observed that carotenoid degradation was accelerated by light and that high temperatures potentiated this effect. They also mentioned that the influence of temperature−light

Table 7. Effect of Feeds on Color Parameters of Fresh Egg Yolks by Light Reflectance Colorimetry[a]

feed	color parameter				
	L	*a*	*b*	hue (*b*/*a*)	chroma $(a^2 + b^2)^{1/2}$
outside light and temp (OLT)	67.0200 b	−6.0033 a	44.4717 b	−7.4173 b	44.8767 b
outside darkness and temp (ODT)	66.1000 ab	−6.1750 a	40.9300 a	−6.6493 c	41.3933 a
inside darkness and temp (IDT)	64.7100 a	−5.5650 b	41.7550 a	−7.6188 b	42.1283 a
AVELUT	65.7100 ab	−5.4417 b	46.3050 b	−8.5294 a	46.6233 b

[a] All values were determined with a Minolta Cr-200 colorimeter and are the average of six measurements. Different letters in the same column indicate singnificant differences.

Table 8. Effect of Feeds on Pigment Content of Egg Yolks (Milligrams per Kilogram)[a]

feed	compound				
	13′-*cis*-lutein	13-*cis*-lutein	*all-trans*-lutein	*all-trans*-zeaxanthin	total pigment
outside light and temp (OLT)	0.7900 a	0.4775 a	14.5438 a	1.9800 a	17.7913 a
outside darkness and temp (ODT)	0.7888 a	0.5000 ab	14.6688 a	2.0388 a	17.9963 a
inside darkness and temp (IDT)	0.8000 a	0.4313 a	15.8613 b	2.1100 a	19.2025 b
AVELUT	0.9963 b	0.5925 b	18.6713 c	2.5713 b	23.5488 c

[a] All values were determined by HPLC and are the average of at least 12 measurements. Different letters in the same column indicate significant differences.

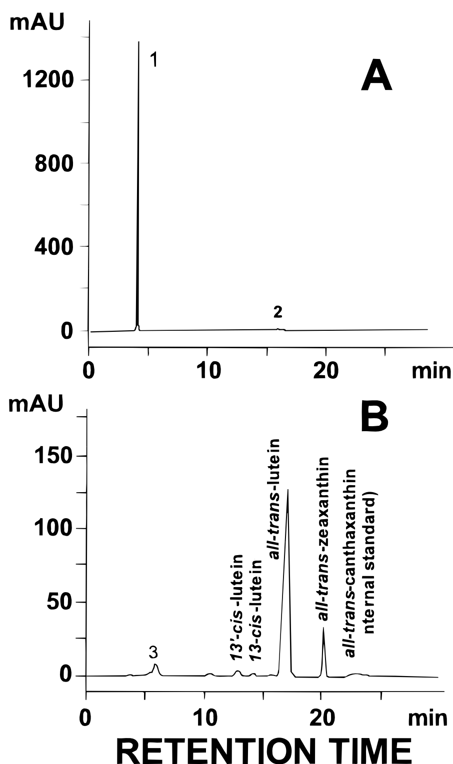

Figure 3. Typical carotenoid HPLC chromatogram of (A) unsaponified and (B) saponified extracts of egg yolks. The peak identities are discussed in the text. Detection was carried out at 450 nm.

combination on carotenoid composition was dependent on sample presentation (solid, liquid). The lack of effect of temperature was probably related to the absence of oxygen in our samples. Goldman et al. (1983) showed that rate of decolorization of β-carotene was favored by high quantities of oxygen. Chandler and Schwartz (1988) suggested that degradation of *all-trans-β*-carotene was associated with an oxidative stress mediated by oxygen.

The HPLC analysis showed lutein isomers and *all-trans*-zeaxanthin as the principal carotenoid components of all our samples; remarkably, illumination did not modify the identities of these compounds (Figure 2). The products of carotenoid degradation could not be detected at 450 nm, probably because the conjugated double-bond systems responsible for color were destroyed. Previously, Mínguez-Mosquera et al. (1994) reported that during processing of paprika there was a global synthesis and also a transformation of carotenoids, into other carotenoids, as an effect of illumination. Clearly, under our experimental conditions we did not observe these phenomena. Therefore, we do not support the proposal of light-promoted conversion of lutein and zeaxanthin to redder pigments (e.g. astaxanthin) that was suggested by Fletcher et al. (1977a). However, the occurrence of isomerization reactions was expected. These reactions have been studied in different systems with HPLC as a main tool. Pesek and Warthesen (1990) used pure *all-trans-β*-carotene and showed the appearance of *cis*-isomers by influence of storage under light or darkness conditions. Doering et al. (1995) worked with pure compounds to show that *cis*−*trans* rearrangements between β-carotene isomers could be possible at temperatures in the range of 37−69 °C. Chandler and Schwartz (1988) studied sweet potatoes under different food processes and concluded that isomerization was the principal chemical reaction with respect to carotenoids. In agreement with those works, we observed that carotenoid isomerization was the main transformation reaction that occurred as a result of treatments on marigold meal pigments, as described below.

In our experiments, we used esterified marigold flower oleoresins, as it is done in industry, because saponification does not improve digestibility in laying hens (Hencken, 1992). Moreover, Philip et al. (1976) showed that lutein fatty acid esters were better utilized by laying hens than pure lutein. In marigold oleoresins the principal isomers were 13′- and 13-*cis*-lutein (Table 3). This result was different from that reported by Pesek and Warthesen (1990), who demonstrated that light favored the formation of 9-*cis*- instead of 13-*cis*-β-carotene. Interestingly, the *all-trans*-zeaxanthin levels in OLT- and ODT-oleoresins suggest that illumination, but not temperature, was a main factor affecting *all-trans*-zeaxanthin level (Table 3). In feeds, **a** higher *all-trans*-zeaxanthin value was also observed in OLT-feed. AVELUT-feed (control) was the other product that showed a high level of *all-trans*-zeaxanthin. OLT- and AVELUT-feeds showed similar levels of *all-trans*-lutein, and this was higher than that observed in ODT-feed. In view of this information and of the better pigmenting efficiencies, in laying hens, reported for the *all-trans* forms of these carotenoids and especially the higher biological availability of *all-trans*-zeaxanthin, a superior pigmenting efficiency of egg yolks was expected for OLT- and AVELUT-feeds (Karunajeeva et al., 1984; Fletcher, 1992; Hencken, 1992).

Fletcher (1992) mentioned that it is necessary to carry out quality control on components and processes leading to a dietary formulation, to achieve certain product specifications more efficiently. Thus, we believe that pigmenting efficiency must be evaluated starting with the color of pigmented feeds, so as to have an idea of the efficiency of the pigmenting agent, establishing at the same time a quality control of the source of pigment and/or different pigment lots in an early step of the process. The color of OLT-feed was in agreement with the pigment composition of OLT-oleoresin (Table 4), resulting in the better pigmented feed. Unexpectedly, chroma in AVELUT-feed was lower than in OLT-feed, indicating a less yellow color.

In the evaluation of egg yolk color, we found that color

Table 9. Effect of Feeds on Variables of Production for Laying Hens[a]

feed	variable				
	egg production (%)	egg mass (g)	daily egg mass/hen (g)	daily feed consumption/hen (g)	feed conversion
outside light and temp (OLT)	73.23 ± 0.78	67.63 ± 0.81	49.7 ± 1.1	104.43 ± 5.68	2.1 ± 0.09
outside darkness and temp (ODT)	70.77 ± 2.26	67.37 ± 0.76	46.77 ± 2.83	113.3 ± 1.66	2.44 ± 0.18
inside darkness and temp (IDT)	73.03 ± 0.78	68.07 ± 0.59	47.67 ±1.73	108.77 ± 0.37	2.29 ± 0.08
AVELUT	70.37 ± 0.72	66.97 ± 1.13	47.47 ± 0.33	106.17 ± 3.88	2.23 ± 0.07
P value	0.3283	0.8266	0.6829	0.3743	0.2711
CV (%)	3.1	2.2	6.3	5.7	8.8

[a] Average of three determinations ± standard error.

reached a stable level by the second week. This result was in agreement with previous studies which established that yolk pigmentation is a rapid process in which a plateau is reached in a period of 9−11 days (Marusich and Baurenfeind, 1981; Avila et al., 1990). The similar subjective egg yolk color produced from OLT- and IDT-feeds (Table 6) was interesting in view of the drastic conditions applied in the OLT processing in contrast with the milder conditions used for the IDT treatment. We did not observe the same effect with egg yolks obtained from ODT-feed, in which MFM was exposed only to high temperatures; this indicates that temperature did not have a significant effect on pigmenting efficiency. These results were reaffirmed by measurements of egg yolk color by reflectance colorimetry (Table 7) that showed high colorations from the OLT treatment, which were similar to those obtained with the AVELUT treatment. Here, it is convenient to remember that feeds were prepared to contain the same carotenoid level, independent of the source of pigment (oleoresin) and of the carotenoid profile. A possible explanation for the differences observed in egg yolk colorations could involve differences in pigment deposition efficiency in egg yolks. Those differences could be caused by changes in minor components of the pigmented feeds, changes promoted by the illumination treatment applied on MFM. With OLT- and ODT-feeds, we observed a decrease in pigment deposition by effect of the extreme illumination and temperature conditions applied to MFM (Table 8). This phenomenon had not been previously reported and was not expected, considering the pigment compositions of the feeds (Table 4). Additionally, production variables for laying hens did not vary by the effects of the different feeds (Table 9). Thus, differences in pigment deposition and color in egg yolks cannot be explained in terms of those variables. This information indicated that illumination of MFM had a positive effect on the pigmentation process of egg yolks. As mentioned above, Fletcher et al. (1977a,b) and Fletcher (1981) found positive effects on pigmentation of egg yolks (1977a) and skins (1977b, 1981) when laying hen feed, broiler feed, or broilers were exposed to light. Fletcher et al. (1977b) used purity of excitation, color intensity, as a measurement of pigment concentration in skin and concluded that although higher pigmentation was obtained by exposure to light, pigment content did not vary. Fletcher (1981) mentioned that when the L value of broiler skins is constant among treatments, it is an indication that total pigments among treatments were about equal. Also, he reported that light-exposed feed resulted in significantly greater redness and yellowness on shank color. Moreover, Fletcher (1981) associated different magnitudes of change in redness and yellowness with modifications in the xanthophyll profile, which result from structural alteration or isomerization of yellow xanthophylls. Finally, this author assigned the observed pigmentation differences to variation in the xanthophyll pattern of feed, although he additionally commented that metabolism of birds could be also contributing to that variation. Our results clearly showed that positive effect of illumination cannot be assigned to the modification of the xanthophyll pattern in feed, and in particular we discarded the presence of redder xanthophylls in the feeds. This prompted us to look for some new xanthophyll(s) produced by the metabolism of laying hens, which could explain the better pigmenting efficiency of OLT-feed. In this respect, the last four wavelength maxima of peak 1 (273, 330.9, 442, and 470 nm) (Figure 3A) indicate the presence of lutein-containing compounds (free lutein, lutein esters) (Delgado-Vargas and Paredes-López, 1996, 1997). However, the retention time corresponds neither to free lutein isomers (Figure 2) nor to lutein isomer esters (which appear at ~45 min) (Delgado-Vargas and Paredes-López, 1996). Maxima at 228 and 273 nm might be due to protein, which could be obtained if lutein forms a lipoprotein complex; this putative complex was broken when the extract was saponified (Figure 3B). Our separation was not in agreement with previous papers (Philip et al., 1976; Schaeffer et al., 1988); Philip et al. (1976) showed that laying hens deacylate lutein derivatives prior to ova deposition. Thus, we assumed that free lutein isomers should be found in egg yolks. Schaeffer et al. (1988) studied the pigmentation of egg yolks resulting from a corn−alfalfa diet or a white corn-purified lutein diet. In their study, extraction of pigments from egg yolks was similar to that used in our work. However, they obtained an unsaponified extract that showed a clear separation between free lutein (>90%) and lutein esters. There are two main differences between their pigment extraction and separation processes and ours; first, they extracted pigments from fresh egg yolks, whereas we used freeze-dried egg yolks; second, they separated pigments with a normal-phase HPLC column, whereas we used a C_{30} reversed-phase column. The consistency in our separations of unsaponified and saponified extracts indicates that our separation was always working correctly. We repeatedly observed peak 1 (putative complex) in unsaponified extracts from freeze-dried egg yolks (Figure 3A) and well-separated lutein isomers in their corresponding saponified extracts (Figure 3B). Hence, a possible explanation for our results is that freeze-drying stabilized the complex of lutein with other lipids and/or proteins, and complex stability did not permit the separation of free lutein isomers during extraction with organic solvents. Peak 2 (Figure 3A) were tentatively identified as 3′-oxolutein. Tyczkowski et al. (1986) isolated 3′-oxolutein initially from egg yolks of laying hens; they showed that 3′-oxolutein was formed by

metabolic oxidation of lutein. However, we could not calculate the ratio of lutein to 3′-oxolutein, to compare with the previously reported ratio (Tyczkowski et al., 1986), because peak 2 was always very small and a free lutein peak was never present in unsaponified extracts. The carotenoids identified in the saponified pigment of egg yolk were the same as those of the other analyzed products (Figure 3B). However, peak 3 showed a spectrum (400 nm) that does not correspond to the typical carotenoid spectra (Goodwin and Britton, 1988), and further research is needed to elucidate the identity of peak 3. In summary, we did not observe any new carotenoid in unsaponified or saponified pigment extracts from egg yolks. Several authors have pointed out that carotenoids found in egg yolk come from the feed. Consequently, if provided carotenoids were deposited in egg yolk, the HPLC carotenoid profiles of pigment extracts from feeds and egg yolks must be very similar (Tyczkowski et al., 1986; Goodwin and Britton, 1988; Schaeffer et al., 1988). On this basis, we conclude that, although the OLT-feed showed an improved egg yolk pigmentation, this improvement cannot be assigned to the appearance of a new redder xanthophyll in egg yolk produced by effect of the metabolic processes in laying hens.

The high pigment deposition and the best efficiency in yolks pigmented with AVELUT may be explained by the fact that this oleoresin is dissolved in a vegetable oil (whose components are unknown to the authors); Karunajeewa et al. (1984) mentioned that inclusion of vegetable oils in laying hen diets improved egg yolk color, and Tyczkowski et al. (1989) showed that inclusion of lipids for the pigmentation of broiler skin produced better coloration.

We have shown above that despite the lower pigment contents in egg yolks obtained from the OLT-feed, egg yolks presented a good color as determined by a subjective evaluation, while reflectance measurements showed colors comparable to those obtained with AVELUT-feed. On the basis of these results, we conclude that sunlight illumination of MFM had a positive effect on egg yolk color, and the principal factors causing the positive effect were not related to the identity and/or quantity of xanthophylls present in laying hen feeds or egg yolks. We propose that sunlight illumination had an effect on other as yet unidentified components of marigold that contribute to pigmenting efficiency on egg yolks. Thus, future research must pay attention to the effect of illumination on minor components and how these components affect the pigmentation process. All of this information will permit recommendations for better storage conditions that lead to more efficient pigmentation with a consequent economical benefit.

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