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Chlorella vulgaris used to Colour Egg Yolk

L Gouveia,[a] V Veloso,[a] A Reis,[b] H Fernandes,[b] J Novais,[a] J Empis,[a]*

[a] Laboratório de Engenharia Bioquímica, Instituto Superior Técnico, 1096 Lisboa Codex, Portugal
[b] Departamento de Energias Renováveis, Instituto Nacional de Engenharia e Tecnologia Industrial, 1699 Lisboa Codex, Portugal

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Abstract: Dry biomass obtained from stressed cells of *Chlorella vulgaris* (rich in carotenoid pigments) was used as such in animal feed, instead of the commercial synthetic pigment. The *in vivo* effect of microalgal biomass as substitute pigment was ascertained with *Hisex* brown hens kept laying during 37 days under conventional conditions, and strongly suggested that yolk pigmentation was comparable to that obtained using commercial pigments, when comparable weight of colourant was formulated into the feed.

Key words: carotenoid pigment, canthaxanthin, lutein, egg yolk, microalgal-biomass, feed, *Chlorella vulgaris*.

INTRODUCTION

Egg yolk colour is an important characteristic when estimating the quality of egg. Besides the usual standards of measurement such as shape and weight, firmness of the shell, freshness and structure of egg white and yolk, the colour of the latter is the decisive criterion in the evaluation of standardised egg quality (Vuilleumier 1968).

As shown by several workers, animals do not have the ability to synthesise carotenoids but they are, however, able to metabolise them. In general, birds chiefly absorb oxycarotenoids. β-Carotene may be converted into mono- and diketo-derivatives, primarily in the liver, while the more highly oxidised xanthophylls are not modified until they reach the feather follicle (Latscha 1990).

Carotenes are found almost exclusively in the retina and liver of birds, and, in small quantities, in body fat, while oxycarotenoids accumulate at various sites, particularly the skin, plumage, fatty tissue, and above all the egg yolk. In the skin, they are mainly deposited in the ester form. Free carotenoids, in contrast, are predominant in the egg yolk, and various carotenoproteins of hydroxy- and ketocarotenoids, in the plumage (Latscha 1990).

The problem of the fixation of colour pigment in egg yolk is dealt in the literature (Williams *et al* 1962). Thus, egg yolk pigment was unsaponifiable, though soluble only in lipidic solvents, and was given the name lutein. Subsequently, it was demonstrated that the nature and amount of carotenoid present in egg yolk depended on the carotenoids fed to the hen, that the pigment of the egg yolk contained zeaxanthin in addition to lutein, and therefore that it was not a single carotenoid as previously thought. Lutein and zeaxanthin were then bound to be more efficiently deposited in the egg yolk than cryptoxanthin and carotene. In 1938 Brown and in 1946 Mann (reported by Williams *et al* 1962), demonstrated that, in general, laying hens will transfer to the egg yolk at least part of the carotenoids consumed and various feed ingredients were found inevitably to affect the colour of yolk (Madiedo and Sunde 1964).

Feed ingredients which have been used for a long time as sources of pigmentation for egg yolk are dehydrated alfalfa meal and yellow corn (Marusich *et al* 1960; Brambila *et al* 1962). However, today, feed mills use low-cost raw materials to provide high-energy rations and control the pigment content by appropriate supplementation. Petals of Aztec marigold (*Tagetes erecta*) have been reported to be very effective as yolk pigmenting agents as has synthetic canthaxanthin (Madiedo and Sunde 1964).

Carotenoids are easily degraded by oxidation and should therefore be provided in encapsulated form. Dry microalgal biomass, conceptually a possible way of conveying carotenoids in an encapsulated form, does not always prove to be an efficient pigmenting agent, due to

* To whom correspondence should be addressed.

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low percentage of utilisable carotenoid (Sommer *et al* 1991).

In an early experiment (Williams *et al* 1962) small amounts of ingested carotenoids were shown to be deposited in the egg yolk within 48 h after the hens were fed a carotenoid-rich diet, and the carotenoid content of the egg yolk increased steadily until the maximum concentration was reached somewhere between the 8th and 10th day.

The experiments reported in this work show the effects of carotenoids present in microalgal biomass upon pigmentation of egg yolk, when this was included in feed, and compares it with the pigmentation obtained when commercially available synthetic pigments were used.

MATERIALS AND METHODS

Microalgae

The microalga *Chlorella vulgaris* was isolated from a *Botryococcus braunii* UC 58 culture where it appeared as a contaminant. The growth medium was Sorokin and Krauss (Richmond 1986) and contained (g litre^{-1}) KNO_3, 1·250; KH_2PO_4, 1·250; $MgSO_4 \cdot 7H_2O$, 1·000; $CaCl_2$, 0·084; H_3BO_3, 0·111; $FeSO_4 \cdot 7H_2O$, 0·050; $ZnSO_4 \cdot 7H_2O$, 0·088; $MnCl_2 \cdot 4H_2O$, 0·014; MoO_3, 0·007; $CuSO_4 \cdot 5H_2O$, 0·016; $Co(NO_3)_2 \cdot 6H_2O$, 0·005; Fe·EDTA, 0·5; pH 6·8.

After the onset of saline stress (NaCl 30 g kg^{-1} solution), nitrogen depletion and increase of luminosity, the orange biomass was freeze-dried and used as such, mixing it into the compound feed. (Gouveia *et al* unpublished results; Japanese Patent 1989).

Animals

A total of 20 *Hisex* brown hens of a commercially available strain, 57 weeks old at the beginning of the experiment, were maintained in laying batteries. They were kept in groups of five (Plate 1), and fed daily 120 g of a base diet for which ingredients and composition are shown in Table 1.

Diet

During the first period—day 1 to day 13—the hens were depleted of colouring ingredients supplies, by feeding them a low-carotenoid base diet.

During the second period—day 14 to day 26—this same diet, supplemented with enough microalgal biomass to attain a level equivalent to that of synthetic pigment present in the corresponding commercially produced feed, was used. For this purpose, the quantity of microalgal biomass to be added to the feed was determined by a trial and error procedure which consisted of comparing the visible spectral absorbance of the acetone extracts of the commercial diet with extracts of diets resulting from the addition of various quantities of microalga to the base feed. When the appropriate level of addition had been determined, a total batch of microalga-enriched feed was prepared from identical starting materials (base diet) and used throughout the whole experiment.

TABLE 1
Composition, analyses (g kg^{-1} as is) and additives of basal diet (industrial feed)

Proximate composition	
Moisture	113
Total protein	170
Total fat	70
Total cellulose	50
Ash	140
Total carbohydrates[a]	457
Analyses	
Calcium	37
Phosphorus	6
Methionine	4
Additives (kg^{-1})	
Vitamin A	10 000 IU
Vitamin D_3	2900 IU
Vitamin E	5 mg
Canthaxanthin	14·7 mg

[a] By difference.

During the third period—day 27 to day 37—the feed used was again the low-carotenoid diet which had been used throughout the first period.

Pigment concentrations in the diets were checked.

The commercial base diet used included cereals and other starchy products, meals (sunflower, soya) and other protein-rich ingredients of vegetable origin, by-products from the cereal transformation industry, animal protein, oil and fat, molasses, mineral substances and aminoacids. These ingredients had been formulated into a base diet, with the composition given in Table 1.

Eggs were picked daily, weighed, their shells broken and their colour evaluated by scoring against the Roche Yolk Colour Fan (90310, 1989). Finally, they were analysed for carotenoid content.

Analysis

Carotenoids of microalga

The carotenoids from the alga were extracted with acetone after breaking the cell walls through mechanical and thermal shocks (about 100 mg of alga with glass beads were put alternately in an ice bath and in a vortex). The separation of carotenoids, for further iden-

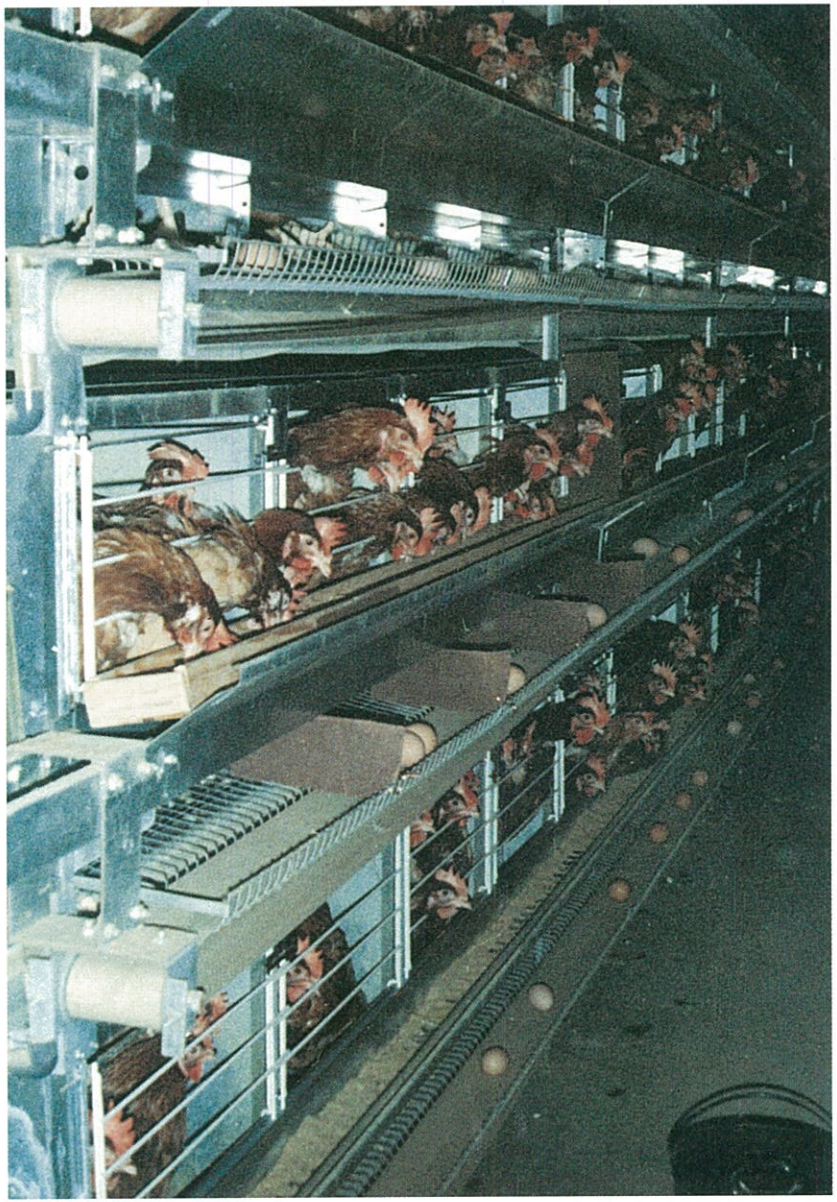

Plate 1. Poultry aviary showing the four cages.

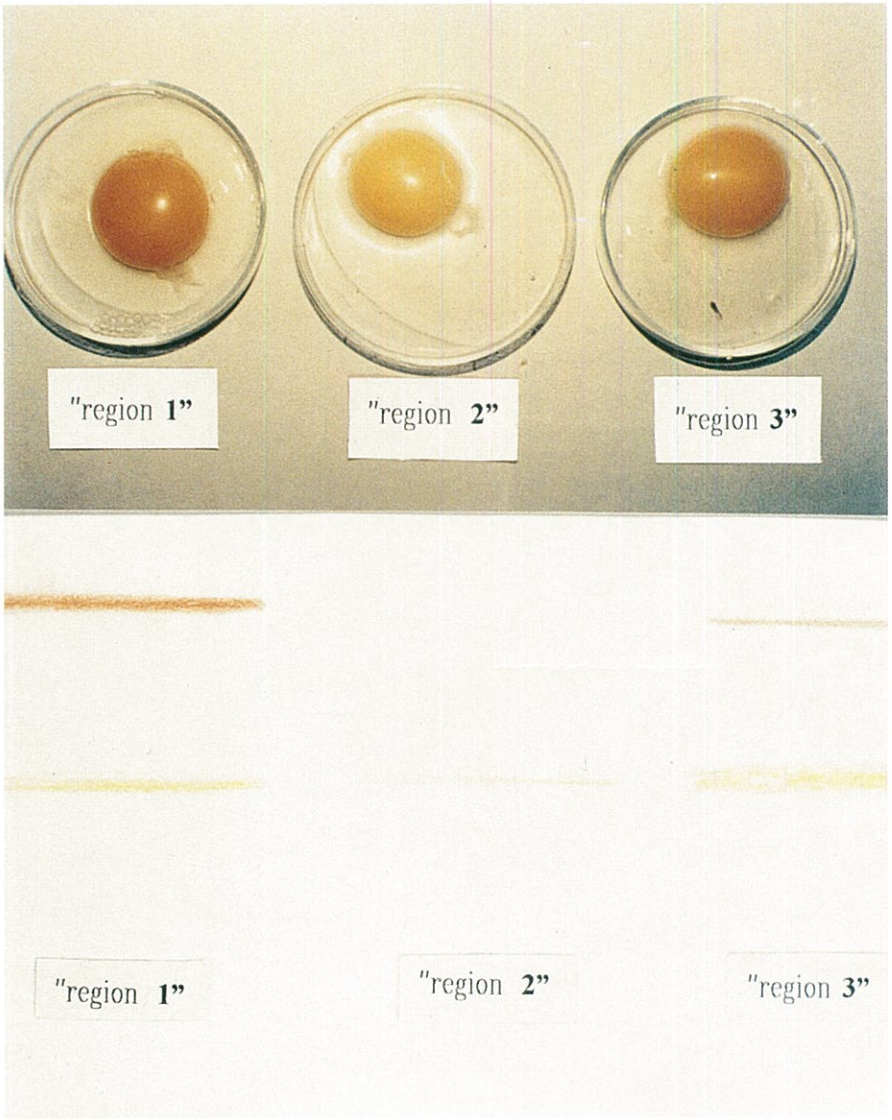

Plate 2. Egg yolks from 'regions 1, 2 and 3' and the corresponding TLC.

TABLE 2
Microalgal carotenoid composition

Component	*mg g*$^{-1}$
Lutein	3
β-Carotene	12
Canthaxanthin	362
Astaxanthin	550
Unidentified pigment	73

tification and quantification, was carried out by thin-layer chromatography (TLC) using plates of silica gel (Merck) of 0·25 mm thickness and an eluent mixture of petroleum-ether (bp 80–100°C)/acetone/diethylamine (10 : 4 : 1, v/v/v). The identification of carotenoids was achieved by the colour, R_f and wavelength at maximum absorbance. The quantification was made by spectrophotometry (Hitachi-2000) according to Choubert and Storebakken (1989), and expressed on a dry basis using extinction coefficients ($E_1^{1\%}{}_{cm}$) of 2200 for canthaxanthin and 2340 for lutein, at their absorption maximum in acetone (Foppen 1971).

Carotenoids of egg yolk

Egg yolk was carefully separated from the albumen, weighed and analysed. For this purpose, approximately 10 ml acetone were added to 1 g yolk and, after vigorous homogenisation, the mixture was centrifuged and the resulting pellet reextracted until white. The acetone extracts were combined (30 ml), the carotenoids transferred to diethyl ether (50 ml) by salting out with 100 g litre^{-1} NaCl solution (20 ml) and the extracts filtered and evaporated to dryness in a rotary evaporator. Absorption was measured at 460 nm in a spectrophotometer using 2 ml acetone solutions. These extracts were also subject to chromatography upon a thin layer of silica gel as described for algal carotenoid analysis.

Total extracts and each of the bands obtained in TLC were subjected to HPLC (Perkin Elmer) with a μ-Bondapak C18 reversed-phase and a detector UV–Vis Waters 481 (λ = 460 nm), using acetonitrile/methanol/water (75 : 25 : 2, v/v/v) as solvent. Methanol and acetonitrile were HPLC grade reagents used without further purification other than filtration and degassing. The pigments were eluted over 30 min with a flow rate 1 ml min^{-1}. Calibration curves were obtained using solutions of standard canthaxanthin and lutein in acetone whose concentrations were previously determined by spectrophotometry.

RESULTS

Microalgal biomass carotenoids consisted of a complex mixture, the main components being canthaxanthin, astaxanthin and its esters, lutein and β-carotene (Table 2).

The broad trends of data obtained in this experiment are detailed in Table 3.

During the first period (base diet without added pigments) the egg yolks show a progressive loss of colour, from Roche grades 13 to 14, 'region 1', to grades 5 to 6, 'region 2', corresponding to a decrease from a mean level of 14·6 mg total carotenoid to 2·1 mg total carotenoid g^{-1} fresh yolk (Table 4).

In the second period (microalgal biomass equivalent in total carotenoids to that present in the commercial diet) egg yolk pigmentation increased to Roche grades 11 to 12, 'region 3', on day 18 (10·8 mg total carotenoid g^{-1} fresh yolk) (Table 4) and this was the maximum pigmentation observed even after increasing further the level of microalgal pigment.

In the third period (same diet as in first period), the decrease of yolk pigmentation was similar to that observed in the first period.

Egg yolks obtained in 'regions 1, 2 and 3' are shown in Plate 2 for comparison.

Each group of chickens yielded 1–5 eggs per day with yolks of 17 g average fresh weight.

Pigments supplemented to commercial diet are 66% canthaxanthin and 34% lutein, and the egg yolks from the corresponding 'region 1' show 53% canthaxanthin and 47% lutein (Table 5).

The TLC results show that when egg yolk carotenoids from 'region 1' were chromatographed the main pigment found was canthaxanthin but also the yellow pigment, lutein, was observed. Egg yolks from 'region 2' only show lutein, present in the base diet.

Diet supplemented with microalgal biomass has 36% canthaxanthin and 0·3% lutein (Table 2), and the correspondly eggs from the 'region 3', show only, 14% canthaxanthin and 86% lutein (Table 5 and Plate 2).

DISCUSSION

The depletion of carotenoid levels in egg yolks observed when hens are fed the carotenoid-poor base diet is relatively quick, and the *ca* 48 h found for the value of time lag or response, is in agreement with results previously reported (Johnson *et al* 1980). The minimum pigment content was reached after 8–10 days deprivation, and its value (5–6 Roche grade) falls no more, presumably because of the carotenoids present in yellow corn (mainly lutein and zeaxanthin) used in the preparation of the base diet.

The supplementation of a carotenoid-poor base diet with microalgal biomass, during the second period, showed a carotenoid increase in the egg yolks until a maximum concentration was reached (also after 8–10 days), which was unaltered even by increasing microalgal carotenoids. This diet proved capable of resulting

TABLE 3
Results of daily determinations of the whole experiment, showing visual scoring of egg yolks (Roche Yolk Colour Fan, 1989)[a]

	Cage 1	*Cage 2*	*Cage 3*	*Cage 4*
Total egg number	131	156	157	131
Average egg weight (g)	62·6	60·6	62·6	64·9
Average yolk weight (g)	16·5	17·4	17·9	16·6

	Cage 1		*Cage 2*		*Cage 3*		*Cage 4*	
Day	*Eggs*	*Roche Yolk Colour Fan*	*Eggs*	*Roche Yolk Colour Fan*	*Eggs*	*Roche Yolk Colour Fan*	*Eggs*	*Roche Yolk Colour Fan*
0	4	12·5 ± 0·5	5	12·8 ± 0·4	4	12·8 ± 0·4	3	13·0 ± 0·0
1	4	13·0 ± 0·0	4	13·0 ± 0·0	3	13·0 ± 0·0	3	13·0 ± 0·0
2	4	11·8 ± 0·4	5	12·0 ± 0·0	5	12·6 ± 0·5	4	12·2 ± 0·4
3	3	11·3 ± 0·5	5	11·4 ± 0·5	5	11·0 ± 0·6	3	12·0 ± 0·8
4	4	10·2 ± 0·4	4	10·5 ± 0·5	4	10·2 ± 0·8	3	9·7 ± 0·5
5	5	8·6 ± 0·5	4	9·0 ± 0·0	4	9·0 ± 0·7	3	8·7 ± 0·9
6	3	7·3 ± 0·5	5	7·2 ± 0·4	4	7·5 ± 0·5	4	7·0 ± 0·7
7	4	6·0 ± 0·7	5	6·0 ± 0·0	3	5·7 ± 0·5	2	6·5 ± 0·5
8	1	5·0 ± 0·0	4	5·8 ± 0·4	5	6·2 ± 1·0	2	6·5 ± 0·5
9	5	6·0 ± 0·0	3	6·0 ± 0·8	4	6·0 ± 0·0	3	5·7 ± 0·5
10	1	6·0 ± 0·0	4	5·5 ± 0·5	5	6·0 ± 0·6	4	6·2 ± 0·8
11	3	6·3 ± 0·5	5	6·6 ± 0·5	5	6·2 ± 0·7	2	6·0 ± 1·0
12	4	7·0 ± 0·7	4	6·0 ± 0·0	5	6·4 ± 0·8	3	6·0 ± 0·0
13	4	6·8 ± 0·4	5	6·0 ± 0·0	4	6·8 ± 0·4	3	6·7 ± 0·5
14	**3**	**7·0 ± 0·0**	**4**	**6·5 ± 0·5**	**3**	**6·3 ± 0·5**	**3**	**6·7 ± 0·5**
15	**3**	**8·3 ± 0·5**	**3**	**8·3 ± 0·5**	**5**	**7·8 ± 0·4**	**3**	**7·7 ± 0·5**
16	**4**	**8·2 ± 0·4**	**4**	**9·0 ± 0·7**	**4**	**9·0 ± 0·7**	**2**	**8·5 ± 0·5**
17	**4**	**10·0 ± 0·7**	**4**	**10·2 ± 0·4**	**3**	**10·3 ± 0·5**	**3**	**10·3 ± 0·5**
18	**2**	**11·5 ± 0·5**	**4**	**11·5 ± 0·4**	**5**	**11·4 ± 0·5**	**3**	**11·0 ± 0·0**
19	**3**	**11·3 ± 0·5**	**4**	**11·5 ± 0·4**	**5**	**12·2 ± 0·4**	**4**	**11·5 ± 0·5**
20	**3**	**11·7 ± 0·5**	**4**	**11·5 ± 0·4**	**3**	**11·0 ± 0·0**	**4**	**11·2 ± 0·4**
21	**4**	**11·2 ± 0·4**	**3**	**11·7 ± 0·5**	**3**	**12·0 ± 0·0**	**4**	**11·2 ± 0·4**
22	**4**	**11·5 ± 0·5**	**4**	**11·8 ± 0·4**	**4**	**11·2 ± 0·4**	**4**	**11·5 ± 0·5**
23	**3**	**11·3 ± 0·5**	**5**	**11·2 ± 0·4**	**4**	**11·0 ± 0·0**	**4**	**11·2 ± 0·4**
24	**4**	**10·5 ± 0·5**	**5**	**11·0 ± 0·0**	**4**	**10·5 ± 0·5**	**4**	**10·5 ± 0·5**
25	**4**	**10·5 ± 0·5**	**3**	**10·0 ± 0·0**	**5**	**10·2 ± 0·4**	**5**	**10·6 ± 0·5**
26	**3**	**11·0 ± 0·0**	**4**	**11·0 ± 0·0**	**3**	**10·7 ± 0·5**	**4**	**11·8 ± 0·4**
27	**5**	**11·0 ± 0·0**	**5**	**10·8 ± 0·4**	**5**	**11·0 ± 0·0**	**4**	**11·8 ± 0·4**
28	2	10·5 ± 0·5	4	10·2 ± 0·4	5	9·8 ± 0·4	2	10·0 ± 0·0
29	3	9·7 ± 0·5	4	9·2 ± 0·4	3	9·3 ± 0·5	4	9·2 ± 0·8
30	4	8·8 ± 0·4	3	8·3 ± 0·5	3	8·3 ± 0·5	4	9·2 ± 0·8
31	4	7·8 ± 0·4	4	7·2 ± 0·4	5	7·0 ± 0·6	3	8·3 ± 0·5
32	1	7·0 ± 0·0	4	7·0 ± 0·0	3	6·7 ± 0·5	5	7·2 ± 0·4
33	4	6·5 ± 0·5	3	6·0 ± 0·0	4	7·0 ± 0·7	1	7·0 ± 0·0
34	4	6·5 ± 0·5	4	6·5 ± 0·5	4	6·2 ± 0·4	3	7·0 ± 0·8
35	5	6·6 ± 0·5	4	6·5 ± 0·5	5	6·0 ± 0·9	3	5·7 ± 0·5
36	3	6·0 ± 0·0	3	6·3 ± 0·5	3	5·7 ± 0·5	4	5·5 ± 0·5
37	3	6·3 ± 0·5	5	6·0 ± 0·6	5	6·2 ± 0·4	4	5·8 ± 0·4

[a] Values in bold indicate a base diet with microalga supplement.

TABLE 4
Carotenoid content ($\mu g\ g^{-1}$ fresh yolk) in egg yolks from the three regions[a]

	1	*2*	*3*
Lutein	6·52 ± 1·4	0·45 ± 0·2	9·36 ± 1·5
Canthaxantin	7·40 ± 0·9	ND[b]	1·48 ± 0·5
Total carotenoids	14·6 ± 1·2	2·1 ± 0·8	10·8 ± 1·0

[a] Each value was the mean ± standard error of four analyses.
[b] ND, not detected.

in a colouring of egg yolks somewhat weaker than that observed when commercial pigment was fed (levels 11–12 in visual Roche score scale were attained 'region 3', against 13–14 in the same scale when using synthetic pigment 'region 1').

In either of the situations depicted as 'region 1' and 'region 3', the total amount of carotenoids in yolk is much less than that which is fed.

When carotenoids present in egg yolk, as depicted in Table 4, are compared with carotenoids in the feed (assuming no carotenoid interconversion) one may see that the transfer of canthaxanthin was 9% and 17% in 'region 1' and in 'region 3', respectively.

Besides the improved absorption of canthaxanthin, lutein incorporation also rose dramatically when microalgal biomass was used as feed supplement. As increased lutein is regarded as beneficial this side-effect will be investigated further.

A closer look at 'region 2' and 'region 3' areas in Table 3 shows a slight compensation tendency—a slow increase in colour level is apparent in 'region 2' and a slow decrease is apparent in 'region 3'. These slight alterations in colour are physiologically in line with adjustments to high (respectively low) diet concentrations of carotenoid amounts fed in excess (respectively default) of daily consumption, and probably reflects rhythms of deposition into (respectively recovery from) pigmented tissues in the body. These phenomena appear to occur with rhythms of similar magnitude, as shown by the similar absolute values of slope of 0·16 for both these deposition and withdrawal situations in the straight lines A and B (Fig 1), obtained by least mean squares adjustment of data.

TABLE 5
Percentage of total carotenoids

	Lutein			*Canthaxanthin*		
	1	*2*	*3*	*1*	*2*	*3*
Carotenoids fed supplement	34	—	0·3	66	—	36
Carotenoids in egg yolk	47	100	86	53	—	14

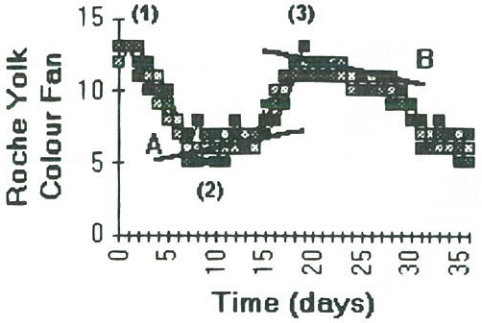

Fig 1. Evolution of egg yolk colour (Roche Yolk Colour Fan, 1989) with the two straight lines A and B in 'region 2' and 'region 3', respectively.

The above reported observations point towards the digestibility and the apparent usefulness of algal biomass as a convenient encapsulated form of transferable carotenoid supplementation in laying hen feed. The data are also good enough to uncover some more subtle information. In the experiment using microalgal biomass, and from 'region 3' onwards, it is possible to determine that yellow carotenoids present in yolk exceed those fed, whereas the reverse is true for the red pigments (for which a 17% transfer was inferred). This indicates that compensation of dietary deviation from expected intake is occurring, and that either depigmentation of the hen tissues in lutein or *in vivo* reduction of red pigment to yellow pigment is occurring. This last situation has not been described for laying hens, but is similar to occurrences mentioned in case of trout (Torrissen 1989; Guillou *et al* 1992a,b).

In any case, the similar depigmentation rate after 'region 1' and 'region 3' may indicate that the organism as a whole does not behave as if it were deprived of carotenoids.

On the other hand, there were no signs of rejection of the diet containing microalgal biomass or any reduction in laying rates. These are not, however, quantifiable observations because the hens' live weight distribution patterns were not followed.

The main carotenoid present in the microalgal biomass used was canthaxanthin, and this unfortunately corresponded to a late algal development stage; an early one would have probably been best for the egg yolk colouring because of the higher levels of lutein as revealed by HPLC (Gouveia *et al* unpublished results). Therefore, the onset and development of carotenogenesis as a result of stress will be studied in order to enable the objective determination of culture conditions best suited to each colouring application, in this case to egg yolk colouring.

The desired yolk colour for domestic use varies with the geographical location and local tastes and consumer preferences but, in general, a fairly light colour is preferred. On the other hand yolk with a high pigment content is in demand for commercially produced bakery

goods, noodles, mayonnaise, and other food products in which a yellow colour is desired and must be provided by egg yolk rather than by the direct addition of colourings (Ashton and Fletcher 1962; Klaui and Bauernfeind 1981). However, the best strategy for the egg producer is to accurately provide and consistently maintain yolk colour within the desired range for his particular market irrespective of the nature of main feed ingredients hence the need for supplementation.

Many studies on regional and national consumer preferences have been reported. In Belgium, deeply coloured yolks with a definite orange tinge are preferred (Marusich and Bauernfeind 1981). In Italy, consumers also preferred deeply coloured yolks. Similar findings were reported in Peru, Israel, Switzerland, Great Britain, Australia, New Zealand, South Africa and the USA (Marusich and Bauernfeind 1981).

In some of these countries, the desired colour for table eggs is related to the visual colour guide mentioned (Roche Yolk Colour Fan), which we used, and the premium price is for egg yolks with colour intensity equal to or greater than a reading of 8 (Marusich and Bauernfeind 1981). The colour 11–12 obtained by us with the microalgal biomass is therefore perfectly satisfactory.

The use of *Chlorella vulgaris* biomass as both food and feed supplement is a relatively widespread practice, especially in the Far East; its virtues as a vehicle for protein and essential fatty acids are used for promotional purposes. The use of this algal biomass, after carotenogenesis, as a carrier of natural pigment, which remains stable within the cell walls before use, for the purpose of colouring the egg yolk has been established in this work. The other trends observed and effective transfer rates may and probably will be clarified by studies with labelled carotenoids.

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